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February 28, 2020

Via FedEx and CD Rom

Dr. David Edwards
Division of Animal Feeds (HFV-220)
Office of Surveillance and Compliance
Center for Veterinary Medicine
MPN 4, Room 2658
1225 Wilkins Avenue
Rockville, Maryland 20852

Re: GRAS Notice for Dried *Methylococcus capsulatus* Product for use in Salmonid Feed

Dear Dr. Edwards:

We respectfully submit the attached generally recognized as safe (GRAS) Notification on behalf of our client, Calysta, Inc. (Calysta) for a dried *Methylococcus capsulatus* product, hereinafter referred to by its tradename FeedKind®, to be used as a protein supplement in salmonid feed. More detailed information regarding product identification, intended use levels, and the manufacturing and safety of the ingredient is set forth in the enclosed GRAS Notification.

Calysta has determined that FeedKind® is GRAS based on scientific procedures in accordance with 21 C.F.R. § 570.30(b) and in conformance with the guidance issued by the Food and Drug Administration (FDA) under 21 C.F.R. § 170.36, 81 Fed. Reg. 54960 (Aug. 17, 2016). Therefore, the use of FeedKind® as described in this GRAS Notification is exempt from the requirement of premarket approval as set forth in the Federal Food, Drug, and Cosmetic Act.

Analytical data and information not already provided in this GRAS Notification are available for FDA review and copying at reasonable times at Keller and Heckman LLP, 1001 G Street, NW, Suite 500W, Washington, DC 20001, or will be sent to FDA upon request. As requested by Dr. Louis Carlacci of CVM on September 17, 2019, we are providing copies of all references cited in section 7 of the GRAS Notification.

Per our conversation with Dr. Carlacci, and described more fully in our submission, Calysta considers some of the information submitted to be confidential business information (CBI) and such information has been placed within a red box to identify it as CBI.

We look forward to the Agency's review of this submission and would be happy to provide Agency officials with any information they may need to complete their assessment. Thank you for your attention to this matter.

Sincerely yours,

Melvin S. Drozen

Enclosure

GRAS Notice for Dried *Methylococcus capsulatus* Product

Prepared for:

Division of Animal Feeds (HFV-220)
Office of Surveillance and Compliance
Center for Veterinary Medicine
Food and Drug Administration
Dr. David Edwards
MPN 4, Room 2658
12225 Wilkins Avenue
Rockville, MD 20852

Submitted by:

Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington, DC 20001

On behalf of our client

Calysta, Inc.
1140 O'Brien Drive
Menlo Park, CA 94025
United States

February 28, 2020

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Part 1 – Signed statements and certification

1.1 Applicability of 21 C.F.R. part 570, subpart E

We submit this Generally Recognized As Safe (GRAS) notice in accordance with 21 C.F.R. part 570, subpart E.

1.2 Name and address of the notifier

Company: Calysta, Inc.
Name: Tomas Belloso
Address: 1140 O'Brien Drive
Menlo Park, CA 94025
Phone: 314-368-7114

All communications on this matter are to be sent to Counsel for Calysta, Inc.

Melvin S. Drozen
Keller and Heckman LLP
1001 G Street, N.W., Suite 500 West
Washington, DC 20001
Phone: (202) 434-4222
Email: drozen@khlaw.com

1.3 Name of the notified substance

Dried *Methylococcus capsulatus* Product, hereinafter for ease of reference we will refer to the Dried *Methylococcus capsulatus* Product as FeedKind®. FeedKind® is a protein-rich single cell protein (SCP) intended for use as a protein supplement in salmonid feed. FeedKind® is produced through the culture of methanotrophic and heterotrophic microbial consortia.

1.4 Applicable conditions of use of the notified substance

FeedKind® is intended for use as a protein source in salmonid species (*e.g.*, Atlantic salmon and rainbow trout) feed with maximum use levels based on published scientific studies and corroborating unpublished data is 18% FeedKind® in the diet.

1.5 Basis for the GRAS determination

Keller and Heckman LLP, on behalf of Calysta, Inc. hereby notifies the Agency of its determination that FeedKind® is Generally Recognized as Safe (GRAS), consistent with Section 201(s) of the Federal Food, Drug, and Cosmetic Act (FDCA). This GRAS conclusion is based on scientific procedures in accordance with 21 C.F.R. §§ 570.30(a) and (b).

1.6 Exclusion from premarket approval

FeedKind® is not subject to the premarket approval requirements of the FDCA based on our conclusion that the notified substance is GRAS when used as a protein source in salmonid feed.

1.7 Availability of data and information

The information for this GRAS conclusion including analytical data, published studies, and information that are the basis for this GRAS determination are available to FDA upon request as required by 21 C.F.R. § 570.225(c)(7)(ii)(A) or (B) by contacting Keller and Heckman LLP at the below address.

Melvin S. Drozen
Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington DC 20005
Tel: 202-434-4222
Fax: 202-434-4646
Email: drozen@khlaw.com

1.8 Applicability of FOIA exemptions

Certain of the data and information in Parts 2 through 7 of this GRAS Notice are exempt from disclosure under the provisions of 5 U.S.C. § 552 (*e.g.* as trade secret or as commercial or financial information that is privileged or confidential). Information claimed as confidential is shown in this document within a red box.

1.9 Certification

We certify on behalf of our client Calysta, Inc. that this GRAS conclusion is based on representative data from Calysta, Inc. required for the safety and GRAS status of FeedKind®. To the best of our knowledge, our GRAS Notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

(b) (6)_ ___2/28/2020____

Melvin S. Drozen Date
Partner

Keller and Heckman LLP

Part 2 – Identity, method of manufacture, specifications, and physical or technical effect

2.1 Scientific data and information that identifies the notified substance

FeedKind® is a biomass product of fermentation which utilizes a consortium of microorganisms to produce a high protein product for use in salmonid feed. The FeedKind® product has been thoroughly tested and characterized. **Figure 1** is a sample data sheet that will be included with the product listing chemical composition.

Figure 1: Label of FeedKind® Product

FeedKind®

For Further Manufacture of Feed

GUARANTEED ANALYSIS

Crude Protein	(min)	68.0	%
Crude Fat	(min)	5.0	%
Crude Fiber	(max)	1.0	%
Moisture	(max)	10.0	%
Ash	(max)	12.0	%

Ingredients: Dried *Methylococcus capsulatus* Product

Storage: Store in a dry and clean place at room temperature.

Directions: For further manufacture of feed for *Salmonidae* fish (18% maximum inclusion rate in final feed).

Lot#

Manufacturing Date:

Expiration Date: 12 months from manufacturing date

Manufactured by:

CALYSTA, Inc
1140 O'Brien Drive
Menlo Park CA, USA 94025
Tel.: (650) 492-6880

Net Weight on Invoice

2.2 Description of the method of manufacture of FeedKind®

2.2.1 Organisms

FeedKind® is produced through the culture of methanotrophic and heterotrophic microbial consortia. *Methylococcus capsulatus* (Bath) is a methanotrophic bacteria that composes 90% of the culture. It is a thermophilic bacterium originally isolated from the hot springs in Bath, England, is widely used as a laboratory culture and has been deposited as NCIMB 11132 at The National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland. *M. capsulatus* (Bath) has optimum growth at 45°C, but growth occurs between 37°C and 52°C. It is a gram-negative nonmotile spherical cell, usually occurring in pairs. The intracellular membranes are arranged as bundles of vesicular discs characteristic of Type I methanotrophs. *M. capsulatus* (Bath) is genetically a very stable organism without known plasmids. It can utilize methane or methanol for growth and ammonia, nitrate, or molecular nitrogen as nitrogen sources for protein synthesis. While only carbon sources containing a single carbon atom are utilized by *M. capsulatus* for growth (i.e., biomass), the organism is capable of oxidizing higher molecular weight hydrocarbons into their corresponding organic acid (e.g., ethane to acetic acid and propane to propionic acid). These higher molecular weight hydrocarbons are largely removed from the methane during processing of natural gas, but may remain in concentrations of 0-15% (ethane) to <5% (propane).

The FeedKind® culture includes three heterotrophic strains, *Cupriavidus sp.* (NCIMB 13287, previously *Alcaligenes acidovorans* DB3), *Aneurinibacillus danicus* (NCIMB 13288, previously *Bacillus brevis* DB4), and *Brevibacillus agri* (NCIMB 13289, previously *Bacillus firmus* DB5), all of which were isolated from mixed cultures growing on methane. The nomenclature changes are indicative of changes in the level of sophistication of bacterial taxonomy, and do not represent any changes to the actual strains utilized to produce FeedKind®. The nomenclature changes are due to a recent whole genome sequencing analysis of the three heterotrophic strains undertaken by the submitter in 2019 (**Appendix 4**). This study found that the DB3 genome's closest known match was to *Cupriavidus gilardii* (91.95% nucleotide identity), indicating that DB3 was a previously undescribed member of genus *Cupriavidus.* Per internationally accepted naming criteria, Calysta intends to name this species *Cupriavidus calystii* per criteria set forth by the International Committee on Systematics of Prokaryotes (ICSP) and to publish these results in The International Journal of Systematic and Evolutionary Microbiology. However, pending peer review and acceptance of these results, this strain will be referred to as *Cupriavidus sp.* in this submission. The sequence of DB4 indicated a 99.54% identity match to *Aneurinibacillus* UBA3580, which is a genome generated via metagenomic datasets, rather than from an actual isolate.[1] *Aneurinibacillus sp.* (NCIMB 13288) was previously renamed to *Aneurinibacillus danicus* based on 16s sequences, however no whole genome sequence was

[1] Parks DH, Rinke C, Chuvochina M, Chaumeil P-A, Woodcroft BJ, Evans PN, Hugenholtz P, and Tyson GW. (2017) Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life. *Nature Microbiology* 2(11): 1533-1542.

available at the time.[2] Finally, DB5 sequence analysis indicated a 99.56% identity match to *Brevibacillus agri*. Matches with greater than 95% nucleotide identity are considered extremely likely to be of the same species.

The purpose of the heterotrophic strains is threefold: 1) to metabolize organic acids (acetate, propionate, butyrate) produced by *M. capsulatus* that have been shown to reduce the efficiency with which *M. capsulatus* converts methane to biomass; 2) to metabolize organic material released via naturally occurring cell lysis during fermentation which could lead to foam formation; and 3) to minimize the risk of culture contamination by undesirable microbes.

Previous regulatory submissions used old taxonomy for the heterotrophic strains, all three of which have been reclassified utilizing modern molecular techniques. However, the strains used have not changed from those previous submissions.

1) *Cupriavidus sp.* (NCIMB 13287) is a gram-negative, aerobic, motile rod. It can utilize ethanol, acetate, propionate and butyrate for growth. *Cupriavidus sp.* accounts for 6-8% of the total cell count of a FeedKind® culture grown on natural gas.
2) *Aneurinibacillus danicus* (NCIMB 13288) is a gram-positive, endospore-forming, aerobic rod. It can utilize acetate, D-fructose, D-mannose, ribose and D-tagatose. It accounts for less than 1% of the cell count during continuous fermentation.
3) *Brevibacillus agri* (NCIMB 13289) is a gram-positive, endospore-forming, motile, aerobic rod. It can utilize acetate, N-acetyl-glucosamine, citrate, gluconate, D-glucose, glycerol and mannitol. It accounts for less than 1% of the cell count during continuous fermentation.

2.2.2 FeedKind® Production

(b) (4)

[redacted]

[2] Goto K, Fujita R, Kato Y, Asahara M, Yokota A. (2004) Reclassification of *Brevibacillus brevis* strains NCIMB 13288 and DM 6472 (=NRRL NRS-887) as *Aneurinibacillus danicus* sp. nov. and *Brevibacillus linophilus* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*. 54 (2):419-427.

Table 1: **Microorganism temperature and residence times.**

Operation	Operating Temperature	Residence Time
Fermenter	(b) (4)	

Centrifuge	(b) (4)	
Heat Treatment		
Evaporator		
Spray Dryer		

As discussed more thoroughly in Part 6.1.1 of this notice, the organisms which are used to produce FeedKind® are safe for consumption. The main production organism, *M. capsulatus,* is a methanotroph and requires single carbon energy sources (e.g. methane or methanol) for growth, as well as elevated temperatures. For these reasons, methanotrophs are not considered to be risks for pathogenicity in animals or humans. Additionally, the three heterotrophic strains were tested in rodents and exhibited no ability to cause infections, even at very high doses (>10^9 cfu/kg bw).[3] Furthermore, tests undertaken by Calysta have shown that the heat treatment step during production is effective at reducing the viable heterotrophic bacterial load by more than 2 logs (99%) in the finished product (Table 2). The safety of the production microorganisms and large reduction in viable cell counts due to post fermentation processing indicate that FeedKind® is not expected to pose any microbiological safety concerns.[4]

Table 2: Comparison of bacterial load in finished FeedKind® without (ST01 and 02) and with heat treatment.

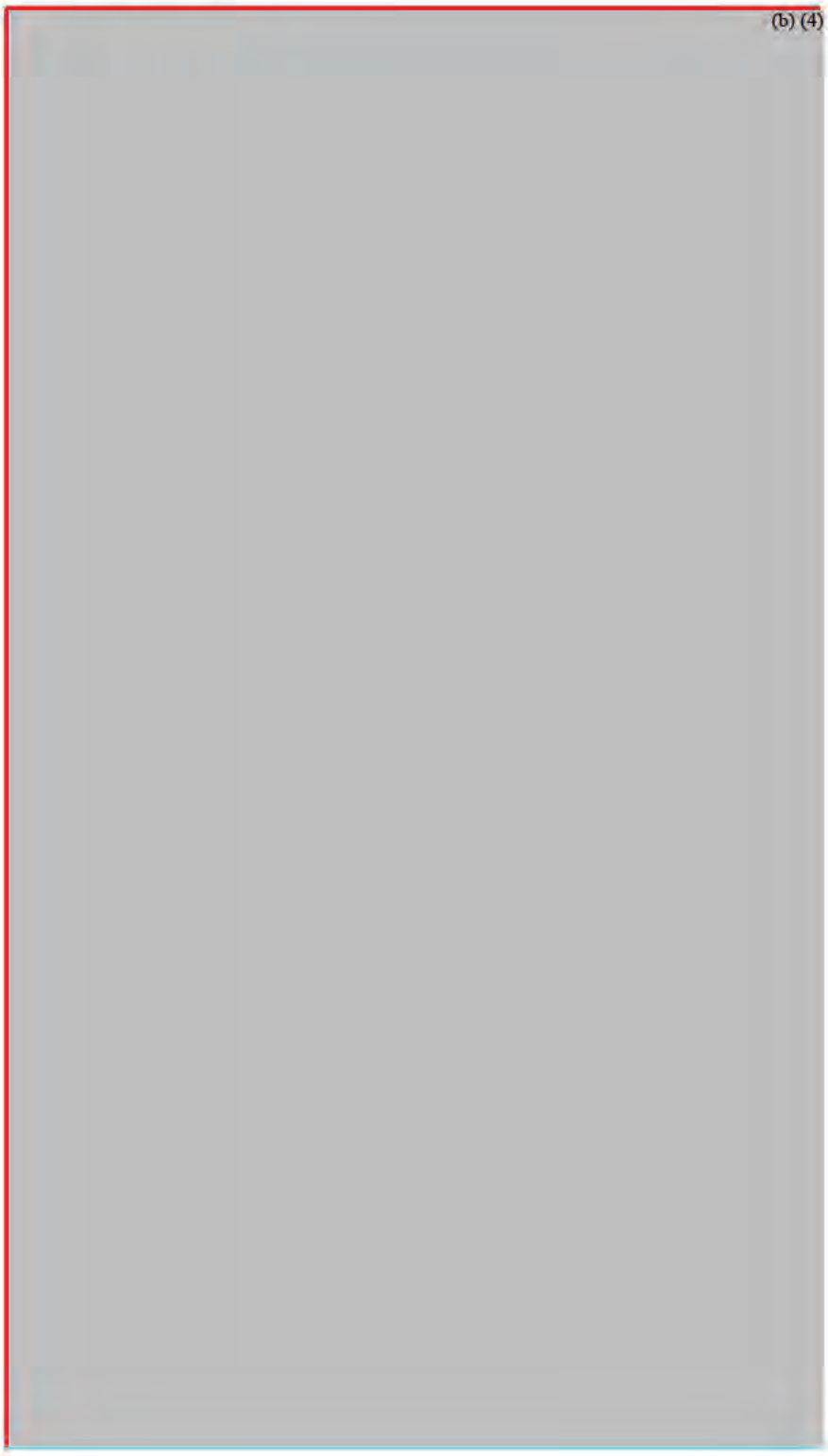
Sample	Anaerobic (cfu/g)	Aerobic (cfu/g)
ST01	6,100	170,000
ST02	6,100	170,000
ST03	280	350
ST04	280	350
ST05	170	240
ST06	170	240
ST07	10	20
ST08	10	20

[3] *See* Part 6.1.1.

[4] *See* Appendix 1.

(b) (4) is the last step in the production of FeedKind®. It undergoes no other technical processes of preparation before use in the formulation of animal feeds.

Figure 2: FeedKind® Production Process



2.2.2.1 Raw Materials and Processing Aids

All raw materials and processing aids, including fermentation media components, utilized in the production of FeedKind® are safe and suitable for use in feed production, and are prepared and handled as feed ingredients. These materials are listed in **Table 3**.

Table 3: Raw materials and (example) processing aids

Raw Material	Function	Authorization Reference	Authorization Limits	Specification
Methane & Natural Gas	Nutrient for culture	None; Safe for use[5]	N/A	*See* **Table 4**
Ammonium Hydroxide	pH control	21 CFR §582.1139	Good Manufacturing or Feeding Practice	25-35%
Sulfuric Acid	pH control	21 CFR §582.1095	GM/FP	0.02% max
Phosphoric Acid	Nutrient for culture	21 CFR §582.1073	GM/FP	31.0-33.0%
Sodium Hydroxide	pH control	21 CFR §582.1763	GM/FP	31.0-33.0%
Potassium Hydroxide	Nutrient for culture	21 CFR §582.1631	GM/FP	46%
Zinc Sulfate Heptahydrate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary supplements	99%
Nickel Chloride Hexahydrate	Nutrient for culture	None; Safe for use[6]	N/A	99.0% min
Cobalt Sulfate Heptahydrate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary	98%
Manganese Sulfate Monohydrate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary	98-102%

[5] *See* discussion in Section 2.2.2.2 of this GRAS Notice.
[6] *See* discussion in Section 2.2.2.4 of this GRAS Notice.

Nitric Acid	pH control and Nutrient for culture	None; GRAS[7]	N/A
Copper Sulfate Pentahydrate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary
Sodium Molybdate Dihydrate	Nutrient for culture	AAFCO Definition # 57.145	N/A
Iron Sulfate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary
Calcium Chloride	Nutrient for culture	21 CFR §582.1193	GM/FP
Magnesium Sulfate Heptahydrate	Nutrient for culture	21 CFR §582.5443	GM/FP; used as a nutrient and/or dietary supplement
Potassium Sulfate	Nutrient for culture	21 CFR §582.1643	GM/FP
Glanapon 2000 Antifoam	Antifoam	21 CFR §§ 172.808, 173.340, 582.4505	*See* Section 2.2.2.6

(b) (4)

All ingredients and processing aids in **Table 3**, including those that rely on 21 CFR Part 582 for an appropriate regulatory status, will be used in accordance with good manufacturing and feeding practice.

FeedKind® will be distributed in lined polypropylene bags, which are widely used within the animal feed industry and which have appropriate regulatory status under 21 CFR § 177.1520.

The current natural gas specifications for FeedKind® are listed in **Table 4**.

Table 4: Current natural gas specifications

Natural Gas	Units	Specification
Nitrogen	Mol %	0-0.5
Methane	Mol %	82-100

[7] *See* Section 2.2.2.5 of this GRAS Notice.

Ethane	Mol %	0-15
Propane	Mol %	0-5
I-Butane	Mol %	0-2
N-Butane	Mol %	0-2
I-Pentane	Mol %	0-0.7
N-Pentane	Mol %	0-0.7
Calorific value – volume	Mol %	37.7-44

2.2.2.2 Natural gas, including methane, safety

Natural gas is composed primarily of methane, although ethane, propane, butane and pentane may also be present.[8] Before refinement, natural gas may also contain 0% to 5% hydrogen sulfide (H_2S) gas and elemental mercury (Hg^0) vapor and noble gases, such as argon (A), helium (He), neon (Ne), or xenon (Xe). After refining, natural gas is essentially pure methane. None of these gaseous or vaporous natural gas constituents are expected to remain in finished FeedKind®. Methane serves as a food source for the bacteria and most, if not all, of the methane and other residual gaseous or vaporous substances that may enter the system with the refined pipeline natural gas, and not be dissolved or otherwise incorporated into the fermentation mix, is vented out of the system during the fermentation, centrifugation, evaporation and re-circulation processes. Furthermore, any residuals of these gases and vapors that may remain in the harvested wet biomass before spray drying will dissipate away from the product during spray drying. Hg^0 is extremely volatile. Thus, no Hg^0 is expected in Feedkind®. However, some of the Hg^0 that may be present in the refined natural gas used to produce FeedKind® may be metabolized by the bacteria to produce methylmercury (MeHg) during fermentation. MeHg has the potential to bioconcentrate in the bacteria and remain in the product after spray-drying. This potential is evaluated in detail in Section 2.2.2.3 below.

2.2.2.3 Mercury safety

Natural gas often contains trace levels of mercury (Hg), predominantly elemental mercury (Hg^0), which must be removed from the gas phase before transport via pipeline to protect downstream heat exchangers from catastrophic failures and catalysts from fouling. The concentrations of Hg in pipeline natural gas is recued to concentrations below <0.01 $\mu g/Nm^3$ by means of current industry practices in the U.S., and Calysta specifications require that natural gas used to produce FeedKind® will contain no more than 0.2 $\mu g/Nm^3$. Inorganic Hg can be metabolized by microorganisms to produce methyl mercury (MeHg), which can then

[8] NATURALGAS.ORG. Available at: https://web.archive.org/web/20140709040340/http://naturalgas.org/overview/background/.

bioaccumulate in the food chain to result in bioconcentration factors (BCFs) as high as 80,000 in fish at the top of the food chain in aquatic environments.

The toxicity of Hg compounds has been well characterized in the literature. MeHg is generally more toxic than inorganic forms of Hg and is of particular concern because it also has the greatest potential to bioaccumulate. The NRC (2005) determined that salmon tolerate chronic MeHg up to 1 µg/kg diet and set a maximum tolerable level of 0.3 µg/kg bw/day for pregnant women to protect children in utero from MeHg from maternal fish ingestion.

We calculated exaggerative estimates of the maximum Hg concentrations based on an example FeedKind® production scenario. In that scenario, (b) (4) tons/year FeedKind® are produced in a single-fermenter production system where a substantially greater faction of the culture medium than required is re-circulated into the production system over each 12-week fermentation cycle. After each fermentation cycle, the system is emptied, cleaned, and prepared to receive fresh bacterial culture and medium to start the next 12-week cycle. The worst-case assumptions incorporated into these calculations include the use of natural gas that invariably contains the maximum concentration of Hg, 100% conversion of the Hg to MeHg by the bacterial consortium, and BCFs as high as 80,000 for MeHg during fermentation.

We also estimated worst-case human exposures to Hg in the edible tissues of salmon and trout fed FeedKind®, assuming that the fish are fed exclusively food containing the maximum level of FeedKind®, all of the Hg in FeedKind® accumulates exclusively in the edible tissues of the fish, consumers eat only salmon or trout fed exclusively food containing the maximum level of FeedKind®, and that high-end consumers eat only salmon or trout at the same daily rate as estimated for the consumption of all finfish, combined, by high-end consumers (i.e. 90th percentile) of finfish.

The worst-case maximum concentration of Hg in salmonid feed containing FeedKind® at the highest use level was 0.289 µg/kg, which is 3460 times lower than the concentration tolerated by salmon determined by NRC (2005). The worst-case exposures to Hg were estimated to be 0.0012 µg/kg bw/day and 0.0015 µg/kg bw/day for exclusive consumers of salmon and trout, respectively, which are ≥ 250 times lower than the maximum tolerable level set by NRC (2005). Thus, the results of these exaggerative exposure calculations demonstrate the there are no significant risks to the target animals or to consumers of the edible tissues of the salmon or trout raised exclusively on food containing FeedKind® at the highest use levels.

This assessment is presented in greater detail in the **Appendix 5**.

2.2.2.4 Nickel chloride hexahydrate safety

Nickel is required in very small amounts (b) (4) mg Ni/kg FeedKind® finished product) as a nutrient in the fermentation media. As the production process for FeedKind® involves re-circulation and reuse of some water recovered from the fermentation media, it is theoretically possible that nickel could accumulate in the fermentation media over time resulting in higher concentrations of nickel in the final finished product. Although this has not been observed in any production run to date, a specification of 10 ppm nickel in the finished product was established.

Nickel from nickel chloride hexahydrate

As discussed in NRC (2005), nickel is an essential element for some lower forms of life. For example, nickel is essential for nitrogen metabolism in plants and for the activity of hydrogenases identified in more than 35 species of bacteria, including nitrogen-fixing bacteria.[9] However, nickel is not considered to be essential for higher animals and humans, although experimental nickel deprivation has been shown to result in subnormal functions that appear to be associated with vitamin B_{12} activity.

Typically, less than 10% of the nickel in food is absorbed in the gastrointestinal tracts of animals and humans, and the small amount that is absorbed is excreted mostly in the urine (NRC 2005).

Nickel compounds are known carcinogens by inhalation exposure, and nickel is a recognized allergen by respiratory and dermal exposure. Allergic effects are possible in sensitized individuals exposed to high levels of nickel in the diet. However, there is no evidence of adverse health effects in humans associated with chronic dietary exposure to nickel. The tolerable upper intake level for humans is 0.017 mg/kg bw/day, based on a NOAEL of 5 mg/kg bw/day in two rat studies (NRC 2005).

In animal studies, the first signs of nickel toxicity appear to be the result of reduced food intake, which is attributable at least in part to reduced palatability of the diet, and gastrointestinal irritation. The most common signs reported after extended exposures include reduced growth, feed intake and feed efficiency, as well as hematological changes and sometimes renal effects. Elevated incidences of the death of offspring have been reported in developmental and reproductive toxicity studies in rats and chickens exposed to soluble nickel salts in drinking water or the diet, indicating the potential for impaired reproductive performance. Some of the effects of long-term excessive oral exposures to nickel are attributable to interference by nickel with the gastrointestinal absorption or use of essential elements, including copper, iron, and zinc, which are more evident when the diet is deficient in these elements. Alterations in cellular redox status, resulting in excessive levels of reactive oxygen species, has also been suggested as a potential mechanism of nickel toxicity (NRC 2005).

On the other hand, the potential for life-threatening toxicity is considered to be low, similar to zinc, chromium, and manganese, for example, because of the existence of effective homeostatic mechanisms for the regulation of nickel.

Generally, toxicity has been observed in animal studies only after chronic exposures to more than 100 ppm water-soluble nickel in the diet of rats, mice, chickens, dogs, rabbits, pigs, ducks, and monkeys. NNRC (2005) suggested maximum tolerable levels of dietary nickel of 100 ppm for cattle, 250 ppm for chickens and pigs, and approximately 1000 ppm for dogs (NRC 2005).

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9 National Research council (NRC) (2005). Nickel. Chapter 22 in: Mineral tolerance of animals. Committee on Minerals and toxic Substances in diets and water for Animals, Board on agriculture and Natural resources, Division on Earth and Life Studies, Second Revised Edition, pp. 276-283.

Most plant-based animal feeds contain relatively high concentrations of nickel and animal-tissue-based feeds contain comparatively low concentrations. Langmyhr and Orre (1980) reported substantial concentrations of 0.7 to 2.8 ppm nickel in five different fish protein concentrates considered for use as a source of protein and trace elements in human nutrition.[10] However, most animal feeds contain less than 10 ppm nickel, which is an order of magnitude less that the lowest maximum tolerated dose of 100 ppm suggested by NRC (2005). Accordingly, Maule et al. (2007) reported an average nickel concentration of 2.35 ppm, ranging from 0.42 to 7.8 ppm, in 55 fish feed samples collected from 11 National Fish Hatcheries between October 2001 and October 2003.[11]

EFSA (2015) reviewed several studies of nickel in fish feed, which support a NOAEL of 10 ppm nickel in feeds for salmonid species.[12] In particular, Ptashinsky et al. (2001 and 2002) reported a LOAEL of 100 ppm and a NOAEL of 10 ppm in Lake Whitefish fed diets supplemented with water-soluble nickel for 10, 31, or 104 days dietary nickel in Lake Whitefish.[13] Histopathological changes in the kidneys were found in the fish fed diets containing ≥100 ppm in this study. In another study, Javed (2013) reported decreased weight gain, fork length, and feed intake in Major Carp fed 73 ppm water-soluble nickel (*i.e.* the lowest concentration tested) for 12 weeks.[14] In addition, Alsop et al. (2014) reported reduced growth in male and reduced total egg production in female Zebrafish fed 116 ppm water-soluble nickel (*i.e.* the lowest concentration tested) for 80 days; Zebrafish are commonly used as an animal model for aquaculture nutrition research.[15]

By comparison, the concentration of nickel would be 1.8 ppm in a salmonid diet containing 18% FeedKind® that contains the specified maximum concentration of 10 ppm nickel. This level (*i.e.* a maximum of 1.8 ppm) is well below the NOAEL of 10 ppm in salmonid species and the maximum tolerable level of 100 ppm suggested by NCC (2005) for domestic animals. Further, comparison with the results published in Maule et al. (2007) indicate that the inclusion of

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[10] Langmyhr FJ, Orre S (1980). Direct atomic absorption spectrometric determination of chromium, cobalt and nickel in fish protein concentrate and dried fish solubles. Analytica Chimica Acta 118: 307-311.

[11] Maule AG, Gannam AL, Davis JW (2007). Chemical contaminants in fish feeds used in federal salmonid hatcheries in the USA. Chemosphere 67: 1308-1315.

[12] EFSA (2015). Scientific Opinion on the risks to animal and public health and the environment related to the presence of nickel in feed. EFSA Journal 13(4): 4074 (59 pp.)

[13] Ptashynski MD, Pedlar RM, Evans RE, Baron CL, Klaverkamp JF (2002). Toxicology of dietary nickel in lake whitefish (Coregonus clupeaformis). Aquatic Toxicology 58: 229–247; Ptashynski MD, Pedlar RM, Evans RE, Wautier KG, Baron CL, Klaverkamp JF (2001). Accumulation, distribution and toxicology of dietary nickel in lake whitefish (Coregonus clupeaformis) and lake trout (Salvelinus namaycush). Comparative Biochemistry and Physiology. Toxicology & Pharmacology 130: 145–162.

[14] Javed M (2013). Chronic effects of nickel and cobalt on fish growth. International Journal of Agriculture & Biology 15: 575–579.

[15] Alsop D, Santosh P, Lall, SP, Wood CM (2014). Reproductive impacts and physiological adaptations of zebrafish to elevated dietary nickel. Comparative Biochemistry and Physiology C 165: 67–75.

up to 1.8 ppm nickel in fish feed via the inclusion of FeedKind® - used as a replacement for other protein sources that might be in fish feed – is likely to result in fish-feed nickel concentrations below the average of 2.35 ppm nickel reported in fish feeds.

Chloride from nickel chloride hexahydrate

As discussed in NRC (2005), chloride is an essential nutrient for essentially all forms of life, most notably because chloride, along with sodium, is critical for maintaining osmotic and acid-balance.[16] The bodies of nearly all animal species maintain extracellular and intracellular osmotic concentrations between 250 and 400 milliosmoles (mOsm). Saltwater fish live in water that typically contains 1,000 mOsm, most of which is attributable to sodium chloride in the water. About 77% of the total dissolved solids in saltwater is composed of sodium chloride. Most saltwater fish excrete sodium and chloride through the gills to maintain normal levels of water in their bodies against the osmotic pressure exerted by the high salt concentration of the water around them. The gills pump sodium against the concentration gradient of the saltwater and chloride follows sodium out of the body.

In contrast, the kidneys of freshwater species excrete very dilute urine to reduce the loss of salt and maintain normal levels of body water against the tendency of the water to diffuse from extracellular fluids to the surrounding freshwater. Freshwater fish typically also have efficient mechanisms for absorbing sodium and chloride from water through the gills. NRC (2005) notes that higher sodium chloride concentrations in saltwater can reduce the toxicity of minerals such as mercury, cadmium, chromium, and zinc, by competing with and reducing the uptake of these minerals through the gills. Accordingly, freshwater fish are generally more sensitive to nitrite than saltwater fish because the chloride ions in saltwater compete with nitrite for absorption through the gills.

As discussed in NRC (2005), excessive amounts of chloride added to the diet as a component of trace metals used in very high amounts to supplement the diet, apart from sodium, has the potential to acidify extracellular fluids, resulting in metabolic acidosis.[17] However, the trace elements in most diets are absorbed in such small amounts that the possibility of altered acid-base status is negligible. In any case, mild acid-base imbalances are amenable to correction through increased renal excretion of cations or anions. Sodium chloride added to the diet generally has essentially no effect on acid-base physiology.

NRC (2005) notes that freshwater fish do not tolerate water containing more than 1500 ppm sodium chloride and saltwater fish do not survive in water containing more than 30,000 ppm.

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[16] National Research council (NRC) (2005). Sodium chloride. Chapter 27 in: Mineral tolerance of animals. Committee on Minerals and toxic Substances in diets and water for Animals, Board on agriculture and Natural resources, Division on Earth and Life Studies, Second Revised Edition, pp. 357- 371.

[17] National Research council (NRC) (2005). Minerals and Acid-base Balance. Chapter 33 in: Mineral tolerance of animals. Committee on Minerals and toxic Substances in diets and water for Animals, Board on agriculture and Natural resources, Division on Earth and Life Studies, Second Revised Edition, pp. 449-452.

As noted by Salman (2008), dietary sodium chloride up to 11.6% (*i.e.* 116,000 ppm) did not impair the growth of rainbow trout when isonitrogenous/isocaloric diets were used.[18] Dietary sodium chloride levels of 1% to 4% (*i.e.* 10,000 to 40,000 ppm) have been demonstrated to have beneficial effects in salmon, carp, trout, and other freshwater fish species, and are commonly used in diets for salmon and trout.[19]

As noted, small amounts of nickel chloride hexahydrate are added as a nutrient to the fermentation medium, which is, thus, present at up to 2 ppm[20] in finished feed. Based on the literature reviewed above, salmonid species tolerate, and even benefit from, dietary chloride concentrations several orders of magnitude greater than the potential contribution of chloride from nickel chloride in FeedKind®. Therefore, the chloride from nickel chloride hexahydrate has no potential to adversely affect the health of these species when fed FeedKind®.

2.2.2.5 Nitric Acid safety

Nitric Acid is a source of nitrogen and a pH control agent. The term nitrate (NO_3^-) refers to salts and esters of nitric acid (HNO_3). As noted in NRC (2005), nitrates are formed naturally in the biological nitrogen cycle (nitrification), through which ammonia in the soil is oxidized by aerobic bacteria to produce nitrite and then nitrate.[21] Plants then use the nitrate to synthesize amino acids and proteins. In contrast, nitrates are not essential nutrients for mammalian species.

Like nitrites (NO_2^-), nitrates are rapidly absorbed in the intestines of nonruminant mammals and the rumen of ruminants.[22] The plasma half-life of nitrate ranges from 4.2 to 4.8 hours in sheep and ponies and up to 44.7 hours in dogs.[23] Nonruminant animals generally excrete more urinary nitrate than ruminants.

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[18] Salman NA (2009). Effect of dietary salt on feeding, digestion, growth and osmoregulation in teleost fish. Chapter 4 In: Osmoregulation and Ion Transport, Volume 1, Handy, Bury and Flick, eds., Society of Experimental Biology UK (SEB).

[19] *See, e.g.*, Salman NA, Eddy FB (1988). Effect of dietary sodium chloride on growth, food intake and conversion efficiency in Rainbow trout (Salmo gairneri Richardson). Aquaculture 70: 131-144; Mzengereza K, Kang'ombe J (2015). Effect of salt (sodium Chloride) supplementation on growth, survival and feed utilization of Oreochromis shiranus (Trewavas, 1941). J. Aquac. Res. Develop. 7(1): 3 pp.

[20] We acknowledge that there are other sources of chloride in the fermentation media, but these are from sources and in uses that are permitted at levels consistent with good manufacturing and feeding practices. The issue addressed in this section is whether the chloride potentially added as part of the nickel chloride hexahydrate poses a health risk to the animals to which it is being fed.

[21] National Research council (NRC) (2005). Nitrates and nitrites. Chapter 34 in: Mineral tolerance of animals. Committee on Minerals and toxic Substances in diets and water for Animals, Board on agriculture and Natural resources, Division on Earth and Life Studies, Second Revised Edition, pp. 453-468.

[22] Walker R (1990). Nitrates, nitrites and N-nitroso compounds: a review of the occurrence in food and diet and the toxicological implications. Food Addit. Contam. 7(6): 717-768.

[23] NRC (2005).

Nitrate itself is not highly toxic. However, nitrate has the potential to be converted to nitrite, which can oxidize hemoglobin in the bloodstream to produce methemoglobin. Unlike hemoglobin, methemoglobin cannot transport oxygen. In ruminants, bacteria in the rumen rapidly convert nitrate to nitrite and nitrite to ammonia, and the ammonia is used by the bacteria to synthesize amino acids and proteins.[24] Thus, nitrate toxicity in ruminants occurs only when the conversion of nitrite to ammonia is disrupted or the nitrate levels in the diet are high enough to saturate the conversion process in the rumen. In nonruminants, methemoglobin in is usually associated with the consumption of high levels of nitrite rather than nitrate.

The clinical signs of acute methemoglobinemia may be evident when 30% to 40% of the hemoglobin in the bloodstream is converted to methemoglobin, including rapid breathing and pulse rate, muscle tremors, and increased urination.[25] Methemoglobinemia may be fatal at methemoglobin levels greater than 80%. The effects of chronic nitrate exposure are difficult to detect in ruminants because these animals can use nitrate as a nitrogen source. However, abortions have been reported in ruminants receiving doses of nitrate high enough to cause clinical signs of toxicity. Reduced feed intake has been reported in beef cattle and sheep receiving more than 10,000 ppm and 30,000 ppm dietary nitrate. Other possible effects mentioned in the literature include methemoglobinemia, placental transfer of methemoglobin, changes in pituitary function, transfer of some nitrate to milk, and effects on vitamin A metabolism in ruminants chronically exposed to nitrate or nitrite. However, as NRC (2005) notes, accumulation of nitrates and nitrites is not expected in the tissues of animals or in the milk of mammals because these substances are generally excreted rapidly.

As reviewed in NRC (2005), reduced body weight gains among nonruminants were reported in chickens and rats fed 3,100 ppm and 2,916 ppm nitrate in the diet, respectively. Increased fetal losses have been reported in guinea pigs treated with nitrate. However, no effect has been observed on the reproductive performance of pigs, and reports of such responses in chickens have been inconsistent.

NRC (2005) suggested a maximum tolerable level of 1,823 ppm nitrate in the diet based on the results of rat studies. In comparison, ruminants exposed to more than 5,000 ppm nitrate in the diet (dry matter basis) have exhibited signs of toxicity.

NRC (2005) suggested that, for drinking water, the EPA guideline of 10 ppm nitrate-N (*i.e.* 44 ppm NO_{3-})[26] in drinking water is a conservative maximum tolerable level because some studies have found no effects in animals exposed to 200 times this guideline and others have reported reduced animal performance only at 20 times the guideline. In comparison, the NRC

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[24] Russell JB (2002). Rumen microbiology and its role in ruminant nutrition. Cornell University, New York state college of Agriculture and Life Sciences, Department of Microbiology, Ithaca NY.

[25] NRC (2005).

[26] 10 ppm nitrate-N x 4.42 grams NO_3^-/gram nitrate-N = 44.2 ppm NO_3^-.

(1974) recommended an upper limit of 100 ppm nitrate-N (*i.e.* 442 ppm NO_3^-) in the drinking water of livestock and poultry.[27]

Nitrate is generally much less toxic to fish and other aquatic organisms than is nitrite.[28] Jensen (1999) noted that the mechanism of toxicity in fish is generally the same as in terrestrial animals, specifically the oxidation of hemoglobin to methemoglobin.[29] The effects of nitrite toxicity in fish include reduced growth rates and suppressed immune function. Nitrite has also been studied for its possible role in the formation of mutagenic and carcinogenic N-nitroso compounds, which have been detected in the muscle and other tissues of nitrite-exposed rainbow trout, for example.

Freshwater fish are generally more sensitive to nitrite than saltwater fish because the chloride ions in saltwater inhibit the uptake of nitrite by competing with nitrite for absorption through the gills. Jensen (1999) noted that fish species with high branchial chloride uptake rates, including rainbow trout, perch, and pike, appear to be more sensitive to nitrite toxicity than species with low uptake rates, such as carp, tench, and eel. However, exposure to nitrite concentrations in the millimolar range can be tolerated by fish for long periods if the water chloride concentrations are elevated sufficiently.

By comparison, the toxicity of nitrate is very low in most aquatic species, with ambient nitrate concentrations of several tens of millimolars required to increase mortality in short-term toxicity tests. Camargo et al. (2005) noted that the relatively low toxicity of nitrate, compared to nitrite and ammonia, is attributable at least in part to the low branchial permeability of nitrate.[30] These authors also noted that long-term exposure to nitrate at the EPA guideline of 10 ppm nitrate-N for drinking water can adversely affect freshwater fish, including Rainbow trout, Cutthroat trout and Chinook salmon. Camargo et al. (2005) cited Kincheloe et al. (1979), who reported elevated mortality of the larvae of these species in water containing 2.3 to 7.6 ppm nitrate-N (*i.e.* 10 to 33.6 ppm, NO_3^-).[31] Based on their review of the literature, Camargo et al. (2005) recommended 2 ppm nitrate-N (*i.e.* 8.8 ppm NO_3^-) as a maximum water concentration to protect the most sensitive freshwater species and 20 ppm nitrate-N (*i.e.* 88 ppm NO_3^-) as a likely

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[27] NRC (1974). Nutrients and Toxic Substances in water for Livestock and Poultry. National academy Press, Washington D.C. (cited by NRC 2005).

[28] Basuyaux O, Mathieu M (1999). Inorganic nitrogen and its effect on the growth of the abalone Haliotis tuberculata Linneaus and the sea urchin Paracentrotus lividus Lamark. Aquaculture 174: 95-107; Colt J, Tchobanoglous G (1976). Evaluation of the short-term toxicity of nitrogenous compounds to channel catfish, Ictalurus punctatus. Aquaculture 8: 209-224; Pierce RH, Weeks JM, Prappas JM (1993). Nitrate toxicity to five species of marine fish. J. World Aquac. Soc. 24: 105-107 (all cited in NRC 2005).

[29] Jensen FB (1999). Physiological effects of nitrite in teleosts and crustaceans. In: Toxicology of Aquatic Pollution Physiological, Molecular and Cellular Approaches, Taylor EW, ed. Cambridge, UK: Cambridge University Press, pp. 169-186 (cited in NRC 2005).

[30] Camargo JA, Alonso A, Salamanca A (2005). Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. Chemosphere 58: 1255–1267.

[31] Kincheloe JW, Wedemeyer GA, Koch DL (1979). Tolerance of developing salmonid eggs and fry to nitrate exposure. Bull. Contam. Toxicol. 23: 575-578.

maximum water concentration for the protection of saltwater species. However, Freitag et al. (2016) found no difference in survival of Atlantic salmon embryos exposed to mean nitrate-N levels of 4 or 93 ppm (*i.e.* 17.7 and 411 ppm NO_3^-).[32]

Davidson et al. (2014) reported that rainbow trout exposed to 80 to 100 ppm nitrate-N (*i.e.* 354 to 442 ppm NO_3^-) for three months demonstrated chronic health and welfare impacts including an increase in abnormal swimming behavior, increased swimming speeds, and mildly reduced survival.[33]
Based on the results, these authors recommended 75 ppm nitrate-N (*i.e.* 332 ppm NO3-) as the upper design limit for water recirculating aquaculture systems used for rainbow trout culture. However, Davidson et al. (2017) reported no effects of chronic (8 months) exposure to 100 ppm nitrate-N (*i.e.* 442 ppm NO_3^-) on survival, swimming behavior or any other measures of a comprehensive set of health variables in post-smolt Atlantic salmon.[34] Davidson et al. (2017) concluded that post-smolt Atlantic salmon can be cultured humanely in aquaculture systems in which the mean nitrate level is maintained at or below 100 ppm nitrate-N (*i.e.*, 442 ppm NO_3^-).

As noted above, nitric acid serves as a nitrogen source and pH control agent in the culture medium used to support the bacterial growth and protein synthesis on which the production of FeedKind® depends. The final concentration nitrate in FeedKind® resulting from nitric acid added to the medium at the start of bacterial growth period is expected to be negligible at the end of this period primarily because most, if not essentially all, of the nitrate will be consumed by the bacteria to synthesize amino acids and proteins. In any case, salmonid species are clearly tolerant of nitrate-N concentrations in water at least up to 2 ppm (*i.e.* 8.8 ppm NO_3^-), and likely at much higher concentrations (*i.e.* up to 100 ppm nitrate-N; 442 ppm NO_3^-), depending on the salinity of the water, life-stage of the fish, and other factors evaluated in the published scientific literature reviewed above. Thus, the contribution of any residual nitrate in FeedKind® resulting from the use of nitric acid in the culture medium to the overall exposure of the fish to nitrates will be negligible in recirculating aquaculture systems operated in accordance with good aquaculture practice.

2.2.2.6 Defoamer authorization

Methylobacterium extorquens protein ("*M. extorquens*" or "KnipBio Meal") appears, per the unredacted portions of AGRN 00026, to use an ethylene oxide-propylene oxide block (EO-PO) copolymer defoamer. *See* AGRN26, **Appendix 2**. The manufacturer of the defoamer used in the manufacture of KnipBio Meal attested to the fact that the defoamer is authorized for use in human food under 21 CFR § 173.340 and that FDA has taken an enforcement discretion policy

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[32] Freitag AR, Thayer LR, Leonetti C, Stapleton HM, Hamlin HJ (2015). Effects of elevated nitrate on endocrine function in Atlantic salmon, Salmo salar. Aquaculture 436: 8-12.

[33] Davidson J, Good C, Welsh C, Summerfelt ST (2014). Comparing the effects of high vs. low nitrate on the health, performance, and welfare of juvenile rainbow trout Oncorhynchusmykiss within water recirculating aquaculture systems. Aquacultural Engineering, 59: 30-40.

[34] Davidson J, Good C, Williams C, Summerfelt ST (2017). Evaluating the chronic effects of nitrate on the health and performance of post-smolt Atlantic salmon Salmo salar in freshwater recirculation aquaculture systems. Aquacultural Engineering 79: 1-8.

for the use of defoamers authorized for use in human food when used in the manufacture of animal feed. *Id.*, AGRN 00026 **Appendix 3** (the "Yingling Letter"). We understand that defoamers listed in the Yingling Letter must meet other specifications on the list and, given FDA's letter of no objection, we presume, though we cannot confirm this, that the defoamer used in the production of KnipBio Meal was on the lists in the Yingling Letter and complied with the supplemental information – for EO-PO copolymers the Yingling Letter matches the listing in 21 CFR § 172.808, including CAS number (9003-11-6).

There is no practical difference between the defoamer used by KnipBio and that used by Calysta (Glanapon 2000 KONZ) - the defoamer used by Calysta in the fermentation of Feedkind® is also an EO-PO copolymer whose manufacturer attests that its use is authorized under 21 CFR §§ 172.808 and 173.340 (including that the CAS number is 9003-11-6).

Beyond the EO-PO copolymer, Glanapon 2000 KONZ also incorporates rape seed oil and fatty acids from rape seed oil, both of which are identified as GRAS by the manufacturer. Mono- and diglycerides of rape seed oil (CAS 93763-31-6) are on the Yingling list and mono- and diglycerides of edible fats or oils and edible fat forming acids are permitted for use in animal feed for use as emulsifying agents consistent with good manufacturing and feeding practice, 21 CFR § 582.4505. It is well established and recognized that triglycerides are metabolized into mono- and diglycerides as well as fatty acids – so the presence of rape seed triglycerides (a precursor) and fatty acids from rape seed oil (a product) are as safe and suitable for use as a defoamer as rape seed oil mono- and diglycerides.

We therefore conclude that Glanapon 2000 KONZ is safe and suitable for use as a defoamer in the manufacture of FeedKind®.

2.2.2.7 Heavy Metal safety

With the exception of the components already identified as being used pursuant to established regulatory authorizations (*e.g.* copper and zinc) or which are safe for use at the levels contemplated (*e.g.* nickel), there is no appreciable risk that heavy metals will be present in the finished product as none of the inputs into the fermentation media are expected to contain heavy metals at above negligible levels.[35] FeedKind® distributed in the United States will be

[35] There may be mercury present in natural gas as it leaves the ground, but, on the basis of the US EPA risk assessment, we do not expect mercury to be present in the natural gas used to manufacture FeedKind®. US EPA (2001) Mercury in petroleum and natural gas: estimation of emissions from production, processing, and combustion. Available at: https://cfpub.epa.gov/si/si_public_record_report.cfm?Lab=NRMRL&dirEntryId=63480.

This risk assessment of mercury found in petroleum and natural gas in the United States acknowledges that, while mercury is a natural component of natural gas, removal strategies are

produced domestically, and therefore mercury contamination from the natural gas feedstock is not expected to occur. Previous testing conducted by Calysta to fully characterize FeedKind® indicates that in most cases mercury is below the detection limit (<0.01 ppm) and in no cases was above 0.03 ppm.

2.3 Specifications for FeedKind®

The FeedKind® product has been thoroughly tested and characterized. The specifications for FeedKind® are included in **Table 5.**

Table 5: FeedKind® Specifications.

Specifications				
Chemical Composition	**Minimum**	**Maximum**	**Units**	**Test Method**
Crude Protein	(b) (4)		% dry weight	DUMAS method
Crude Fat			% dry weight	modified Weibull Acid Hydrolysis Method
Crude Fiber			% dry weight	AOCS Ba 6a-05, Ba 6-84 AOAC 962.09, S 1022 using Gravimetry
Ash			% dry weight	AOAC 942.05, S 1024 using Gravimetry

employed and residual mercury in US pipeline gas was present at a negligible level (<0.03-0.3 ppb). As levels of mercury in the raw materials used in the media are orders of magnitude lower than the recommended permissible level of 2 ppm from the AAFCO "Official Guidelines for Contaminant Levels Permitted in Mineral Feed Ingredients," Table 2, there is no cause for concern from mercury in the product. AAFCO, 2018 Official Publication, "Official Guidelines for Contaminant Levels Permitted in Mineral Feed Ingredients," Table 2, located at page 298.

Moisture	(b) (4)	w/w	AOAC 934.01, 930.15, S 1024 using Gravimetry
Nickel		*mg/kg*	ICP-OES

Microbiological Limits	**Limits**	**Test Method**
Mesophilic Aerobic Plate Count	(b) (4)	AOAC 2011.03, 2003.09
Mold		AOAC 997.02; FDA/BAM Chapter 18
Yeast		AOAC 997.02; FDA/BAM Chapter 18

Analysis of three (3) non-consecutive batches of FeedKind® can be found in **Table 6**. Test methods for the analysis are listed in **Table 5**, above.

Table 6: Result of Batch Analysis for 3 non-consecutive batches

BATCH NUMBER	TEES-009/63	TEES-009/102	TEES-009/84
Crude Protein (g/100 g)	(b) (4)		
Crude Fat (g/100 g)			
Crude Fiber (g/100 g)			
Moisture (g/100 g)			
Ash (g/100 g)			
Nickel (mg/kg)[36]			

36 Because the specification for nickel was recently set to address possible safety concerns arising from the use of nickel chloride hexahydrate in the fermentation media, original batch analyses did not include tests for nickel. Four batches of product used in the stability testing (see below) were tested specifically for nickel and the results are presented in **Table 7**.

Aerobic Plate Count (TVC) (cfu/g)	(b) (4)
Molds (cfu/g)	
Yeasts (cfu/g)	
Salmonella (/25 g)	
Listeria species (/25 g)	
Bacillus cereus (cfu/g)	
Escherichia coli (cfu/g)	
Pepsin digestibility (%)	
Alanine (g/100 g) [37]	
Arginine (g/100 g)	
Aspartic acid (g/100 g)	
Glutamic acid (g/100 g)	
Glycine (g/100 g)	
Histidine (g/100 g)	
Isoleucine (g/100 g)	
Leucine (g/100 g)	
Lysine (g/100 g)	
Phenylalanine (g/100 g)	
Proline (g/100 g)	
Serine (g/100 g)	
Threonine (g/100 g)	
Tyrosine (g/100 g)	
Valine (g/100 g)	
Tryptophan (Total) (g/100 g)	
Methionine (g/100 g)	
Cysteine +Cystine (g/100 g)	
Salt (from chloride) (g/100 g)	
Ether Extract (g/100g)	
Sodium (g/100 g)	
Calcium (g/100 g)	
Phosphorus (g/100 g)	
Copper (mg/kg)	
Zinc (mg/kg)	
Manganese (mg/kg)	
Iron (mg/kg)	
Magnesium (g/100 g)	

Table 7: Result of Nickel Analysis

BATCH NUMBER	TEES-004/29	TEES-004/29A	TEES-004/11	TEES-005/28
Nickel (mg/kg)	(b) (4)			

[37] Amino acid content determined using AOAC 994.12.

2.4 Stability Testing

Two separate samples of four individual lots of FeedKind® have been stored under controlled conditions for stability testing. Storage will continue for 156 weeks (which is longer than the expected shelf life for FeedKind®) and will generate sufficient data to accurately set a shelf life of FeedKind®. Samples have been chosen at random from different batches of FeedKind® during production runs on March 27, 2017 (TEES004/11), April 11, 2017 (TEES004/29 and TEES004/29a), and September 21, 2017 (TEES005/28). A single sample from each batch was separated into 20 samples of 500g each. One sample was tested for the 0-week timepoint and the remaining samples (9 each) were placed in temperature and humidity-controlled cabinets at 25°C/60%RH to represent real time testing or 40°C/75%RH to represent accelerated testing. The sample containers used are HDPE, to replicate the polyethylene bulk sacks that may be used at a commercial scale. Holes have been drilled in the lids to allow air into the sample container to represent leakage or absorption at full scale, such that all test conditions represent 'worst-case' conditions. Only real time results are reported here as they are most representative of actual shelf life conditions. Full interim results are available in **Appendix 1.** Sample designations are given in **Table 8**.

Table 8: Samples for Stability Testing

Test Number	Batch	Test Conditions
Stability Test 01	TEES004/29	(b) (4)
Stability Test 03	TEES004/29a	
Stability Test 05	TEES004/11	
Stability Test 07	TEES005/28	

***** Not heat treated

Batch TEES004/29 was not subjected to the final UHT step in order to determine if this step affected shelf-life. The testing plan is given in **Table 9**. Proximate testing refers to testing for crude protein, crude fat, ash, moisture and crude fiber.

Table 9: Test Plan

Time	Testing
0 Weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines

4 Weeks	Proximate, microbiology, biogenic amines
8 Weeks	Proximate, microbiology, biogenic amines
12 Weeks	Proximate, microbiology, biogenic amines
26 Weeks	Proximate, microbiology, biogenic amines
39 Weeks	Proximate, microbiology, biogenic amines
52 Weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines
78 Weeks	Proximate, microbiology, biogenic amines
104 Weeks	Proximate, microbiology, amino acid profile, biogenic amines, fatty acid profile
156 weeks	Proximate, microbiology, amino acid profile, biogenic amines, fatty acid profile

Proximate and microbiological test results for real time testing through week 52 are available and given in **Table 10**. Full test results including for accelerated testing through week 52 are available in **Appendix 1.**

Table 10: 52 Week Stability Testing Results

Batch TEES004/29 25°C/60%RH (no UHT; real time)

Nutritional Analysis

Duration (Weeks)	Moisture (Max 10%)	Crude Fat (Min 5%)	Crude Protein (Min 68%)	Crude Fiber (Max 1%)	Ash (Max 12%)
0	(b) (4)				
4					
8					
12					
26					
39					

52	(b) (4)					

Microbiological Analysis

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Molds cfu/g	Test Duration (Weeks)
0	(b) (4)				
4					
8					
12					
26					
39					
52					

Batch TEES004/29a 25°C/60%RH (real time)

Duration (Weeks)	Moisture (Max 10%)	Crude Fat (Min 5%)	Crude Protein (Min 68%)	Crude Fiber (Max 1%)	Ash (Max 12%)
0	(b) (4)				
4					
8					
12					
26					
39					
52					

Microbiological Analysis

[38] This sample was inadvertently tested using a method that lacked sufficient precision.

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	(b) (4)			
4				
8				
12				
26				
39				
52				

BatchTEES004/11 25 C/60%RH

Nutritional Analysis

Duration (Weeks)	Moisture (Max 10%)	Crude Fat (Min 5%)	Crude Protein (Min 68%)	Crude Fiber (Max 1%)	Ash (Max 12%)
0	(b) (4)				
4					
8					
12					
26					
39					
52					

Microbiological Analysis

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	(b) (4)			
4				

8	(b) (4)
12	
26	
39	
52	

Batch TEES005/28 25ºC/60%RH

Nutritional Analysis

Duration (Weeks)	Moisture (Max 10%)	Crude Fat (Min 5%)	Crude Protein (Min 68%)	Crude Fiber (Max 1%)	Ash (Max 12%)
0	(b) (4)				
4					
8					
12					
26					
52					

Microbiological Analysis

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	(b) (4)			
4				
8				
12				
26				
39[39]				

[39] Data for this sample at this time point is missing.

52		(b) (4)

The initial findings of the shelf life study show FeedKind® to be a stable but hygroscopic product. The data for real time storage when fully open to atmosphere support stability for at least 52 weeks under normal conditions, and accelerated testing confirms that no safety issues are observed with degraded product. The only significant changes observed in the composition of the samples under either real-time or accelerated conditions are the moisture and protein levels, although, aside from a single moisture level at 52 weeks that was (b) (4) instead of the (b) (4) maximum, they remain within specification for the real time aged product. It is also important to note that the sample containers were open to atmosphere under the test conditions such that water absorption from the atmosphere is not unexpected. As the FeedKind® moisture content increases, relatively less of the other components are present in the sample, consistent with the amount of moisture gained diluting the other components. There is no significant change in the protein level when calculated on a dry matter basis. The commercial packaging for FeedKind® will be sealed to help maintain the moisture and protein content within specification.

In an older stability testing performed in 1994 (**Appendix 2**) on product manufactured with the same bacteria and using the same methods, the storage of FeedKind® was monitored for 64 weeks at 22°C and 37°C. A 10 kg sample was divided into nine subsamples with one sample being analyzed immediately. The remaining 8 subsamples were packed in airtight polyethylene bags and stored in an incubator at the indicated temperature (4 each). Bags were removed and analyzed at 4, 16, 32, and 64 weeks for moisture, crude protein, crude fat, free fatty acids, and amino acids (cysteine, methionine, threonine, and lysine). The analyses indicated that the moisture content changed over time (increased at 22°C and decreased at 37°C), but that protein as a percent of dry matter remained steady. Slight decreases in crude fat and increases in free fatty acids were observed and indicate a slow oxidative deterioration of fat over the storage period. Though not all current specifications were tested, these results suggest satisfactory storage stability for the proposed shelf life of 1 year for FeedKind® and that, when properly stored, FeedKind® will not pick up substantial amounts of moisture from the atmosphere.
Further, because the more recent testing was performed under circumstances by which the material was open to the atmosphere, we believe that the single out of specification moisture measurement is a result of an overly-aggressive test protocol rather than an indication that, when properly stored, the material is not viable up to (and beyond) 52 weeks.

With regard to the absence of nickel testing, because nickel is not expected to be gained or lost during storage, the adherence to the new specification at the time of production is sufficient to confirm adherence to this specification after long-term storage.

2.5 Information on the technical effect of FeedKind®

FeedKind® is a biomass to be used as a protein source for animal feed. FeedKind® is intended for use in the species and at the levels listed in Section 1.4 above.

Part 3 – Dietary exposure

FeedKind® will be included in formulated diets for salmonid species as a replacement for traditional protein sources (*e.g.* soy meal, fish meal, etc.) at inclusion rates of up to 18%. **Table 11** is a comparison of the essential amino acid content of FeedKind® and traditional protein sources commonly found in animal feeds and indicates that FeedKind® is an appropriate replacement for other sources of protein.

Table 11: Essential amino acid composition of protein sources for animal feed. (g/100g dry matter)

Amino Acid	Fish Meal[40]	Soy Meal[41]	FeedKind®[42]
Arginine	4.0	3.43	4.35
Histidine	1.38	1.22	1.5
Isoleucine	2.65	2.1	3.04
Leucine	4.54	3.57	5.22
Lysine	4.78	2.99	3.9
Methionine	1.74	0.68	1.84
Phenylalanine	2.57	2.33	2.9
Threonine	2.83	1.85	2.92
Tryptophan	0.70	0.65	1.1
Tyrosine	2.07	0.40	1.78
Valine	3.00	2.26	3.84

Section 6 includes detailed summaries of well conducted safety studies on salmonids. The No Observed Adverse Effect Levels (NOAELs) from those studies, as expressed as a percent of the diet are:

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[40] Ween O, *et al* 2017. Nutritional and functional properties of fishmeal produced from fresh by-products of cod (Gadus morhua L.) and saithe (Pollachius virens). *Heliyon*. 3(7): e00343

Ween *et al* is used because it is the most complete reference, but the values vary based on type of fish used to generate the fish meal. For example: cod (Ween 2017) vs. pollock in Folador JF *et al*. (2006) Fish meals, fish components, and fish protein hydrolysates as potential ingredients in pet foods. *J Anim Sci*. 84: 2752-2765.

[41] US Soybean Export Council (2015) https://ussec.org/wp-content/uploads/2015/10/US-Soybean-Meal-Information.pdf

[42] Average of three batch analysis in **Table 6.**

- Atlantic salmon: 19.3%
- Rainbow trout: 18%

We conservatively utilize the lowest NOAEL of all of the salmonid species tested (18% in rainbow trout) when determining the maximum use level. Exposure to FeedKind® will therefore be no more than 18% of the diet for salmonids.

3.1 Human Exposure Through Consumption of Target Animals

None of the substances in FeedKind® fed to animals is expected to be transferred, intact, to people consuming the edible products of any of the food-producing animals. The composition of FeedKind® is like that of other common animal feeds, including amino acids, phospholipids, and lipopolysaccharides. Therefore, FeedKind® consumed by the target animals will be digested and converted to biomass and as such there is no expected exposure to FeedKind® for humans *via* consumption of target animals fed FeedKind®.

Part 4 – Self-limiting levels of use

Farmers, aquaculturists, and feed manufacturers using FeedKind® will limit the inclusion of FeedKind® in feed to levels that will not harm or reduce growth rates in the animals being fed. Use will be further limited to 18% in salmonid species, consistent with this GRAS Notice.

Part 5 – Experience based on common use in food before 1958

N/A

Part 6 – Narrative

6.1 Target Animal Safety Summary

Several published studies have evaluated the safety and efficacy of feed formulations containing FeedKind®.[43] These studies typically refer to the test article as "bacterial protein meal" (BPM), which is the biomass product of a bacterial consortium grown on natural gas (methane) as the sole carbon source. The consortium consists of a majority (~90%) of *Methylococcus capsulatus* (Bath) with the remaining 10% consisting of three heterotrophic strains: *Cupriavidus sp.* (previously *Alcaligenes acidovorans* DB3), *Aneurinibacillus danicus* (previously *Bacillus brevis* DB4), and *Brevibacillus agri* (previously *Bacillus firmus* DB5), all of which were isolated from mixed cultures growing on methane.[44]

The data and literature presented in this notification support Calysta's conclusion that use of FeedKind® is safe and GRAS when incorporated at 18% or less of aquaculture feed for salmonid species. This conclusion is corroborated by a number of studies described below, in which salmonids were fed FeedKind® with no adverse effects and no effect on the growth of the animals. This conclusion is also corroborated by ample evidence from the literature and other experimental data derived by Calysta and others.

Regarding the use of FeedKind® in aquaculture, Calysta views Atlantic salmon and rainbow trout as representative models for all salmonid species, including Arctic char and Coho salmon, for example.[45] Among the important considerations for defining representative species include the life cycle, diet consumed in nature, physiology and metabolism, available background information, and commercial relevance.

Calysta believes that the data from investigations of the common salmonids Oncorhynchus mykiss (rainbow trout) and Salmo salar (Atlantic salmon) are sufficient to support the broader use of the notified substance for all aquaculture feed for salmonid species, with these two test species fulfilling all of the criteria required for covering all of the species of the phylogenetic family Salmonidae, including the requirement that test subjects be well-studied, sensitive to testing, and commercially-relevant.

Salmonid test species generally serve as good surrogates in nutritional studies because species in this phylogenetic family are characteristically sensitive to allergenic substances added to their diets. Gastrointestinal inflammation (gastroenteritis) is a well characterized effect observed in salmonids fed diets containing terrestrial proteins such as soy protein. Salmonids (*i.e.*

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[43] The bacteria used for the test articles in the animal studies and for the product manufactured today are the same strains and come from a culture bank. Aside from a slight widening of tolerances for the pH and temperature of the fermenter, further the fermentation parameters have not been changed. We may therefore conclude that the finished product is the same.

[44] As described in Section 3.2, BPM and FeedKind® are identical. The BPM nomenclature is used as a vestige of the nomenclature used in some of the animal studies.

[45] A similar approach was taken in AGRN 26 for application to all finfish species on the basis of studies in several species.

salmon and trout) represent a substantial fraction of the total commercial value for the industry (Mente et al. 2006; Glencross et al. 2007; Gjedrem et al. 2012; Ababouch et al. 2016).

For these reasons, Calysta believes that the data and information presented on rainbow trout and Atlantic salmon in this notification are sufficient to support the finding that the notified substance is Generally Recognized as Safe for use in any aquaculture feed for salmonid species, when incorporated at 18% or less of the feed.

6.1.1 Safety of the Microorganisms[46]

A detailed search of the public literature did not find any reports of pathogenicity, infections, or toxin production by any of the members of the consortia utilized to produce FeedKind®. Both old and new taxons were searched. Searches return hits for various *Bacillus* species (*i.e. anthracis* and *cereus*) though there are no organisms currently classified as "*Bacillus*" in FeedKind® and none of the hits for *Bacillus* species implicates the organisms in FeedKind® as potential pathogens. A variety of *Cupriavidus* species have been reported to cause opportunistic infections in humans. *C. metallidurans*[47] and *C. gilardii*[48] have been associated with sepsis in elderly patients with other underlying pathology (diabetes, etc.). *C. pauculus* appears to be associated with the most cases of human disease, with more than 30 cases reported in the literature.[49] While most cases are reported in the very young or very old with or without underlying disease, several appear to be associated with otherwise apparently healthy patients. The effects of *C. pauculus* infection appear to be more severe than those seen with other *Cupriavidus* species with *C. pauculus* being associated with cases of meningitis, respiratory tract infections, septicemia, and at least 3 deaths. As with the literature search hits for *Bacillus*, the species of *Cupriavidus* for which the literature indicates potential pathogenicity are not the same as is used in FeedKind®. There is a single report of a urinary tract infection (UTI) caused by a *Brevibacillus* organism that was later determined to be *Brevibacillus agri* through 16s sequencing. However, additional characterization of the strain which caused the infection

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[46] The data supporting the safety of the microorganisms can be found summarized in the April 28, 1995 Scientific Committee for Animal Nutrition report, found in **Appendix 3**. The accepted nomenclature for these bacteria has changed based on modern molecular (*i.e.* sequencing) techniques. However, the bacteria used to produce FeedKind® has not changed relative to those used to conduct the animal studies. Further, a recent literature search to confirm that these bacteria are not known to be human or animal pathogens did not yield any new results that might change the conclusion reached in 1995.

[47] Langevin S, Vincelette J, Bekal S, and Gaudreau C. (2011) First case of invasive human infection caused by *Cupriavidus metallidurans*. *J. Clin. Microbiol.* 49 (2): 744-745.

[48] Kobayasi T, Nakamura I, Fujita H, Tsukimori A, Sato A, Fukushima S, Ohkusu K, Matsumoto T. (2016) First case report of infection due to *Cupriavidus gilardii* in a patient without immunodeficiency: a case report. *BMC Infectious Diseases*. 16: 493; Zhang Z, Deng W, Wang S, XuL, Yan L, Liao P. (2017) First case report of infection caused by *Cupriavidus gilardii* in a non-immunocompromised Chinese patient. *IDCases*. 10:127-129.

[49] Yahya R, and Mushannen A. (2019) *Cupriavidus pauculus* as an emerging pathogen: a mini-review of reported incidents associated with its infection. *EC Pulmonology and Respiratory Medicine* 8(9): 633-638.

indicated that the *B. agri* strain in question had acquired genes related to pathogenicity, likely through horizontal transfer, including hemagglutination and serum resistance that are not typically present in *Brevibacillus agri*.[50] There were no reports of infections caused by any *Methylococcus* or *Aneurinibacillus*. The pathogenicity of the heterotrophic strains is further addressed by the rodent studies described below.

Methanotrophic bacteria are not known to be human or animal pathogens. They require single-carbon energy sources (*e.g.* methane or methanol) for growth and, thus, are not believed to pose any danger to humans or animals. *M. capsulatus* (Bath) has a high optimal growth temperature (45°C), which is substantially greater than the normal body temperatures of mammalian species,[51] providing another indication that it is unlikely to become a human or animal pathogen.

Cupriavidus sp., *Aneurinibacillus danicus,* and *Brevibacillus agri* showed no signs of pathogenicity in mice injected with 9.5 x 10^9, 5.3 x 10^9, and 2.9 x10^9 viable cells/kg body weight, respectively, which were the highest doses tested. *Cupriavidus sp.* has a high optimal growth temperature (44°C) similar to that of *M. capsulatus* and is restricted to utilization of carbon sources with chain lengths from 2-4 carbons. The highest dose of both *Brevibacillus agri* and *Aneurinibacillus danicus* did induce signs of acute toxicity. However, these effects were transient (lasting a few hours to 3 days after exposure) and were consistent with effects expected following injection of large amounts of organic material. The pathogenicity studies indicate that none of the constituent organisms used in the FeedKind® production process is pathogenic to animals. The studies are summarized below:[52]

Cupriavidus sp. was administered intravenously to 5 male and 5 female mice at doses of (b) (4) Animals were observed for 14 days and then killed and subjected to pathological examination. There were no clinical signs of reaction to the treatment during the observation period or upon necropsy. As a Gram-negative organism, *Cupariavidus sp.* contains endotoxins associated with the cell wall. However, as there are no adverse effects indicated by this assay, they clearly pose no safety concerns.

Aneurinibacillus danicus was administered intravenously to 5 male and 5 female mice at doses of (b) (4) viable cells/kg bw. Animals were observed for 14 days and then killed and subjected to pathological examination. Clinical signs including

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50 Suneeva SC, *et al* (2014) Transformation of *Brevibacillus,* a soil microbe to an uropathogen with hemagglutination trait. *World J Microbiol Biotechnol.* 30 (6) 1837-1844.

51 For example, normal human body temperatures range from 36°C to 37°C.

52 (b) (4)

(b) (4)

piloerection and depression were seen in all dosed groups but were transient. Signs lasted for 3 hours, 1 day, and 3 days respective to increasing dose. Males generally exhibited signs for longer than females, however one female in the highest dose group died approximately 1 hour after dosing. No clinical signs of pathogenicity were observed during the study or during necropsy.

Brevibacillus agri was administered intravenously to 5 male and female mice at doses of (b) (4) viable cells/kg bw. Animals were observed for 14 days and then killed and subjected to pathological examination. Similar to *Aneurinibacillus danicus*, clinical signs including piloerection and depression were seen in the medium and high dose groups but were transient. Signs lasted for 1 day or 3 days respective to increasing dose. Males generally exhibited signs for longer than females, however females exhibited a greater degree of depression immediately after dosing. No clinical signs of pathogenicity were observed during the study or during necropsy.

6.1.2 Salmonid Species

One freshwater and two saltwater feeding studies were performed in Atlantic salmon at the Institute for Aquaculture Research in Norway and published in the peer-reviewed journal Aquaculture from 2004 through 2006. These studies are summarized below.

In the freshwater study, Storebakken *et al.* (2004) fed groups of Atlantic salmon (*Salmon salar*) (n=600/group; 3 groups/diet; average initial body weight 0.2 g/fry) 0%, 5%, 10%, 19.3%, or 37% BPM equivalent to FeedKind® in the diet for 364 days, starting with the first feeding at the fry stage of the life cycle. The BPM added to the feed replaced an equivalent amount of high-quality fish meal in the feed in each group of exposed animals.[53]

After the first 112 days of exposure, the salmon fed 5% BPM exhibited the greatest average body weight (bw = 3.69 ± 0.07 g) and specific growth rate (SGR =2.64 ± 0.02), both of which were statistically significantly greater than the corresponding control values (i.e. bw = 3.44 ± 0.22 g; SGR = 2.57 ± 0.06).[54] As well, the average bw and SGR were statistically-significantly elevated in the fish fed 5% BPM compared to fish fed BPM at any of the other inclusion levels.[55] Both of these parameters were statistically significantly reduced in the fish fed 37% BPM (bw = 2.63 ± 0.08; SGR = 2.33 ± 0.03), compared with controls. However, the SGRs of Atlantic salmon fed 5%, 10% or 19.3% BPM for 112 days were not statistically-significantly different from the SGR of the fish fed the control diet.

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[53] Storebakken T, *et al.* (2004) Bacterial protein grown on natural gas in diets for Atlantic salmon, *Salmo salar*, in freshwater. *Aquaculture*. 241: 413-425.

[54] Bulk weight of all fish in each tank was measured every 28 days, mean weight per fish (w) was calculated from the bulk weight and the number of fish remaining in the tank, and specific growth rate (SGR) was calculated; SGR = $100(e^{x}-1)$, where x = (ln (w_{final} – ln w_{start}) – ln w_{start}) ÷ days fed.

[55] SGR of fish fed 5% BPM was 4.5%, 6.8% and 12% greater than the SGR for fish fed BPM at 10%, 19.3%, and 37% in the diet, respectively, for 112 days.

From day 113 to 253 of the exposure period, the SGRs were statistically significantly lower in fish fed 19.3% BPM (SGR = 0.60 ± 0.05) and 37% BPM (SGR = 0.51 ± 0.05), compared with controls (SGR = 0.74 ± 0.03). By comparison, the SGRs calculated for fish exposed to 5% or 10% BPM were not statistically different from each other or from the control values.

However, from day 254 to 364, the SGRs calculated for fish fed 5%, 10%, or 19.3% BPM were not statistically significantly different from controls and the final average body weights were statistically significantly elevated compared with controls (e.g., bw = 46.2 ± 1.6 g for fish fed 19.3% BPM compared with 38.2 ± 3.0 g for controls). The final average bw and SGR were statistically significantly reduced only for fish fed 37% BPM (bw = 28.0 ± 2.3 g; SGR = 0.82 ± 0.06), compared with controls (bw = 38.2 ± 3.0 g; SGR = 0.97 ± 0.03). Likewise, cumulative survival rate for the day 113 to 364 exposure period was statistically significantly reduced only for fish fed 37% BPM (98.0 ± 0.4%) compared with the controls (99.6 ± 0.00%).

In sum, bw and SGR values were indistinguishable or improved in salmon receiving feed containing 5%, 10% or 19.3% BPM in the diet, compared with controls, over the first 112 days of the exposure period in the study conducted by Storebakken et a. (2004). These values were statistically significantly reduced during the exposure period extending from day 113 to day 252 but recovered to be indistinguishable from controls during the final period extending from day 254 to day 364. The reduced bw and SGR measurements observed over the 113-day to 252-day period are attributable to two factors related to the experimental protocol of this study, including:[56]

- Infrequent size grading of the fish, which was done to keep undersized salmon in the population and, thus, increase the probability of detecting any long-term histopathological effects of BPM on the salmon
- Pellet sizes too large for fish at earlier life stages, which should have been changed to correspond with the increasing size of the fish, but the pellet sizes used were selected to minimize the potential for feed batch variations to affect the results

Generally, there is substantial body size variability in farmed fish of the same age. Periodically grading and sorting the fish based on body size enables feeding the fish food pellet sizes that are appropriate for their body size, which improves feed conversion efficiency among other beneficial effects of this practice. In comparison, Storebakken et al. (2004) graded and sorted the fish only on day 253 of the exposure period. During the first 112 days of the exposure period, Storebakken et al. (2004) fed the fish experimental diets that were prepared by cold-pelleting the feed formulation through a 5-mm die on a laboratory mill, and then crumbling the pellets with a coffee grinder and sieving the particles to produce the appropriate pellet sizes for fish. From day 113 to day 364 of the exposure period, the fish received the diets that were cold-pelleted through a 3-mm die, and these pellets were not crumbled before feeding to the fish. Thus, the reduced average bw and SGR measurements calculated for the fish during day 113 to day 252 of the exposure period are attributable to feeding the fish pellet sizes that were too large for many, if not most, of the fish, especially during the early days of this period. The complete recovery of the fish during the final day 252 to day 354 exposure period lend considerable weight to this conclusion. Gut-to-body-weight ratio and whole-body fat tended to increase with

[56] Storebakken T, *et al.* (2004) Bacterial protein grown on natural gas in diets for Atlantic salmon, *Salmo salar*, in freshwater. *Aquaculture*. 241: 413-425.

increasing dietary BPM concentration, and gut-to-body-weight ratios and liver-to-body weight ratios were slightly, but statistically-significantly, elevated in the fish fed 37% BPM in the diet for 364 days. However, histological evaluations revealed no evidence of disease and no systematic differences in the tissues of the fish exposed to 5% or 37% BPM for 252 days, except for reduced hepatocellular vacuolization in the fish fed 37% BPM.[57]

Storebakken et al. (2004) assessed nutrient digestibility indirectly by feeding salmon (n=50/group, 3 groups/concentration; mean body weight 60 g) diets containing 0%, 5%, 10%, 19.3%, or 37% BPM equivalent to FeedKind® for 14 days. Cr_2O_3 was added to the diets as a marker before pelleting the formulations. As in the main study, the BPM added to the feed replaced an equivalent amount of fish meal in the feed of each group of exposed animals. The feces were collected by manual stripping after the exposure period. Total concentrations of nitrogenous substances (including proteins and nucleic acids) and fat were measured in feces, and apparent digestibility coefficients (ADCs) were calculated for nitrogenous compounds (i.e. "nitrogen digestibility") from the nutrient-to-marker ratios of the diet and the feces.

The ADCs for nitrogen digestibility were statistically significantly lower in the salmon receiving BPM in the diet, compared with controls. The ADCs for nitrogen digestibility were 89.9%, 88.1%, 88.3%, 86.7%, and 84.2% for salmon receiving 0%, 5%, 10%, 19.3%, and 37% BPM in the diet, respectively. Thus, the ADCs were lower than the control value by 1.8%, 1.6%, 3.2%, and 5.7% in the salmon fed diets containing 5%, 10% or 19.3%, and 37% FeedKind®, respectively. The authors were able to fit the ADCs for nitrogen reasonably well to a straight line (r=0.95) after omitting the ADC for the salmon exposed to 5% BPM. They estimated the nitrogen digestibility to be 78.4% from this curve, assuming that 100% replacement of fish meal with BPM in the diet.[58] Although this estimate was not corrected for differences in the content of nitrogenous non-protein substances (i.e. mainly nucleic acids, including 2.2% DNA and 7.3% RNA in BPM), the authors indicated that this value represents the digestibility of crude protein from BPM. They noted that this result is consistent with the 81.9% total nitrogen digestibility reported by Skrede et al. (1998), who fed salmon BPM as the sole source of protein.[59]

The ADCs for fat digestibility were approximately 96.4%, 96.2%, 95.8%, and 95% for salmon receiving 0%, 5%, 10%, 19.3% and 37% BPM in the diet, respectively. Although there appears to be a downward trend in the ADC with increasing BPM, only the ADC for fat digestibility for salmon fed 37% BPM was statistically significantly lower than the control value. The authors noted that Storebakken et al. (1998) and others found no effect on the ADC for lipid digestibility in salmon fed a diet in which BPM replaced fish meal. They noted that their results, particularly in the animals fed the diet containing 37% BPM, may be attributable to the presence of relatively high levels of non-starch polysaccharides, which are known to reduce the absorption of fats in the diet.

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[57] Fish (n=10/2 of 3 replicate tanks/diet) exposed to 5% or 37% BPM for 252 days were sampled for histological examination to include poorly growing fish that were discarded during size grading.

[58] ADC for nitrogen = 89.76-(0.113 x 100) = 78.46%;

[59] Skrede A, Berge GM, Storebakken T, Herstad O, Aarstad KG, Sundstol F (1998). Digestibility of bacterial protein grown on natural gas in mink, pigs, chicken, and Atlantic salmon. Animal Feed Sci. Technol. 76(1-2): 103-116.

In a saltwater experiment, Berge *et al.* (2005) fed groups of Atlantic salmon (n=1000/pen; 2 pens/diet; average initial body weight = 1.39 kg) 0%, 10%, or 20% BPM equivalent to FeedKind® in the diet (equivalent to 0%, 17.2% and 33.1% dietary nitrogen, respectively) for 5 months.[60] As in the freshwater study, the BPM added to the feed replaced an equivalent amount of high-quality fish meal in the feed.[61]

During the initial 3 weeks of the exposure period, mortalities were frequent regardless of the BPM content of the feed (i.e., 13.9 ± 1.3%, 17.3 ± 3.6% and 9.0 ± 0.1% at 0%, 10%, and 20% BPM in the diet, respectively) but were not statistically significant among the control and BPM-exposed groups. Mortality was low throughout the rest of the study. No statistically significant effects were found on mean body weights, growth rates, feed intake, or feed conversion ratio (FCR) measured after 2 months and after 5 months of exposure and analyzed by analysis of variance (ANOVA).

However, Berge et al. (2005) noted that body weights and thermal growth coefficients were inversely correlated with dietary BPM concentration at 2 months and 5 months when the data were analyzed by linear regression.[62] However, Aas et al. (2006a) re-analyzed the data reported by Berge et al. (2005) and reported that there were no statistically significant differences in the mean body weights of the fish fed 10% or 20% BPM in the diet for 2 months or 5 months, compared with controls.[63] Aas et al. (2006a) found that the only statistically-significant difference in body weights was between the fish fed 10% BPM and those fed 20% BPM for 2 months, and there was no statistically significant difference in body weights between the fish fed 10% BPM and the fish fed 20% BPM at the end of the 5-month exposure period.

Carcass and visceral dry matter and visceral fat and dry matter contents were also statistically-significantly inversely correlated with dietary BPM levels, but there were no detectable effects on dressed-out carcass, liver, or intestine weight-to-body-weight ratios. There appeared to be trends of decreasing digestibility of nitrogen, fat, and energy with increasing BPM concentration in the diet, but none of the trends were statistically significant in ANOVA or regression analysis of the data. There were no statistically significant differences in whole-body nitrogen retention among any of the animal groups.

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60 Berge GM, *et al*. (2005) Bacterial protein grown on natural gas as protein source in diets for Atlantic salmon, *Salmo salar*, in saltwater. *Aquaculture*. 244: 253-240.

61 Daily feed intake was quantified for each pen as the difference between the ration fed and the collected excess feed corrected for leaching in sea water. Salmon were counted and weighed individually at the start of the experiment and 2, 4, and 5 months thereafter, and salmon were sampled (n=10/pen at start and 5/pen thereafter) for body composition analyses. Salmon (n=10/pen) were sampled for fat-content estimation via computerized tomography. Digestibility was estimated based on the results of analyses of feces collected at 4 months and fish collected (minimum n=60 fish samples pooled/pen). Histological examination and hematocrit measurement were performed on fish collected after 5 months of exposure (n=5/pen).

62 At 5 months, mean body weights were 3889 ± 32, 3776 ± 101, 3649 ± 63 g/fish and thermal growth coefficients were 2.89 ± 0.03, 2.79 ± 0.09, and 2.67 ± 0.00 for fish exposed to 0%, 10%, and 20% BPM in the diet, respectively.

63 Aas TS, et al. (2006a) Improved growth and nutrient utilization in Atlantic salmon (Salmo salar) fed diets containing a bacterial protein meal. Aquaculture. 259: 365-376.

Histological examinations indicated that the mucosa of the distal intestines was generally normal, including absorptive vacuoles in the enterocytes of the intestinal folds and moderate amounts of leucocytes infiltrating the mucosa and submucosa across the animal groups.

Only one fish, a male fed 10% BPM in the diet, exhibited severely inflamed intestinal mucosa, heavy leucocyte infiltration of the mucosa, and no absorptive vacuoles, without the reduction in mucosal-fold height reported to be induced by soybean meal.[64] However, the body weight and length of this fish was close to the tank means, and the fish had no external signs of disease and had normal hematocrit measurements after 364 days of exposure to BPM. In general, none of the fish examined exhibited signs of allergic reaction to BPM in the distal intestinal mucosa, even those exposed to 20% BPM in the diet for 364 days, and all of the fish had hematocrit measurements within the normal range.

Likewise, evaluation of carotenoid concentration and sensory characteristics of the flesh showed no differences between controls and the fish fed BPM at any concentration.

Berge et al. (2005) used the same indirect method as Storebakken et al. (2004) to assess the digestibility of nitrogen, fat, and energy, except that Y_2O_3 was used as the marker, rather than Cr_2O_3. Salmon (n=60/pen minimum) were fed diets containing 0%, 10%, or 20% BPM for 4 months, after which fecal samples were collected by manual stripping and the samples from each pen were pooled, homogenized and analyzed. The nutrient digestibility estimated for salmon raised in inner pens were statistically significantly greater than the corresponding values estimated for salmon raised in the outer pens, which is consistent with the observation that the salmon in the inner pens were less stressed, because of reduced exposure to the open sea, than the salmon in the outer pens. In any case, the were no statistically significant differences between the exposed salmon and the controls in the ADC measurements for nitrogen (ADC = 86.8%, 84.9%, and 83.1% for 0%, 10%, and 20% BPM, respectively), fat (ADC = 88.5%, 86.0%, and 84.0% for 0%, 10%, and 20% BPM, respectively), and energy (ADC = 84.4%, 82.0%, and 78.6% for 0%, 10%, and 20% BPM, respectively), and no statistically significant concentration-response relationship was evident by regression analysis in this study.

Berge et al. (2005) found no statistically significant differences in nitrogen retention in the salmon receiving BPM in the diet, compared with controls, or any evidence of a concentration response trend in this parameter.

Like Storebakken et al (2004), Berge et al. (2005), noted that the mean ADCs for nitrogen digestibility were lower in salmon fed BPM than in the controls (e.g., 83.1% in salmon fed 20% BPM vs. 86.8% in salmon fed at 0% BPM in the diet). The ADCs for nitrogen digestibility

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[64] A related study showed that increasing dietary concentrations of BPM combined with 200 g/kg solvent-extracted soybean meal attenuated the typical soybean-meal-induced disturbances observed in the distal intestines of Atlantic salmon; the levels of inflammatory regulators $CD8\alpha^+$ T lymphocytes and MHC II-reactive cells observed in the intestinal tissues of the soya-extract-expose animals were normalized by sufficient inclusion of BPM in the diet, the regulatory mechanisms for these effects are not yet known. *See* Romarheim et al. (2012 online). Prevention of soya-induced enteritis in Atlantic salmon (Salmo salar) by bacteria grown on natural gas is dose dependent and related to epithelial MHC II reactivity and $CD8\alpha^+$ intraepithelial lymphocytes. Brit. J. Nutr. March 2013: 1-9.

reported by Berge et al. (2005) for salmon receiving diets containing 0%, 10% and 20% BPM are comparable to those reported by Storebakken et al. (2004) for salmon, which ranged from 84.2% in salmon fed 37% BPM to 89.9% in salmon fed at 0% BPM in the diet. Like Storebakken et al. (2004), Berge et al (2005) noted that their results illustrate a tendency for poorer digestibility of the crude protein of BPM. However, Berge et al. (2005) acknowledged that a tendency for reduced nitrogen digestibility of BPM, compared with high quality fish meal, can be attributed to the presence in BPM of bacterial cell walls and membranes that are resistant to enzymatic digestion.

In another saltwater experiment, Aas *et al.* (2006a) fed Atlantic salmon (n=18/group; 3 groups/diet; average initial body weight 170 g) 0%, 4.5%, 9%, 18%, or 36% BPM equivalent to FeedKind® in the diet for 48 days.[65] In a parallel digestibility study, salmon (n=3/group; initial body weight 494 g) were fed 0%, 18%, or 36% BPM in the diet for 15 days. There were no mortality or health problems observed in any of the fish exposed to BPM in the diet for up to 48 days.

The SGRs of the fish fed 18% or 36% BPM were statistically-significantly greater than the rates of the controls or the fish fed 4.5% BPM.[66] BPM did not affect feed intake.

Increased dietary BPM levels were also associated with reduced branchial[67] and/or renal nitrogen and energy losses and energy spent on activity and maintenance.[68] The liver-to-body-weight and viscera-to-body weight ratios were statistically-significantly lower in the salmon fed 4.5% and 4.5% or 9% BPM, respectively, compared to the controls and to the fish fed higher concentrations of BPM in the diet.[69]

The copper concentrations were greater in the viscera of fish fed 36% BPM,[70] but there were no adverse effects of copper on growth or survival and no discernable differences in copper or phosphorous concentrations in the liver or carcass, dry-matter, fat, nitrogen, ash, or energy contents of the liver, viscera, or carcass, or amino acid content of the whole body. The authors attributed the elevated visceral concentration of copper levels measured in the fish fed 36% BPM

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65 Aas TS, *et al.*(2006a) Improved growth and nutrient utilization in Atlantic salmon (*Salmo salar*) fed diets containing a bacterial protein meal. *Aquaculture*. 259: 365-376.

66 For example, body weights measured on day 52 averaged 33 ± 12.3, 327 ± 10.7 and 360 ± 3.2 g in fish exposed to 0%, 4.5%, and 36% BPM for 48 days, respectively.

67 Branchial means of or related to the gills.

68 The authors concluded that the reduction in the energy used for activity and maintenance per kg body growth is attributable to the greater growth rates at the highest dietary BPM concentrations tested.

69 For example, the liver-to-body ratios were 1.33 ± 0.04, 1.20 ± 0.02, and 1.38 ± 0.03 in fish fed 0%, 4.5%, and 36% BPM, respectively; the corresponding viscera-to-body-weight ratios were 7.37 ± 0.13, 6.91 ± 0.16, and 7.52 ± 0.09, respectively.

70 Mean [Cu] = 0.1 ± 0.1 mg/kg and 0.2 ± 0.1 mg/kg in viscera of fish exposed to 0% and 36% dietary BPM, respectively.

to the supplementary copper added to all of the diets in this study (5 mg/kg), as well as to the greater copper content of the BPM compared to that of the fish meal used.[71]

Aas et al. (2006a) assessed nutrient digestibility by feeding salmon (20.4 kg biomass/group, 3 groups/concentration; mean body weight 494 g) diets containing 0%, 18%, or 36% BPM equivalent to FeedKind® for 14 days. Y_2O_3 served as the marker. Like Berge et al. (2005), Aas et al. (2006) calculated ADCs for nitrogen, lipid, and energy, as well as nitrogen retention. In addition, Aas et al. (2006) calculated ADCs for individual amino acids and for the sum of amino acids.

The ADCs calculated for nitrogen digestibility were consistent with those reported by Storebakken et al. (2004) including lower values in the exposed animals compared with controls and a downward trend with increasing BPM concentration in the diet (ADC for nitrogen = 87.8%, 86.6%, and 84.8% for 0%, 18%, and 36% BPM, respectively). In addition, Aas et al. (2006) found comparable trends in the ADCs of individual amino acids, as well as in the ADCs for the sum of amino acids. However, the ADCs for the sum of amino acids (ADC for sum of amino acids = 90.7%, 89.3%%, and 87.6% for 0%, 18%, and 36% BPM, respectively) and ADCs for individual amino acids were greater than the corresponding ADCs for nitrogen digestibility. These results showed that using total nitrogen ADCs to represent the ADCs for protein or amino acids results in the underestimation of these values for BPM. The authors suggested that this effect may be attributable to the relatively indigestible cell walls in BPM, which are not present in the high-quality fish meal used in these studies.

Furthermore, Aas et al. (2006a) found that ingested and digested nitrogen retention and energy retention and ingested lipid retention were statistically significantly elevated in the salmon fed diets containing 18% or 36% BPM, compared with controls. The authors attributed the absence of adverse effects on mortality rates, growth rates and other indices of health in salmon exposed to up to 36% BPM to the improved utilization of feed containing BPM.In addition to the published studies in Atlantic salmon summarized above, there is a published study in another species of the Salmonidae (salmonid) family, namely the rainbow trout. In the rainbow trout (*Oncorhynchus mykiss*) experiment, Aas *et al.* (2006b) fed triplicate groups of the fish (n=11/group; initial average body weight = 361 g) 0%, 9%, 18%, or 27% BPM equivalent to FeedKind® or 9% BPM autolysate[72] in the diet for 71 days. The BPM or BPM autolysate replaced the equivalent levels of fish meal and starch of the base diet.[73]

One fish in the group receiving 27% BPM in the diet died. However, there were no statistically significant differences after the 71-day exposure period across the groups in mean

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[71] Aas et al. (2006a) reported the copper concentration to be 87.9 ppm in the BPM tested; the copper concentrations in the test diets containing 0%, 4.5%, 9%, 18%, and 36% BPM were 9.6 ppm, 14.4 ppm, 14.9 ppm, 20.4 ppm and 35.6 ppm, respectively.

[72] Autolysis, aka self-digestion, is the destruction of cells through the action of the enzymes of the cells. The authors provided no details about the production of the BPM autolysate used in the study, except to note that the BPM autolysate and BPM represented two different batches of bacterial biomass, which helps to explain some of the differences in the compositions of the two products tested.

[73] Aas TS, *et al.*(2006b). Effects of diets containing a bacterial protein meal on growth and feed utilization in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*. 261: 357-368.

body weights, SGRs, feed intake, or FERs, or liver- or viscera-to-body-weight ratios evaluated by ANOVA. Regression analysis suggested increasing liver-to-body-weight ratio with increasing dietary BPM content ($p=0.044$). However, the relationship between liver-to-body-weight ratio and dietary BPM content did not appear to be linear ($r^2=0.35$), indicating that this result may be a statistical artifact.

There were no statistically significant differences in nitrogen, crude-lipid, dry-matter, ash, or energy levels in the liver, carcass or viscera across the groups, except for a slightly elevated ash content of the liver of the animals exposed to 27% BPM in the diet. There were no statistically significant differences in the mineral contents of the liver, including copper, in the BPM-exposed animals compared with the controls, and no effects on whole-body amino-acid composition, based on ANOVA. Linear-regression analysis indicated increasing whole-body histidine and decreasing whole-body methionine concentrations with increasing dietary BPM concentration.[74]

Uric acid levels in plasma were also elevated in trout exposed to 27% BPM, compared to controls and trout exposed to 18% BPM in the diet.[75] However, there were no exposure-related effects on urea levels measured in plasma, liver or muscle.[76]

The ADCs for copper were lower than control values and for phosphorus greater than control values for all groups exposed to BPM, and these differences were statistically significant.[77]

There were no statistically significant differences in retention of digested lipid, energy, nitrogen or amino acids among the groups. No exposure-related effects were found in the ANOVA analysis of nitrogen-budget parameters, except for elevated fecal-nitrogen loss. However, regression analysis indicated increasing nitrogen intake per kg body growth with increasing dietary BPM concentration. Fecal energy loss also increased with increasing dietary BPM content above 9%, and the energy used for activity and maintenance[78] was greater in trout

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[74] For example, whole-body histidine concentrations were 2.79% ± 0.06% and 2.94% ± 0.05% in fish fed 0% and 27% BPM, respectively; the corresponding values for methionine were 3.30% ± 0.03% and 3.23% ± 0.01%, respectively.

[75] Plasma uric acid concentrations were 29.04 ± 3.15, 29.56 ± 2.16, and 43.68 ± 4.47 μmol/l in fish receiving 0%, 18%, and 27% BPM in the diet, respectively.

[76] The authors noted a previous study in Atlantic salmon fed high dietary levels of BPM that found elevated urate oxidase activity in the liver and urea concentrations in the plasma, liver, and muscle without statistically-significant elevations in plasma uric acid levels, indicating that uric acid is less stable in trout than in Atlantic salmon; see Anderson *et al*. (2006). Purine-induced expression of urate oxidase and enzyme activity in Atlantic salmon (*Salmo salar*); Cloning of urate oxidase in liver cDNA from three teleost species and the African lungfish Protopterus annectens. FEBS J. 273: 2839-2850.

[77] For example, ADCs for copper were 73.2 ± 1.9 and 47.7 ± 3.7 for fish receiving 0% and 27% BPM, respectively; the corresponding values for phosphorus were 55.0 ± 0.7 and 63.9 ± 0.6, respectively.

[78] Energy cost of maintenance and activity was calculated as the difference between heat loss and heat increment.

receiving 27% BPM and heat increment[79] was lower in trout receiving 9% autolyzed BPM compared with the corresponding values for controls and fish exposed other concentrations of BPM. The energy spent on total heat increased statistically significantly with increasing dietary BPM content, based on regression analysis of the data.

No diet-related morphological changes were observed in the digestive tract, and differences in degree of vacuolization of the epithelial cells of the villous folds of the pyloric caeca were observed in nearly all fish, regardless of the presence or absence of BPM in the diet.

Overall, there were no significant differences in the results obtained with autolyzed BPM compared with BPM.

Aas et al. (2006b) assessed nutrient digestibility by analyzing feces collected during weeks 6 to 9 of the 10-week exposure period. Y_2O_3 (0.1 g/kg) in the diets served as the marker. As in the salmon studies, the ADCs calculated for nitrogen, energy, and sum of the amino acids, exhibited downward trends with increasing dietary BPM concentrations (e.g., ADC for nitrogen = 95.5%, 94.7%, 94.0%, and 90.7% for 0%, 9%, 18%, and 27% dietary BPM, respectively; ADC for sum of amino acids = 96.8%, 96.3%, 96.2%, and 92.4% for 0%, 9%, 18%, and 27% dietary BPM, respectively). Notable reductions in these parameters were observed only in the trout fed diets containing 27% BPM. The ADCs calculated for the sum of amino acids and lipids were statistically significantly lower than the respective controls only in salmon receiving the diet containing 27% BPM.

The ADCs for the sum of amino acids and individual amino acids were greater than the corresponding ADCs for nitrogen digestibility in trout, as Aas et al. (2006a) reported for salmon, indicating that the protein fraction is digested more effectively than non-protein fraction. These authors noted the potential for the cell wall to reduce the nitrogen digestibility of BPM compared with that of fish meal. However, BPM had no significant effect on digested lipid, energy, nitrogen or amino acids retention in trout at any test concentration, compared with the elevated retention of nutrients reported in salmon by Aas et al. (2006a).

6.1.3 Immunogenicity

Generally, dietary proteins affect the immune-system status of gut associated lymphoid tissue (GALT) when ingested. Accordingly, unpublished subchronic oral exposure studies in rats have demonstrated that BPM can cause moderate elevations of mesenteric lymph node (MLN) weight and macrophage/neutrophil levels of MLNs, which suggests stimulation of the innate immune system.[80] However, BPM levels less than 15% in the diet yielded minimal-to-no

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79 Heat increment was calculated as the difference in heat loss between fed fish and fasted fish.

80 (b) (4)

(b) (4)

evidence of colonic or intestinal inflammation, no indication of severe colitis or tissue destruction, and no signs of secondary endotoxemia, pain, distress, or overt inflammation in these studies.

Christensen et al. (2003) showed that BPM-specific total Ig, IgA, IgG1, and IgG2A antibodies were elevated in blood samples and BPM-specific IgA antibodies were elevated in saliva samples from mice exposed to BPM in the diet.[81] They noted also that: (1) IgG1 antibody production is supported by T-helper cell type 2 (Th-2 cells) of humoral immunity, and (2) IgG2A antibody production is supported by Th-1 cells of cell-mediated immunity. The sustainment of IgG2A antibody levels observed after the cessation of exposure to BPM in this study suggests that factors supporting the Th-1 response in these mice may be cleared less efficiently than those supporting the Th-2 response. Th-1-type cytokines tend to produce pro-inflammatory responses that can lead to tissue damage if excessive. Th-2-type cytokines, on the other hand, are associated with anti-inflammatory responses. Thus, an optimum response to an immune challenge is generally a balanced Th-1 and Th-2 response.

Furthermore, IgG2A antibodies may have affinity for lipopolysaccharides (LPS). Christensen et al. (2003) noted that the main bacteria of BPM (*M. capsulatus*) contains LPS as an integral part of the cell membrane, which are likely candidates as adjuvants in BPM. However, Christensen et al. (2003) explained that LPS, which are abundant in the mucosal lumen, can enhance oral tolerance rather than potentiate the immunogenicity of an antigen. On the other hand, the sustained elevation of IgG2A antibody levels, accompanied by the decrease in IgG1 antibody in the blood of mice after the cessation of BPM exposure, as reported by Christensen et al. (2003), suggests the potential that chronic exposure to BPM in the diet may pose a risk for long-term inflammatory responses in mammalian species.

As noted above (Section 6.1.2. Salmonid Species), FeedKind® has been tested in Atlantic Salmon in one freshwater and two saltwater feeding studies published in a peer-reviewed journal.[82] Among these studies, Storebakken et al. (2004) showed that there were no histopathological changes in the anterior intestines, pyloric sacs or posterior intestines of Atlantic salmon fed up to 37% BPM in the diet starting at the fry stage and for 252 days thereafter in freshwater. As well, there were no histopathological changes observed in the cross section of the carcass (muscle, skin, kidney) or the liver, except for reduced hepatocellular vacuolization in the fish fed 37% BPM. Likewise, Berge et al (2005) found no histopathological changes in the intestines of Atlantic salmon fed up to 20% BPM in the diet starting from 1.39 kg initial body weight and for 150 days thereafter in seawater. Only one fish fed 10% BPM exhibited severely inflamed intestinal mucosa, leucocyte infiltration of the mucosa, and absence of absorptive vacuoles, but without the reduction in mucosal-fold height typical of the immunogenic response

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[81] Christensen HR, Larsen LC, Frokiaer H (2003). The Oral Immunogenicity of BioProtein, a Bacterial Single-Cell Protein, is Affected by its Particulate nature, Brit. J. Nutr. 90: 169-178; WBC-specific total Ig, IgG_1, and IgG_{2A} antibodies were not measured in the saliva samples in this study.

[82] Storebakken T, *et al.* (2004) Bacterial protein grown on natural gas in diets for Atlantic salmon, *Salmo salar*, in freshwater. *Aquaculture*. 241: 413-425; Berge GM, *et al.* (2005) Bacterial protein grown on natural gas as protein source in diets for Atlantic salmon, *Salmo salar*, in saltwater. *Aquaculture*. 244: 253-240; Aas TS, et al. (2006a) Improved growth and nutrient utilization in Atlantic salmon (Salmo salar) fed diets containing a bacterial protein meal. Aquaculture. 259: 365-376.

induced by soy protein in these fish. Furthermore, Aas et al. (2006) found increased copper concentrations in the viscera of salmon fed 36% BPM in the diet for 48 days in seawater, but no adverse effects on growth and survival, no effects on copper contents of any other tissues or on energy contents of viscera or any other tissue. These authors attributed the elevated copper levels in the viscera to the copper levels of the basal diet (5 ppm) plus the greater copper content BPM (87.9 ppm) compared to the fish meal it replaced (3.7 ppm). Overall, these studies showed that chronic exposure to diets containing up to 37% BPM does not induce an inflammatory response in the intestines of salmon, in contrast to soybean meal extracts used as a protein source in salmonid aquaculture.

Two scientific studies published in the peer-reviewed literature were performed to assess the potential for dietary BPM to produce immunogenic or immunotoxicological effects in Atlantic salmon. These studies are summarized below.

Romarhein *et al*. (2011) fed triplicate groups of juvenile Atlantic salmon (n=75/group; initial mean body weight = 133 g/fish) control fish-meal diet (*i.e.* 0% solvent-extracted soybean meal [SBM] and 0% BPM) or a diet in which the fish meal was incrementally replaced to contain 20% SBM, 30% BPM, or 20% SBM plus 30% BPM for 80 days.[83] Conventional solvent-extracted SBM is considered to be a suitable protein source for farmed salmonids, although dietary inclusion levels as low as 7.6% are known to cause SBM-enteritis in salmonid species, which is characterized by inflammation of the distal intestines. The mechanism for this reversible effect appears to involve impaired immune tolerance to SBM caused by alcohol-soluble components of SBM, such as saponins.

As expected, the fish fed 20% SBM for 80 days in this study developed enteritis, lacked carbonic anhydrase 12 in the epithelial cells of the brush border of the distal intestines,[84] and had greater numbers of epithelial cells reacting to proliferating nuclear antigen compared with the fish fed the other diets. The fish fed control, 30% BPM, or 20% SBM plus 30% BPM showed no signs of inflammation in the distal intestines on histopathological examination of the tissues.

Furthermore, the fish fed 20% SBM in the diet exhibited statistically-significantly reduced final body weight, thermal growth coefficient (TCG), and FCR, compared to fish fed the control diet. On the other hand, 30% BPM in diet resulted in a statistically significant increase in FCR but no statistically significant differences in final body weight or TCG.[85]

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[83] Romarheim OH, *et al.* (2011) Bacteria grown on natural gas prevent soybean meal-induced enteritis in Atlantic salmon. *J Nutr*. 141: 124-130.

[84] Carbonic anhydrases (CAs) are zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide, participate in a variety of biological processes, and are highly expressed in normal tissues.

[85] For example, final body weights were 362, 319, 344, and 328 g/fish for fish fed the control, 20% SBM, 30% BPM, and 20% SBM plus 30% BPM diets, respectively.

Digestibility of crude protein and lipids was statistically-significantly reduced in the fish receiving 30% BPM in the diet, compared with controls,[86] but total gut, liver, stomach, and mid- and distal-intestine weights, were elevated relative to body weight in these animals.[87]

The authors concluded that BPM counteracts or neutralizes SBM-induced enteritis in Atlantic salmon.

In a follow-on study (Romarhein *et al*., 2012), duplicate groups of juvenile Atlantic salmon (n=50/group; initial mean body weight = 273 g/fish) a control fish-meal diet (*i.e.* 0% SBM and 0% BPM) or a diet in which the fish meal was incrementally replaced to contain 20% SBM plus 0%, 2.5%, 5%, 10%, 15%, 20% or 30% BPM for 47 days.[88] Only one fish died after the experiment was started.

Fish fed 20% SBM in the diet developed SBM-enteritis, as expected, but this effect decreased with increasing BPM levels in the diets containing 20% SBM. Likewise, the number of clusters of $CD8\alpha^+$ intraepithelial lymphocytes in fish fed 20% SBM decreased with increasing BPM inclusion levels.[89] Morphometric evaluation revealed that intestinal stretches stained for proliferating-cell nuclear-antigen in the fish fed 20% SBM plus ≥15% BPM were indistinguishable from those of fish fed the control diet, as was the number of clusters of $CD8\alpha^+$ intraepithelial lymphocytes at the base of the intestinal epithelium in fish fed 20% SBM plus ≥20% BPM. Staining for major histocompatibility complex class II (MHC II) revealed numerous reactive leucocytes in the brush border and other areas of the intestinal epithelium in salmonids fed 20% SBM plus 0%, 1.2%, or 5% BPM, but this effect decreased in a concentration-dependent manner in salmonids were fed 20% SBM plus 10%, 20% or 30% BPM. Single and isolated lymphocyte aggregates consisting primarily of leucocytes were found in otherwise normal intestines in 2 of 12 and 1 and 12 fish fed 20% SBM plus 20% BPM and 20% SBM plus 30% BPM, respectively.

There were no significant differences in feed consumption or growth across all groups, although 20% and 30% BPM in the diet were associated with slightly reduced protein digestibility[90] and increased relative weight of the distal intestines.[91] The authors suggested that

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[86] Mean digestibility of crude protein was 86.6%, 86.1%, 83.3%, and 84.6% and mean digestibility of crude lipid was 96.1%, 94.7%, 92.2%, and 95.7% for fish fed the control, 20% SBM, 30% BPM, and 20% SBM plus 30% BPM diets, respectively.

[87] For example, mean relative liver weights were 16.1, 15.3, 17.2, and 19.3 g/kg body weight for fish fed the control, 20% SBM, 30% BPM, and 20% SBM plus 30% BPM diets, respectively.

[88] Romarheim OH, *et al.* (2012). Prevention of soya-induced enteritis in Atlantic salmon (*Salmo salar*) by bacteria grown on natural gas is dose dependent and related to epithelial MHC II reactivity and $CD8\alpha^+$ intraepithelial lymphocytes. *Br J Nutr*. 109 (6): 1062-1070.

[89] Mobilization of CD8α+ T cells indicates that SBM-induced enteritis is a T-cell-mediated inflammatory response to SBM.

[90] The authors suggested that the reduced protein digestibility of BPM is attributable to cell-wall components of BPM, as it is for yeast products fed to salmonids, as well as the extensive intracytoplasmic membranes of M. capsulans grown on natural gas.

[91] Mean crude-protein digestibility was 83.7%, 84.9%, 84.1%, 84.5%, 83.8%, 84.8%, 83.6%, and 82.2% in fish fed 20% SBM plus 0%, 2.5%, 5%, 10%, 15%, 20% or 30% BPM, respectively,

the continued increase in the relative weights of the distal intestines at dietary BPM levels greater than levels that prevented SBM-induced enteritis indicates that BPM stimulates intestinal growth in the affected fish. This could be because, for example, BPM contains relatively high purine and pyrimidine levels that can serve as substrates supporting the growth of intestinal epithelial cells.

As in the previous experiment, there was no effect on the mean relative weights of the total gut, liver, stomach, pyloric region or mid-intestines. The authors suggested that the mechanism by which BPM counteracts pro-inflammatory responses in salmonids exposed to 20% SBM in the diet is related to immune-system mechanisms that are also responsible for ensuring tolerance to feed antigens and to commensal intestinal microbiota.

Overall, the results of studies of salmonid species chronically exposed to up to 37% BPM in the diet are uniformly negative for any signs of an inflammatory response that can be attributed to BPM exposure. These studies demonstrate that chronic exposures to BPM, even at very high levels in the diet, do not produce the exposure-related chronic inflammatory responses suggested based on the changes in antibody titers reported in mice orally exposed to BPM.

6.1.4 Liver Weight and Prothrombin Time

In two Scientific Opinions published in 2017, the European Food Safety Authority (EFSA) Panel on Additives and Products or Substances in Animal Feed (FEEDAP) expressed uncertainties about the safety of genetically-modified (GM) *Escherichia coli* (*E. coli*) and other gram-negative bacterial biomasses intended to be fed to food producing mammalian species.[92] Specifically, the EFSA Panel noted that the mechanisms for the increased liver weights reported in pigs and reduced prothrombin time reported in multiple species fed biomasses produced by the GM *E. coli* (gram negative) strains are not known. However, the Panel acknowledged that these effects were small and clearly not attributable to the systemic absorption of endotoxins or lipopolysaccharides (LPS) from these biomasses in the digestive tracts of the animals tested, and dietary variation is a plausible explanation for the effects reported.

The Panel noted that other products derived from other gram-negative microorganisms may pose similar issues, without citing evidence to implicate gram-negative organisms other than *E. coli* as having any potential to cause adverse effects, and without providing any specific rationale for this assertion. On the contrary, the Opinions of the EFSA Panel provided few or no assertions that the *E. coli* biomasses would have adverse effects on the animals or on humans ingesting products derived from food-producing animals fed these biomasses. The Panel's

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for 47 days; corresponding average distal-intestine-to-body-weight ratios were 5.8, 5.6, 5.2, 5.6, 6.3, 6.1, 6.8, and 7.0, respectively.

[92] EFSA FEEDAP Panel (2017). Scientific Opinion on the safety and nutritional value of a dried killed bacterial biomass from *Escherichia coli* (FERM BP-10941) (PL73 (LM)) as a feed material for pigs, ruminants and salmonids. EFSA Journal. 15:4935. Available at: https://doi.org/10.2903/j.efsa.2017.4935; EFSA FEEDAP Panel (2017). Scientific Opinion on the safety and nutritional value of a dried killed bacterial biomass from *Escherichia coli* (FERM BP-10942) (PT73 (TM)) as a feed material for pigs, ruminants and salmonids. EFSA Journal.15:4936. Available at: https://doi.org/10.2903/j.efsa.2017.4936.

conclusions in both Opinions stated that "the recipient strain E. coli K-12S B-7 is considered to be safe."

Like *E. coli*, the *M. capsulatus* that serves as an integral microorganism of the consortium used to produce FeedKind® is a gram-positive bacterium. However, there is no evidence in any of the numerous, substantial studies that have been performed with BPM, equivalent to FeedKind®, in pigs, rats, and other mammalian species, as well as in salmonids, suggesting that short-term or long-term exposures to FeedKind® is inherently dangerous or unsuited for use in salmonids at the proposed feeding levels for these endpoints (i.e. increased liver weight or decreased prothrombin time). There are biological, physiological and taxonomic differences between *M. capsulatus* and *E. coli*. There is no evidence that *M. capsulatus* produces harmful endotoxins, lipopolysaccharides (LPS), or any other substance identical or similar to such substances produced by some strains of *E. coli* and other gram-negative microorganisms that are known to be pathogens. There is no evidence in the literature implicating *M. capsulatus* as having any pathogenic, toxic or other negative characteristics, as discussed in Section 6.1.1.

Furthermore, gram-negative microorganisms are used as fish feed or to produce fish feed substances. For example, a species of *M. extorquens* is GRAS for use in fish feed (product name: KnipBio; AGRN26).

6.1.5 Human Toxicity

None of the substances in FeedKind® fed to animals is expected to be transferred, intact, to people consuming the edible products of any of the food-producing animals. The composition of FeedKind® is like that of other common animal feeds, including amino acids, phospholipids, and lipopolysaccharides. Therefore, FeedKind® consumed by the target animals will be digested and converted to biomass and as such there is no expected exposure to FeedKind® for humans via consumption of target animals. There is no evidence indicating that the nucleic acids in FeedKind® would be incorporated into human food products to be transferred to consumers. There is no evidence that any hazardous substances are formed during the production of FeedKind®. Further, the effects observed in studies in which animals were fed diets containing relatively high concentrations of FeedKind® (generally reduced growth rates and final body weight) are not expected to affect the health of people consuming such products. Thus, the human health risks associated with the consumption of products from animals fed FeedKind® are negligible at the dietary concentrations tested in the studies summarized above.

6.1.6 Conclusion

Table 12 presents the NOAELs and LOAELs from the key safety studies summarized above to support specific FeedKind® inclusion levels in diets for salmonid species, together with brief statements of the adverse effects observed at each LOAEL.[93]

[93] The critical effect is defined as the first adverse effect, or its known precursor, that occurs in a test species as the dose or exposure concertation increases.

Table 12: Safety study summaries for FeedKind® (BPM) inclusion rates

Species	NOAEL (% of diet)	LOAEL (% of diet)	Exposure Duration (days)	Critical Effect(s)	Ref
Atlantic salmon	19.3	37	364	Reduced body weight, specific growth rate, and survival rate	Storebakken *et al.* (2004)
Atlantic salmon	20	N/D*	150	None	Berge *et al.* (2005)
Atlantic salmon	36	N/D*	48	None	Aas *et al.* (2006a)
Rainbow trout	18	27	71	Reduced apparent digestibility coefficients for N, lipid, energy, amino acids; elevated energy used for activity and maintenance	Aas *et al.* (2006b)

*N/D = not determined; there were no adverse health effects observed at the highest dietary FeedKind® inclusion rate tested.

The most conservative species-specific NOAELs determined from the results of the key feeding studies include the following:

- Atlantic salmon: 19.3%
- Rainbow trout: 18%

The results of the studies indicate that the NOAEL for Atlantic salmon during the saltwater phase of its lifecycle is 36%, which is nearly twice as high as the NOAEL observed during the early or juvenile freshwater phase. The lower value reported for Atlantic salmon during the freshwater phase can be attributed to testing with a feed pellet size too large for the size of the fish and other experimental conditions, rather than to effects attributable specifically to the FeedKind® in the feed. Nevertheless, Calysta utilizes the most conservative NOAEL derived from well-conducted, well-reported studies, including a chronic- and two subchronic-exposure studies on Atlantic salmon and a subchronic-exposure study on rainbow trout to conclude that the studies summarized above support specific FeedKind® inclusion levels up to 18% in diets for salmonid species.

Therefore, we believe the above summarized data fully supports the safe use of FeedKind® at inclusion rates not to exceed 18% in salmonid species.

6.1.7 Summary of Safety Argument; Assertion of GRAS Status

Calysta concludes that the generally available data and information that establish safety, as discussed above, provide a basis that the notified substance is generally recognized among qualified experts to be safe under the conditions of its intended use for the target animal species and for humans consuming human food derived from food producing animals.

The notified substance is a fermentation of naturally occurring microorganisms that have not been reported to be a safety concern in the company's literature searches. Manufacture of FeedKind® will use a consistent growth medium with standard fermentation procedures. Raw materials of suitable purity will be used in manufacture and manufacture will be occur under Good Manufacturing Practice. There are not expected to be any impurities in FeedKind® relevant to the health or safety of the target species to which FeedKind® will be fed. Finally, the safety studies conducted by Calysta and others indicate that the product is safe for use at the level contemplated.

Use of FeedKind® will not result in any adverse health effects in humans consuming animals that have been fed FeedKind®, because humans will not be exposed to any unique components or compounds. FeedKind® will be fully metabolized by the target species and, when incorporated in the flesh of the target species, the amino acids and other components of FeedKind® will be indistinguishable from the same components derived from other sources. There are not expected to be any impurities that would impact the target species or which would persist in the flesh of the target species and pose a risk to human health when consumed. Based on the above, Calysta concludes that the notified substance is Generally Recognized as Safe for use in aquaculture feed for salmonid species when used as an additive of up to 18% by weight in the animal feed.

6.2. Basis for GRAS Conclusion for Intended Use of FeedKind®

As described above, the safety of FeedKind® for use in salmonid feed at the levels indicated within this submission is demonstrated by published and supported by unpublished toxicity studies and in supportive feeding studies.

6.3. Safety of Constituents

FeedKind® is the only constituent for which a GRAS Notice is being submitted. Safety of FeedKind® has been addressed above.

Part 7 – List of supporting data and information

Calysta has disclosed all safety data of which it is aware and have found none that is inconsistent with the GRAS determination.

7.1 References

AAFCO, 2018 Official Publication, "Official Guidelines for Contaminant Levels Permitted in Mineral Feed Ingredients," Table 2, located at page 298.

Aas TS, et al. (2006a) Improved growth and nutrient utilization in Atlantic salmon (Salmo salar) fed diets containing a bacterial protein meal. Aquaculture. 259: 365-376.

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APPENDICES

Appendix 1 Stability testing report

Appendix 2 Historic stability testing

Appendix 3 1995 Scientific Committee for Animal Nutrition report

Appendix 4 Genome sequencing report for DB3, DB4, DB5

Appendix 5 Mercury concentration assessment

APPENDIX 1. Stability testing report

Ian Higgins
May 2019

Shelf Life Testing of FeedKind® Interim Report

Introduction

Samples of FeedKind® have been stored under controlled conditions for 52 weeks. Samples remain under test conditions for each batch being tested and the final samples are expected to be removed from test after 156 weeks. The shelf life trial will be conducted over 156 weeks which is longer than the expected shelf life of FeedKind® and will generate sufficient data to accurately predict the shelf life of FeedKind®. This interim report will focus on the stability of the proximate components of FeedKind® crude protein, crude fat, crude fibre, ash and moisture. The final report will include details of the amino acids, fatty acids and microbiology over the full test period.

Experimental plan

Samples have been chosen at random from different batches of FeedKind® produced at the Teesside UK Market Introduction Facility (MIF) during each production run:

Reference Number	Batch	Test Conditions	Start Date
Stability Test 01	TEES004/29	25°C/60%RH*	12 October 2017
Stability Test 02	TEES004/29	40°C/75%RH*	12 October 2017
Stability Test 03	TEES004/29a	25°C/60%RH	18 October 2017
Stability Test 04	TEES004/29a	40°C/75%RH	18 October 2017
Stability Test 05	TEES004/11	25°C/60%RH	19 October 2017
Stability Test 06	TEES004/11	40°C/75%RH	19 October 2017
Stability Test 07	TEES005/28	25°C/60%RH	10 January 2018
Stability Test 08	TEES005/28	40°C/75%RH	10 January 2018

*Not heat killed.
The MIF broth is processed through a heat kill step before spray drying to kill any active bacteria this step was omitted on TEES004/29 as a trial to investigate if removing the heat kill step has an impact on the final FeedKind®.

A single bag from each batch was separated into 20 X 500g samples one sample was tested and the remaining samples placed in temperature and humidity controlled cabinets at 25°C/60%RH and 40°C/75%RH.
The sample containers used are HDPE, to replicate the PE bulk sacks that may be used at a commercial scale. Holes have been drilled in the lids to allow air into the sample container to represent leakage or absorption at full scale.

The sample plan below is being followed:

0 Weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.
4 Weeks	Proximate, microbiology, biogenic amines.
8 Weeks	Proximate, microbiology, biogenic Amines.

12 Weeks	Proximate, microbiology, biogenic Amines.
26 Weeks	Proximate, microbiology, biogenic Amines.
39 Weeks	Proximate, microbiology, biogenic Amines.
52 Weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.
78 Weeks	Proximate, microbiology, biogenic amines.
104 Weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.
156 weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.

NOTE: Proximate testing is for crude protein, crude fat, ash, moisture and crude fibre.

Results

The results summary below includes data for the proximate testing on all the samples under test for the first 12 months of the stability test.

Stability Test 01 TEES004/29 25°C/60%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min 68%	Max 1%	Max 12%
0					(b) (4)
4					
8					
12					
26					
39					
52					

Table 1

Stability Test 02 TEES004/29 40°C/75%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min 68%	Max 1%	Max 12%
0					(b) (4)
4					
8					
12					
26					
39					
52					

Table 2

Stability Test 03 TEES004/29a 25°C/60%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min 68%	Max 1%	Max 12%
0					(b) (4)
4					
8					
12					
26					
39					
52					

Table 3

Stability Test 04 TEES004/29a 40°C/75%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min 68%	Max 1%	Max 12%
0					(b) (4)
4					
8					
12					
26					
39					
52					

Table 4

Stability Test 05 TEES004/11 25°C/60%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min 68%	Max 1%	Max 12%
0					(b) (4)
4					
8					
12					
26					
39					
52					

Table 5

Stability Test 06 TEES004/11 40°C/75%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min 68%	Max 1%	Max 12%
0	[redacted] (b) (4)				
4					
8					
12					
26					
39					
52					

Table 6

Stability Test 07 TEES005/28 25°C/60%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min 68%	Max 1%	Max 12%
0	[redacted] (b) (4)				
4					
8					
12					
26					
52					

Table 7

Stability Test 08 TEES005/28 40°C/75%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min 68%	Max 1%	Max 12%
0	[redacted] (b) (4)				
4					
8					
12					
26					
52					

Table 8

Discussion

(b) (4)

FeedKind® absorbs moisture from the atmosphere over time which can lead to an out of specification result. Table 9 below (b) (4)

(b) (4)

Test	Moisture	Protein
Weeks to Out of Specification Result		
ST01	(b) (4)	N/A
ST02		(b) (4)
ST03		N/A
ST04		(b) (4)
ST05		N/A
ST06		(b) (4)
ST07		N/A
ST08		N/A

Table 9

The higher moisture content has an impact on the protein level in the sample as the FeedKind® moisture content increases during storage from say 8 to 10% the amount of moisture present in a given sample means less of the other components are present in the sample.

E.g. 100g FeedKind® with 8% moisture contains 8g of water and 92g of other components.

100g FeedKind® with 10% moisture contains 10g of water and 90g of other components.

Consequently, less of the other components are present in the sample with a higher moisture content this appears to have an impact on the protein level in the sample. The sample does not lose protein but gains moisture.

However, there is no significant change in the protein level when calculated on the dry basis.

The protein levels calculated on a dry basis are summarised in table 10.

Test	Protein (dry Basis)	
	Week 1	Week 52
ST01	(b) (4)	
ST02		
ST03		
ST04		
ST05		
ST06		
ST07		
ST08		

Table 10

The packaging material used for the FeedKind® can be selected to minimise the moisture the finished product is exposed to and help to maintain the moisture content within specification.

Conclusion

The initial findings of the shelf life study show FeedKind® to be a stable but hydroscopic product. The final product can be tested before use and any increase in moisture can be compensated for during feed production if necessary.
The data from the shelf life study will be used to confirm the shelf life of FeedKind® and determine the specification for the FeedKind®.

The final report will be issued when the shelf life tests have been completed.

Ian Higgins
Michelle Lindley
May 2019

Supplemental FeedKind® Shelf Life Report

Introduction

Samples of FeedKind® have been stored under controlled conditions for 52 weeks. Samples remain under test conditions for each batch being tested and the final samples are expected to be removed from test after 156 weeks. The shelf life trial will be conducted over 156 weeks which is longer than the expected shelf life of FeedKind® and will generate sufficient data to accurately predict the shelf life of FeedKind®. This supplemental report will focus on the stability of the amino acids, fatty acid, biogenic amines and microbiology of FeedKind®. The final report will be issued when the samples have completed the full test period of 156 weeks.

Experimental Plan

Samples have been chosen at random from different batches of FeedKind® produced at the Teesside UK Market Introduction Facility (MIF) during each production run:

Reference Number	**Batch**	**Test Conditions**	**Start Date**
Stability Test 01	TEES004/29	25°C/60%RH*	12 October 2017
Stability Test 02	TEES004/29	40°C/75%RH*	12 October 2017
Stability Test 03	TEES004/29a	25°C/60%RH	18 October 2017
Stability Test 04	TEES004/29a	40°C/75%RH	18 October 2017
Stability Test 05	TEES004/11	25°C/60%RH	19 October 2017
Stability Test 06	TEES004/11	40°C/75%RH	19 October 2017
Stability Test 07	TEES005/28	25°C/60%RH	10 January 2018
Stability Test 08	TEES005/28	40°C/75%RH	10 January 2018

*Not heat killed.
The MIF broth is processed through a heat kill step before spray drying to kill any active bacteria this step was omitted on TEES004/29 as a trial to investigate if removing the heat kill step has an impact on the final FeedKind®.

A single bag from each batch was separated into 20 X 500g samples one sample was tested and the remaining samples placed in temperature and humidity controlled cabinets at 25°C/60%RH and 40°C/75%RH.
The sample containers used are HDPE, to replicate the PE bulk sacks that may be used at a commercial scale. Holes have been drilled in the lids to allow air into the sample container to represent leakage or absorption at full scale.

The sample plan below is being followed:
0 Weeks Proximate, microbiology, amino acid profile, fatty acid profile,

	biogenic amines.
4 Weeks	Proximate, microbiology, biogenic amines.
8 Weeks	Proximate, microbiology, biogenic Amines.
12 Weeks	Proximate, microbiology, biogenic Amines.
26 Weeks	Proximate, microbiology, biogenic Amines.
39 Weeks	Proximate, microbiology, biogenic Amines.
52 Weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.
78 Weeks	Proximate, microbiology, biogenic amines.
104 Weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.
156 weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.

NOTE: Proximate testing is for crude protein, crude fat, ash, moisture and crude fibre.

Amino acid results summary up to 52 week time point.

ST01 TEES004/29 25°C/ 60%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	5.7	1.7	2.9	2.2	7.3	3.4	4.6	3.6
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	2.9	5.0	1.6	2.8	1.4	3.7	4.2	2.8	1.0
52	(b) (4)								

Table 1

ST02 TEES004/29 40°C/ 75%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	5.7	1.7	2.9	2.2	7.3	3.4	4.6	3.6
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	2.9	5.0	1.6	2.8	1.4	3.7	4.2	2.8	1.0
52	(b) (4)								

Table 2

ST03 TEES004/29a 25°C/ 60%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	5.8	1.7	2.9	2.2	7.5	3.4	4.7	3.7
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	3.0	5.1	1.7	2.9	1.5	3.8	4.3	2.8	0.9
52	(b) (4)								

Table 3

ST04 TEES004/29a 40°C/ 75%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	5.8	1.7	2.9	2.2	7.5	3.4	4.7	3.7
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	3.0	5.1	1.7	2.9	1.5	3.8	4.3	2.8	0.9
52	(b) (4)								

Table 4

ST05 TEES004/11 25°C/ 60%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	5.6	1.7	2.9	2.2	7.3	3.3	4.5	3.6
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	2.9	5.0	1.5	2.8	1.4	3.6	4.1	2.8	0.9
52	(b) (4)								

Table 5

ST06 TEES004/11 40°C/ 75%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	5.6	1.7	2.9	2.2	7.3	3.3	4.5	3.6
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	2.9	5.0	1.5	2.8	1.4	3.6	4.1	2.8	0.9
52	(b) (4)								

Table 6

ST07 TEES005/28 25°C/ 60%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	6.4	1.9	3.2	2.4	7.9	3.7	5.1	4.1
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	3.3	5.5	2.0	3.1	1.6	4.2	4.5	3.0	
52	(b) (4)								

Table 7

ST08 TEES005/28 40°C/ 75%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	6.4	1.9	3.2	2.4	7.9	3.7	5.1	4.1
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	3.3	5.5	2.0	3.1	1.6	4.2	4.5	3.0	
52	(b) (4)								

Table 8

Amino Acid Discussion

The amino acid profile of the samples being tested under both storage conditions showed no significant changes in the first 52 weeks of the shelf life study.

The amino acid profile will be tested again at the 104 & 156 week time points.

Fatty acid profile results summary up to 52 week time point.

ST01 TEES004/29 25°C/ 60%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	1.79	3.50	2.17	0.07
52				(b) (4)

Table 9

ST02 TEES004/29 40°C/ 75%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	1.58	3.53	2.42	0.00
52				(b) (4)

Table 10

ST03 TEES004/29a 25°C/ 60%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	1.91	3.40	2.38	0.03
52				(b) (4)

Table 11

ST04 TEES004/29a 40°C/ 75%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	1.91	3.40	2.38	0.03
52				(b) (4)

Table 12

ST05 TEES004/11 25°C/ 60%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	2.14	3.36	2.28	0.03
52				(b) (4)

Table 13

ST06 TEES004/11 40°C/ 75%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	2.14	3.36	2.28	0.03
52				(b) (4)

Table 14

ST07 TEES005/28 25°C/ 60%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	1.58	3.15	2.36	0.02
52	(b) (4)			

Table 15

ST08 TEES005/28 40°C/ 75%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	1.58	3.15	2.36	0.02
52	(b) (4)			

Table 16

Fatty Acid Discussion

The fatty acids profile showed no significant changes over the first 52 weeks of the shelf life test. The fatty acid profile will be tested again at the 104 & 156 weeks time points.

Biogenic amine results summary up to 52 Week time point.

ST01 TEES004/29 25°C/ 60%RH

Duration Weeks	Putrescine mg/kg	Histamine mg/kg	Cadaverine mg/kg	Spermidine mg/kg	Tyramine mg/kg	Spermine mg/kg
0	<5	<5	231	5150	<5	<5
4	(b) (4)					
8						
12						
26						
39						
52						

Table 17

ST02 TEES004/29 40°C/ 75%RH

Duration Weeks	Putrescine mg/kg	Histamine mg/kg	Cadaverine mg/kg	Spermidine mg/kg	Tyramine mg/kg	Spermine mg/kg
0	<5	<5	231	5150	<5	<5
4	(b) (4)					
8						
12						
26						
39						
52						

Table 18

ST03 TEES004/29a 25°C/ 60%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	7	4599	<5	<5
4	(b) (4)					
8						
12						
26						
39						
52						

Table 19

ST04 TEES004/29a 40°C/ 75%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	7	4599	<5	<5
4	(b) (4)					
8						
12						
26						
39						
52						

Table 20

ST05 TEES004/11 25°C/ 60%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	<5	4471	<5	<5
4	(b) (4)					
8						
12						
26						
39						
52						

Table 21

ST06 TEES004/11 40°C/ 75%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	<5	4471	<5	<5
4	(b) (4) redacted					
8						
12						
26						
39						
52						

Table 22

ST07 TEES005/28 25°C/ 60%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	<5	4953	<5	<5
4	(b) (4) redacted					
8						
12						
26						
52						

Table 23

ST08 TEES005/28 40°C/ 75%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	<5	4953	<5	<5
4	(b) (4) redacted					
8						
12						
26						
52						

Table 24

Biogenic Amine Discussion

The putrescine concentration starts to increase after 26 weeks on test however the spermidine concentration decreases over time. The other biogenic amines histamin, cadaverine, tyramine and spermine remained below detection limits.

Cadaverine is present in ST01 and ST02 which is the material which was not heat killed during production the concentration of cadaverine will be monitored for the duration of the shelf life study.

The biogenic amines will be tested again at the 78, 104 & 156 weeks time points.

Microbiology results summary up to 52 week time point.

Stability Test 01 TEES004/29 25ºC/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	6100	170000	<10	40
4	(b) (4)			
8				
12				
26				
39				
52				

Table 25

Stability Test 02 TEES004/29 40ºC/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	6100	170000	<10	40
4	(b) (4)			
8				
12				
26				
39				
52				

Table 26

Stability Test 03 TEES004/29a 25ºC/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	280	350	<10	10
4	(b) (4)			
8				
12				
26				
39				
52				

Table 27

Stability Test 04 TEES004/29a 40ºC/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	280	350	<10	10
4	(b) (4)			
8				
12				
26				
39				
52				

Table 28

Stability Test 05 TEES004/11 25°C/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	170	240	<10	<10
4	(b) (4)			
8				
12				
26				
39				
52				

Table 29

Stability Test 06 TEES004/11 40°C/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	170	240	<10	<10
4	(b) (4)			
8				
12				
26				
39				
52				

Table 30

Stability Test 07 TEES005/28 25°C/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	10	20	70	<10
4	(b) (4)			
8				
12				
26				
52				

Table 31

Stability Test 08 TEES005/28 40°C/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	10	20	70	<10
4	(b) (4)			
8				
12				
26				
52				

Table 32

Discussion

Overall there is no significant change in the microbiology of the samples under test. There is an increase in total viable count (TVC) at weeks 26 and 52 for Stability tests 07 and 08. Nevertheless, more time points are required to determine if this is a significant trend. A high number of yeasts was observed at week 12 in Stability test 08. However, compared to the results from other time points in this test, it appears spurious in nature.
The microbiological activity in the samples will be tested again at the 78, 104 & 156 weeks time points.

Conclusion

The initial findings of the shelf life study show FeedKind® to have a stable amino acid and fatty acid profile. No significant change has been observed in the biogenic amines and microbiological components of the samples under test.

The final report will be issued when the shelf life tests have been completed.

Shelf Life Testing of FeedKind® Interim Micro Report

Introduction

Samples of FeedKind® have been stored under controlled conditions for 52 weeks. Samples remain under test conditions for each batch being tested and the final samples are expected to be removed from test after 156 weeks. The shelf life trial will be conducted over 156 weeks which is longer than the expected shelf life of FeedKind® and will generate sufficient data to accurately predict the shelf life of FeedKind®.

This interim report covers the microbiology results of the study to date.

Results

Stability Test 01 TEES004/29 25ºC/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	6100	170000	<10	40
4	(b) (4)			
8				
12				
26				
39				
52				
78				

Stability Test 02 TEES004/29 40ºC/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	6100	170000	<10	40
4	(b) (4)			
8				
12				
26				
39				
52				

Stability Test 03 TEES004/29a 25°C/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	280	350	<10	10
4	(b) (4)			
8				
12				
26				
39				
52				

Stability Test 04 TEES004/29a 40°C/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	280	350	<10	10
4	(b) (4)			
8				
12				
26				
39				
52				

Stability Test 05 TEES004/11 25°C/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	170	240	<10	<10
4	(b) (4)			
8				
12				
26				
39				

52	(b) (4)

Stability Test 06 TEES004/11 40ºC/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	170	240	<10	<10
4	(b) (4)			
8				
12				
26				
39				
52				

Stability Test 07 TEES005/28 25ºC/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	10	20	70	<10
4	(b) (4)			
8				
12				
26				
52				

Stability Test 08 TEES005/28 40ºC/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	10	20	70	<10
4	(b) (4)			
8				
12				

26	(b) (4)
52	

Discussion

Overall there is no significant change in the microbiology of the samples under test. There is an increase in total viable count (TVC) at weeks 26 and 52 for Stability tests 07 and 08. Nevertheless, more time points are required to determine if this is a significant trend. A high number of yeasts was observed at week 12 in Stability test 08. However, compared to the results from other time points in this test, it appears spurious in nature.

APPENDIX 2. Historic stability testing

Storage stability of BioProtein

V.4

(b) (4)

STORAGE STABILITY OF BIOPROTEIN

Summary

The storage stability of BioProtein containing about 6 percent of moisture have been monitored at 22 and 37 °C. The samples were stored in polyethylene bags the moisture content during the storage period of 64 weeks increased at 22 °C and decreased at 37 °C due to moisture diffusion. The content of fat, protein and the essential amino acids remained fairly constant during 64 weeks of storage. Free fatty acids increased from 13 to 19 % of total fat during the first 16 weeks and then remained constant for the last 48 weeks.

Samples and storage conditions

A 10 kg sample of bioprotein, label 93010635, was received from (b) (4)

The sample was divided into nine subsamples on January 6th 1993. One of the subsamples was analysed immediately and the others were packed in airtight-closed polyethylene bags and stored for later analysis. The bags were placed in thermostated incubators - four bags at 21-23 °C and the other four at 36-38 °C. One bag was removed from each incubator and analysed after storage for 4, 16, 32 and 64 weeks.

The bags was made from 0.07 mm polyethylene, which is permeable for oxygen and carbon dioxide, but is a fairly effective barrier for water vapour.

Analytical programme

The analytical programme comprised the nutrients: Protein, essential amino acids and fat, together with the deterioration products from fat: Free fatty acids and peroxides. The approved EEC-methods were used wherever possible as stated below:

-	Moisture	EF(71/393/EØF)
-	Crude protein	EF(72/199/EØF)
-	Crude fat (acid hydrolysis)	EF(84/4/EØF)
-	Cystine, methionine, threonine and lysine	Landbr.min.met. 1.2.1
-	Free fatty acids (FFA)	BI-medd. 2, 1980
-	Peroxide value	BI-medd. 2, 1980

(b) (4)

Results and discussion

The analytical results on BioProtein after storage at 22 and 37 °C is shown in Table 1 and graphed in figure 1. The last column in table 1 shows the analytical precision of the results measured as the standard deviation of repeated analyses of a reference sample during a long period of time. The precision of peroxide value is just estimated by experience, because it is impossible to keep a constant value in a sample for a long time.

The variance of the present results were compared to the analytical precision by a chi-squared test at a significance level of 0.05. Only moisture and free fatty acids showed a significant variation during the storage period.

The significant changes in moisture content show that the polyethylene bags were not entirely impermeable to water vapour. The samples absorbed 1 % moisture from the surroundings at 22 °C and lost 2 % moisture at 37 °C during 64 weeks. In order to obtain comparable values, all the other parameters are calculated on dry matter basis or as percentage of protein or fat.

Crude protein and the amino acids, cystin, methionine, threonine and lysine, remained fairly constant during the storage period of 64 weeks, irrespective of the storage temperature. This shows an excellent storage stability of the protein and essential amino acids, which constitutes the main part of the feed value of BioProtein.

The content of crude fat was determined by acid hydrolysis. The rather low value after 4 weeks of storage at 22 °C deviates significantly from the other values but the deviation is not significant compared with the analytical precision. If this low value is ignored, a slight decrease of fat content during the storage period is evident from figure 1 and it might be explained by a slow deterioration of the lipids in BioProtein.

Free fatty acids is determined by titration and calculated as oleic acid, but might comprise other acids produced by hydrolysis or oxidation of lipids. The results show significantly increasing values during the first 16 weeks of storage and then constant values for the next 48 weeks. The peroxide value is very low and remains constant or slightly decreasing during storage. This shows a satisfactory oxidative stability of the fat in BioProtein and the increased level of free fatty acids is probably not of any importance for the feed value.

TABLE 1. Storage stability of BioProtein at 22 and 37 °C - Analytical results.

Storage temperature, °C:		22				37				Precision of analysis
Storage time, weeks:	0	4	16	32	64	4	16	32	64	SEM
Moisture, %	6.2									
Crude protein, % in DM	72.1									
Crude fat, % in DM	10.3									
Free fatty acids, % of fat	12.8									
Peroxide value, meq/kg fat	2.3									
Cystin, g/16 g N	0.54									
Methionine, g/16 g N	2.60									
Threonine, g/16 g N	4.45									
Lysine, g/16 g N	6.04									(b) (4)

(b) (4)

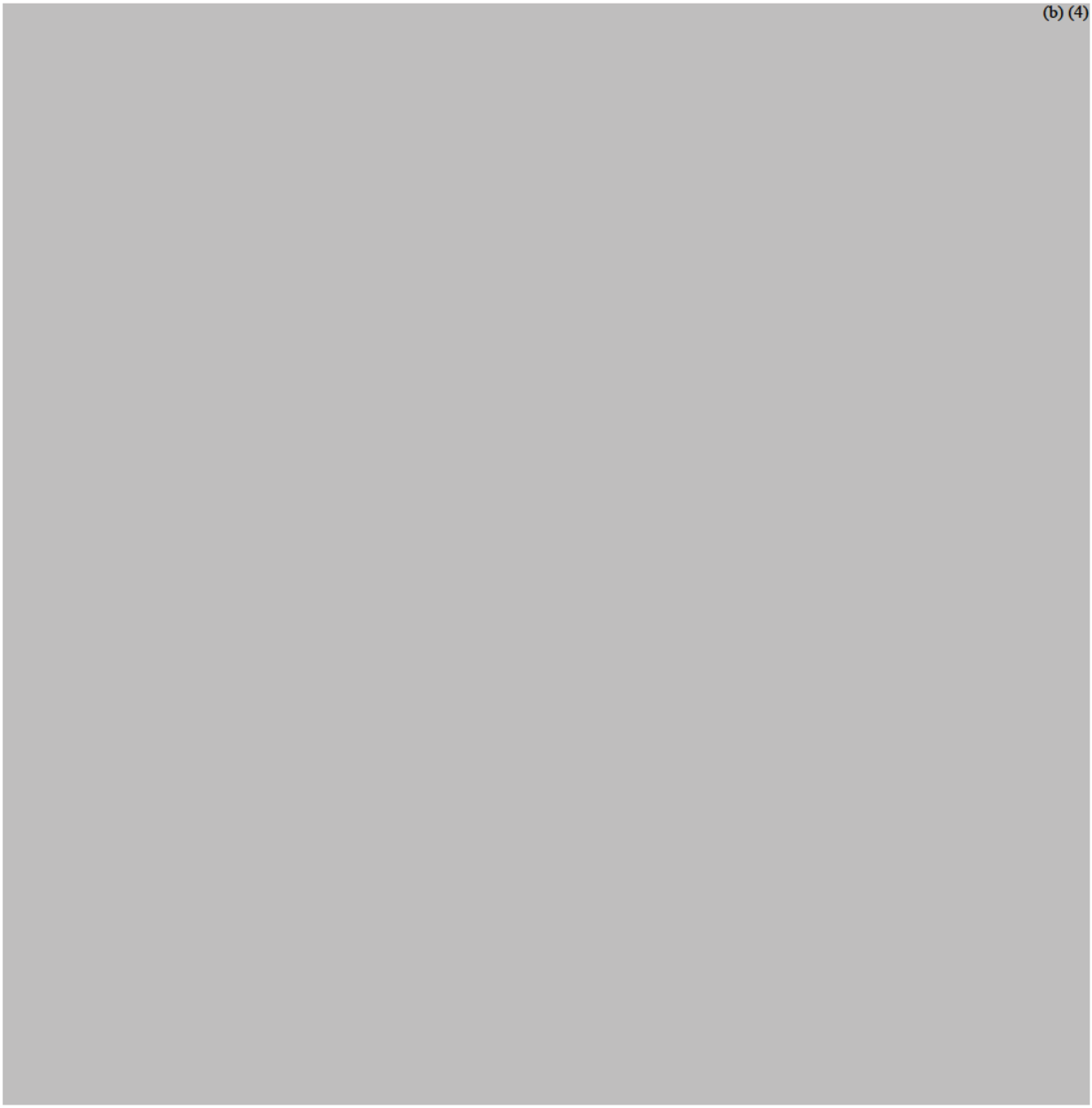

Figure 1. Quality of Bioprotein during storage at 22 C (solid line) and at 37 C (dotted line).

APPENDIX 3. 1995 Scientific Committee for Animal Nutrition report

First report of the Scientific Committee for Animal Nutrition on Question 69 by the Commission on the use of protein products of fermentation from natural gas obtained by culture of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans, Bacillus brevis, Bacillus firmus*, the living cells of which have been killed
(Opinion expressed on 28 April 1995)

TERMS OF REFERENCE (May 1994):

The Scientific Committee for Animal Nutrition (SCAN) and the Scientific Committee for Food (SCF) are requested to give their opinion on the following questions:

1. Does the protein product of fermentation from natural gas obtained by culture of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans, Bacillus brevis, Bacillus firmus,* the living cells of which have been killed, have a nutritional value for the animal because it provides nitrogen or protein?

2. Can the use in animal nutrition of the protein product of fermentation from natural gas obtained by culture of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans, Bacillus brevis, Bacillus firmus,* the living cells of which have been killed, result in risks for humans (consumer or user) or the animal health, or be prejudicial to the environment?

3. Does the use of the protein product of fermentation from natural gas obtained by culture of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans, Bacillus brevis, Bacillus firmus,* the living cells of which have been killed, harm the consumer by impearling the distinctive features of animal products?

4. Can the above-mentioned protein product be monitored in feedingstuffs?

BACKGROUND

The Council of the European Union, when adopting Directive 82/471/EEC[1] considered it essential, before including a new product in one of the groups listed in the annex of this directive, to establish that it has the required nutritional value and that, when used sensibly, it has no detrimental effect on human or animal health or on the environment; and does not harm the consumer by impairing the distinctive features of animal products. With a view to providing all necessary guarantees, the Community procedure adopted should in certain cases of amendment of the annex make provisions for the compulsory consultation of the Committees created by Commission Decisions 74/234/CEE[2] and 76/791/EEC[3].

In accordance with the provisions laid down in the Article 6, amendments to be made to the annex as a result of developments in scientific or technical knowledge shall be adopted by the Standing Committee of Animal Nutrition in accordance with the procedure laid down in Article 13.

According to this article the Commission representative shall submit to the Standing Committee a draft of the measures to be adopted, and the Standing Committee shall deliver

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1 Concerning certain products used in animal nutrition (O.J. No. L213, 21.07.82, p. 8)
2 Instituting a Scientific Committee for Food (SCF). (O.J. No. L136, 20.05.74, p.1)
3 Instituting the Scientific Committee for Animal Nutrition (SCAN). (O.J. No. L279, 09.10.76, p.35)

their opinion on the draft within a time limit set by the chairman according to the urgency of the matter, and shall decide by majority votes. In order to ensure that the product concerned complies with the principles set out in Directive 82/471/EEC[1], a dossier prepared in accordance with the provisions of Council Directive 83/228/EEC[4] should be prepared and, if requested, be the subject of consultation of members of the above-mentioned Scientific Committees set up by the Commission. This consultation is made compulsory for bacteria and yeasts by Article which 6 establishes that in the case of the products referred to in sections 1.1 (bacteria) and 1.2 (yeasts) of the annex, the Commission shall consult the SCF and the SCAN.

A request has been made to register the protein product of fermentation from natural gas obtained by culture of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans, Bacillus brevis, Bacillus firmus,* the living cells of which have been killed according to the conditions set out in the attached table. It should be noted that, a product of similar nature was examined previously by the SCAN, and the opinion of the Committee expressed on 23 September 1985[5] in a report established jointly by the Scientific Committee for Animal Nutrition and the Scientific Committee for Food on the use in animal nutrition of protein products obtained from bacteria of the *Methylococcaceae* family[6].

A first report on the submission for registration of the protein product of fermentation from natural gas was considered both by the SCAN and the SCF in May 1994. In this report the documentation submitted in March 1993 was reviewed and certain additional information was requested by both committees at that time before a final answer to question No. 69 could be given to the Commission. A first supplementary dossier was provided in November 1994 which answered some of the questions of the committees. At the same time the submission was changed by restricting the use of the protein product in the feed of young growing animals, e.g. piglets, calves and fish in the early stages of growth. No application for the use in chicken was made, although data for this species were included in the original submission. Even this first supplementary dossier left some of the questions previously put by the SCAN and the SCF inadequately answered. The points still at issue were again transmitted to the submitter and a further supplementary dossier was submitted in February 1995. All additional information now supplied is included in this final report.

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4 On the fixing of guidelines for the assessment of certain products used in animal nutrition. (O.J. No. L126, 13.05.83, p. 13)

5 Fifth Series 1986; Report EUR 1041EN. Catalogue N° CD-NK-86-003-EN-C. p.51

6 In particular from *Methylophilus* cultivated in methanol (Pruteen)

Proposal for inclusion into the annexes of Directive 82/471

1	2	3	4	5	6	7
Name of product group	Name of Product	Designation of nutritive principle or identity of the micro-organism	Nutrient substrate (specifications if any)	Composition characteristics of the product	Animal species	Special provisions
1.1. Bacteria 1.1.2Bacteria cultivated on natural gas	1.1.2.1 Protein product of fermentation from natural gas obtained by culture of: *Methylococcus capsulatus* (Bath) *Alcaligenes acidovorans*, *Bacillus brevis*, *Bacillus firmus*, - the cells of which have been killed	*Methylococcus capsulatus (Bath)* NCIMB strain 11132, *Alcaligenes acidovorans* NCIMB strain 12387, *Bacillus brevis* NCIMB strain 13288, *Bacillus firmus* NCIMB strain 13280	Natural gas, (91% methane, 5,1% ethane 1,9% propane 0,4% isobutane 0,5% n-butane 1,1% other minor components), ammonia, mineral salts	Nitrogen expressed as crude protein minimum 65%	- Pigs - Calves - Fish	Declaration to be made on the label or packaging of the product - the name: "Protein product obtained by fermentation of natural gas" - nitrogen expressed as crude protein - crude ash - crude fat - moisture content - instructions for use - declaration of the words "avoi inhalation" - For each animal species: recommended and maximum inclusion level expressed as percentag of the total nitrogen content of th complete feedingstuffs Declaration to be made on the label or packaging of the com-

						pound feedingstuffs: -the name: "Protein product obtained by fermentation of natural gas" - amount of the product contained in the feedingstuff"

OPINION OF THE COMMITTEE

The protein product presently being considered is another example of an edible bioprotein produced by cultivation of bacteria of the Methylococcaceae family together with certain other bacteria, for which product data on performance in livestock feeding have become available to the Committees and for which appropriate dossiers were supplied. BioProtein* is obtained by growing *Methylococcus capsulatus* (Bath), strain NCIMB-11123, aerobically on natural gas composed of 91% methane, 5.1% ethane, 1.9% propane, 0.4% isobutane, 0.5% n-butane and 1.1% other minor components. The bacteria oxidise methane through intermediate steps ending with CO_2 and thereby form a biomass. Ammonia is used as nitrogen source and appropriate mineral salts are added to satisfy the culture requirements.

The oxidation products arising during the fermentation process can inhibit, however, the growth of *Methylococcus capsulatus*, and it is therefore necessary to include other bacteria in the production line, which are able to utilise these inhibitory oxidation products. These are *Alcaligenes acidovorans DB3* (strain NCIMB 12387), *Bacillus brevis* DB4 (strain NCIMB 13288) and *Bacillus firmus DB5* (strain NCIMB 13280). These 4 bacterial strains form an ecosystem which in part ensures a stable production performance and in part protects the fermenting culture against unwanted contamination with other bacteria by occupying all niches which might be available to intruding micro-organisms.

Nutritional value

The protein product is marketed as a free-flowing non-dusty agglomerate (particle diameter 100-300 im). The crude protein content (Nx6.25) of the product is approximately 70% on a dry weight basis and includes minor contributions of nitrogen compounds other than protein, notable nucleic acids. The true protein content is 66% of dry weight. It also contains 9% crude fat, 7% crude ash, 7% nucleic acid, and a maximum of 100mg/kg copper. The N-free fraction is composed of 4.5% glucose, 2.4% starch and variable amounts of unidentified cellwall polysaccharides, and about 7% crude ash. The amino acid pattern, typical of a single cell protein, consists of lysine 6.5, methionine 2.8, methionine-cysteine 3.4, threonine 4.7. tryptophan 2.2, arginine 6.2, tyrosine 3.4 g/&§g N and a number of others in smaller amounts.

The true digestibility of the protein is 78.5% and the biological value 84% as determined in the rat. The NPU (net protein utilisation) is about 66% of the biological value with PU (protein utilisation = NPU x protein content) being 44.7%.

Further information on the amino acid profile of the protein product and their digestibility has now been supplied. the protein product is nutritionally characterised by a high protein content and a well balanced amino acid profile compared with other protein sources used in animal feeds.

The content of essential and semi-essential amino acids has been compared with that of fish meal, meat and bone meal, meat meal, blood meal, soybean meal, soyprotein concentrate and rape seed meal. The levels of methionine and cystine, the S-containing amino acids, and the lysine and arginine are somewhat lower, while the levels of threonine, tryptophan, leucine, isoleucine and phenylalanine are higher than those found in fishmeal (Pedersen et al., 1994).

The digestibility of the amino acids has also been studied in mink, pigs and poultry (Skrede *et al.*, 1994) and values for veal calves and salmon are supplied in the second supplementary dossier (February, 1995). The D-amino acids are most likely to derive from the peptidoglycan murein that is present in the bacterial wall. They do not present a nutritional problem for the target species.

Information has now been supplied on the composition of the lipid fraction of the protein product. New data were obtained by using gas chromatography combined with mass spectrometry and a full list of all fatty acids present including cyclopropanoic acid (cycC17:0 or 9,10 methylene hexadecanoic acid) is now available. The corrected cyclopropanoic acid content is now calculated to be about 1.6%. In a three months feeding study in pigs no accumulation of cyclopropanoic acids was demonstrable in the body fat. The content of n-3 fatty acids in fish fed the protein product shows only a small reduction in C 22:5 and C 22:6 fatty acids when the diet included 37% of the protein product, allowing the conclusion that the cyclopropanoic acid does not interfere significantly with the elongases and desaturases involved in the synthesis of the nutritionally important n-3 fatty acids in salmon fed the protein product.

Apart from the sensory evaluation of chicken meat which was supplied in the original submission sensory evaluation data have now been made available for salmon fed for 2 years four diets containing the protein product and also for the back fat of pigs fed the protein product for 100 days. In neither instance was a negative effect on the quality of the edible products from animals and fish fed the protein product noted.

The protein product has been tested for its efficacy and nutritive value only in trials conducted under laboratory conditions. No field trials with pigs and veal calves are available, and no trials at all with calves starting at birth weight The adverse effect of 12% inclusion for veal calves is probably the result of nutritional imbalance. The protein product is proposed to be used at 8% in the feed of calves as a replacement of some of the skimmed milk in milk feeds. An experiment was carried out over 10 days to study the acceptability and functionality of calf milk replacers containing 10%, 20% and 30% protein product.

The results showed no acceptability problems arising even with a dietary incorporation of 30% bioprotein. But these experiments were not carried out with calves at birth weight. The protein product was, however, found to be unstable in calf milk replacers and caused faeces to be stiffer and to have an aberrant grey green colour.

In a second experiment on the digestibility of including either 10% or 20% of the protein product in calf feed there was a significant reduction in apparent digestibility of the crude protein (72% against 92%) and a similar reduction in the digestibility of carbohydrates (38% against 98%) but no effect on the digestibility of fat (95%) compared to the control diet. Only at the 10% inclusion level were daily weight gains and feed conversion ratios comparable to the control diet. The digestibilities of the amino acids were well below (10%-20%) those of skim milk.

In a third combined balance and growth study in calves 4%, 8%, 12%, 18% and 24% of the protein product were used in combination with 44%, 38%, 32%, 26% and 14.6% skimmed milk powder.

The feeds were also corrected for lysine, methionine and threonine content. Inclusion levels above 12% reduced the digestibility of the test diets. Weight gain, nitrogen retention, metabolisable energy content (2.8 Mcal/kg bioprotein against 4.5 Mcal/kg casein) and feed conversion ratio were reduced.

The nutritive value of the protein product for pigs in terms of protein and energy value was studied in piglets and compared with that of fishmeal. In a second experiment the protein digestibility of the bioprotein was 72% and the net energy was 8.96 MJ/kg.

In a 28 day study, using 120 weaned piglets aged 28 days, either 4%, 8% or 12% of the protein product were included in the feed. Daily weight gain was highest when 4% bioprotein were included (1 kg bioprotein replaced 0.85 kg LT fishmeal + 0.15 kg cereals, extra lysine was added to meet Danish recommendations). Inclusion of 12% yielded a reduced feed conversion ratio. These results support the inclusion of 8% protein product in pig feed as replacement for fishmeal, a level causing no adverse effects on the performance and health of piglets. However no dose-response studies over longer periods are available and no large field trials exist with 8% inclusion levels in pig feeds in EU countries.

The nutritive value of bioprotein for Atlantic salmon was studied in several trials. Fish weighing 70 g were maintained in seawater and fed feed with inclusion levels of 6.5%, 13% and 26% of the protein product. Growth was good and showed no difference in daily weight gains and feed conversion ratio between the groups. The highest inclusion level produced a tendency for reduced growth but no explanation is given for this effect.

In another dose-response trial in fresh water using fish of average weight of 650 g, up to 36% replacement of fish protein by the protein product caused no significant depression in growth rate compared to controls. Feed conversion ratio was also unaffected and no pathological effects attributable to the bioprotein were noted. At higher inclusion levels weight gain and feed conversion ratio were impaired.

Significant differences in growth were observed with atlantic salmon, in sea water with 19,3% of the protein product inclusion against 36% inclusion in the diet for 14 months from first feeding. Optimal inclusion levels averages 20%. This means that 25% of dietary amino acids can be provided by the protein product.

In a trial on juvenile fish weighing 60 g the protein and amino acid digestibility decreased linearly with increasing inclusion levels of 5%, 9.9%, 19.3% and 37% of the protein product. Fat digestibility and metabolisable energy of the diet also decreased but starch digestibility increased. The palatability of the composite feed was as good as that of the conventional fishmeal diet.

In a further diet performance trial over 4 periods, each of 28 days, in juvenile salmon weighing 0.2 g similar growth was obtained with up to 20% protein replacement by the protein product. Inclusion of 37% reduced growth and caused higher though unexplained mortality during the starting feeding period. The digestibility of the protein product was calculated to be 79%, of the fat 78% and of the starch 68%. There is insufficient information about the metabolism of the non-protein nitrogen fraction to estimate the metabolisable energy or net energy.

In another test using salmon weighing between 50 and 64 g there was no difference from controls with regard to growth and digestibility of the protein product for the first month. After 3 months the group fed the protein product showed better growth and better digestibility of amino acids. No adverse effects were seen with inclusion of 19% after 6 months. Fin erosion was noted in all groups.

The inclusion level of 19-20% in salmon diets (this means an inclusion of 14% protein) from Bioprotein seems the optimum. Seawater fishes support 35% inclusion level without differences in growth rate and feed conversion rate.

On the basis of the foregoing information the Committees concluded that the protein product appears to have a good nutritional value and to be an adequate protein source for fish. In pigs and calves adverse results occurred when the inclusion in feeds exceeds certain values.

The role of the comparatively high nucleic acid content and the high ash content is unclear and with some species, such as chicken, a palatability problem is apparent. No quantitative information has been supplied on the presence of D-amino acids but their presence is not regarded as of nutritional importance. The available efficacy trials are few in number, are only carried out in experimental groups, and are of short duration. No field trials have been reported. There are no efficacy trials in calves at birth weight nor in piglets weaned before 28 days of age.

Evaluation of risks

Methylococcus capsulatus (Bath), the bacterial strain used for the production of the protein product, is the type species for the family of Methylococcaceae. It represents about 90% of the stock culture. It occurs naturally in aerobic environments where methane is available. Growth occurs at temperatures between 37°C and 52°C. It is not known to be pathogenic or toxicogenic. Because it grows only on methane and at high temperatures pathogenic effects cannot be expected.

Alcaligenes acidovorans DB3 belongs to the family of *Pseudomonadaceae* and represents about 8% of the stock culture. The description *Comamonas acidovorans DB3* does not appear in Bergey's Manual (1984). This bacterium also occurs naturally and it is not known to carry virulence plasmids. The non-pathogenicity of this bacterium is further supported by the results of experiments carried out on mice by i.v. injection of 10^4 - 10^9 viable cells/kg b.w. Weight gain was not influenced by this treatment. No treatment-related macroscopic pathological changes were observed.

Bacillus brevis DB4 belongs to the genus Bacillus, but the species used is heterogeneous. It represents about 1% of the stock culture. The strain used in the manufacture of the protein product also occurs naturally and does not carry any known virulence plasmids. The non-pathogenicity is supported by the results of experiments carried out on mice by t.v. injection of 10^4 - 10^9 viable cells/kg b.w. There were no adverse effects on weight gain. One mouse died with signs of shock following the i.v. injection. No treatment-related macroscopic pathological changes were observed. However, food poisoning has been performed with a suspension of washed bacteria and oral test for toxin production is needed.

Bacillus firmus DB5 belongs to the genus Bacillus and the species used is heterogeneous. The strain occurs naturally and does not carry any known virulence plasmids. It represents about 1% of the stock culture. the non-pathogenicity is supported by the results of experiments on mice using i.v. injection of 10^4 - 10^9 viable cells/kg b.w. There were no adverse effects on weight gain and no mortalities. No treatment-related macroscopic pathological changes were observed.

The protein product stock and production cultures are kept freeze-dried and are regularly tested for composition and for the presence of pathogenic contaminants. Pathogenic risks for man or animals can only be expected, if viable cells of the production strains escape the fermenter or the sterilization procedure of the final product is inadequate. Because the optimum temperatures of the bacterial strains used all exceed 37°C and of the need for special growth requirements as well as the experimental evidence of non-pathogenicity it is unlikely that these bacteria can act as human or animal pathogens.
Heat-stable toxic substances are not expected in the sterilised product because of the absence of adverse effects in the feeding trials with calves, piglets, chicken and salmon.

The protein product is a sterile product as concerns the bacteria used for production of the biomass. The commercial product is highly contaminated but the type and number of contaminating micro-organisms do not differ from those found in similar bioproteins.

All production strains are genetically stable and unlikely to mutate into antibiotic-producing or toxin-producing strains. The fermentation product is regularly checked for the presence of toxins and antibiotics. The protein product does not constitute a microbiological risk to animals or man. Accidental release into the environment will have no deleterious consequences as all organisms of the production culture are already present in natural habitats. Nevertheless, the *Bacillus brevis* strain used should be tested orally to exclude the possibility of toxin production.

No immunological monitoring of workers exposed to the protein product has been carried out. No data have been supplied on possible exposure to dust from the spray-dried biomass or the agglomerated product.

Effects in target species

A feeding study in calves, starting at weight 110 kg, extending over 7 weeks, with 4%-24% protein product in the diet produced no significant toxicological effects, although inclusion levels at and above 18% reduced weight gain and feed conversion ratio and caused difficulties with digestion. The latter showed itself as faeces of reduced stiff consistency and a reduced feed intake. Apparent faecal digestibility coefficients of dry matter, ash, organic matter, crude protein, crude fat, carbohydrates, and iron decreased with increasing inclusion percentage. Nitrogen utilisation decreased similarly. Utilisation of ingested iron increased with increasing inclusion of the bioprotein. No clinical parameters were reported. The NEL appeared to be the inclusion level of 12% in the diet.

A feeding study in piglets, aged 28 days and weighing 25 kg, extending over 4 weeks, with 4%-12% protein product in the diet showed no significant effects on health, appetite and daily weight gain. At the 12% inclusion level the feed conversion ratio was significantly smaller. No

clinical parameters were reported. The NEL appeared to be a replacement level of 8% fishmeal in the diet.

Some 5 feeding studies in salmon, extending over 14-20 weeks, with 5%-70% protein product in the diet showed good growth and no effect on the feed conversion ratio up to the 33% inclusion level. No signs of hepatotoxicity attributable to the protein product were noted. The palatability test indicated a preference for the diets containing the protein product but the digestibility of protein and fat were reduced with increasing incorporation levels.

From these data it can be concluded that the inclusion of the protein product in the feeds of the above 4 animal species carries no appreciable risks for animal health, when added at a rate of up to 12% in the diet of calves weighing 110 kg but not at that level as milk protein replacer in calves at birth weight, up to 8% in the diet of pigs weighing 25 kg but not at that level in suckling pigs, up to 3% in the diet of broiler chicken, and up to 33% in that of salmon.

The statement, that in none of the feeding trials did any health problems, related to a disturbance in the microbial flora of the gut, arise is not substantiated by any experimental evidence.

Effects on the quality of animal products

As the protein product actually used as additive contains only normal feed ingredients, e.g. proteins, fat, carbohydrates and minerals, no toxic or other adverse residues are assumed to be present in the edible animal products. Tests for the presence of possible antibiotics and known toxins are carried out routinely on the added protein product. The organoleptic properties of the meat obtained from animals fed the protein product have been tested and found to be satisfactory.

Effects in laboratory animals

The nutritional studies provide evidence that this protein product is metabolized in the same way as conventional proteins.. Hence rigorous toxicological, metabolic and residue studies appear not to be meaningful.

No available data show that the bacterial strains used in production do not elaborate toxins and are not pathogenic. The analysis of the chemical composition of protein product has shown that nitrosamines are not present at the detection limit of 0.3ig/kg dry matter, and that methanol and polycyclic aromatic hydrocarbons are not present at the detection limit of 5 mg/kg dry matter. No significant toxicity was noted in the feeding studies in target animals.

A 4-week oral feeding study in rats was carried out using 5 groups of Wistar rats, each of 5 males and 5 females, fed a standard diet containing added 0%, 5%, 10% and 15% of the protein product. A positive control group received added 15% of a standard protein. Only a few incidental clinical changes were noted. Bodyweight gain of all groups was comparable to controls. There were no consistent differences between the groups regarding food consumption. Serum urea levels of males and females in the top test and positive control groups were increased. Serum creatinine levels in females of these groups were reduced. The relative kidney weights of the 10% and 15% (females only) test groups and the positive control group were increased. These changes were the expected consequences of high protein intake. Gross pathology and limited histopathology showed no treatment-related changes.

A 90-day oral feeding study in rats was carried out using 5 groups of Wistar rats, each of 10 males and 10 females, fed a standard diet with added 0%, 5%, 10% and 15% of the protein product, the positive control group receiving added 15% standard protein.

No adverse clinical symptoms related to treatment were noted. Bodyweight, food consumption, water consumption and food conversion ratios were comparable to controls. Haematology was unremarkable.

The two top test groups showed a small rise in serum alanineaminotransferase levels indicating slight hepatocellular dysfunction. Serum ornithine carbamyltransferase levels were unchanged and hepatic histology was normal. Serum urea levels were increased in the top test and positive control groups. Urinary excretion of N-acetyl-Â-D-glucosaminase was increased in the top test groups and in males of the 10% test and positive control groups without any associated renal histopathology. This slight leakage of renal tubular enzymes was probably due to the high protein load but an additional toxic effect of the protein product cannot be excluded. The increase in female relative kidney weights of the top dose were probably related to the higher bodyweight. Gross and histopathology showed no treatment-related findings.

A 90-day oral feeding study in minipigs was carried out in 5 groups, each of 4 male and 4 female minipigs, given a standard diet with added 0%, 5%, 10% and 15% of the protein product, the positive control group receiving a corresponding high-protein diet.

There were no adverse clinical signs and no toxicologically significant changes in bodyweight, food consumption, haematology, clinical chemistry, urinalysis and organ weights compared to controls. Gross pathology and histopathology showed no treatment-related changes.

No studies have been carried out in laboratory animals on multigeneration-reproduction, teratogenicity, chronic toxicity and carcinogenicity. However, no toxic effects were noted on the reproductive organs in the laboratory animals examined. The submitter has undertaken to carry out reproduction studies, if use of the protein product is to be extended to animals capable of reproduction.

The protein product was examined for genotoxicity in a salmonella reverse mutation test using strains TA98, TA100, TA1535 and TA1537 +/- S9 and dose levels varying fro 0.63mg to 10 mg/plate. No increase in revertants was found, thereby confirming absence of mutagenic activity in this system.

The protein product was also tested in a mouse micronucleus test at dose levels of 1.25 g, 2.5 g and 5 g/kg b.w. No significant increase in polychromatic erythrocytes with micronuclei was seen, thereby confirming absence of mutagenic potential in this test system.

The absence of significant toxicity including genotoxicity and the anticipated digestive breakdown of the protein product appear to indicate that it is toxicologically safe as protein source in animal feeds at the proposed levels. Further animal testing is not deemed necessary.

Protective measures against dust inhalation for the production workers and the users are recommended pending information to be provided as to the allergenic potential of this bioprotein.

Effects on man

Skin and eye irritation potential was examined in rabbits and showed that the protein product was non-irritant to the skin and eyes. The allergenic potential of the protein product has not been investigated.

Effects on the environment

The protein product contains no substrate residues or heavy metal contaminants except for a maximum copper content of 100 mg/kg, dry matter. It carried no viable production organisms because of the sterilisation step in the production of the final product. Any escape into the environment of the production organisms from the fermenter causes no hazard as these organisms all occur naturally, are non-pathogenic, and carry no virulence plasmids.

Monitoring in foodstuffs

The protein product can be determined in feedingstuffs qualitatively by indirect immunofluorescence using antibodies specific against *Methylococcus capsulatus* (Bath) and quantitatively by a spectrofluorimetric method with a sensitivity better than +/- 1%. Techniques to determine the proportions of each of the constituent organisms are not described in the dossier.

Conclusions

The protein product obtained from methylotrophic bacteria has been the subject of a basic dossier and two supplementary dossiers prepared in accordance with the guidelines for the assessment of certain products used in animal nutrition. This report is therefore limited to the assessment of this particular protein product, in the light of the information provided.

The product examined has an acceptable nutritional value as a source of protein for feeding to animals provided the inclusion levels in feeds do not exceed the values set out in the suggested conditions of use. It is not suitable for ewe milk replacers and further extensions should fill the remaining gaps in the general nutritional information.

On the base of the information provided by the Firm, the product examined carries no appreciable risks for livestock, if the levels of incorporation do not exceed 8% in the ration of pigs starting at weight 25 kg, does not exceed 8% in the feed of veal calves starting at weight 80 kg, does not exceed 19% in the feed of freshwater salmon and 33% in the feed of seawater salmon up to 3 years. There were no data to protein level of inclusion in the feed of piglets and non ruminant calves.

It poses no appreciable risk on presently available evidence for the health of workers involved in its production, distribution and use, if adequate precautions are taken to prevent exposure to dust. Because the allergenic potential of the bioprotein has not been investigated a warning should be included on the label, that the dust may cause sensitisation by inhalation and may give rise to respiratory allergic reactions in susceptible people.

It carries no microbiological risks because of its origin from non-pathogenic naturally occurring bacteria, known not to produce antibiotics or toxins and not being present as viable organisms in the final product because of its sterilisation.

It has no adverse toxicological or genotoxic effects although reproductive toxicology, teratogenicity, chronic toxicity and carcinogenicity have not been specifically investigated in laboratory animals, and is free from harmful contaminants arising from the culture medium or manufacturing process but can contain up to 100 mg/kg dry weight of copper, which would be diluted when mixed into composite feeding stuffs. Its use in animal feed does not result in appreciable risks for the environment.

It carries no appreciable risk for the consumer from the consumption of products obtained from animals fed with a diet containing this protein product. The characteristics and organoleptic properties of such animal products from chicken, pigs and salmon have been investigated and show no deleterious properties.

It can be monitored in feedstuffs, although no techniques are described to determine the proportions contributed by each of the constituent 4 bacteria to the biomass.

Suggested conditions of use

During the examination of the registration files it has been observed growth depression in some target animal species, and that, based upon the information provided by the firm, it exists a lack of data concerning the metabolisms of cyclopropanoics and other non identified fatty acids, that are present in the product and may be present in the animal product lipids.

Further to these observations, the Committee has judged that it will be prudent to limit the conditions of usage of this product as follows:

- Growing pigs from 25 kg up to a 100 kg live weight.

- The quantity of inclusion in complete feedingstuffs should no exceed
  - 8% for piglets starting at 25 kg
  - 8% for veal calves starting at 80 kg
  - -19% for salmon fish in fresh water
  - -33% for salmon fish in seawater

- The amount of protein provided by the product should be expressed as percentage of the total protein content of the compound feedingstuffs

These declarations are to be made on the label or packaging of the compound feedingstuffs, and should be introduced in the usage conditions requested by the company (See annexed table)

Future extension of use to other species or type of animals

If in the near future the firm asks for an extension of use to other species or type of animals providing edible products to the human consumer the following information should be provided.

For chickens for fattening, data concerning the availability of the individual aminoacids at different inclusion levels to be able to elucidate the cause and mechanisms of the fall in performances and sufficient results to assess the optimum inclusion level to exclude nutritional imbalances from an overdosage of the product in their diet. For pigs: more field trials are required

For all animals providing edible products to the consumer, analysis of the true content of cyclopropanoic acid in their lipids, instead of the expected content by calculation.

References

Dossiers on BioProtein*, Sections I-IV (1994) by Dansk Bioprotein A/S, Denmark
Pedersen A.-T., Skrede A., 011i J. & Eggebo L.M. (1994) Report submitted Oct. 15, 1994.
Skrede A., Herstad O., Sundstol F., Overland M. & Mroz, Z. (1994) Report submitted Oct. 15, 1994.
Supplement (VII) dossier by Danks Bioprotein A/S (1995).

Suggested conditions of use

1	2	3	4	5	6	7
Name of roduct group	Name of product	Designation of nutritive principle or identity of the micro-organism	Nutrient substrate Specifications if any	Composition characteristics of the product	Animal species	Special provisions
1. Bacteria						
1.2. Bacteria ıltivated on ıtural gas	1.1.2.1 Protein product of fermentation from natural gas obtained by culture of: *Methylococcus Capsulatus* (Bath) *Alcaligenes Acidovorans*, *Bacillus brevis*, *Bacillus*	*Methylococcus capsulatus* (Bath) NCIMB 11132, *Alcaligenes acidovorans* NCIMB 12387, *Bacillus brevis* NCIMB 13288, *Bacillus firmus* NCIMB 13280	Natural gas, 91% methane, 5,1% ethane 1,9% propane 0,4% isobutane 0,5% n-butane 1,1% other minor components), ammonia, mineral salts	Nitrogen expressed as crude protein minimum 65%	- Pigs from 25 kg - Calves from 80 - Salmon Fish.	Declaration to be made on the label or packaging of the product: - the name: "Protein product obtained by fer- - nitrogen expressed as crude protein - crude ash - crude fat - moisture content - instructions for use - declaration of the words "avoid inhalation" - The quantity of inclusion in complete - 8% for piglets starting at 25 kg - 8% for veal calves starrting at 80 kg - 19% for salmon fish in fresh water - 33% for salmon fish in sea water Declaration to be made on the label or packaging of the compound feedingstuffs:

	firmus, -the cells of which have been killed					- the name: "Protein product obtained by fer- - Amount of the product contained in the - Amount of protein provided by the product

APPENDIX 4. Genome sequencing report for DB3, DB4, DB5

CALYSTA REPORT

2019-10-28

Genome Assembly of DB3, DB4, DB5

By (b) (4)

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Summary

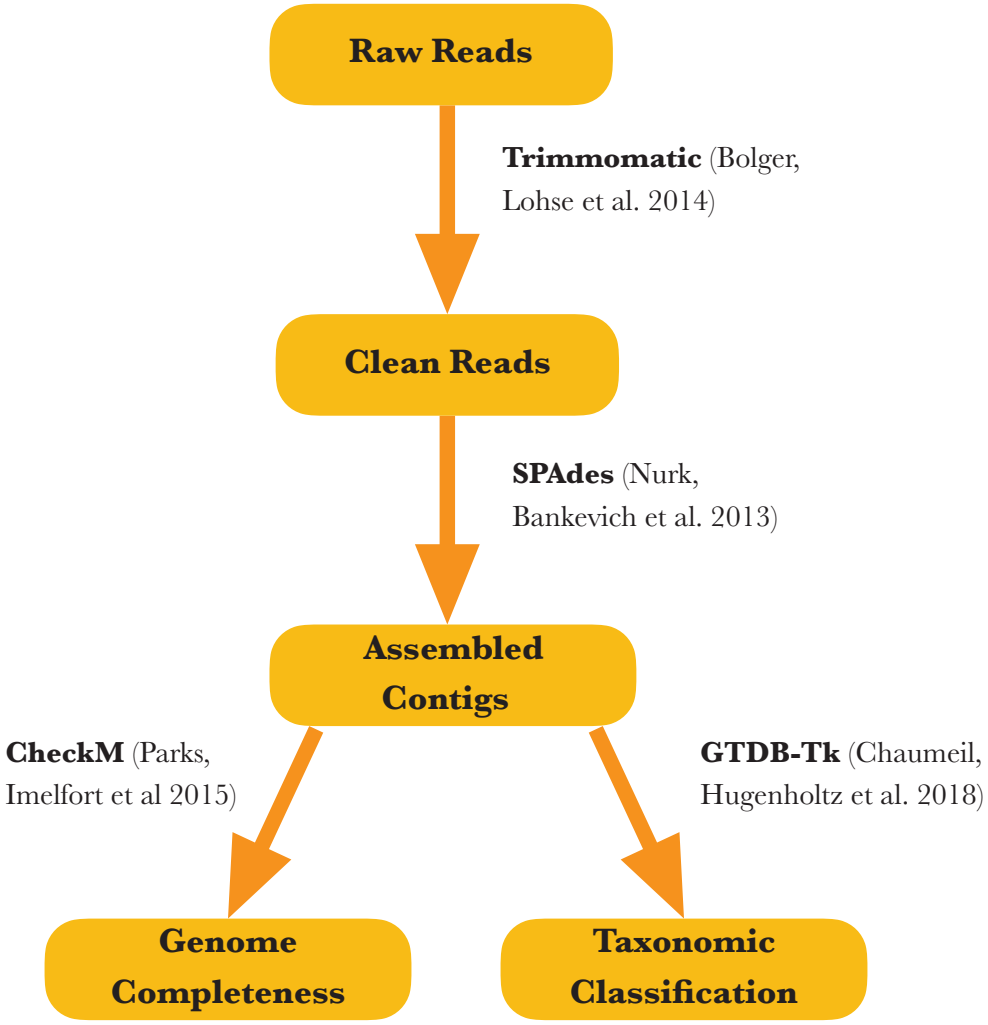
The datasets consisted on three genomes (**DB3**, **DB4**, and **DB5**). Each of these genomes was sequenced by (b) (4), using the (b) (4) platform, with 2x150bp reads.

This reports shows the results of the *de novo* assembly and phylogenomic classification of the three genomes. In addition, each genome was compared to a previously selected reference genome (**DB3**, *Cupriavidus gillardii*. **DB4**, *Brevibacillus brevis*. **DB5**, *Brevibacillus agri*).

Using *de novo* assembly, we were able to assemble the three genomes with good results. Based on the presence of single copy gene markers all of the genomes were sequenced to completion. Phylogenomics analysis allowed the taxonomic classification of each genome to the best possible taxonomic category. **DB3** was classified up to the genus level (*Cupriavidus*). **DB4** to the species level, where the most similar species was *Anaeurinibacillus* sp002375825, a genome assembled from a metagenome dataset but with no reported isolate. **DB5** was classified up to the species level, as *Brevibacillus agris*.

Average nucleotide identity (ANI) analysis of the three genomes with the selected references, showed that **DB3** had a 91.95% mean ANI value with *Cupriavidus gillardii*, supporting the result that **DB3** is a new species within the *Cupriavidus* genus. **DB4** had a 74.61% mean ANI value with *Brevibacillus brevis*, which strongly suggest that both genomes come from taxonomically unrelated microorganisms. **DB5** had a 99.56% mean ANI value, supporting the classification of this genome as a strain of *Brevibacillus agri*.

Analysis Workflow



Genome Assembly Results

Table 1 shows the assembly results for the three genomes. Overall, the assembly results are good. Because all of the genomes were sequenced using short reads (Illumina sequencing), is not possible to assembled them into a single chromosome.

Table 1. Genome assembly statistics

	DB3	DB4	DB5
Total N° contigs	673	470	520
Total N° contigs, over 500 bp.	71	178	160
Largest contig	375,320	154,135	446,056
Total length (all contigs)	5,930,539	4,589,507	5,492,175
Total length (contigs over 500bp.)	5,777,668	4,521,391	5,407,915
N50	163,990	52,744	84,594
L50	12	27	15
%GC	68	46.61	53.7

Based on the CheckM (Parks, Imelfort et al. 2015) results (which checks for the presence of single copy genes in the assembled genome), all of the genomes were sequenced to completion (Table 2), with low levels of contamination, which suggest that in all of the cases there was no contamination from another organism in the sequence information.

Table 2. CheckM results

	DB3	DB4	DB5
Genome completeness (%)	99.89	99.20	99.73
Contamination (%)	4.96	1.64	1.6

Taxonomic Analysis

Taxonomic classification of each genome was done using GTDB-tk (Chaumeil, Hugenholtz et al. 2018), which uses 120 bacterial markers to perform a phylogenomic analysis and compare the genome of interest against a collection of 145,904 genomes from the Genome Taxonomy Database (Parks, Chuvochina et al. 2018). The results (Table 3)

	DB3	**DB4**	**DB5**
Phylum	Proteobacteria	Firmicutes	Firmicutes
Class	Gammaproteobacteria	Bacilli	Bacilli
Order	Burkholderiales	Anauerinibacillales	Brevibacillales
Family	*Burkholderiaceae*	*Anaeurinibacillaceae*	*Brevibacillaceae*
Genus	*Cupriavidus*	*Aneurinibacillus*	*Brevibacillus*
Species		*Aneurinibacillus* sp002375825	*Brevibacillus agri*

DB3

In the case of **DB3**, the best classification that can be achieved using this method was up to Genus level, in this case to *Cupriavidus*. This suggest that this genome may represent a novel species within the *Cupriavidus* genus, as no similar species was found using a phylogenomic approach.

An additional comparison against a reference genome for *Cupriavidus gilardii* was performed, to confirm that **DB3** is indeed a different species. Figure 1 shows the mapping of all **DB3** against the genome of *C. gilardii* CR3 .

The alignment shows that although there are reads that map to the genome, the coverage is uneven (in particular in the second chromosome). In addition the coverage is a multiple levels of sequence identity (from 75% going up), suggesting that the genomes are similar (probably from the same genus), but are not closely related. For example, if we want to classify **DB3** as a strain of *C. gilardii*, we should expect a higher and even coverage of all the genome (both chromosomes), and also at higher identity levels (ideally close to a 100%). This is not observed on Figure 1, supporting the phylogenomic results that suggest that **DB3** is a novel species within the *Cupriavidus* genus.

Figure 1. Mapping of the **DB3** reads against the two chromosomes of *C. gilardii*.

Another analysis that can be performed to confirm the observation that **DB3** is a novel species, is to directly compare their genome sequences. Using a metric called average nucleotide identity (ANI) (Goris, Konstantinidis et al. 2007), we can compared both genomes and determine the average nucleotide identity between them. In general, values above 95% can be considered to be genomes that belong to the same species. In this case (Figure 2), the ANI value between **DB3** and *C. gilardii* is only 91.95%, also supporting the classification of **DB3** as a novel species within the *Cupriavidus* genus.

Figure 2. Distribution showing the frequency of hits at different identity thresholds for the genomes of **DB3** and *C. gilardii*. The results shows that the mean average nucleotide identity between the two genomes is 91.95%.

DB4

The phylogenomic analysis allowed classification of this genome up to the species level. The closest species found was a genome called *Anaeurinibacillus* UBA3580. This correspond to a metagenome-assembled genome, a sequence obtained by assembling and curating metagenomic datasets, and not from an actual isolate. This genome was generated in a recent study (Parks, Rinke et al. 2017), by assembling more than 8,000 metagenomic samples. Because of the large-scale nature of that study, it is difficult to track the origin of the sample were *Anaeurinibacillus* UBA3580 was obtained. More information about that genome can be found on its NCBI page (https://www.ncbi.nlm.nih.gov/assembly/GCA_002375825.1).

ANI analysis (Figure 3), supports the phylogenomic findings. The ANI value between the two genomes is 99.54%, which strongly suggest that they are the same species. Based on this evidence, **DB4** represents (based on the available information) the first isolate for this species. In contrast, when the comparison was performed between **DB4** and *Brevibacillus brevis*, the ANI value between the two genomes was 74.61%, which is outside the detection limit of this approach, showing that these two genomes are unrelated at the species and probably at the genus level as well.

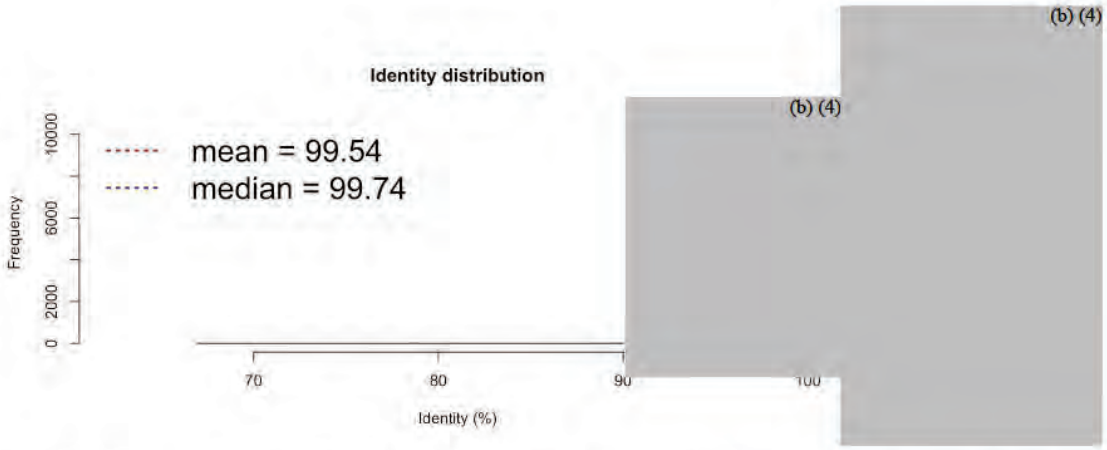


Figure 3. Distribution showing the frequency of hits at different identity thresholds for the genomes of **DB4** and *Anaeurinibacillus* UBA3580. The results shows that the mean average nucleotide identity between the two genomes is 99.54%

DB5

The phylogenomic analysis allowed classification of this genome up to the species level. The closest species match was to *Brevibacillus agri*. This is the same species used as a reference.

Comparing the *B. agri* genome with **DB5** (Figure 4), shows that the ANI value between both genomes is 99.56%, supporting the phylogenomic results and allowing the classification of **DB5** as a strain of *B. agri*.

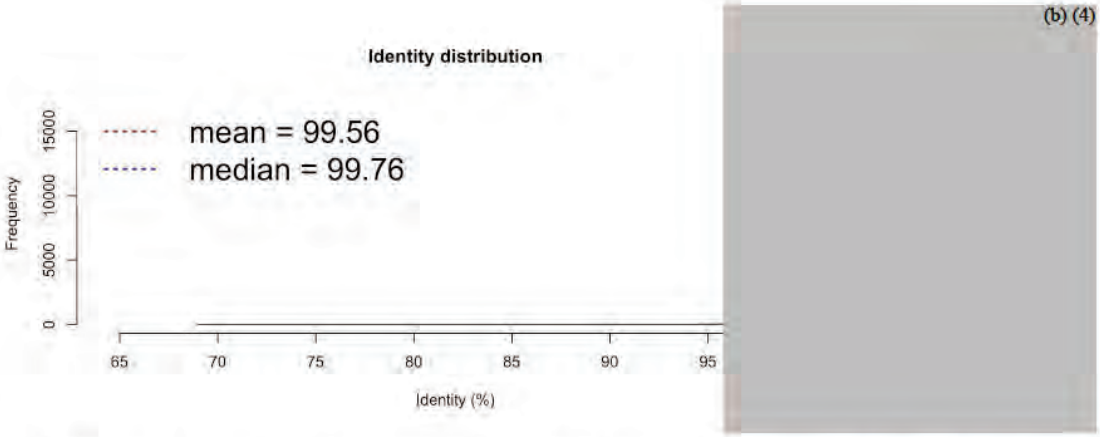


Figure 3. Distribution showing the frequency of hits at different identity thresholds for the genomes of **DB5** and *B. agri*. The results shows that the mean average nucleotide identity between the two genomes is 99.56%

Software packages

- Quality trimming: Trimmomatic 0.36 (Bolger, Lohse et al. 2014)
- Genome assembly: SPAdes 3.12.0 (Nurk, Bankevich et al. 2013)
- Genome completeness: CheckM v1.0.18 (Parks, Imelfort et al. 2015)
- Phylogenomic analysis: GTDB-Tk v0.3.2 (Chaumeil, Hugenholtz et al. 2018)

References

Bolger, A. M., M. Lohse and B. Usadel (2014). "Trimmomatic: a flexible trimmer for Illumina sequence data." Bioinformatics (Oxford, England) **30**(15): 2114-2120.

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Goris, J., K. T. Konstantinidis, J. A. Klappenbach, T. Coenye, P. Vandamme and J. M. Tiedje (2007). "DNA DNA hybridization values and their relationship to whole-genome sequence similarities." International journal of systematic and evolutionary microbiology **57**(1): 81-91.

Nurk, S., A. Bankevich, D. Antipov, A. Gurevich, A. Korobeynikov, A. Lapidus, A. Prjibelsky, A. Pyshkin, A. Sirotkin, Y. Sirotkin, R. Stepanauskas, J. McLean, R. Lasken, S. R. Clingenpeel, T. Woyke, G. Tesler, M. A. Alekseyev and P. A. Pevzner (2013). Assembling Genomes and Mini-metagenomes from Highly Chimeric Reads, Berlin, Heidelberg, Springer Berlin Heidelberg.

Parks, D. H., M. Chuvochina, D. W. Waite, C. Rinke, A. Skarshewski, P.-A. Chaumeil and P. Hugenholtz (2018). "A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life." Nature biotechnology **36**: 996-1004.

Parks, D. H., M. Imelfort, C. T. Skennerton, P. Hugenholtz and G. W. Tyson (2015). "CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes." Genome research **25**(7): 1043-1055.

Parks, D. H., C. Rinke, M. Chuvochina, P.-A. Chaumeil, B. J. Woodcroft, P. N. Evans, P. Hugenholtz and G. W. Tyson (2017). "Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life." Nature Microbiology **2**(11): 1533-1542.

APPENDIX 5. Mercury concentration assessment

Natural gas production often generates hydrocarbon streams containing trace levels of mercury (Hg), predominantly elemental mercury (Hg^0) in the gas phase.[1] For example, Corvivni et al. (2002) reported Hg concentrations ranging from below detection limits up to 120 μg/Nm^3.[2] However, the Hg that may be present must be removed from natural gas to be transported by pipeline to protect downstream aluminum (Al) heat exchangers from catastrophic mechanical failure and gas leakage caused by the amalgamation of Hg with the Al of the exchangers over time.[3] As well, Hg must be removed from natural gas to prevent catalyst deactivation in the production of ethylene from the ethane or propane of the natural gas, for example. Chao and Attari (1993) did not detect Hg in pipeline natural gas in a survey of gas samples across the gas distribution system in the US using a measurement method for which the detection limits for Hg ranged from 0.02 to 0.2 μg/Nm^3.[4] Current industry practices reduce Hg concentrations to < 0.01 μg/Nm^3.

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[1] Porcheron F, Barthelet K, Schweitzer JM, Daudin A (2012). Mercury traces removal from natural gas: Optimization of guard bed adsorption properties. Conference paper presented at the 2012 American Institute of Chemical Engineers (AIChE) Annual Meeting, Environmental Applications of Adsorption I: Gas Phase, 1 November 2012: https://www.aiche.org/conferences/aiche-annual-meeting/2012/proceeding/paper/632e-mercury-traces-removal-natural-gas-optimization-guard-bed-adsorption-properties.

[2] Corvini G, Stiltner J, Clark K (2002). Mercury removal from natural gas and liquid streams. UOP LLC, Houston TX; https://web.archive.org/web/20110101194809/http:/www.uop.com/objects/87MercuryRemoval.pdf; Nm^3 = volume in m^3 at normal temperature and pressure; the International Standard Metric Conditions for natural gas and similar fluids are 288.15 K (15.00 °C; 59.00 °F) and 101.325 kPa; https://www.iso.org/standard/20461.html.

[3] *See also* Aly MAEl E, Mahgoub IS, Nabawi M, Ahmed MAA (2008). Mercury monitoring and removal at gas-processing facilities: case study of Salam gas plant. SPE Proj. Facilit. Construct. 3(1): 1-9: https://www.researchgate.net/publication/250091182_Mercury_Monitoring_and_Removal_at_Gas-Processing_Facilities_Case_Study_of_Salam_Gas_Plant; Porcheron F, Barthelet K, Schweitzer JM, Daudin A (2012). Mercury traces removal from natural gas: Optimization of guard bed adsorption properties. Conference paper presented at the 2012 American Institute of Chemical Engineers (AIChE) Annual Meeting, Environmental Applications of Adsorption I: Gas Phase, 1 November 2012: https://www.aiche.org/conferences/aiche-annual-meeting/2012/proceeding/paper/632e-mercury-traces-removal-natural-gas-optimization-guard-bed-adsorption-properties.

[4] Chao SS, Attari A (1993). Characterization and Measurement of Natural Gas Trace Constituents, Volume II: Natural Gas Survey, Part 1. Institute of Gas Technology Report to Gas Research Institute, Contract No. 5089-253-1832 (November), GRI, Chicago, IL. Available at: https://www.osti.gov/biblio/71153-characterization-measurement-natural-gas-trace-constituents-volume-natural-gas-survey-final-report-otober-october; For summary, see: Wilhelm SM (2001). Mercury in petroleum and natural gas: estimation of emissions from production, processing, and combustion. Prepared by the National Risk Management Research Laboratory for US EPA Office of Air Quality Planning and Standards. US EPA EPA/600/R-01/066. Pp 61-62, including table 7-20.

Mercury vapor (Hg^0), inorganic mercury compounds (Hg^{2+}), and methylmercury (MeHg) are well studied environmental toxicants.[5] Atmospheric Hg^0 vapor is derived from natural degassing of the earth crust and through volcanic eruptions as well as from anthropogenic sources.[6] Eventually, atmospheric Hg^0 is oxidized to water-soluble inorganic forms (Hg^{2+}) and returned to the surface in rainwater, from which Hg^{2+} can be reduced back to Hg^0 and returned to the atmosphere, or the Hg^{2+} may be methylated by microorganisms to produce MeHg in the sediments of freshwater and saltwater bodies. The MeHg produced in this way can enter the food chain starting with plankton, and then bioaccumulate in the food chain through herbivorous fish up to carnivorous fish and sea mammals. The bioaccumulation of Hg can result in MeHg levels in the tissues of fish and mammals at the top of the food chain that are from 1800 up to 80,000 times greater than the corresponding Hg concentrations in the water in which these animals live and feed. In turn, the bioaccumulation of MeHg can result in human exposures through the consumption of fish, especially fish at the top of the food chain (e.g., swordfish and shark) in which MeHg tissue concentrations are typically substantially greater than the concentrations in fish at lower levels of the food chain (e.g., salmon and trout).[7]

MeHg and other organomercurial compounds are generally recognized to be more potent toxicants than inorganic Hg compounds in fish and mammals, including humans, especially for exposures that occur during early life stages of development. NRC (2005) noted that salmon and poultry tolerate chronic exposures to MeHg at up to 1 mg Hg/kg diet, and the NRC (1980) established a dietary MeHg level of 2 mg Hg/kg diet as safe for swine and ruminants.[8] On a per-kg-body-weight (bw) basis, nonreproducing rodents and cats tolerated chronic MeHg exposure to 0.1 mg/kg bw/day MeHg (i.e. 100 µg/kg bw/day). During reproduction, rodents, nonhuman primates and cats tolerated chronic MeHg exposure to 5 µg Hg/kg bw/day in all studies reviewed. For human exposure to Hg in fish, NRC (2005) set a maximum tolerable level

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[5] For reviews, see: Goyer RA and Clarkson TW (2001). Toxic Effects of Metals: Iron (Fe). Chapter 23 In: Casarett and Doull's Toxicology: The Basic Science of Poisons. 6th Edition. Klaassen CD Editor. McGraw-Hill. Pp. 834-837; Tokar EJ, Boyd WA, Freedman JH, Waalkes MP (2013). Toxic Effects of Metals: Iron (Fe). Chapter 23 In: Casarett and Doull's Toxicology: The Basic Science of Poisons. 8th Edition. Klaassen CD Editor. McGraw-Hill. Pp. 996-999.

[6] For reviews, see id.

[7] For example, see: US FDA (undated). Mercury levels in commercial fish and shellfish (1990-2012): https://www.fda.gov/food/metals/mercury-levels-commercial-fish-and-shellfish-1990-2012; US FDA (2019). Technical information on development of FDA/EPA advice about eating fish for women who are or might become pregnant, breastfeeding mothers, and young children: https://www.fda.gov/food/metals/technical-information-development-fdaepa-advice-about-eating-fish-women-who-are-or-might-become.

[8] National Research council (NRC) (2005). Mercury. Chapter 20 in: Mineral tolerance of animals. Committee on Minerals and toxic Substances in diets and water for Animals, Board on agriculture and Natural resources, Division on Earth and Life Studies, Second Revised Edition, pp. 276-283.

(MRL)[9] of 0.3 μg Hg/kg bw/day for human exposure based on the potential for effects on the neurodevelopment of children exposed in utero to MeHg from maternal fish ingestion.[10]

The specification for Hg in pipeline gas used in the production of FeedKind® is 0.02 μg/Nm3 maximum, and Calysta will not accept or use pipeline natural gas that is not certified to contain Hg ≤ 0.02 μg/Nm^3 for manufacturing this product.

Exaggerative estimates of exposures to mercury were calculatedassuming that salmon or trout are raised on feed containing the maximum proposed use level of FeedKind® (i.e. 18%) and other conservative assumptions. FeedKind® is manufactured in 12-week fermentation cycles using pipeline natural gas containing mercury at the maximum concentration defined by specification in this GRASN (i.e. 0.02 μg/Nm^3). Our estimates were calculated based on an example production scenario in which FeedKind® is synthesized continuously by the bacterial consortium in a fermenter during a 12-week cycle, and fractions of the biomass and associated culture medium are constantly conveyed to a centrifuge in which the biomass is separated from the culture medium. The biomass is processed further downstream to produce FeedKind®. The culture medium that is separated from the biomass is recycled to the fermenter. The fermenter and all downstream manufacturing equipment will be emptied and cleaned at the end of each 12-week cycle and prepared to receive fresh bacterial culture and medium at the start of the next 12-week cycle.

Approximately 5.5 x 10^6 Nm^3 natural gas will be consumed during each 12-week cycle to produce 2,308 tons (2.1 x 10^6 kg) dry biomass (i.e. FeedKind®) during each 12-week cycle.[11] If the concentration of Hg is assumed to be constant at the maximum 0.02 μg/Nm^3 in the pipeline gas during production, then approximately 109 mg Hg will have been delivered to the reactor during the 12-week cycle.[12]

The Hg introduced into the reaction vessel from the natural gas will partition into two principal fractions of the bacterial culture, including the aqueous growth medium (i.e. the medium fraction) and the bacterial cells (i.e. the biomass fraction) during fermentation. Most of the Hg in the natural gas will be inorganic. However, the bacterial cells will likely convert at least some of the inorganic Hg to MeHg, which is much more toxic and hydrophobic than the

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9 MRL = maximum tolerable level = the dose that can be ingested for a lifetime without significant risk of adverse effects; the MRL for MeHg assumes 70-kg maternal BW.

10 In comparison, the UN FAO and WHO set a maximum mercury intake of 0.23 μg/kg bw/day to protect the developing fetus and ATSDR recommended that pregnant women do not consume fish containing more than 250 μg Hg/kg; NRC (2005) noted that these agencies "stressed that public health authorities should keep in mind that fish play a key role in meeting nutritional needs in many countries"; US FDA's "action level" for MeHg is 50 μg/kg for fish in interstate commerce.

11 10,000 tons FeedKind® produced per year; (10,000 tons/year ÷ 52 weeks/year) x 12 weeks/cycle = 2,308 tons FeedKind® produced per cycle; 2,365 Nm^3 natural gas consumed per ton; 2,308 tons FeedKind®/cycle x 2,365 Nm^3 natural gas/ton FeedKind® = 5.4584 x 10^6 Nm^3 natural gas/cycle; 2,308 tons FeedKind® x 907.185 kg/ton = 2.0938 x 10^6 kg FeedKind®

12 5.45842 x 10^6 Nm^3 natural gas/cycle x 0.02 μg Hg/Nm^3 natural gas = 1.09 x 10^5 μg Hg/cycle = 1.1097 x 10^5 μg Hg/cycle ÷ 1000 μg/mg = 109 mg Hg/cycle.

inorganic forms of Hg. The much greater bioconcentration factors (BCFs) and bioaccumulation factors (BAFs) typically reported for MeHg, compared with inorganic Hg compounds, is attributable to the substantially greater lipophilicity of MeHg.[13] For example, US EPA (1995) estimated BCFs of 52,175 kg/l and 2,998 kg/l for MeHg and inorganic Hg, respectively, based on the results of laboratory tests with MeHg and highly soluble forms of inorganic Hg.[14] These BCFs are within the 1800 to 80,000 range generally reported for Hg in carnivorous fish and sea mammals at the top of the food chain.[15]

As noted above, the fermenter will receive approximately 109 mg Hg during the production of 2.09 x 10^6 kg dry FeedKind® in each 12-week cycle if the Hg concentration in the natural gas is always equal to the specified maximum of 0.02 µg/Nm^3 throughout the cycle. FeeedKind® will be produced in a re-circulating system in which the bacterial culture is continuously harvested, the harvested culture is centrifuged to separate the wet biomass from most of the medium, the wet biomass fraction is spray dried to produce FeedKind®, and the medium fraction is returned to the fermenter.

For the following calculations, the concentration of wet biomass in the harvested culture is assumed to be 2 g/100 ml (i.e. 2%) and the wet biomass production rate is 5192 kg/hour.[16] It follows that the concentration of medium in the harvested culture will be 98 g/100 ml (i.e. 98%).[17] The concentration of wet biomass in the harvested culture will increase from 2% to 35% through centrifugation and evaporation. Thus, 96.2% of the medium in the harvested culture will be returned to the fermenter, assuming conservatively and for simplicity, that the concentration to 35% wet biomass is achieved solely through centrifugation and that 100% of the separated medium is returned to the fermenter.[18] The calculations demonstrate a worst-case conservative

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[13] Bioconcentration Factor (BCF): ratio of the concentration of a substance in an organism to the aqueous concentration as a result of direct uptake from the water; Bioaccumulation factor (BAF): ratio of the concentration of the substance in an organism to the aqueous concentration as the result of uptake from all exposure routes, including diet; BCFs and BAFs are often expressed as the ratio of mg of chemical per kg of organism to mg of chemical per liter of water (i.e. l/kg).

[14] *See* New York State (1998). Human Health Fact Sheet: Ambient Water Quality Value Based on Human Consumption of Fish. March 12. 1998, https://www.epa.gov/sites/production/files/2015-06/documents/ny_hh_202_f_03121998.pdf; US EPA (1995). Great Lakes water Quality Initiative technical Support Document for the Procedure to Determine Bioaccumulation Factors. Office of Water 4301. EPA-820-B95-005.

[15] Tokar EJ, Boyd WA, Freedman JH, Waalkes MP (2013). Toxic Effects of Metals: Iron (Fe). Chapter 23 In: Casarett and Doull's Toxicology: The Basic Science of Poisons. 8th Edition. Klaassen CD Editor. McGraw-Hill. p. 997.

[16] The production of 5192 kg wet biomass/hour = 5192 kg/hour x 0.2 kg dry biomass/kg wet biomass = 1038 kg dry biomass/hour, assuming conservatively that the cells contain, by volume, 20% and 80% dry biomass and water, respectively; 1038 kg dry biomass/hour x 2016 hours/12-week cycle = 2.09 x 10^6 kg dry biomass/12-week cycle; for discussion of bacterial dry matter content see: Bratbak G, Dundas I (1984). Bacterial dry matter content and biomass estimations. Appl. Environ. Microbiol. 744-757.

[17] 100% culture = 98% medium + 2% wet biomass.

[18] For example, every 100 ml culture harvested will contain 2 g wet biomass and 98 ml medium before centrifugation. After centrifugation, the separated biomass fraction will contain

production scenario, as evidenced by the fact that only approximately 85%, rather than 96.2%, of the culture medium will be returned to the reactor after centrifugation and 15% will remain with the separated biomass to be concentrated through evaporation and spray drying to produce FeedKind®.

Based on these assumptions, the increase in the Hg concentration in the wet biomass (i.e. µg Hg/kg wet biomass) with the time of harvest (hours) over the 12-week cycle follows an exponential growth curve of the following form (correlation coefficient = 1.00):[19]

$$y = a(1-\exp(-bx)$$

where,

- y = concentration of Hg in wet biomass produced (µg Hg/kg)
- a = curve-fitting coefficient
- b = curve-fitting coefficient
- x = time of harvest (cumulative hours after cycle initiation)

These curves rise rapidly over the first few hours of each 12-week cycle, depending on the BCF assumed for Hg, and then much more slowly over the remaining hours of the 12-week cycle as the system approaches a dynamic equilibrium between the Hg that continues to enter the fermenter with the pipeline gas and the Hg that continues to be removed from the fermenter with the harvested wet biomass. **Table 13** presents the maximum concentrations of Hg in the wet biomass and dry biomass (i.e. FeedKind®) calculated assuming 1800 and 80,000 as the BCF for Hg and 2016 hours total cycle time (i.e. 12 weeks).

Table 13. Maximum Hg Concentrations in Wet Biomass and in FeedKind®

BCF	Coefficient a	Coefficient b	Hg Concentration in Wet Biomass (µg/kg)	Hg Concentration in FeedKind® (µg/kg)[20]
1800	1.041×10^{-2}	6.683×10^{-1}	1.041×10^{-2}	5.205×10^{-2}
80,000	1.042×10^{-2}	6.937×10^{-1}	1.042×10^{-2}	5.212×10^{-2}

Thus, the highest concentration of Hg, which will be in the last kg of 2.1×10^6 kilograms of FeedKind® produced during the 12-week production cycle, is approximately 0.052 µg/kg, assuming, conservatively, that the BCF for Hg in the fermenter is the highest BCF reported for

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[2 g wet biomass ÷ (3.71 ml medium + 2 g wet biomass)] x 100 = 35% wet biomass; it follows that the volume of the harvested medium returned to the fermenter will be (100 ml harvested culture – 5.71 separated culture) = 94.29 ml, which is (94.29 ml returned medium ÷ 98 ml harvested medium) x 100 = 96.2% of the harvested medium returned to the fermenter.

[19] Curve fitted using CurveExpert Professional (v.2.6.5); $5.79 \times 10^{-2} \times (1-\exp(-2.3 \times 2.1 \times 10^6) = 5.79 \times 10^{-2}$

[20] Hg concentration in FeedKind® = Hg concentration in wet biomass ÷ 0.2, assuming conservatively that the cells contain, by volume, 20% and 80% dry biomass and water, respectively.

Hg in fish and sea mammals at the top of the food chain and that no Hg vapor escapes the fermenter, centrifuge, spray dryer, or other elements of the production system during the cycle.[21]

It follows that salmonid feed containing 18% FeedKind® will contain no more than 0.289 µg Hg/kg feed.[22] This value is 3460 times lower than the 1 mg Hg/kg diet tolerated by salmon exposed chronically to dietary MeHg.[23] Thus, the risks to salmonids from chronic exposure to any Hg from pipeline natural gas in FeedKind® at up to the maximum use levels in fish feeds (i.e. 18%) is virtually non-existent.

High-end MeHg exposures were estimated for human consumption of salmon and trout raised on diets containing 18% FeedKind®, and assuming that 100% of the Hg in FeedKind® is in the form of MeHg. Again, the estimates were based on the highest calculated Hg concentration in the salmonid feed (i.e. 0.289 µg/kg feed). Additional assumptions included:

- Cumulative feed consumed by the target animal per weight of edible tissue (i.e. 1.77 and 2.14 kg feed/kg edible body weight for Atlantic salmon and trout, respectively)[24]
- 100% of the Hg intake from the feed accumulates in the edible fish tissue
- High chronic daily consumption of salmon or trout by humans is equal to the 90th percentile daily ingestion level of all finfish (i.e., 0.17 kg/day)[25]
- Body weight 70 kg[26]

Based on these highly exaggerative assumptions, the estimated daily intake (EDI) of Hg is 0.0012 and 0.0015 µg/kg bw/day for salmon and trout, respectively.[27] NRC (2005) specified an MRL of 0.3 µg Hg/kg bw/day for the protection of human health, for a 70-kg person, based on the potential for neurodevelopmental effects in children exposed in utero to methylmercury from maternal fish ingestion. Thus, the EDI is 250 and 200 times less than the MRL for the consumption of salmon and trout, respectively.[28]

Thus, the risks to consumers from chronic exposure to any Hg from pipeline natural gas in FeedKind® is negligible or virtually non-existent as well, even assuming that 100% of the fish

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21 For comparison, the calculated maximum Hg concentrations in FeedKind® assuming BCF=1 and BCF=10 is 0.018 µg/kg and 0.044 µg/kg, respectively.

22 0.052 µg Hg/kg FeedKind® ÷ 0.18 kg FeedKind®/kg feed = 0.289 µg Hg/kg fish feed

23 1 mg Hg/kg diet x 1000 µg/mg ÷ 0.289 Hg/kg fish feed = 3460.

24 *See* Table 2 in Fry JP, Mailloux NA, Love DC, Milli MC, Cao L (2018). Feed conversion efficiency in aquaculture: do we measure it correctly? Environ. Res. Lett. 13: 024017: https://iopscience.iop.org/article/10.1088/1748-9326/aaa273/pdf.

25 *See* Table 2.055 in Smiciklas-Wright H, Mitchell DC, Mickle SJ, Cook AJ, Goldman JD (2002). USDA 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII 1994-1996).

26 NRC (2005) specifies an MRL of 0.0003 mg Hg/kg bw/day for a 70-kg person

27 For example, [0.052 µg Hg/kg FeedKind® ÷ 0.18 kg FeedKind®/kg feed x 2.14 kg feed/kg edible trout tissue x 0.17 kg trout/day]/70 kg bw = 0.0015 µg Hg/kg bw/day.

28 MOE = MRL/EDI; for salmon, 0.3 µg/kg bw/day ÷ 0.0012 µg/kg bw/day = 250; for trout, 0.3 µg/kg bw/day x ÷ 0.0012 µg/kg bw/day = 200.

consumed by high-end fish consumers are salmon or trout raised exclusively on salmonid feed containing the maximum level of FeedKind® (i.e. 18%), all of which was produced using pipeline natural gas invariably containing the maximum possible concentration of Hg (i.e. 0.02 $\mu g/Nm^3$).

From: Cerrito, Chelsea
To: Adams, Carissa
Cc: Carlacci, Louis; Wong, Geoffrey K
Subject: FW: GRN Amendment
Date: Friday, July 17, 2020 3:22:30 PM
Attachments: 07.17.20_Amendment to FeedKind GRASN.zip

Hey Carissa,

I won't get to logging this one in before I'm on leave. Can you combine the files in the zip folder, make a new sub form, put it in the folder for the DCU, and send them an e-mail please?

Thanks for your help!
Chelsea

From: Carlacci, Louis <Louis.Carlacci@fda.hhs.gov>
Sent: Friday, July 17, 2020 2:12 PM
To: Wong, Geoffrey K <Geoffrey.Wong@fda.hhs.gov>; Cerrito, Chelsea <Chelsea.Cerrito@fda.hhs.gov>
Subject: FW: GRN Amendment

Hi. This amendment for M-85 just came in. Submission M-85 has not yet been filed as a GRAS notice. I opened the zip file and each of the documents inside the zip file with no problems.

Chelsea would you submit this amendment to DCU.

Geoff, the evaluators for M-85 are CMC: Lou; TAS: Becky; UTL: Diego.
I am going to create a folder in my draft folder on the shared drive and let the evaluators find the documents there until it is available on DCU.

Thanks.
Lou

Louis Carlacci, Ph.D.
Chemist
Ingredient Safety Team (HFV-224)
Division of Animal Feeds
Center for Veterinary Medicine
Ph 240-402-2921

From: Drozen, Melvin S. <Drozen@khlaw.com>
Sent: Friday, July 17, 2020 1:31 PM
To: Carlacci, Louis <Louis.Carlacci@fda.hhs.gov>
Subject: GRN Amendment

Dr. Lou,

On behalf of Calysta, Inc., attached please find the Amendment to GRAS Notice for Dried *Methylococcus Capsulatus* product (hereinafter "FeedKind®"), which was originally submitted to CVM on February 28, 2020. The Amendment addresses the questions raised by CVM during the April 23, 2020 telelconference regarding (1) the safety of FeedKind®'s raw material specifications and (2) concerns regarding the elevation in serum IgG2a levels reported in mice in Christensen et al. (2003) to pose a risk of chronic inflammation in salmonids.

The attached zip file contains (1) the Amendment to the GRASN, (2) a revised Part 7 reference list with additional studies highlighted in yellow, and (3) copies of those additional studies. For your convenience, we've also included copies of the following three studies, although such studies were previously provided to CVM with the February 28, 2020 submission: (a) Svedsen and Damm-Jorgensen (1992), (b) Takawale (2004), and (c) Thestrup (2004).

Please let us know if you have any questions or if you have any difficulty accessing the materials. We look forward to confirmation of your receipt of this email and the filing of the GRASN.

Have a good weekend.

Best,

Mel.

Melvin S. Drozen
Partner
tel: +1 202.434.4222 | fax: +1 202.434.4646 | drozen@khlaw.com
1001 G Street NW, Suite 500 West | Washington, DC 20001

From: Carlacci, Louis <Louis.Carlacci@fda.hhs.gov>
Sent: Thursday, July 9, 2020 10:13 AM
To: Drozen, Melvin S. <Drozen@khlaw.com>
Cc: Mahoney, Jill M. <mahoneyj@khlaw.com>
Subject: RE: Update on Calysta's amendment

Hi Mel.

Please send the documents via email(s). For your convenience, we accept zip files.
Thanks.

Lou

Louis Carlacci, Ph.D.
Chemist
Ingredient Safety Team (HFV-224)
Division of Animal Feeds
Center for Veterinary Medicine
Ph 240-402-2921

From: Drozen, Melvin S. <Drozen@khlaw.com>
Sent: Thursday, July 09, 2020 9:28 AM
To: Carlacci, Louis <Louis.Carlacci@fda.hhs.gov>
Cc: Mahoney, Jill M. <mahoneyj@khlaw.com>
Subject: RE: Update on Calysta's amendment

Hi Lou,

We plan to provide an amendment to the GRAS notice shortly as discussed below. There will be some additional references as well. Can we submit everything by email, although for the references it would be good to be able to upload to a link if possible which we can provide to you to download. Let us know when you can please. Thanks very much. Mel.

From: Carlacci, Louis <Louis.Carlacci@fda.hhs.gov>
Sent: Monday, July 6, 2020 1:36 PM
To: Drozen, Melvin S. <Drozen@khlaw.com>
Subject: RE: Update on Calysta's amendment

Hi Mel.

Thanks for updating us. Best wishes.

Lou

Louis Carlacci, Ph.D.
Chemist
Ingredient Safety Team (HFV-224)
Division of Animal Feeds
Center for Veterinary Medicine
Ph 240-402-2921

From: Drozen, Melvin S. <Drozen@khlaw.com>
Sent: Monday, July 06, 2020 1:34 PM
To: Carlacci, Louis <Louis.Carlacci@fda.hhs.gov>
Subject: RE: Update on Calysta's amendment

Hello Lou,

When we spoke on May 18 I thought it would take us about 2-3 weeks to submit the amendment. Various circumstances have intervened to delay that unfortunately. We hope to have the amendment to you by the end of next week or early the week of July 20. Our apologies for the delay. Regards. Mel Drozen.

From: Carlacci, Louis <Louis.Carlacci@fda.hhs.gov>
Sent: Monday, May 18, 2020 10:18 AM
To: Drozen, Melvin S. <Drozen@khlaw.com>
Subject: Update on Calysta's amendment

Hi Mel.
Would you give us a status update on when we should expect the amendment to Calysta's GRAS notice? It has been three weeks since our teleconference to request the amendment.

I tried calling the number (202) 434-4222 but was unable to connect.

Thanks.
Lou

Louis Carlacci, Ph.D.
Chemist
Ingredient Safety Team (HFV-224)
Division of Animal Feeds
Center for Veterinary Medicine
Ph 240-402-2921

AMENDMENT TO GRAS NOTICE FOR DRIED *METHYLOCOCCUS CAPSULATUS* PRODUCT

Submitted by: Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington, DC 20001

On behalf of our client

Calysta, Inc.
1140 O'Brien Drive
Menlo Park, CA 94025
United States

July 17, 2020

I. Introduction

The purpose of this amendment is to address questions raised by the U.S. Food and Drug Administration's Center for Veterinary Medicine (FDA-CVM) during the April 23, 2020 teleconference regarding the February 28, 2020 submission of the Generally Recognized As Safe (GRAS) Notice for Calysta, Inc.'s Dried *Methylococcus capsulatus* Product (hereinafter "FeedKind®"). Specifically, FDA-CVM requested raw material specifications for FeedKind® to confirm the safety of the raw materials should they concentrate over the course of FeedKind®'s continuous fermentation process. Thus, Part A of this amendment provides the raw material specifications used during the production of FeedKind®. Part A also provides test results that demonstrate there is no concentration of any raw materials of concern (i.e., heavy metals) over the course of FeedKind®'s multiple continuous fermentation runs. Therefore, it can be concluded that FeedKind®'s raw materials do not pose any potential contamination or safety concerns.

Second, FDA-CVM expressed concern that elevation in serum IgG2a levels reported in mice in Christensen *et al.* (2003)[1] suggests the potential for long-term dietary exposures to FeedKind® (equivalent to Bioprotein) to pose a risk of chronic inflammation in salmonids. Part B of this amendment addresses this issue in a statement by Drs. Judith T. Zelikoff and Daniel Wierda, experts in the field of fish and mammalian immunotoxicology.

In summary, these experts reviewed the published rodent and salmonid studies, together with supporting unpublished studies, and have concluded that the concern postulated by FDA-CVM is not valid and is not supported by the current scientific literature. Collectively, the studies they reviewed and discuss provide *reasonable certainty of no harm to salmonids* exposed to FeedKind® (equivalent to BP). These experts conclude that based on the published studies as supported by the unpublished studies, FeedKind® is generally recognized as safe (GRAS) in the diet of salmonids at the intended use concentrations. The reasons for Drs. Zelikoff and Wierda's conclusions are discussed in their statement. All of the relevant studies have either been provided to FDA-CVM with the original GRAS Notice submission on February 28, 2020 or, with regard to several studies referenced by Drs. Zelikoff and Wierda, are also being transmitted with this amendment to our GRAS Notice. We are also including a revised Part 7 reference list, with the additional studies being transmitted with this amendment highlighted in yellow.

[1] Christensen HR, Larsen LC, Frøkær H (2003). The oral immunogenicity of BP, a bacterial single-cell protein, is affected by its particulate nature. Brit. J. Nutr., 90: 169-178.

Part A – Safety of FeedKind®'s Raw Material Specifications

I. Raw Material Specifications

The FeedKind® GRASN provides the list of raw materials and (example) processing aids in Table 3 on page 14. Raw materials specifications have been added to a revised Table 3, provided below, with the exception of incoming pipeline natural gas which is addressed in Table 4 on page 15 of the GRASN.

Based on our review of the raw materials, the only contaminants of potential concern in FeedKind® are heavy metals, specifically lead (Pb), arsenic (As), and cadmium (Cd). These metals are listed as potential contaminants with maximum allowed specifications for several of FeedKind®'s starting materials. The metals serve no nutritional purpose for the microbes and, therefore, have the potential to accumulate over the course of the continuous fermentation run. However, given the raw material specifications and as demonstrated by Calysta's contaminant testing and monitoring (discussed in Section III below), we believe it is reasonable to assume there would be no long term harm to the salmonid species fed FeedKind®.

The revised Table 3 with the raw material specifications is provided below.

REVISED Table 3: Raw material specifications and (example) processing aids

Table 3 GRASN					**Raw Material Specification**		
Raw Material	**Function**	**Authorization Reference**	**Authorization Limits**	**Specification**	**Specification**	**Units**	**Value**
Methane & Natural Gas	Nutrient for culture	None; Safe for use[5]	N/A	*See* **Table 4**			
Ammonium Hydroxide	pH control	21 CFR §582.1139	Good Manufacturing or Feeding Practice	(b) (4)	Grade of material	NA	(b) (4)
					Ammonia (NH_3, aq)	wt%	
Sulfuric Acid	pH control	21 CFR §582.1095	GM/FP		Grade of material	N/A	
					Sulfuric Acid (H_2SO_4)	wt%	
Phosphoric Acid	Nutrient for culture	21 CFR §582.1073	GM/FP		Grade of material	N/A	
					Phosphoric Acid	wt%	
					Chloride, as Cl	ppm	
					Iron, as Fe	ppm	
					Arsenic, as As	ppm	
					Heavy metals, as Pb	ppm	
					Sulfate, as SO_4	ppm	
Sodium Hydroxide	pH control	21 CFR §582.1763	GM/FP		Grade of material	NA	
					Sodium Hydroxide (NaOH)	wt%	

				(b) (4)	Chloride, as Cl	ppm	(b) (4)
					Iron, as Fe	ppm	
					Heavy metals, as Pb	ppm	
Potassium Hydroxide Solution	Nutrient for culture	21 CFR §582.1631	GM/FP		Grade of material	NA	
					Potassium Hydroxide (KOH)	wt%	
					Carbonate; as K2CO3	wt%	
					Sodium, as Na	wt%	
					Chloride, as Cl	ppm	
					Sulfate, as SO4	ppm	
					Iron, as Fe	ppm	
Zinc Sulfate Heptahydrate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary supplements		Grade of material	N/A	
					Zinc Sulfate Heptahydrate (ZnSO4*7H2O)	wt%	
					Cadmium, as Cd	ppm	
					Arsenic, as As	ppm	
					Heavy Metals, as Pb	ppm	
Nickel Chloride Hexahydrate	Nutrient for culture	None; Safe for use[6]	N/A		Grade of material	N/A	
					Nickel Chloride Hexahydrate (NiCl2*6H2O)	wt%	
					Iron, as Fe	ppm	

					Arsenic, as As	ppm	(b) (4)
					Copper, as Cu	ppm	
					Cadmium, as Cd	ppm	
					Lead, as Pb	ppm	
Cobalt Sulfate Heptahydrate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary	(b) (4)	Grade of material	N/A	
					Cobalt Sulfate Heptahydrate ($CoSO_4*7H_2O$)	wt%	
					Iron, as Fe	ppm	
					Chloride, as Cl	ppm	
					Copper, as Cu	ppm	
					Nickel, as Ni	ppm	
Manganese Sulfate Monohydrate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary		Grade of material		
					Manganese Sulfate Monohydrate ($MnSO_4*H_2O$)	wt%	
					Iron, as Fe	ppm	
					Chloride, as Cl	ppm	
					Arsenic, as As	ppm	
					Heavy Metals, as Pb	ppm	
Nitric Acid	pH control and Nutrient for culture	None; GRAS[7]	N/A		Grade of material		
					Nitric Acid (HNO_3)	wt%	
					Chloride, as Cl	wt%	
Copper Sulfate Pentahydrate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional		Grade of material Copper Sulfate	NA wt%	

			dietary		Pentahydrate (CuSO4*5H2O) pH (via sulfuric acid) Iron, as Fe Arsenic, as As Heavy metals, as Pb Nickel, as Ni	 ppm ppm ppm ppm	(b) (4)
Sodium Molybdate Dihydrate	Nutrient for culture	AAFCO Definition # 57.145	N/A	(b) (4)	Grade of material		
					Sodium Molybdate Dihydrate (Na2MoO4*2H2O)	wt%	
					Iron, as Fe	ppm	
					Arsenic, as As	ppm	
					Chloride, as Cl	ppm	
					Cadmium, as Cd	ppm	
					Lead, as Pb	ppm	
Iron Sulfate	*Nutrient for culture*	21 CFR §582.80	GFP; added as nutritional dietary		Grade of material	NA	
					Grade of material	wt%	
					Iron Sulfate Heptahydrate (FeSO4*7H2O)	wt%	
					pH (via sulfuric acid)		
					Chloride, as Cl	wt%	
					Arsenic, as As	wt%	
					Sodium, as Na	wt%	
					Copper, as Cu	wt%	
					Heavy Metals, as Pb	wt%	

Calcium Chloride Solution (Prepared From Calcium Chloride Dihydrate In De-Mineralized Water)	Nutrient for culture	21 CFR §582.1193	GM/FP	(b) (4)	Grade of material		(b) (4)
					Calcium Chloride Dihydrate ($CaCl_2*2H_2O$)	wt%	
					Calcium Hydroxide, as $Ca(OH)_2$	wt%	
					pH (via sulfuric acid)		
					Sodium Chloride, as NaCl	wt%	
					Magnesium Chloride, as $MgCl_2$	wt%	
					Calcium Sulfate, as $CaSO_4$	wt%	
					Iron, as Fe	wt%	
Magnesium Sulfate Solution (Prepared From Magnesium Sulfate Heptahydrate In De-Mineralized Water And Sulfuric Acid) (Note: this can be a substitute for Manganese Sulfate Monohydrate)	Nutrient for culture	21 CFR §582.5443	GM/FP; used as a nutrient and/or dietary supplement		Grade of material		
					Magnesium Sulfate Heptahydrate ($MgSO_4*7H_2O$)	wt%	
					pH (via sulfuric acid)		
					Chloride, as Cl	ppm	
					Iron, as Fe	ppm	
					Heavy metals, as Pb	ppm	
					Arsenic, as As	ppm	
Glanapon 2000 Antifoam	Antifoam	21 CFR §§ 172.808, 173.340, 582.4505	*See* Section 2.2.2.6		Grade of material	NA	
					Mixture of fatty acid esters and EO-PO-Copolymers	wt%	

FDA-CVM also raised questions regarding the levels and possible concentration of nickel (Ni) and mercury (Hg) in FeedKind®. We provided a specification for nickel in Table 5 on page 26 of the GRASN, and the safety of potential nickel in FeedKind® was previously discussed in section 2.2.2.4 on page 17 of the GRASN. As for mercury, a specification has been set in revised Table 5, provided below. Note also that the safety of potential mercury in FeedKind® was previously discussed in section 2.2.2.3 on page 16 of the GRASN.

For human exposure to Hg in fish, NRC (2005) set a maximum tolerable level (MRL)[2] of 0.3 µg Hg/kg bw/day for human exposure based on the potential for effects on the neurodevelopment of children exposed in utero to methyl mercury (MeHg) from maternal fish ingestion.[3]

A conservative upper limit (and specification) for the Hg concentration in FeedKind® was calculated for the protection of human health based on the MRL for human exposure, assuming that salmon or trout are raised on feed containing the maximum proposed use level of FeedKind® (i.e., 18%), exclusively, and that 100% of the Hg in FeedKind® is in the form of MeHg. Additional assumptions included:

- Cumulative feed consumed by the target animal per weight of edible tissue (i.e., 1.77 and 2.14 kg feed/kg edible body weight for Atlantic salmon and trout, respectively)[4]
- 100% of the Hg intake from the feed accumulates in the edible fish tissue
- High chronic daily consumption of salmon or trout by humans is equal to the 90th percentile daily ingestion level of all finfish (i.e., 0.17 kg/day)[5]
- Body weight 70 kg[6]

Based on these exaggerative assumptions, the upper limit of the Hg concentration in salmon or trout is 10 µg/kg (i.e., 0.01 mg/kg), which was derived as follows.

- **General Equation:** MRL (µg/kg bw/day) x bw (kg) ÷ [Edible Fish Tissue Consumption (kg/day) x Fish Feed Consumed per Edible Tissue Produced (kg/kg)] x Maximum Feedkind® Concentration in Fish Feed (%) = Hg Concentration Limit in FeedKind® (µg/kg)
- **For Salmon:** 0.3 µg/kg bw/day x 70 kg ÷ (0.17 kg x 1.77 kg/kg) x 18% = 12.6 µg/kg

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[2] MRL = maximum tolerable level = the dose that can be ingested for a lifetime without significant risk of adverse effects; the MRL for MeHg assumes 70-kg maternal BW.

[3] In comparison, the UN FAO and WHO set a maximum mercury intake of 0.23 µg/kg bw/day to protect the developing fetus and ATSDR recommended that pregnant women do not consume fish containing more than 250 µg Hg/kg; NRC (2005) noted that these agencies "stressed that public health authorities should keep in mind that fish play a key role in meeting nutritional needs in many countries;" NRC (2005) also stated that US FDA's "action level" for MeHg is 500 µg/kg for fish in interstate commerce. However, FDA's Compliance Policy Guide (CPG) 540.600, which was updated in 2007, now states that the "action level" for MeHg has increased to1000 µg/kg.

[4] *See* Table 2 in Fry JP, Mailloux NA, Love DC, Milli MC, Cao L (2018). Feed conversion efficiency in aquaculture: do we measure it correctly? Environ. Res. Lett. 13: 024017: https://iopscience.iop.org/article/10.1088/1748-9326/aaa273/pdf.

[5] *See* Table 2.055 in Smiciklas-Wright H, Mitchell DC, Mickle SJ, Cook AJ, Goldman JD (2002). USDA 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII 1994-1996).

[6] NRC (2005) specifies an MRL of 0.0003 mg Hg/kg bw/day for a 70-kg person

- **For Trout:** 0.3 µg/kg bw/day x 70 kg ÷ (0.17 kg x 2.14 kg/kg) x 18% = 10.4 µg/kg

Rounding down from the lowest of these values (i.e., 10.4 µg/kg) yields a limit of 10 µg/kg for Hg in KeedKind®.

For the protection of the health of the fish, NAS (2005) specified an MTL of 1 mg/kg (i.e. 1000 µg/kg) for MeHg in the diet (but no MTL for inorganic Hg), which is two orders of magnitude greater than the 10 µg/kg specification derived for total Hg in FeedKind®.

The revised Table 5 with the mercury specification is provided below.

REVISED Table 5: FeedKind® Specifications

Specifications				
Chemical Composition	**Minimum**	**Maximum**	**Units**	**Test Method**
Crude Protein	(b) (4)		% dry weight	DUMAS method
Crude Fat			% dry weight	modified Weibull Acid Hydrolysis Method
Crude Fiber			% dry weight	AOCS Ba 6a-05, Ba 6-84 AOAC 962.09, S 1022 using Gravimetry
Ash			% dry weight	AOAC 942.05, S 1024 using Gravimetry
Moisture			w/w	AOAC 934.01, 930.15, S 1024 using Gravimetry
Nickel			*mg/kg*	ICP-OES

Mercury	(b) (4)	*mg/kg*	ICP-MS
Microbiological Limits	**Limits**		**Test Method**
Mesophilic Aerobic Plate Count	(b) (4)		AOAC 2011.03, 2003.09
Mold			AOAC 997.02; FDA/BAM Chapter 18
Yeast			AOAC 997.02; FDA/BAM Chapter 18

For convenience, below we provide the sections and page numbers for all of the raw material safety analyses discussed in the GRASN:

- Section 2.2.2.2 (page 16) – Safety of Natural gas, including methane
- Section 2.2.2.3 (page 16) – Mercury safety
- Section 2.2.2.4 (page 17) – Nickel chloride hexahydrate safety
- Section 2.2.2.5 (page 21) – Nitric acid safety
- Section 2.2.2.6 (page 24) – Defoamer authorization
- Section 2.2.2.7 (page 25) – Heavy metal safety

II. Testing for Potential Contaminant Concentration

During production runs, Calysta conducts daily testing for compositional analyses (e.g. protein, ash, moisture, etc.) as well as periodic (approximately weekly) testing and monitoring of the FeedKind® product. This periodic testing includes analyses of potential heavy metal contaminant concentration in the continuous fermentation run, including for lead (Pb), cadmium (Cd), and arsenic (As). Test results from three separate continuous fermentation runs (i.e., TEES-004/1-59, TEES-005/01-54, and TEES-009/1-102) are provided below in Table A, which summarizes the first, last, and range of analytical results obtained from the three fermentation runs. Samples are numbered sequentially (i.e. TEES-004/1 is from Day 1, and TEES-04/59 is from day 59). Results for TEES-09 begin after an initial experimental period of 33 days in which the early batches were not subjected to a heat kill step. These batches were deemed to be not suitable due to undesirable microbial growth and were discarded. The heat kill step is included in the production process for all commercial batches. Subsequent batches, for which results are reported in Figure C, were subjected to the heat kill step. Results for mercury (Hg) and nickel (Ni) are also provided, where available.

Table A: Summary of heavy metal concentrations during continuous fermentation runs

TEES-004/1-59					
Analyte (mg/kg)	**Pb**	**Cd**	**As**	**Hg**	**Ni**
First	(b) (4)				
Last					
Range	0.015-0.032	0.001-0.006	0.006-0.017	<0.001-0.003	N/A
TEES-005/01-54					
First	(b) (4)				
Last					
Range	<0.09-0.11	<0.02-<0.02	<0.05-<0.05	<0.01-0.03	1.12-1.89
TEES-009/1-102					
First	(b) (4)				
Last					
Range	<0.09-<0.11	<0.02-0.03	<0.05-0.051	<0.01-<0.01	1.94-5.25

Figures A-C plot the concentration of the specified heavy metals during the three continuous fermentation runs.

Figures A-C: Concentration of heavy metals during three separate continuous fermentation runs. Nickel concentrations in Figure B and Figure C are plotted on the right axis.

Figure A

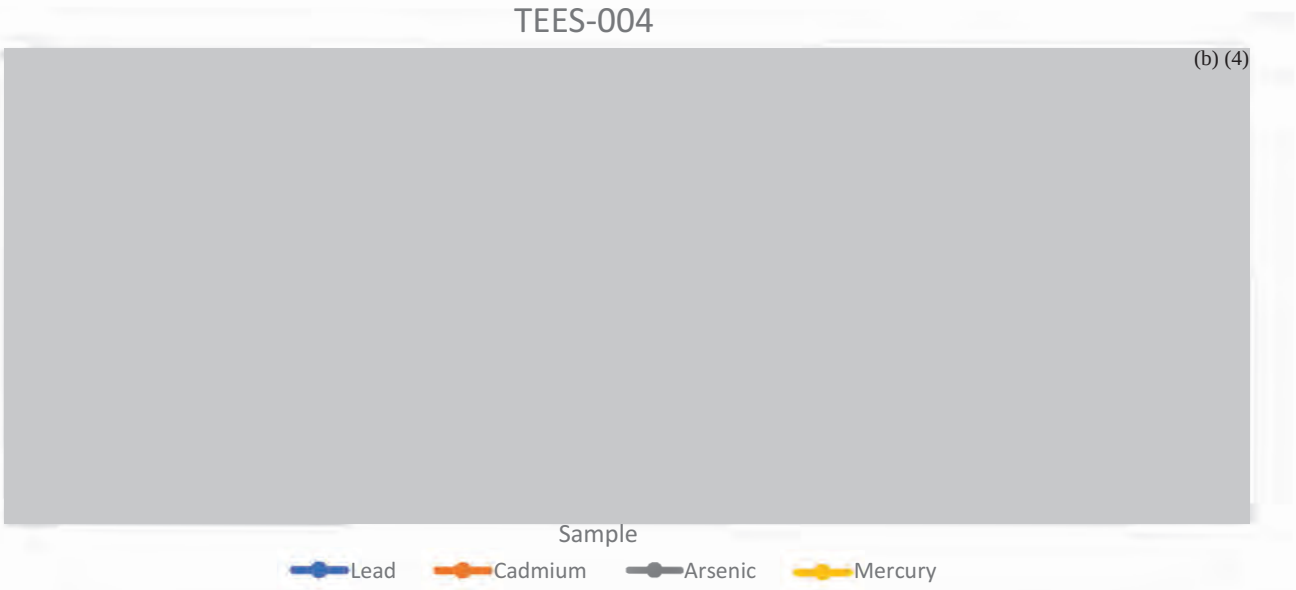


Figure B

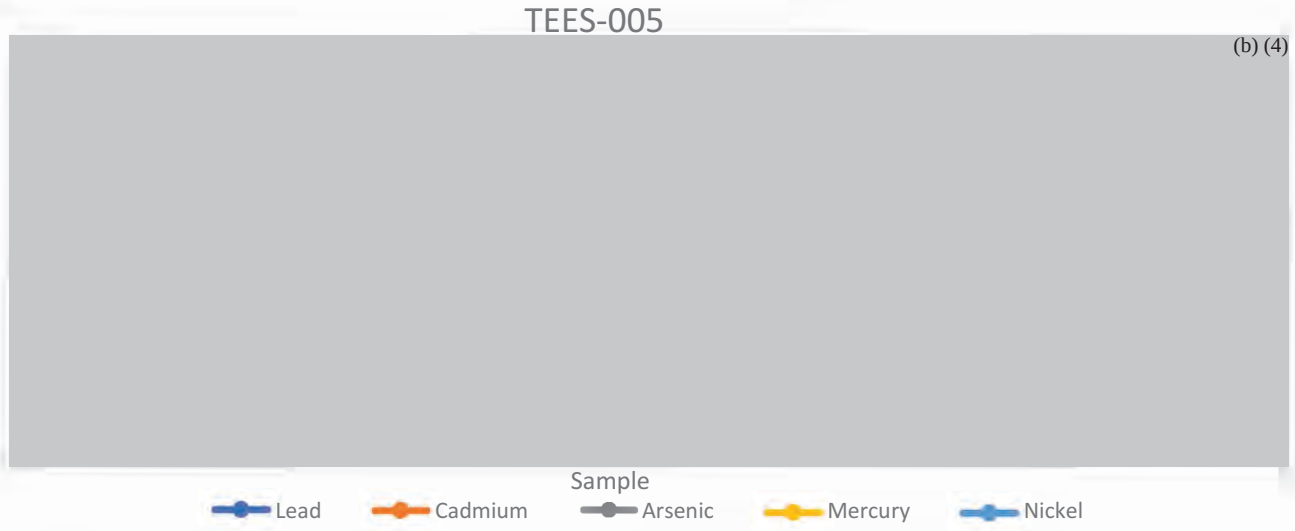
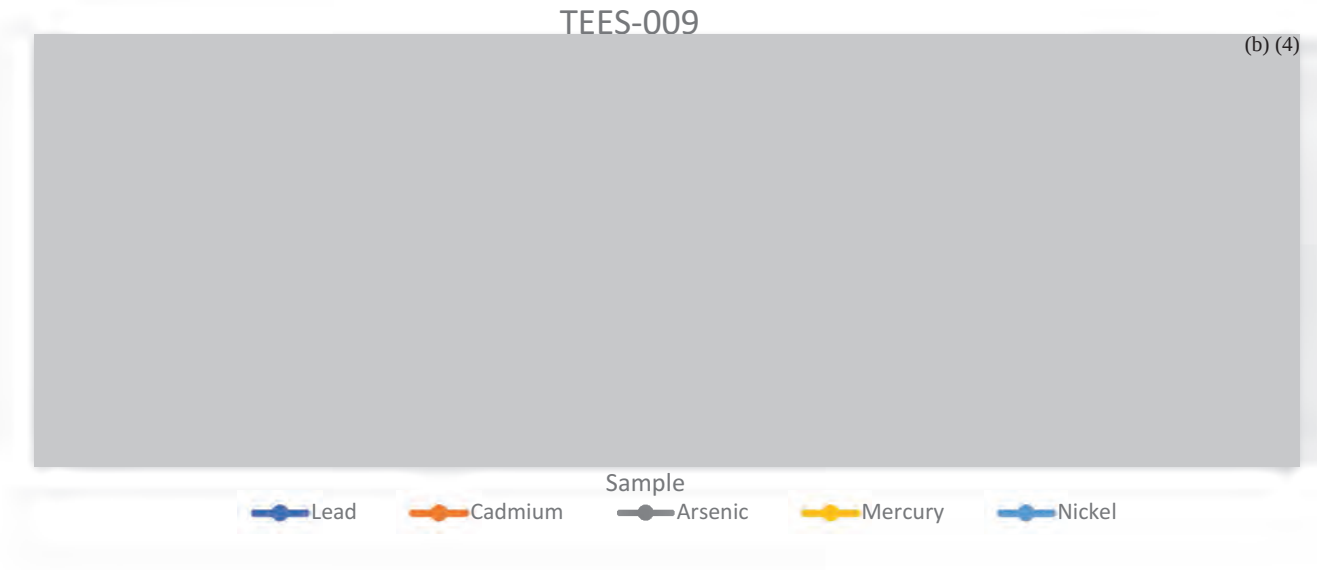


Figure C



The test data and figure plotting clearly indicates that there is no concentration of any heavy metals over the course of multiple continuous fermentation runs.

The NAS (2005) MTLs for the protection of the health in fish is 5, 10, 10, 1, and 50 mg/kg in fish feed for arsenic, cadmium, lead, methyl mercury, and nickel, respectively. The feed will contain no more than 18% FeedKind®. Thus, FeedKind® containing no more than 27.8, 55.6, 55.6, and 277.8 mg/kg arsenic, cadmium, lead, methyl mercury, and nickel, respectively,[7] cannot increase the concentrations of these metals in the feed above their respective MTLs. As noted above, a conservative upper limit specified for chronic exposure to Hg in FeedKind®, based on the NAS (2005) MRL for MeHg for the protection of human health and a compilation of exaggerative exposure assumptions, is 0.01 mg/kg. As shown in Table A and Figures A, B, and C, the concentrations of arsenic, cadmium, lead, and nickel in the FeedKind® samples analyzed were well below their respective NAS (2005) MTLs, and the Hg concentrations were well below the MTL for MeHg and the specification for Hg derived above from the NAS (2005) MRL for MeHg for the protection of human health.

Therefore, it can be concluded that the heavy metals do not pose any potential contamination or safety concerns. To ensure future products contain similar levels to the data provided above, Calysta will maintain current testing and monitoring protocols, in addition to adhering to the raw material specifications in revised Table 3.

III. Conclusion

Based on the provided raw material specifications and available test results for potential heavy metal contaminant concentration in the continuous fermentation run, we conclude that

[7] For example, feed containing 18% FeedKind® containing 27.8 mg As/kg would have no more than 5 mg As/kg if the arsenic concentration in the other ingredients of the feed, combined, is no more than 5 mg As/kg (i.e. the MTL for As).

there is no concentration of heavy metals in the finished FeedKind® product and therefore additional finished product specifications are not required. We further conclude that there are no other raw materials that present potential contamination concerns. Thus, the use of the raw materials in the continuous fermentation process as indicated in the GRASN do not pose any safety concerns.

Part B - Statement of Drs. Judith T. Zelikoff and Daniel Wierda

I. Statement Introduction

We have been requested by Calysta, Inc., to review relevant safety data, much of which is part of the pending GRAS notice submitted to FDA. We have reviewed these and other data (referenced below) and offer the following statements and conclusion.

By way of background, we understand from Calysta that the US Food and Drug Administration Center for Veterinary Medicine (CVM) expressed concern that elevation in serum IgG2a levels reported in mice[8] suggests the potential for long-term dietary exposures to FeedKind® (equivalent to Bioprotein) to pose a risk of chronic inflammation in salmonids.

Specifically, Christensen *et al.* (2003) characterized the serum and salivary antibody profiles of mice exposed to Bioprotein (BP) and nucleic-acid reduced Bioprotein (NRBP) in the diet. These investigators focused part of their study on IgA production, because of its association with gut immunity, and another part of their study on IgM, IgG1 and IgG2a production. Christensen *et al.* (2003) reported that the IgG1 response abated, but the IgG2a response was sustained in mice after the diet containing NRPB was replaced with the control diet. From this observation, we understand that CVM postulated a cause for concern that chronic feeding of FeedKind® to animals may be detrimental because IgG2a can be a marker for inflammation.

Extensive published chronic and sub-chronic exposure studies demonstrate that salmonids thrive on diets containing up to 18% or more BP, with no signs of inflammation or other adverse health effects that can be attributed to FeedKind®.[9] In addition, in other published studies, dietary BP antagonizes the well-known inflammatory responses to dietary soybean meal (SBM) observed in the gut of salmon.[10] Calysta concluded from these studies that FeedKind® does not produce adverse health effects in salmonids through an immunogenic mechanism. We understand that CVM responded that it would be acceptable to address their concern by providing a statement from the published literature confirming that the toxicity endpoints of the existing salmonid studies are appropriate for demonstrating no adverse health effects attributable to antibody-mediated (*i.e.* humoral) responses to FeedKind® in the diet.

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[8] Christensen HR, Larsen LC, Frøkær H (2003). The oral immunogenicity of BP, a bacterial single-cell protein, is affected by its particulate nature. Brit. J. Nutr., 90: 169-178.

[9] Berge GM, *et al*. (2005) Bacterial protein grown on natural gas as protein source in diets for Atlantic salmon, *Salmo salar*, in saltwater. *Aquaculture*. 244: 253-240; Aas TS, et al. (2006a) Improved growth and nutrient utilization in Atlantic salmon (Salmo salar) fed diets containing a bacterial protein meal. Aquaculture. 259: 365-376; Aas TS, *et al.*(2006b). Effects of diets containing a bacterial protein meal on growth and feed utilization in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*. 261: 357-368; Storebakken T, *et al.* (2004) Bacterial protein grown on natural gas in diets for Atlantic salmon, *Salmo salar*, in freshwater. *Aquaculture*. 241: 413-425; See summaries of these studies in the GRAS Notice for Dried *Methylococcus capsulatus* Product (submitted to US FDA CVM on 2/28/2020)

[10] Romarheim OH, et al. (2011) Bacteria grown on natural gas prevent soybean meal-induced enteritis in Atlantic salmon. J Nutr. 141: 124-130; Romarheim OH, et al. (2012). Prevention of soya-induced enteritis in Atlantic salmon (Salmo salar) by bacteria grown on natural gas is dose dependent and related to epithelial MHC II reactivity and CD8α+ intraepithelial lymphocytes. Br J Nutr. 109 (6): 1062-1070.

Calysta uncovered no literature that explicitly addresses the concern that increased serum IgG2a levels or other humoral effects in animals fed diets containing protein products derived from bacteria or from other sources can cause inflammation. We are also aware of no such literature. Thus, Calysta solicited our opinion as independent specialists in the fields of fish and mammalian immunotoxicology to determine whether the data from the available studies supports a conclusion of a *reasonable certainty of no harm* to salmonids fed FeedKind® at the maximum intended use level of 18% in the diet. Our curriculum vitae are attached to this amendment.

We have reviewed the published rodent and salmonid studies, together with supporting unpublished studies, and conclude that the concern postulated by CVM is not valid and is not supported by the current scientific literature. Collectively, the studies we have reviewed and discussed below provide *reasonable certainty of no harm to salmonids* exposed to FeedKind® (equivalent to BP). We, therefore, conclude that based on the published studies as supported by the unpublished studies, FeedKind® is generally recognized as safe (GRAS) in the diet of salmonids at the intended use concentrations The published and unpublished studies are briefly summarized, below, followed by a discussion of the relevant information and rationale leading to the conclusion of this opinion.

II. Pertinent Rodent Studies

Christensen *et al.* (2003) fed mice for 56 days a diet containing one of the following: 24% casein (controls), 6% NRBP, 24% NRBP, or 24% BP. The NRBP was prepared by adding Fe_2SO_4 to BP and subjecting the mixture to heat-shock treatment to activate endogenous RNase and DNase, and then incubating at 60°C to allow the activated enzymes to degrade the nucleic acids. Christensen *et al.* (2003) found that BP-specific total Ig, IgA, IgG1, and IgG2a antibody titers in blood and BP-specific IgA antibody levels in saliva were significantly elevated in mice exposed to BP or to NRBP, compared with controls. No statistically significant differences were found in these parameters in the mice exposed to 24% BP compared with the mice exposed to 24% NRBP. The authors found that the treatment performed to reduce the nucleic acid content had no effect on the oral immunogenicity of BP, although homogenization to remove the particulate phase of the BP reduced the systemic immunogenicity of the compound.

An additional group of mice in the same study received 24% NRBP for 14 days followed by a control diet for 42 days. After cessation of exposure to NRBP, serum IgA levels declined precipitously to control levels by day 28, total serum Ig and IgG1 levels declined steadily to approximately 85% and 75%, respectively, by day 56, and IgA in saliva and Ig2a in serum remained at approximately 82% and 100%, respectively, by day 56. Christensen et al (2003) concluded that BP-specific IgA levels were induced solely at mucosal sites, without a systemic IgA response, and that the factors supporting a Th-2/IgG2A response may be more efficiently cleared from the immune system than the factors supporting a Th-1/IgG1 response. In mammals, Th1 cells are assumed to be associated with generation of IgG1. Christensen et al (2003) also suggested that prolonged exposure to BP or NRBP in the diet may result in the maintenance of a balance between the levels of IgG1 and IgG2A and, thus, between Th-1-type (pro-inflammatory) cytokines and Th-2-type (anti-inflammatory) cytokines. Such a balance could reduce or eliminate any risk of chronic inflammation that might exist in mammalian target

species fed diets containing BP.

Christensen *et al.* (2003) noted that the systemic antibody responses observed in their study in mice exposed to BP or NRBP may be related to the accumulation of foamy macrophages in the mesenteric lymph nodes (MLNs) as reported by Mølck *et al.* (2002) in rats exposed NRBP in the diet for 90 days.[11] Related effects were reported by Glerup (1999)[12] in rats receiving BP in the diet for 56 days. However, these and other sub-chronic oral exposure studies demonstrated that BP levels up to and including 15% in the diet of rats yielded minimal-to-no evidence of inflammation in the intestines or other organs examined and no signs of endotoxemia.[13]

Accordingly, Glerup (2002) reported similar results in an unpublished study in rats receiving up to and including 22% Brewer's yeast for 28 days. Christensen *et al.* (2004)[14] reported that mice exposed to soy protein in the diet or in drinking water across 3 generations (F_0, F_1, and F_2) exhibited antibody responses coinciding with the induction of oral tolerance, and that these appear to be commonly-seen responses to the ingestion of soy protein. The immunogenicity of orally administered BP in mice may be analogous to those of Brewer's yeast, soy protein and/or other protein sources commonly used in animal feeds, including the induction of oral tolerance.[15]

Thestrup (2004)[16] evaluated the serum antibody data from two unpublished studies in which rats received BP or Brewer's yeast in the diet. The studies included a one-generation reproductive toxicity study[17] in which the animals were fed a diet containing 6% or 12% BP or Brewer's yeast, as well as a 90-day study[18] in which juvenile rats were fed a diet containing 12% BP.[19]

The oral exposures in these studies produced elevation of BP-specific serum levels of IgA, IgG, IgG1, and IgG2Aa (ranging from 1-fold for IgA to 3-fold for IgG1 compared with

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[11] Mølck A-M, Poulsen M, Christensen HR, Lauridsen ST, Madsen C (2002). Immunotoxicity of nucleic acid reduced BP – a bacterial derived single cell protein – in Wistar rats. Toxicology 174: 183-200.

[12] Glerup P (1999). Eight-week lymph node toxicity study in the rat. Scantox test report, prepared for Dansk BP A/S, Lab No. 30864, 20 September, 73 pp.

[13] Glerup P (1999). Eight-week lymph node toxicity study in the rat. Scantox test report, prepared for Dansk BP A/S, Lab No. 30864, 20 September, 73 pp; Svendsen O, Damm-Jørgensen K (1992). Single cell protein: three-month oral toxicity study in the rat. Scantox test report, prepared for Dansk BP A/S, Lab. No. 12960, July 31, 91 pp; Takawale P (2004). BP: Study in juvenile rats. Scantox test report, prepared for Norferm A/S, Study no. 52692, 20 October, 166 pp; Thestrup HN (2004). BP antibody responses in feeding studies. Internal report, Norferm Denmark, 14 October, 12 pp.

[14] Christensen HR, Brix S, Frøkær H (2004). Immune response in mice to ingested soya protein: antibody: antibody production, oral tolerance, and maternal barrier. Brit. J. Nutr., 91: 725-732.

[15] For example, see: https://www.feedipedia.org/node/72; https://feedipedia.org/node/674.

[16] Thestrup HN (2004). BP antibody responses in feeding studies. Internal report, Norferm Denmark, 14 October, 12 pp.

[17] Takawale P (2004). BP: Study in juvenile rats. Scantox test report, prepared for Norferm A/S, Study no. 52692, 20 October, 166 pp.

[18] Clausing and Bøgh (2002). BP: One generation reproduction toxicity study in rat. Scantox test report, prepared for Norferm A/S, Lab No. 25995, 22 January, 263 pp.

[19] See also Noferm AS (2004). Supplement to BP® Dossier. Submitted for registration of BP® in the EU under directive 82/471/EEC.

controls). The antibody responses were one to two orders of magnitude weaker than the responses observed in control rats challenged with BP by *i.p.* injection. In addition, the antibody responses to orally administered BP were elevated in the parental animals, but not in the offspring. In the rats exposed to Brewer's yeast, serum IgA, IgG1, and IgG2a levels specific for Brewer's yeast produced essentially the same pattern of antibody responses as the BP-specific antibody levels observed in the rats exposed to BP.

In the 90-day study, the antibody responses in rats fed BP beginning at 3-weeks-of-age were significantly lower than those observed in rats fed BP beginning at 7 weeks of age.

As noted by Thestrup (2004), the results of the one-generation study and the 90-day study in rats, taken together, could indicate that immunological tolerance to BP was induced in rats exposed orally to BP. This interpretation is consistent with the results of other studies reporting minimal-to-no evidence of inflammation in the intestines or other organs of rats receiving BP in the diet.

In other experiments, Christensen *et al.* (2003) showed that spleen cells from mice exposed to NRBP exhibited a statistically significant increase in splenocyte (lymphocyte) proliferation, indicating that T-lymphocytes were activated in the exposed animals, and that the mucosal immunogenicity, but not the systemic immunogenicity, of the NRBP was retained in a whole-cell-free BP homogenate, implying that the particulate nature of BP plays a crucial role in the systemic immunogenicity of ingested BP. The latter effects can be attributed to the more efficient transfer of larger particles from the mesenteric lymph nodes (MLNs) to the spleen, which could elicit systemic IgG responses, compared with smaller particles in the digestive tract. The bacterial cells associated with BP are optimum in size for partial systemic translocation through the MLNs and partial retention in the Peyer's patches of the digestive tract of mammalian species, which helps to explain why BP could induce a systemic as well as a mucosal immune response in mammals.

However, these results are not inconsistent with the reported absence of inflammatory responses attributable to BP in rats exposed to BP in the diet. As Christensen *et al.* (2003) noted, the lipopolysaccharides (LPS) of the cell membrane of *Methylococcus capsulatus* may be responsible for the adjuvant component of BP. They also noted that the mucosal adjuvant activity of LPS is quite complex; could enhance oral tolerance to antigens; and varies substantially from one bacterial species to another in their effects on antigen presenting cells.

III. Pertinent Salmonid Studies

Berge *et al.* (2005) fed groups of saltwater-maintained Atlantic salmon a diet of 0%, 10%, or 20% BP for 5 months.[20] Histological examinations indicated that the mucosa of the distal intestines was generally normal across all of the groups, including the numbers of absorptive vacuoles in the enterocytes of the intestinal folds and amounts of leucocytes infiltrating the mucosa and submucosa. One fish in the 10% BP group exhibited a severely

[20] Berge GM, Bæverfjord G, Skrede A, Storebakken T (2005). Bacterial protein grown on natural gas in diets for Atlantic salmon, Salmo salar, in saltwater. Aquaculture, 244: 233-240.

inflamed intestinal mucosa, but the body weight and length of this fish was close to the tank means and there were no external signs of disease. The authors concluded in their published study that there were no signs of any allergic-like reaction to BP, even in fish exposed to 20% BPM in the diet for 5 months.

Aas *et al.* (2006a) fed Atlantic salmon BP in the diet for 48 days.[21] The authors attributed the absence of adverse effects on mortality, growth rates and other indices of health in the salmon exposed to up to 36% BP in this peer-reviewed published study to improved utilization of the feed containing BP.

Aas *et al.* (2006b) fed rainbow trout 0%, 9%, 18%, or 27% BP or 9% BP autolysate in the diet for 71 days.[22] There were no statistically significant differences across the groups in mean body weight, specific growth rate (SGR), feed intake, or feed efficiency ratio (FER), or liver- or viscera-to-body-weight ratio at the end of the exposure period. Histopathological examinations revealed no exposure-related changes in morphology in any region of the gastrointestinal tract of the fish receiving up to 36% BP. In addition, there were no significant differences observed between fish fed autolyzed BP and those fed BP.

Romarheim *et al.* (2011) fed juvenile Atlantic salmon for 80 days a control high-quality fish meal (FM) diet or a diet in which the FM was incrementally replaced to contain 20% solvent-extracted soybean meal (SBM), 30% BP, or 20% SBM plus 30% BP.[23] Morphological, morphometric, and immunohistochemistry examinations in this published study revealed normal intestinal tissue in salmon receiving FM alone, BP, or SBM plus BP in the diet. In contrast, salmon fed diets containing SBM without BP exhibited SBM-induced enteritis in the distal intestine, including atrophy of simple and complex folds, loss of epithelial vacuolation, decreased epithelial-cell height, and prominent inflammatory-cell infiltration of the mucosa. Staining for proliferating cell nuclear antigen (PCNA, a marker of cell proliferation) revealed that the length of the stained sections of the distal intestines decreased in fish fed (in descending order):

(1) 20% SMB plus 40% FM
(2) 20% SMB plus 30% BP plus 10% FM
(3) 30% BP plus 30% FM
(4) 60% FM alone

A brush border reaction for carbonic anhydrase 12 (CA12) was conspicuously absent in the fish fed 20% SBM plus 40% FM, in contrast to the normal reactivity expressed in fish fed any one of the other diets.

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[21] Aas TS, Grisdale-Helland B, Terjesen BF, Helland SJ (2006a). Improved growth and nutrient utilization in Atlantic salmon (Salmo salar) fed diets containing a bacterial protein meal. Aquaculture 259: 365-376.

[22] Aas TS, Hatlen B, Grisdale-Helland B, Terjsen BF, Bakke-McKellep AM, Helland SJ (2006b). Effects of diets containing bacterial protein meal on growth and feed utilasation in rainbow trout (Oncorhynchus mykiss). Aquaculture 261: 357-368.

[23] Romarheim OH, Øverland M, Mydland LT, Skrede A, Landsverk T (2011) Bacteria grown on natural gas prevent soybean meal-induced enteritis in Atlantic salmon. J Nutr. 141: 124-130.

Romarheim *et al.* (2011) reported in another published study that fish fed 20% SBM in the diet exhibited significantly reduced final body weight, thermal growth coefficient (TGC), and feed conversion ratio (FCR) compared to fish fed the FM control diet. In contrast, 30% BP resulted in a statistically significant increase in FCR, but no statistically significant differences in final body weight or TGC. The authors concluded that the addition of BP counteracts or neutralizes SBM-induced enteritis in Atlantic salmon.

Romarheim *et al.* (2013) fed juvenile Atlantic salmon for 47 days a control FM diet or a diet in which the FM was incrementally replaced to contain 20% SBM plus 0%, 2.5%, 5%, 10%, 15%, 20% or 30% BP.[24] Enteritis was observed in all fish fed the diet containing 20% SBM without BP, which was reflected by accumulation of leucocytes in the lamina propria and widening of the lamina propria and submucosa that was attributable to cellular infiltration and edema, among other pathologies. These morphological changes decreased with increasing concentration of BP in the 20% SBM diet, with no evidence of enteritis in the fish receiving 20% or 30% BP with 20% SBM.

Romarheim *et al.* (2013) found that $CD8\alpha^+$ lymphocytes were prevalent at the base of the intestinal epithelium in salmon receiving a diet containing 20% SBM without BP, indicating that SBM-induced enteritis is a T-cell-mediated inflammatory response. Like the morphological changes, the mobilization of $CD8\alpha^+$ lymphocytes decreased with increasing concentration of BP, with no significant difference in the density of $CD8\alpha^+$ intraepithelial lymphocytes in fish receiving 20% or 30% BP in the diet containing 20% SBM, compared with the FM-fed controls. Intense staining for MHC-2 in the leukocytes at the base of the intestinal epithelium was indistinguishable from controls in fish fed 20% SBM plus 30% BP, unlike the staining observed in the fish fed the other diets containing SBM. The lengths of stretches of PCNA-stained sections of the distal intestines of the salmon fed 20% SBM decreased with increasing BP concentration and were indistinguishable from controls in the fish receiving ≥15% BP in the 20% SBM diet.

Romerheim *et al.* (2013) suggested in this same study that the most likely mechanism by which BP counteracts pro-inflammatory responses in salmonids exposed to 20% SBM in the diet is related to immune-system mechanisms that are also responsible for ensuring tolerance to feed antigens and to commensal intestinal microbiota. They noted that regulatory and CD8+ and CD4+ T-lymphocytes that express the Foxp3 transcription factor are known to play a key role in the prevention of inflammatory responses to food antigens and commensal bacteria in mice and in humans.

IV. Discussion

The immune system of fish has been extensively studied in only a few species, including

[24] Romarheim OH, Hetland D, Skrede A, Øverland M, Mydland LT, Skrede A, Landsverk T (2013). Prevention of soya-induced enteritis in Atlantic salmon (Salmo salar) by bacteria grown on natural gas is dose dependent and related to epithelial MHC II reactivity and CD8α+ intraepithelial lymphocytes. Br J Nutr. 109 (6): 1062-1070.

salmon, trout, and zebrafish.[25] In general, these studies show that fish share the basic components of the immune system with all other jaw vertebrates. However, there are known differences in the immune systems of fish, compared with those of mammalian species, as well as a plethora of unknowns in fish immunology. One major difference between bony fish and mammalian species resides in the adaptive, humoral arm of the immune response. The 3 major antibody/immunoglobulin types identified to date in teleost fish (depending on the species) are IgM, IgT/Z, and IgD as compared to the 5 classes (and several sub-classes) in mammals (*i.e.* IgG, IgD, IgM, IgE, IgA) each with distinct effector type functions. IgM constitutes the main systemic immunoglobulin in fish, IgT plays a key role in mucosal surfaces, and the role of IgD in fish immunity remains to be fully elucidated. Along with the lack of an IgG isotype, bony fish also lack Ig class switching recombination (Barreto et al., 2005).

CVM postulated a cause for concern that chronic feeding of FeedKind® to animals may be detrimental because IgG2a (as a result of IgG galactosylation) can be used as a biomarker for predicting inflammation.[26] While this is true for mammals, the evidence does not support this for bony fish. The widely accepted consensus of many research groups in this field is that the proinflammatory activity of IgG2 in mammals depends predominantly upon the presence of cellular Fc receptors (*e.g.*, FcγRs). Blockage of these receptors minimizes antibody-mediated inflammation.[27] Activated complement component C5a modulates the expression of FcγRs. Mice deficient in the C5a-receptor demonstrate a loss of antibody-mediated inflammation. In comparison, relatively few FcRs are found in fishes. In addition, fish have both teleost-specific receptor families (*i.e.*, novel immune-type receptors, NITRs) and receptor families that are distantly related to various mammalian immunoregulatory receptors belonging to the immunoglobulin superfamily (IgSF) (*i.e.* leukocyte immune-type receptors, LITRs), which presents a formidable challenge for determining the precise roles of all of the receptor-types in teleost immunity.[28] Furthermore, the complement system in bony fish, which is essential in mammals for Ig-mediated inflammation, is similar in many ways, but also quite distinct from that of mammals, and may not act in a way that is similar to the complement-activation/opsonization system of mammals.[29] Thus, from a mechanistic perspective it cannot be concluded that increases in serum IgG2 levels in mammals, or a possible equivalent in fish, will (or even could) lead to an inflammatory response.

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[25] For review see, for example, Magadan S, Sunyer OJ, Boudinot P (2015). Unique features of fish immune repertoires: particularities of adaptive immunity within the largest group of vertebrates. Results Probl. Cell. Differ. 57: 235-264. Barreto VM, Pan-Hammarstrom Q, Zhao Y, Hammarstrom L, Misulovin Z, Nussenzweig MC. (2005). AID from bony fish catalyzes switch class recombination. J. Exp. Med. 202(6): 733-738.

[26] Plomp R, Ruhaak LR., Uh H-W, Reiding KR, Selman M, Houwing-Duistermatt JJ, Slagboom PE, Beekman M, Wuhrer M. (2017). Subclass-specific IgG glycosylation is associated with markers of inflammation and metabolic health. Scientific Reports. 7:12325.

[27] Aschermann S, Lux A, Baerenwaldt A, Biburger M, Nimmerjahn F. 2010. The other side of immunoglobulin G: suppressor of inflammation. Clin. Exper. Immunol. 160(2): 161–167.

[28] Fei C, Pemberton JG, Lillico DME, Zwozdesky MW, Stafford JL. Biochemical and Functional Insights into the Integrated Regulation of Innate Immune Cell Responses by Teleost Leukocyte Immune-Type Receptors. Biology (Basil). Mar; 5(1): 13.

[29] Nonaka M, Smith SL. (2000). Complement system of bony and cartilaginous fish. Fish Shellfish Immunol. 2000 Apr;10(3):215-28.

As noted by Magadan *et al.* (2015), fish have the most extensive and complex mucosal surfaces among vertebrates, which include the skin as well as the gills and gut mucosa, the immunities of which are poorly understood. However, the protection of mucosa in fish appears to primarily involve IgT, which is analogous to the role played by IgA in mammals. Furthermore, fish mount protective immune responses despite the lack of lymph nodes and germinal centers that initiate immune responses in mammals. Instead, adaptive responses and T-/B-cell cooperation probably take place in the spleen of fish. Fish also lack Peyer's patches or similar encapsulated structures found in the gastrointestinal tract mammals. Thus, the gut associated lymphocyte tissue (GALT) simply comprise the macrophages, B and T lymphocytes and granulocytes of the digestive tract in fish. These substantial differences in the immune systems of fish species compared with mammalian species indicate that the mechanisms resulting in the systemic immune responses to dietary BP in mice and rats are not likely mechanisms associated with fish. For example, the absence of MLNs or analogous tissues in fish indicates that none of the particulates of BP in the gastrointestinal tract of fish can migrate to the spleen of the fish to stimulate a systemic immune system response.

It is also important to distinguish between the direct effects of a test substance on the immune system, which is the study of immunotoxicology, from immunological responses to the test substance that can cause inflammation and other indirect adverse health effects, which is the subject of this opinion.[30] The former, direct effects result from direct interactions of the test substance with molecules or cells of the immune system that lead to immunomodulation, immunoenhancement, or immunosuppression that lead, in turn, to subsequent adverse health effects. The latter, indirect effects result from the immunogenicity of the test substance.

The results of the studies summarized in the preceding sections, in which salmonids were exposed for up to 5 months to diets containing up to 36% BP, were consistently negative for signs of adverse inflammatory effects that can be attributed to FeedKind®. In salmonids, FeedKind® did not produce the exposure-related inflammatory responses that were suggested as possible based on the changes in the IgG2a titers in mice reported by Christensen *et al.* (2003). This conclusion is supported by unpublished studies in which salmon were fed diets containing up to 37% FeedKind® for up to 364 days without affecting body weight or other toxicity endpoints indicative of adverse health effects.[31]

The findings reported by Romarheim *et al.* (2012, 2013) provide substantial support for this conclusion. Conventional solvent-extracted SBM is considered a suitable protein source for farmed salmonids, although dietary inclusion levels as low as 7.6% are known to cause SBM-enteritis (characterized by inflammation of the distal intestines) in salmon. The mechanism for this reversible effect appears to involve impaired immune tolerance to SBM caused by alcohol-soluble components of SBM, such as saponins. In their published review, Martin *et al.* (2017) noted that SBM is now commonly used as a model for inducing gut inflammation (*i.e.* enteritis

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[30] For review, see Rehberger K, Werner I, Hitzfeld B, Segner H, Baumann L (2017). 20 Years of fish immunotoxicology – what we know and where we are. Crit. Rev. Toxicol. 47(6): 516-542.

[31] Storebakken T, Bæverfjord G, Skrede A, Olli JJ, Berge GM (2004). Bacterial protein grown on natural gas in diets for Atlantic salmon, *Salmo solar*, in freshwater. Aquaculture. 241: 413-425.

and associated histopathological changes in the intestines).[32] Romarheim *et al.* (2011, 2013) showed that BP effectively counteracted the severe pro-inflammatory responses to 20% SBM in the intestinal mucosa of these fish, such that the responses were completely abated in the fish fed diets containing 20% SBM plus 15%, 20%, or 30% BP.

IgG2a is an implied biomarker for the immunogenicity of FeedKind® in *mammalian species*, as reported by Christensen *et al.* (2003). Bacterial antigens, which are present in feed ingredients derived from bacteria, are known to stimulate the production of IgG2a and other antibodies in mammals because bacteria stimulate toll-receptors on lymphocytes, which can induce Th-1-type responses. These responses include immunoglobulin class switching in B cells to produce IgG2a antibodies that optimize the clearance of extracellular bacteria and viruses.

However, stimulating antibody production, *per se*, does not mean that there will be pathological consequences to the host. For example, antibodies produced against the bacterial antigens and the adjuvants of orally administered vaccines do not cause host pathologies. The *i.m.* or *s.c.* injection of such vaccines typically cause no more than temporary inflammation at the injection site, which is not antibody-mediated. For instance, the production of IgG2a antibodies in mammals immunized parenterally against *Salmonella typhimurium* is stimulated through a Th-1 response. Likewise, anti-drug antibodies generated against monoclonal-antibody therapeutic agents counteract the pharmacological activity of the agents and remain in the patients in the long term, but generally do not cause any adverse health effects in the patients.

Accordingly, an increase in IgG2a in mammals is not a necessary corollary or an indicator of adverse inflammatory processes, including tissue damage, especially in bony fish (see information above).

As noted above, Christensen *et al.* (2004) found that IgG2a was produced in mice fed SBM. It is possible that antibodies analogous to IgG2a were produced in fish fed a diet containing SBM plus BP, as well as in the fish fed diets containing SBM without BP, in the studies by Romarheim *et al.* (2011, 2013). Nevertheless, the morphological, morphometric, and immunohistochemistry data revealed normal intestinal tissue in the salmon receiving the diet containing BP or the diet containing SBM plus BP, as well as in fish receiving the FM control diet, in contrast to fish fed a diet containing SMB without BP. This indicates that the postulated humoral immune-system response in fish fed diets containing BP, is likely not analogous to the production of IgG2A reported in mice by Christensen *et al.* (2003) and does not appear to be associated with consequent adverse inflammatory processes in the fish.

The studies summarized in the preceding sections show clearly that both BP and Brewer's yeast cause similar age- and sex-specific immune responses in fish and rodents. The humoral responses in fish are reflected in the changes in Ig levels and histological parameters observed in the spleen, which is considered to be the counterpart to mammalian lymph nodes. However, the outcomes of salmon growth rate and body weight measurements and intestinal inflammation studies indicate no overall toxicity. Thus, there is no indication that humoral

[32] Martin AM, Król E (2017). Nutrogenomics and immune function in fish: new insights from omics technologies. Develop. Compar. Immunol. 79: 86-98.

responses activated by BP (or Brewer's yeast), including the changes in Ig levels, lead to adverse effects or Ig-mediated inflammation.

V. Conclusion

Review of the published rodent and salmonid studies, together with the supporting unpublished studies and acknowledgement of mechanism differences between the mammalian immune response and that of bony fish, results in the conclusion that the concern postulated by CVM is not supported by the current scientific literature and that the studies provide *reasonable certainty of no harm to salmonids* exposed to FeedKind® (equivalent to BP). Thus, we conclude that that this ingredient is GRAS at the intended use concentrations up to a maximum of 18% in the diet.

Respectfully submitted,

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Appendix – Expert Curriculum Vitae

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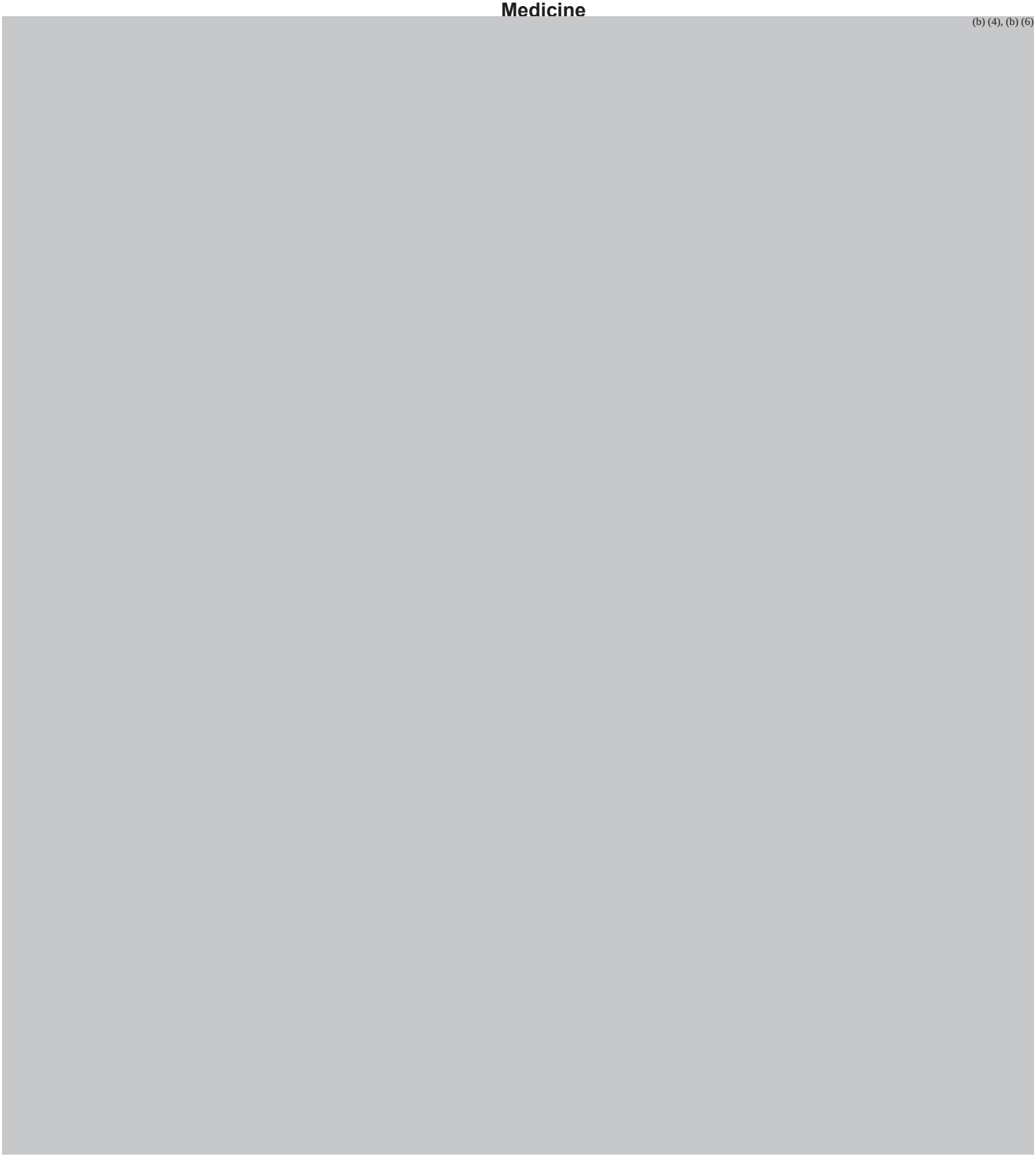
   Tenured Professor
   NYU Grossman School of Medicine
   Department of Environmental Medicine

2. Daniel Wierda, M.S., Ph.D., Fellow ATS
   Wierda Toxicology Consulting, Inc.

JUDITH TERRY ZELIKOFF, Ph.D.

Tenured Professor, NYU Grossman School of Medicine; Dept. Environmental Medicine



Curriculum Vitae of

DANIEL WIERDA, M.S., Ph.D., Fellow ATS

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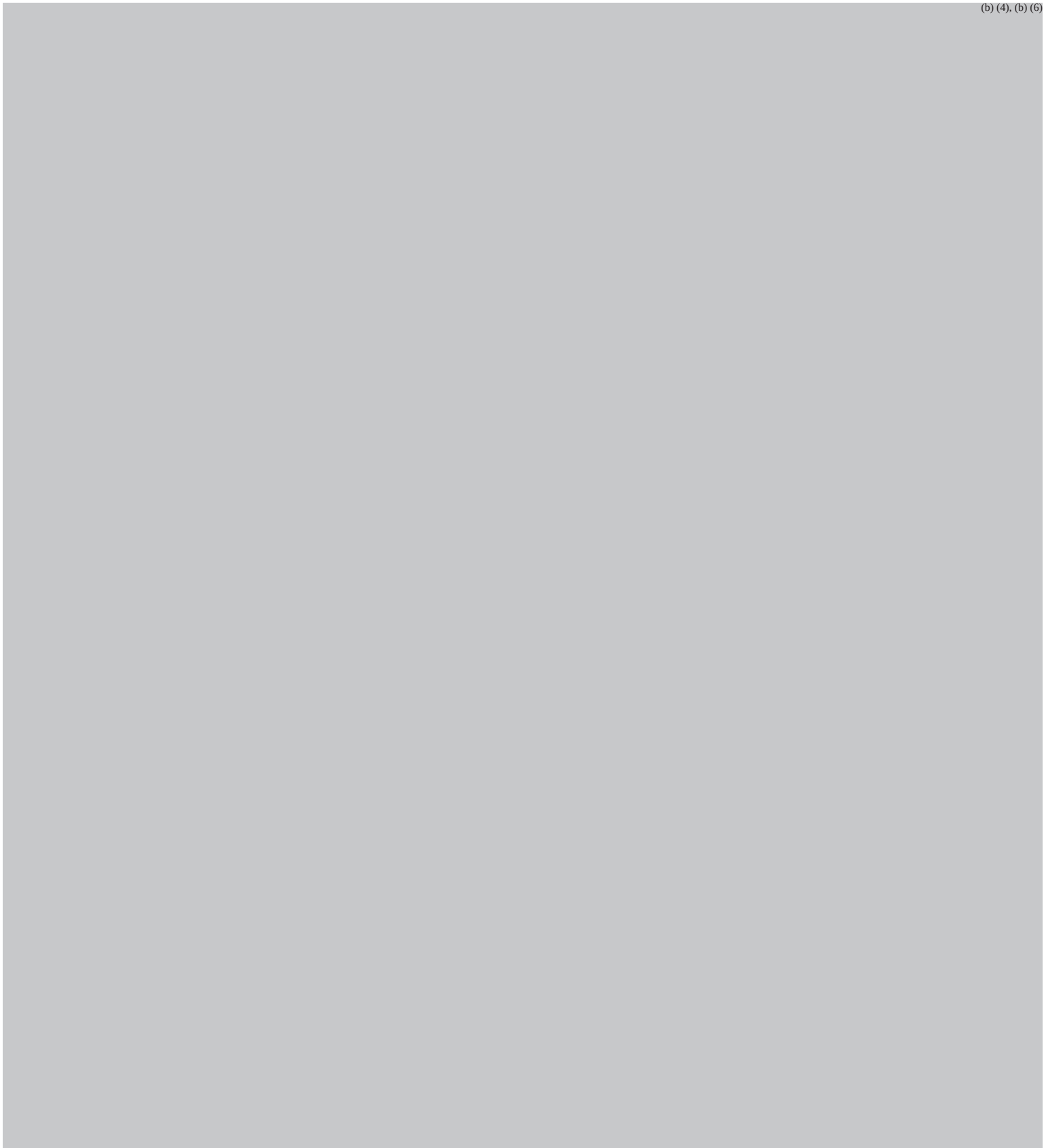

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(b) (4)

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TEST REPORT

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

(b) (4) **Lab No:** 25995

Date: 22 January 2002

Authors: (b) (4)

Number of pages: 263

Sponsor: (b) (4)

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The investigation described in this report "BioProtein - One-Generation Reproduction Toxicity Study in the Rat" was carried out under my supervision and responsibility and in accordance with the OECD principles of Good Laboratory Practice (as revised in 1997) which are in conformity with:

EC Principles of Good Laboratory Practice, Directive 1999/11/EC,
United States Food and Drug Administration, Title 21, CFR, Part 58, and
Japanese Ministry of Health and Welfare, PAB Notification No. 424.

The report is a complete and accurate account of the methods employed and the data obtained.

(b) (4)

22 January 2002

(b) (4)

QUALITY ASSURANCE STATEMENT

The Quality system at [redacted] (b) (4) complies with the OECD Principles of Good Laboratory Practice (as revised in 1997) and the European Standard EN45001.

This study "BioProtein - One-Generation Reproduction Toxicity Study in the Rat" has been inspected by the Quality Assurance Unit in compliance with the principles of Good Laboratory Practice. Inspection reports have been communicated to the Study Director and to the management of Scantox.

Protocol reviewed on	08 June 2000	
Study-based inspections:	Performed on	Reported on
	13 June 2000	13 June 2000
	14 June 2000	14 June 2000
	02 August 2000	02 August 2000
	09 August 2000	09 August 2000
	22 August 2000	22 August 2000
	11 September 2000	11 September 2000
	12 September 2000	12 September 2000
	03 October 2000	03 October 2000
	Audited on	Reported on
Report	26 February 2001 to 15 March 2001	15 March 2001
	22 January 2002	No report

The report has been audited by the Quality Assurance Unit and was found to be an accurate description of the methods and procedures used during the conduct of the study and an accurate reflection of the raw data.

22 January 2002

[redacted] (b) (4)

PERSONNEL RESPONSIBLE FOR THE STUDY

(b) (4)

Study Director until 31 July 2001

Study Director from 01 August 2001

Pathology

Peer review of pathology

Statistics

Quality Assurance

Study Monitor

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SUMMARY

This study was conducted at (b) (4) (b) (4) in accordance with OECD guideline 415 and in compliance with the Principles of Good Laboratory Practice. The objective of the study was to obtain information concerning the effects of BioProtein on male and female reproductive performance, including gonadal function, oestrous cycle, mating behaviour, conception, pregnancy, parturition, lactation, weaning and on the growth and development of the offspring up to weaning.

Ninety-six male and 96 female Wistar (Mol:Wist) rats, aged 5-6 weeks (males) or 8-9 weeks (females) at start of treatment were allocated to four groups of 24 animals/sex/group. The animals were treated orally by dietary administration with concentrations of 0% (group 1), 5.5% (group 2), 11% (group 3) or 22% (group 4) BioProtein in the diet. Males were treated for 10 weeks before mating and during the mating period. Females were treated from 2 weeks before mating until weaning the offspring on postnatal day 21. Selected male offspring from group 1 and 4 were allocated to either control diet condition or BioProtein diet condition (16.5% dietary concentration) and were treated for 4 or 12 weeks after weaning.

After the pre-mating period, parental animals were paired on a one to one basis (male and female from the same group) until signs of successful mating (copulation plugs) had been recorded or three weeks had elapsed. After completion of mating the males were killed and necropsied. Females were allowed to litter normally and rear their progeny to weaning. After weaning the females were killed and subjected to gross pathological examination (see page 9).

Clinical signs were recorded daily for all animals, body weight for each female was recorded on arrival, on the first day of treatment and weekly thereafter. The dams were weighed on gestational days 0, 7, 14 and 20, and on lactational days 1, 4, 7, 14 and 21. Males were weighed on arrival and weekly in the pre-mating and mating period. Pups were weighed collectively (i.e. the litters, males and females separately), on days 1, 4, 7 and 14 and individually on postnatal day 21. Food consumption was recorded weekly.

Time to mating, the length of the gestation period, fertility and gestation rate were calculated. The following parameters recorded between birth and weaning were included:

1. Number and sex of pups, number of stillbirths and live births as soon as possible after delivery (day 0-1).
2. Litter size including dead pups on the morning after birth (day 1).
3. Number of male and female pups (day 1).
4. Number of survivors on day 1, 4, 7, 14 and 21.
5. Number of pups with grossly visible abnormalities.
6. Number of pups with physical or gross behavioural abnormalities after parturition and during the lactation period.

At necropsy, blood samples from all parental animals and selected offspring were collected for possible serological analyses to be reported separately by the Sponsor and the weight of the mesenteric lymph nodes was recorded. Macroscopic abnormalities, cervix, coagulation gland, epididymides, mesenteric lymph nodes, ovaries, prostate, pituitary, seminal vesicles, testes, uterus, vagina were collected from all parental animals, and histological examination was performed on these tissues/organs of the control and high dose group.

Food consumption was dose-dependently reduced in both sexes of parental animals. There were no other effects of treatment on any of the in-life parameters that were recorded.

The weight of mesenteric lymph nodes was significantly higher in parental females of the medium and high dose group and a similar trend was observed in parental males. However, in the offspring no weight change of mesenteric lymph nodes was seen at weaning or after 12 weeks of postweaning treatment with BioProtein at a dietary concentration of 16.5%. Treatment-related findings observed in the mesenteric lymph nodes of parental animals consisted of focal/multifocal granuloma.

In conclusion, dietary administration of BioProtein at dietary concentrations of 5.5, 11 or 22% to Wistar (Mol: Wist) rats during the pre-mating, gestation and lactation period had no effects on reproductive parameters. Previously observed effects of BioProtein on mesenteric lymph nodes in parental animals were confirmed. However, no changes were seen in the weight of mesenteric lymph nodes of offspring treated with BioProtein at a dietary concentration of 16.5% for 4 or 12 weeks after weaning. The dose-dependently lower food consumption in males and females of the medium and high dose group was mainly observed in the premating period and was therefore considered of no relevance for the reprotoxicological outcome of the study.

INTRODUCTION

The objective of this study was to obtain general information concerning the effects of BioProtein on male and female reproductive performance, such as gonadal function, oestrous cycle, mating behaviour, conception, pregnancy, parturition, lactation, weaning and on the growth and development of the offspring up to weaning. The present study was conducted in accordance with the OECD Guideline No 415 (adopted 26 May 1983).

The rat was selected as the test model because of its proven suitability in this type of study. The Wistar (Mol:Wist) rat in particular was used, because of the historical data available at Scantox on this strain.

The oral route of administration was chosen in order to comply with the possible route of exposure when marketing the product.

The dose levels were selected by the Sponsor.

This study was conducted at [redacted] (b) (4)

The males arrived on 07 June 2000, the females arrived on 03 August 2000. Treatment commenced on 12 June 2000 (males) and 07 August 2000 (females). The in-life phase was completed on 28 December 2000.

MATERIALS AND METHODS

Test article

The test article BioProtein, (batch Nos as presented in Table 1, page 26), was supplied by the Sponsor as a constituent in the diets purpose-made for this study. At the request of the Sponsor, the test and control diets were manufactured by [redacted] (b) (4) Test article characterisation (purity, stability) was the responsibility of the Sponsor. Batch Nos 9/99; 10/99; 11/99 and 12/99 were expired before the first day of treatment. At [redacted] (b) (4) the diets were stored at room temperature in the dark.

Prior to the commencement of the study, the Sponsor supplied a BioProtein Data Sheet indicating test material identity, purity, stability, appearance, handling, safety instructions and storage conditions.

To certify that the diets remained stable under the storage conditions at (b) (4) a sample of each batch of the diet formulations was collected on the first and last day of treatment and returned to the Sponsor for analysis after completing the treatment period (Table 1). However, the Sponsor decided not to perform these analyses. Therefore such results were not included in this final report.

The data obtained during the conduct of this study related to the test article supplied by the Sponsor.

Animals

The experiment was performed in 96 male and 96 female SPF Wistar (Mol:Wist) rats from (b) (4) (b) (4) At the start of their acclimatisation period the male rats were 5 - 6 weeks old and their weight was 114 - 143 g, the female rats were 8-9 weeks old and their weight was 157 - 193 g. The weight of a few males was slightly below the weight limit defined in the protocol (120-150 g). This was not considered to have an effect on the outcome of the study.

Twelve animals (six of each sex) were available until completion of the acclimatisation period for replacement purposes.

An acclimatisation period of 4 days for females and 5 days for males was allowed in order to reject animals in poor condition or at extremes of the weight range.

Because the breeder of the animals informed on 19 June 2000 that the males came from a colony seropositive for Kilham rat virus, blood samples for serology were collected from 3 males of each group to give at least 0.2 ml serum per animal. Blood samples were collected from the retro-orbital plexus under CO_2 anaesthesia. For approximately 30 minutes the samples were stored and thereafter centrifuged at room temperature (for 10 minutes at 1270G), transferred to cryotubes, labelled properly and stored at approximately-18°C until dispatch with dry ice to: (b) (4) (b) (4) The results of this analysis showed a low positive titer for Kilham rat virus, which was not considered to pose a problem for the study.

Housing

The study took place in animal room No 4, 14 and 203 provided with filtered air at a temperature of 21°C ± 3°C and relative humidity of 55% ± 15%. On 11 days during the study, the relative humidity was temporarily and - in most instances - slightly above the upper limit of the permissible range. This was not considered to have an impact on the outcome of the study. The ventilation system of the animal room had been designed to give 10 air changes per hour. The room was illuminated to give a cycle of 12 hours light and 12 hours darkness. Light was on from 06:00 to 18:00 h.

The temperature and relative humidity in the animal room were recorded hourly during the study and the records have been retained.

During the acclimatisation period and the pre-mating period the rats were kept in polycarbonate (macrolone type III) cages (floor area 810 cm^2) with two in each cage, males and females separated. During mating, one male and one female were housed together and a wire mesh grid was placed on the bottom of the cage. There was no bedding in the cage. Diet and drinking water were offered *ad libitum*. During pregnancy and lactation, the dams were housed one per cage. The cages were cleaned and the bedding changed at least twice per week.

Before the animals arrived, the animal room was cleaned and disinfected with Glu-Cid®. During the study the animal room was cleaned regularly and rinsed with water.

Bedding

The bedding was softwood sawdust (b) (4) (b) (4) Regular analyses for relevant possible contaminants are performed. Certificates of analysis have been retained.

Diet

During the acclimatisation period, all animals received the control diet (Batch No 9/99) *ad libitum*. Thereafter, the animals received the special diets containing BioProtein *ad libitum* as specified in the paragraph "Treatment".

Drinking water

The animals had free access to bottles with domestic quality drinking water acidified with hydrochloric acid to pH 2.5 in order to prevent microbial growth. Analyses for relevant possible contaminants are performed regularly. Certificates of analysis have been retained.

Animal randomisation and allocation

On the day of arrival the animals were randomly allocated to four groups using a randomisation scheme.

Animal and cage identification

Each animal was identified by punched earmarks (see table below).

Each cage was identified by a colour-coded cage card marked with study number (Lab No 25995), cage number, group number, sex and animal ear number.

Treatment

F_0-generation

The groups, dose levels, animal numbers and colour codes were as follows:

Group	Concentration of BioProtein in the diet (%)	Animal Nos		Colour code
		Male	Female	
1	0	1 - 24	101 - 124	White
2	5.5	25 - 48	125 - 148	Blue
3	11.0	49 - 72	149 - 172	Green
4	22.0	73 - 96	173 - 196	Red

Treatment period and route of treatment

BioProtein was offered continuously as a constituent of the diet to the males daily for 10 weeks before mating and during the mating period, and to the females for 2 weeks before mating and throughout the mating, pregnancy and lactation periods.

In each group, treatment was continued after mating for the first 10 successfully mating males. This was done to have another male of the same group of proven fertility available in case some females had not successfully mated within three weeks. The number of these males of proven fertility was reduced in accordance with the mating success in each group.

After having successfully mated with their first partner, these males were returned to individual housing and their food consumption was recorded on each Monday and until they were terminated.

Mating procedure

Each female was paired on a one to one basis with a male from the same group until pregnancy occurred or three weeks had elapsed.

Each morning and afternoon, the bottom of the cage under the grid was examined for ejected copulation plugs and the female was examined for the presence of a vaginal plug.

The plug-positive day was designated day 0 of pregnancy.

Procedure after mating

After completion of mating the males were killed and necropsied.

The females were allowed to litter normally and rear their progeny to weaning. After weaning the females were killed and subjected to gross pathological examination.

Clinical signs

All visible signs of ill health and any behavioural changes of all animals (including pups) were recorded daily. Any deviation from normal was recorded with respect to time of onset, duration and intensity.

Mortality

All animals (including pups) found dead or killed for humane reasons were subjected to macroscopic examination with the aim of identifying the cause of death/poor condition.

Body weight

Parental animals (F_0)

The weight of each female was recorded on arrival, on the first day of treatment and weekly thereafter. The dams were weighed on gestational days 0, 7, 14 and 20, and on lactational days 1, 4, 7, 14 and 21.

Males were weighed on arrival and weekly in the pre-mating and mating dosing period.

Pups (F_1)

The morning after birth was designated day 1 of lactation. The live pups were weighed collectively (i.e. the litters, males and females separately), on lactational days 1, 4, 7 and 14. On lactational day 21 offspring was weighed individually.

Food consumption

During the pre-mating period, food consumption was recorded weekly. During pregnancy, weekly food consumption was recorded for gestational days 0 - 7, 7 - 14 and 14 - 20. During the lactational period, food consumption was recorded on the same day as the weighing of the pups.

Time to mating and gestational period

The time to mating was calculated for each male and female rat. The gestational period was calculated as the period between the day of mating sign and the day of parturition.

Litter data

The following parameters were recorded between birth and weaning:

1. Number and sex of pups, number of stillbirths and live births as soon as possible after delivery (day 0 - 1)

2. Litter size including dead pups on the morning after birth (day 1).

3. Numbers of male and female pups (day 1).

4. Numbers of survivors on day 1, 4, 7, 14 and 21.

5. Numbers of pups with grossly visible abnormalities.

6. Numbers of pups with physical or gross behavioural abnormalities after parturition and during the lactation period.

Reproduction parameters

The fertility and the gestation rates were calculated. The fertility rate was determined as the percentage of mated females that became pregnant. Pregnancy was demonstrated on the basis of implantation sites observed at necropsy of all female rats.

The gestation rate was determined as the percentage of pregnant females that gave birth.

Blood sampling, necropsy and histopathology

From all F_0-animals at necropsy and from 2 offspring/litter (if possible 1 male and 1 female) on lactational day 21, blood samples were collected from the retro-orbital plexus under barbiturate anaesthesia. The blood samples were collected to give at least 0.2 ml serum per sample. Samples were stored at room temperature for approximately 30 minutes, centrifuged at room temperature for 10 minutes at 1270G, transferred to cryotubes, labelled properly and stored at approximately -18°C until dispatch with dry ice to the Sponsor on 10 January 2001.

The results of the analysis of the samples will be reported separately by the Sponsor.

After recording their body weight (i.e. on the day of necropsy), all F_0 animals were killed when they were no longer necessary for assessment of reproductive effects (males after completion of mating and females at weaning of offspring). At necropsy, mesenterial lymph nodes were dissected and weighed. Absolute and relative weight of lymph nodes were reported.

After recording their body weight, F_1 offspring was killed when weaned, except for 4 males per litter from the first 10 appropriate litters of group 1 and 4 which were subjected to the procedures described in the paragraph "Additional investigations in selected offspring". The remaining offspring, after recording their body weight, was killed at weaning, the mesenteric lymph nodes were collected and their weight recorded from up to four animals per litter if possible from two males and two females.

Gross necropsy

At the time of sacrifice or death during the study all parental animals (F_0) were examined macroscopically for any structural abnormalities or pathological changes, with special attention being paid to the organs of the reproductive system. The implantation sites were counted.

Dead or moribund pups were examined for defects.

Sampling of organs

From all F_0-animals, representative specimens from the organs and tissues listed below were collected and, except for the testes, fixed in phosphate buffered neutral 4% formaldehyde. The testes were fixed in Bouin's fixative. The fixative for long-term preservation was phosphate buffered neutral 4% formaldehyde for all tissues.

Abnormalities
Cervix
Coagulation gland
Epididymides
Mesenteric lymph nodes
Ovaries
Prostate
Pituitary
Seminal vesicles
Testes
Uterus
Vagina

Processing and microscopic examination

After fixation, the organs and tissues sampled for microscopic examination were trimmed and representative specimens were taken for histological processing. The specimens were embedded in paraffin and cut at a nominal thickness of approximately 5 µm, stained with haematoxylin and eosin and examined under the light microscope. Paired organs were processed together.

At the request of the Sponsor an additional set of histological slides from the mesenteric lymph nodes of the parental animals (F_0-generation) were prepared and shipped to (b) (4) (b) (4) on 05 January 2001. Results on possible examinations of these slides will be reported separately by the Sponsor.

All pathological findings were entered directly onto the Path Data computer system.

Histological alterations were graded on a 5 grade system:

Grade 1	-	Minimal/Very few/Very small
Grade 2	-	Slight/Few/Small
Grade 3	-	Moderate/Moderate number/Moderate size
Grade 4	-	Marked/Many/Large
Grade 5	-	Massive/Extensive number/Extensive size
Present	-	Finding present/Severity not scored

The following organs and tissues were examined microscopically:

1) All organs and tissues from all control (group 1) and high dose animals (group 4).
2) All gross lesions from all animals.

Tissues not examined microscopically were held in fixative and stored at Scantox.

Peer review

A peer review was performed on slides from all organs from two males and two females from the high dose (group 4) and from two males and two females from the control group (group 1), on mesenteric lymph nodes from all animals, on organs showing alterations from individual animals and on selected other slides. Diagnostic discrepancies were resolved by discussion.

Additional investigations in selected offspring

Selection and housing of animals

At weaning (lactational day 21), 4 male offspring per litter from 10 litters each of groups 1 and 4 were selected and transferred to macrolone type III cages. If more than 10 litters with 4 male pups were available, the first ten litters (according to weaning date) were used. The offspring was housed two animals per cage. General housing conditions were as described in the protocol.

Animal and cage identification

Each animal was identified by earmarks with a punched number (see table below).

Each cage was identified by a group-wise colour coded cage card marked with study number (Lab No 25995), cage number, group number, sex and animal ear number.

Treatment (F_1-generation)

Two males from each selected litter of group 1 were allocated to group 1C and the other two were allocated to group 1B. Two males from each selected litter of group 4 were allocated to group 4C and the other two were allocated to group 4B.

The groups, dose levels, animal numbers and colour codes were as follows:

Group	Nominal Concentration of BioProtein in the diet (%)	Animal Nos (Males)	Colour code
1C	0	201 up to 220	White
1B	16.5	221 up to 240	Blue
4C	0	241 up to 260	White/red margin
4B	16.5	261 up to 280	Blue/red margin

The first digit of the group number indicated the pre-weaning history, the second digit of the group number indicated the current treatment. The letter C indicated control diet administration and the letter B indicated bioprotein diet administration.

Preparation of bioprotein diet

Test diet for groups 1B and 4B was identical and was prepared by mixing identical amounts of diet from group 3 (11% bioprotein) and group 4 (22 % bioprotein) resulting in a nominal concentration of 16.5% bioprotein using a Turbula Shaker Mixer (b) (4) (b) (4) The Shaker-Mixer was run for at least 6.5 minutes.

Characterisation of the new dietary admixture

Characterisation of the new dietary admixture was the responsibility of the Sponsor. One sample each (approximately 50 g/sample) was collected on the first and last day of treatment of offspring. The samples were sent to the Sponsor on 04 October 2000 to analyse the content of bioprotein. However, the Sponsor decided not to perform these analyses.

Treatment period and route of treatment

All animals were offered bioprotein or control diet for 4 weeks. After treatment for 28 days, one male from each group and litter was terminated. Treatment of the other males per group and litter was continued for a total of 12 weeks. Treatment of all animals was continued until the day of necropsy.

Procedures similar to those executed with parental (F_0)-animals

Daily recording of clinical signs, procedures in case of mortality and weekly recording of body weight and food consumption were as described for parental (F_0)-animals.

Blood sampling, necropsy and histopathology

On the day of necropsy, blood samples were collected from all animals of this phase of the study. Serum samples for possible serological analyses by the Sponsor were prepared and handled as described in the protocol for F_0-animals.

After recording their body weight, all animals of this phase of the study were killed, mesenteric lymph nodes were dissected collected and weighed. Thereafter, these lymph nodes were fixed in phosphate buffered neutral 4% formaldehyde. Histological slides of lymph nodes from offspring treated for 12 weeks after weaning were prepared and shipped to [redacted] (b) (4) on 30 January 2001.

Statistics

For the gestational period and for litter data, non-pregnant rats were excluded from statistics. For continuous variables, descriptive statistics were performed (sample size, mean values and standard deviations). Each continuous variable was tested for homogeneity of variance with Bartlett's test. If the variance was homogeneous, possible intergroup differences were assessed with Dunnett's test. If the variance was heterogeneous, each variable was tested for normality by the Shapiro-Wilk method. In case of normal distribution, possible intergroup differences were identified with Student's t-test. Otherwise the possible intergroup differences were assessed by Kruskal-Wallis's test. If any significant intergroup differences were detected, the subsequent identification of the groups was carried out with Wilcoxon Rank-Sum test.

Categorical (frequency) data were analysed with Chi-square test or Fisher's Exact Test. The sex ratio was first tested for homogeneity within groups before data were pooled for a between-group comparison performed with a Chi-square test.

Entries for the statistical analyses of offspring body weights were average litter values for each sex. A possible significant interaction between sex and treatment was investigated. If no interaction was found, the mean of the offspring body weights of the two sexes was analysed together. Otherwise the offspring body weights for each sex were analysed separately.

The statistical analyses were made with SAS® procedures (version 8.1) described in "SAS/STAT® User's Guide, SAS OnlineDoc®, 1999, SAS Institute Inc., Cary, North Carolina 27513, USA and StatXact® 4 for Windows User Manual, 1998, Cytel Software Corporation, Cambridge, MA 02139, USA.

Archives

For a period of 10 years the following material relating to the study will be retained in archives of (b) (4)

Protocol, protocol amendments and correspondence
Test material receipts
Sample of test article
Animal records
All original data
Wet tissues, blocks and slides
Final report

Histological slides of mesenteric lymph nodes shipped to the Sponsor will not be archived at Scantox.

At the end of the storage period (b) (4) will contact the Sponsor for instructions whether the material should be transferred, retained or destroyed.

Deviations from protocol

The following deviations from protocol occurred which are not considered to have an impact on the integrity of the study.

1. During blood collection for serology for the following animals EDTA-glass tubes instead of plain glass tubes were used erroneously: 201, 203, 205, 207, 209, 221, 223, 225, 227, 229, 241, 243, 245, 247, 261, 263, 265, 267.
2. During pipetting the serum of animal No 277 was lost. Therefore the serum sample for this animal is lacking.

RESULTS

Clinical signs and mortality (F_0: Appendix I, F_1: Appendix II)

No treatment-related clinical signs were observed. One dam (No 168) became pregnant without mating signs being noticed and therefore was excluded from part of the statistics. Offspring No 270 (group 4B) was terminated on postnatal day 53 for humane reasons.

Body weight

Parental animals (Tables 2 and 3, Appendices III and IV)

The body weight and body weight gain in males of group 3 was significantly higher from day 43 to 78 of treatment. This was considered incidental, because it was not dose-dependent and the relative difference to the control group remained constant (4-5% higher). On one single day for each of groups 2 and 4, group mean body weight was significantly lower than that of the control group. This was also considered incidental. No significant group mean differences were observed for females, neither during the pre-mating period, nor during gestation and lactation.

Offspring (Tables 4, 5 and 6, Appendices V, VI and VII)

No significant group differences were seen during the preweaning period. A transiently lower mean body weight was observed in group 4C (preweaning BioProtein/postweaning control condition) as compared to group 1C (preweaning control/postweaning control condition). This was considered incidental, because the transiently lower body weight in group 4B (preweaning BioProtein/postweaning BioProtein condition) was less pronounced and observed only on two occasions (postnatal day 21 and 35).

Food consumption

Parental animals (Tables 7 and 8, Appendices VIII and IX)

Food consumption was significantly lower in males of group 2 (week 1 and 10), group 3 (week 1 and 3-5) and group 4 (week 1-5 and 10), as well as in females of group 3 (week 1-2) and group 4 (week 2). Food consumption was dose-dependently lower in both sexes and was therefore considered test article-related.

Offspring (Table 9, Appendix X)

In week 5, a significantly lower food consumption was observed for group 1B (preweaning control/postweaning BioProtein condition) and 4B (preweaning BioProtein/postweaning BioProtein condition). Because this was an isolated incidence, it was considered incidental. In week 9, the food consumption of groups 1B, 4C and 4B was significantly lower as compared to group 1C. This was attributed to an incidentally high food consumption in group 1C, which was about 20% higher in week 9 as compared to week 8 and 10.

Reproduction parameters (Table 10, Appendix XI)

No significant group differences were seen in any of the reproduction parameters (mating period, gestation period, number of implantations, post-implantation loss). The fertility rate was 100% in groups 1, 3 and 4. In group 2 the fertility rate was 92% (2 of 24 females not pregnant), which was considered incidental. The gestation rate was 100% in all groups.

Litter data (Tables 11 and 12, Appendix XII)

No group differences were observed in litter size from LD 0-1 to LD 21, number of male and female offspring on LD 0-1 and at weaning, and number of surviving pups on LD 1, 4, 7, 14 and 21.

None of the pups showed any grossly visible physical or behavioural abnormalities after parturition and during the lactation period. During the lactation period, two pups in group 1 (dam No 112, dam No 114), one pup in group 2 (dam No 144), three in group 3 (two from dam No 165, one from dam No 171) and two pups in group 4 (dam No 178, dam No 185) appeared underweight.

Weight of mesenteric lymph nodes

Parental animals (Table 13, Appendix XIII)

A dose dependent increase of the weight of mesenteric lymph nodes was observed in both sexes. The group difference, however, became statistically significant only in females of group 3 and 4. The increase itself was more pronounced in females (up to 150% of the control mean) as compared to males (up to 126% of the control mean). The increase of the weight of mesenteric lymph nodes is known from previous studies with BioProtein. In investigations with other compounds (food-grade white oils) which caused a weight increase of mesenteric lymph nodes, it has also been reported that females were more sensitive (BALDWIN et al., Toxicologic Pathology 20, 1992: 426-435; SMITH et al., Toxicologic Pathology 24, 1996: 214-230).

Offspring (Table 14, Appendix XIV)

The weight of mesenteric lymph nodes was unaffected in offspring. No significant group differences were observed at weaning. No significant group differences compared to group 1C (preweaning control/postweaning control condition) were observed, neither after 4 weeks nor after 12 weeks of postweaning treatment with BioProtein at a dietary concentration of 16.5%.

Macroscopic findings (Addendum A)

The very few macroscopic changes observed were considered incidental for rats of this strain and age.

Microscopic findings (Addendum A)

Treatment-related findings observed in the mesenteric lymph nodes of the parental animals consisted of focal/multifocal granuloma formation in the cortical and medullar area characterised by clusters of large pale- (or sometimes eosinophilic) staining histiocytes. This type of granuloma can be seen in lymph nodes of control animals at minimal severity and incidence presenting the normal background level of control animals. However, the severity and frequency of this finding was higher in the animals treated with BioProtein. A summary of these findings is shown in the following overview:

Group	1 (control)		4 (22% BioProtein)	
Sex	Male	Female	Male	Female
No animals examined	24	24	24	24
Granuloma formation in mesenteric lymph nodes:				
Minimal grade	2	2	4	3
Slight grade	-	-	4	3
Moderate grade	-	-	4	-

Minimal to moderate diffuse cortical/medullar histiocytosis, minimal to slight focal/multifocal sinusoidal haemorrhage and minimal to slight focal sinusoidal dilatation were seen to the same extent in the two groups. Focal chronic active inflammation in the lymphoid follicle was noted in animal No 81 of group 4.

Other microscopic findings seen in the organs examined were minor changes normally seen in rats of this strain and age.

CONCLUSION

Dietary administration of BioProtein at dietary concentrations of 5.5, 11 or 22% to Wistar (Mol:Wist) rats during the pre-mating, gestation and lactation period had no effects on reproductive parameters. Previously observed effects of BioProtein on mesenteric lymph nodes in parental animals were confirmed. However, no changes were seen in the weight of mesenteric lymph nodes of offspring treated with BioProtein at a dietary concentration of 16.5% for 4 or 12 weeks after weaning. The dose-dependently lower food consumption in males and females of the medium and high dose group was considered of no relevance for the reprotoxicological outcome of the study.

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Test and control diets supplied by the Sponsor

Batch No	Arrival date	Expiry date	Conc. of TA	Treatment			Date of preparation of dose formulation	Date of pre-dose sampling	Date of post-dose sampling	Date of dispatch of pre-and post dose dietary samples to the Sponsor
				Group No	Animal No	Date of opening of first and last bag of test diet				
09-99 *	23-12-99	17-05-00	0	1	1-24 101-124	07-06-00/03-10-00	18-11-99	19-06-00	-	19-06-00
07-2000	07-08-00	25-01-01	0	1	1-24 101-124	11-09-00/22-09-00	27-07-00	-	08-01-01	08-01-01
10-99	23-12-99	17-05-00	5.5%	2	25-48 125-148	12-06-00/20-09-00	18-11-99	19-06-00	08-01-01	19-06-01
11-2000	29-08-00	16-02-01	5.5%	2	25-48 125-148	28-09-00/	17-08-00	-	08-01-01	10-01-01
11-99	23-12-99	17-05-00	11%	3	49-72 149-172	12-06-00/05-09-00	18-11-99	19-06-00	-	19-06-01
12-2000	29-08-00	16-02-01	11%	3	49-72 149-172	20-09-00/03-10-00	17-08-00	-	08-01-01	10-01-01
12-99	23-12-99	17-05-00	22%	4	73-96 173-196	12-06-00/03-10-00	18-11-99	19-06-00	-	19-06-01
13-2000	29-08-00	16-02-01	22%	4	73-96 173-196	12-09-00/03-10-00	17-08-00	-	08-01-01	10-01-01

* all animals received control diet during the acclimatisation period

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Test and control diets used for treatment of offspring

Batch No	Final concentration	Date of first and last treatment	Animal No	Preparation at (b) (4)	Disp.
09-99 07-2000	0%	03-10-00 28-12-00	201-220 241-260	-	04-10-00
12-2000 12-99 13-2000	16.5%	03-10-00 28-12-00	221-240 261-280	03-10-00 27-11-00 01-12-00	04-10-00

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental animals

Group mean values - Premating and mating period

Males

GROUP	ON ARRIVAL				DAY 1				DAY 8				DAY 15				DAY 22			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	128.9	5.0	24		160.7	6.1	24		193.8	11.8	24		232.8	10.2	24		255.8	14.7	24	
2	128.3	6.8	24		160.9	8.7	24		178.9	21.1	24	**	230.1	20.7	24		259.5	20.0	24	
3	129.9	6.2	24		162.7	5.5	24		189.8	17.6	24		236.8	11.2	24		264.4	12.5	24	
4	130.7	4.5	24		162.5	5.7	24		189.0	15.9	24		224.2	15.6	24	*	255.2	13.3	24	

GROUP	DAY 29				DAY 36				DAY 43				DAY 50				DAY 57			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	270.0	17.3	24		292.9	16.6	24		319.7	18.6	24		336.8	21.9	24		352.1	23.1	24	
2	274.0	23.9	24		290.1	29.8	24		320.0	29.5	24		337.6	32.1	24		353.8	32.9	24	
3	265.3	26.0	24		303.6	22.4	24		331.4	17.5	24	*	352.4	17.7	24	**	368.8	18.1	24	**
4	257.8	18.6	24		287.5	23.6	24		320.6	19.9	24		339.5	19.8	24		353.8	21.5	24	

* means p<0.05, versus control group
** means p<0.01, versus control group

S.D. = standard deviation N = number of animals

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental animals

Group mean values - Premating and mating period

Males

GROUP	DAY 64				DAY 71				DAY 78				BODY WT GAIN 1-78			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	364.4	25.1	24		373.6	24.2	24		376.5	27.7	24		215.8	24.9	24	
2	367.0	34.3	24		376.0	34.0	24		381.7	35.0	24		220.8	30.8	24	
3	384.1	19.0	24	**	388.0	21.1	24		398.8	21.6	24	**	236.2	22.0	24	*
4	365.1	19.9	24		378.6	20.9	24		383.8	21.1	24		221.2	19.4	24	

* means p<0.05, versus control group
** means p<0.01, versus control group

S.D. = standard deviation N = number of animals

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental animals

Group mean values - Premating period

Females

GROUP	ON ARRIVAL				DAY 1				DAY 8				DAY 15				BODY WT GAIN 1-15			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	174.2	7.3	24		183.5	11.5	24		192.2	8.8	24		195.0	10.7	24		11.5	10.1	24	
2	173.1	8.1	24		182.7	8.2	24		192.9	9.7	24		197.2	11.8	24		14.5	8.5	24	
3	177.3	7.0	24		187.3	8.1	24		191.6	7.9	24		199.1	10.7	24		11.8	7.7	24	
4	171.3	6.8	24		184.3	7.3	24		192.0	11.5	24		197.8	11.8	24		13.5	8.9	24	

p>0.05, versus control group

S.D. = standard deviation N = number of animals

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental females

Group mean values - During gestation

GROUP	GD 0				GD 7				GD 14				GD 20				BW GAIN GD 0-20			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	205.4	10.5	24		230.4	12.0	24		260.9	15.5	24		321.7	16.5	24		116.3	13.3	24	
2	203.5	8.8	21		231.5	15.3	22		265.7	17.5	21		324.1	20.7	22		118.8	15.1	22	
3	207.7	13.3	23		233.2	12.6	22		266.0	18.2	23		323.4	25.0	23		115.7	14.8	23	
4	206.0	9.7	24		232.7	11.4	24		264.8	16.9	24		323.0	19.2	24		116.9	15.8	24	

p>0.05, versus control group

S.D. = standard deviation N = number of animals

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental females

Group mean values - During lactation

GROUP	LD 1				LD 4				LD 7			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	242.7	18.2	24		247.7	17.5	24		263.9	15.8	24	
2	243.5	19.9	22		253.1	15.2	22		270.5	19.8	22	
3	246.9	17.7	24		256.5	18.1	24		268.9	18.4	24	
4	249.5	18.5	24		249.3	15.0	24		262.0	14.6	23	

GROUP	LD 14				LD 21				BW GAIN LD 1-21			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	259.0	23.3	24		262.2	18.0	24		19.5	17.4	24	
2	272.0	17.9	22		270.5	20.3	22		27.0	18.6	22	
3	275.0	25.1	24		271.7	16.8	24		24.8	15.9	24	
4	260.0	25.6	24		259.5	17.2	24		10.0	21.6	24	

p>0.05, versus control group

S.D. = standard deviation N = number of animals

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g) PND1 to 14- Offspring - Preweaning period

Group mean values

GROUP	PND 1				PND 4				PND 7				PND 14			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	6.0	0.6	24		8.8	1.2	24		13.7	1.9	24		24.8	3.9	24	
2	6.2	0.7	22		8.9	1.6	22		13.3	2.5	22		25.5	4.4	22	
3	6.0	0.5	24		8.6	1.5	24		12.8	2.3	24		24.1	3.8	23	
4	6.1	0.7	24		9.0	1.4	24		13.5	2.0	23		22.8	4.6	24	

PND = postnatal day

p>0.05, versus control group

S.D. = standard deviation N = numbers of cages

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g) on PND21, absolute (mg) and relative (% of b.wt.)
weight of mesenteric lymph nodes - Offspring at weaning

Group mean values

GROUP	BODY WEIGHT BOTH SEXES PND 21				MESENT. LYMPH NODE, ABS. MALE				MESENT.LYMPH NODE, ABS. FEMALE				MESENT. LYMPH NODE, REL. MALE				MESENT.LYMPH NODE, REL. FEMALE			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	39.4	4.7	24		65.3	17.0	21		69.2	20.4	24		0.162	0.031	21		0.185	0.065	24	
2	41.1	7.4	22		72.4	15.2	22		76.5	20.7	22		0.172	0.024	22		0.184	0.031	22	
3	39.7	6.4	24		65.2	17.5	24		68.4	18.0	24		0.164	0.040	24		0.170	0.035	24	
4	34.8	7.9	24		61.9	19.5	24		60.1	14.5	24		0.174	0.037	24		0.177	0.029	24	

PND = postnatal day

p>0.05, versus control group

S.D. = standard deviation N = number of animals

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Selected offspring

Group mean values - From weaning to postnatal day 49

Males

GROUP	PND 21				PND 28				PND 35			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1C	38.5	5.0	20		64.6	6.4	20		110.7	8.6	20	
1B	37.2	5.6	20		65.0	8.2	20		104.8	9.7	20	
4C	29.9	6.2	20	**	57.5	9.2	20	*	98.2	13.6	20	**
4B	31.3	6.0	19	**	60.1	8.8	19		101.2	12.2	19	*

GROUP	PND 42				PND 49				BODY WT GAIN 21-49			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1C	156.2	10.9	20		198.4	14.2	20		159.9	10.6	20	
1B	149.4	13.6	20		188.2	19.2	20		151.0	14.8	20	
4C	140.4	17.5	20	**	180.2	21.8	20	*	150.4	16.6	20	
4B	146.8	16.3	19		184.7	21.1	19		153.4	17.1	19	

PND = postnatal day

* means p<0.05, versus control group
** means p<0.01, versus control group

S.D. = standard deviation N = number of animals

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Selected offspring

Group mean values - From postnatal day 56 to 105

Males

GROUP	PND 56				PND 63				PND 70				PND 77				PND 84			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1C	238.4	21.2	10		273.8	21.5	10		296.3	22.6	10		319.1	26.3	10		335.9	28.6	10	
1B	227.8	22.4	10		264.8	25.5	10		291.0	28.2	10		308.9	25.2	10		327.4	26.0	10	
4C	225.4	24.2	10		260.4	24.7	10		281.6	23.1	10		303.2	24.9	10		320.9	31.4	10	
4B	224.3	28.3	9		259.8	30.0	9		276.9	21.6	8		306.6	33.7	9		320.9	39.0	9	

GROUP	PND 91				PND 98				PND 105				BODY WT GAIN 21-105			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1C	355.4	28.4	10		363.5	30.2	10		369.4	30.8	10		331.1	28.9	10	
1B	342.9	29.1	10		352.0	29.5	10		359.2	29.0	10		322.7	27.3	10	
4C	338.7	28.4	10		345.8	30.8	10		355.3	29.5	10		325.4	25.0	10	
4B	337.9	36.0	9		343.8	34.3	9		354.2	35.0	9		322.8	30.5	9	

PND = postnatal day

p>0.05, versus control group

S.D. = standard deviation N = number of animals

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental animals

Group mean values per animal per cage - Premating period

Males

GROUP	WEEK 1				WEEK 2				WEEK 3				WEEK 4			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	196.3	27.0	24		201.4	28.5	24		210.8	20.9	24		197.1	24.3	24	
2	159.7	34.4	24	**	208.0	22.6	24		201.1	14.4	24		198.1	18.6	24	
3	169.7	18.9	24	**	193.9	17.7	24		193.0	17.5	24	**	161.5	19.4	24	**
4	159.2	14.0	24	**	183.5	21.3	24	*	184.9	18.4	23	**	157.3	15.4	24	**

GROUP	WEEK 5				WEEK 6				WEEK 7				WEEK 8			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	228.9	19.7	24		263.4	29.1	23		270.0	39.5	24		239.3	38.5	24	
2	231.8	10.9	24		253.2	27.8	24		299.0	41.9	24		242.5	45.2	24	
3	217.9	14.4	24	*	260.8	23.8	24		283.5	52.0	24		260.0	41.2	24	
4	218.8	17.8	24	*	248.7	34.0	23		261.8	45.2	24		240.6	43.5	24	

* means p<0.05, versus control group
** means p<0.01, versus control group

S.D. = standard deviation N = numbers of cages

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental animals

Group mean values per animal per cage - Premating period

Males

GROUP	WEEK 9				WEEK 10				TOTAL 1-10			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	246.8	34.7	24		273.8	39.8	24		2343.5	173.3	23	
2	248.7	27.9	24		221.4	28.2	23	**	2251.4	170.9	23	
3	255.7	39.2	24		253.6	28.8	24		2249.4	182.6	24	
4	232.0	21.6	24		232.5	23.6	24	**	2098.8	152.4	22	**

** means p<0.01, versus control group

S.D. = standard deviation N = numbers of cages

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental animals

Group mean values per animal per cage - Premating period

Females

GROUP	WEEK 1				WEEK 2				TOTAL 1-2			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	154.0	7.8	12		150.7	9.5	12		304.7	15.0	12	
2	154.0	6.4	12		141.3	16.0	12		295.3	16.8	12	
3	144.2	9.6	12	*	135.0	12.1	12	**	279.2	16.3	12	**
4	152.7	8.6	12		131.1	8.6	12	**	283.8	8.8	12	**

* means p<0.05, versus control group
** means p<0.01, versus control group

S.D. = standard deviation N = numbers of cages

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental females

Group mean values - During gestation

GROUP	GD 0-7				GD 7-14				GD 14-20				TOTAL 0-20			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	204.6	43.2	24		196.3	35.7	24		196.3	36.5	24		597.2	66.0	24	
2	218.0	34.5	21		215.7	37.2	22		184.1	32.4	22		613.6	82.4	21	
3	208.0	35.4	23		213.3	31.7	23		189.0	26.1	23		610.3	67.8	23	
4	204.7	47.9	24		213.7	28.4	24		201.6	28.9	24		620.0	63.1	24	

p>0.05, versus control group

S.D. = standard deviation N = numbers of cages

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental females

Group mean values - During lactation

GROUP	LD 1-4				LD 4-7				LD 7-14				LD 14-21				TOTAL LD 1-21			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	133.0	32.4	24		180.5	31.4	24		380.5	74.1	24		564.2	61.1	24		1258.3	148.2	24	
2	128.6	30.4	22		176.1	33.3	22		402.1	59.8	21		545.5	61.8	22		1244.7	131.1	21	
3	146.0	37.5	24		183.7	41.3	24		410.5	101.7	24		531.5	68.6	24		1271.6	174.9	24	
4	128.6	27.2	22		173.8	29.4	23		365.1	106.5	24		489.1	63.7	24	**	1160.7	155.6	21	

** means p<0.01, versus control group

S.D. = standard deviation N = numbers of cages

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Selected offspring

Group mean values per animal per cage From weaning to week 15

Males

GROUP	WEEK 4				WEEK 5				WEEK 6				WEEK 7				WEEK 8			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1C	60.0	5.4	10		114.5	11.0	10		167.1	32.9	10		206.9	41.5	10		336.0	100.3	10	
1B	62.3	5.4	10		103.4	9.3	10	*	143.8	21.9	10		200.4	53.0	10		254.3	90.8	10	
4C	95.2	54.4	10		107.2	7.6	10		159.0	26.6	10		203.0	35.4	10		300.1	85.5	10	
4B	63.1	4.4	9		101.8	10.6	9	*	162.6	21.6	9		195.0	21.4	9		265.8	46.3	9	

GROUP	WEEK 9				WEEK 10				WEEK 11				WEEK 12				WEEK 13			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1C	400.8	110.7	10		357.9	89.6	10		391.4	96.3	10		418.5	111.5	10		373.1	113.7	10	
1B	287.7	76.7	10	*	312.3	81.3	10		299.7	87.5	10		315.3	58.8	10		295.4	45.4	10	
4C	303.6	79.6	10	*	329.7	85.4	10		308.2	63.8	10		358.4	73.8	10		329.2	58.0	10	
4B	297.2	78.6	9	*	300.4	79.0	9		309.3	72.4	9		354.1	88.0	9		302.2	69.9	9	

* means 0.01<p<0.05, versus control group

S.D. = standard deviation N = numbers of cages

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Selected offspring

Group mean values per animal per cage From weaning to week 15

Males

GROUP	WEEK 14				WEEK 15				TOTAL 4-15			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1C	375.4	94.6	10		472.9	138.6	10		3674.3	774.5	10	
1B	309.6	59.6	10		438.4	114.6	10		3022.5	492.1	10	
4C	317.5	51.5	10		532.0	82.6	9		3376.7	540.5	9	
4B	305.1	73.2	9		479.9	56.1	9		3136.6	486.9	9	

p>0.05, versus control group

S.D. = standard deviation N = numbers of cages

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Reproduction parameters

Group mean values

GROUP	MATING PERIOD (DAYS)				GESTATION PERIOD (DAYS)				NO OF IMPLANTATIONS				POST-IMPLANTATION LOSS, %			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	3.0	3.2	24		21.4	0.5	24		13.4	1.6	24		8.5	8.5	24	
2	2.3	1.0	22		21.5	0.6	22		13.5	1.8	22		13.4	13.6	22	
3	2.6	1.2	22		21.3	0.5	23		13.2	2.5	24		8.0	12.4	24	
4	2.4	1.1	24		21.3	0.5	24		12.9	2.3	24		10.8	13.3	24	

p>0.05, versus control group

S.D. = standard deviation N = numbers of cages

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Litter size (LS including dead pups), number of live and dead pups, number of surviving pups (S) on Day 1, 4, 7, 14 and 21

Group mean values

GROUP	LS DAY 0-1				LS MALE DAY 0-1				LS FEMALE DAY 0-1				LIVE DAY 0-1				DEAD DAY 0-1			
	Mean	S.D.	N	p	Mean	S.D.	N	p	Mean	S.D.	N	p	Mean	S.D.	N	p	Mean	S.D.	N	p
1	12.4	2.0	24		5.9	2.4	24		6.5	2.1	24		12.3	1.9	24		0.1	0.3	24	
2	12.0	2.5	22		6.0	1.7	22		5.9	1.7	22		12.0	2.5	22		0.0	0.0	22	
3	12.1	2.6	24		5.8	2.2	24		6.3	1.7	24		12.1	2.6	24		0.0	0.0	24	
4	11.9	2.6	24		5.8	2.4	23		6.1	2.2	23		11.8	2.7	24		0.1	0.3	24	

GROUP	S DAY 1				S DAY 4				S DAY 7				S DAY 14				S DAY 21			
	Mean	S.D.	N	p	Mean	S.D.	N	p	Mean	S.D.	N	p	Mean	S.D.	N	p	Mean	S.D.	N	p
1	12.0	1.7	24		11.8	1.8	24		11.6	1.7	24		11.3	1.8	24		11.3	1.8	24	
2	11.8	2.3	22		11.5	2.0	22		11.3	1.9	22		10.9	1.5	22		10.8	1.4	22	
3	12.0	2.6	24		11.6	2.5	24		11.4	2.2	24		11.0	2.2	24		11.0	2.2	24	
4	11.8	2.7	24		11.6	2.7	24		11.6	2.7	24		11.5	2.6	24		11.4	2.5	24	

p>0.05, versus control GROUP

S.D. = standard deviation N = numbers of cages

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Number of male and female offspring at weaning

Group	MALE	FEMALE	TOTAL
1	122	147	269
2	116	121	237
3	122	141	263
4	131	144	275
Total	491	553	1044

p>0.05, versus control group

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes

Group mean values

Parental animals - Males

GROUP	BODY WEIGHT (g)				MESENTERIC LYMPH NODE, ABSOLUTE				MESENTERIC LYMPH NODE, RELATIVE			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	386.9	30.8	24		323.7	131.2	24		0.0833	0.0327	24	
2	387.3	33.9	24		374.6	93.7	24		0.0968	0.0221	24	
3	406.9	27.2	24		391.5	99.5	24		0.0963	0.0238	24	
4	390.2	22.5	24		406.3	108.3	24		0.1042	0.0268	24	

p>0.05, versus control group

S.D. = standard deviation N = number of animals

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes

Group mean values

Parental animals - Females

GROUP	BODY WEIGHT (g)				MESENTERIC LYMPH NODE, ABSOLUTE				MESENTERIC LYMPH NODE, RELATIVE			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1	262.2	18.0	24		360.5	93.6	24		0.1374	0.0342	24	
2	267.8	22.1	24		435.3	125.6	24		0.1629	0.0492	24	
3	271.7	16.8	24		539.6	140.5	24	**	0.1988	0.0516	24	**
4	259.5	17.2	24		495.8	132.6	24	**	0.1908	0.0473	24	**

** means p<0.01, versus control group

S.D. = standard deviation N = number of animals

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes

Selected offspring after 4 weeks of treatment

Group mean values

GROUP	BODY WEIGHT				MESENTERIC LYMPH NODE, ABSOLUTE				MESENTERIC LYMPH NODE, RELATIVE			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1C	200.5	14.9	10		451.5	70.0	10		0.226	0.035	10	
1B	190.8	20.0	10		493.6	109.6	10		0.257	0.040	10	
4C	177.6	21.7	10		385.2	117.9	10		0.215	0.050	10	
4B	185.6	19.8	10		438.3	103.0	10		0.234	0.037	10	

p>0.05, versus control group

S.D. = standard deviation N = number of animals

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes

Offspring after 12 weeks of treatment

Group mean values

GROUP	BODY WEIGHT				MESENTERIC LYMPH NODE, ABSOLUTE				MESENTERIC LYMPH NODE, RELATIVE			
	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
1C	370.6	31.0	10		543.2	154.3	10		0.147	0.043	10	
1B	359.5	29.4	10		574.2	112.1	10		0.161	0.038	10	
4C	356.4	28.8	10		532.8	170.6	10		0.148	0.040	10	
4B	356.2	39.9	9		564.0	112.3	9		0.158	0.026	9	

p>0.05, versus control group

S.D. = standard deviation N = number of animals

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Clinical signs - Individual Observations

Parental animals (F0-generation)

Group 1: 0% BioProtein (Control)

Animal Nos 1-24 (males)

No adverse clinical signs.

Animal Nos 101-124 (females)

No adverse clinical signs.

Group 2: 5.5% BioProtein

Animal No 25 (male)

Day 15-19:	Left eye dry and slightly swollen.
Day 20-22:	Left eye swollen with opacity.
Day 23-27:	Left eye - cornea damaged, prolaps of anterior chamber, not light sensitive.
Day 28:	No adverse clinical signs.

Animal Nos 26-48 (males)

No adverse clinical signs.

Animal Nos 125-148 (females)

No adverse clinical signs.

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Clinical signs - Individual Observations

Parental animals (F0-generation)

Group 3: 11.0% BioProtein

Animal Nos 49-61 (males)

No adverse clinical signs.

Animal No 62 (male)

Day 85-92: Right eye slightly opaque and enlarged.

Animal Nos 63-68 (males)

No adverse clinical signs.

Animal No 69 (male)

Day 73-74: Separated from female No 169, because of clinical signs (piloerection, swollen head, unbalanced locomotions).
Day 75-79: Paired with female No 169 again, although clinical signs still present.
Day 79: Terminated after successful mating, necropsied.

Animal Nos 70-72

No adverse clinical signs.

Animal Nos 149-167 (females)

No adverse clinical signs.

Animal No 168 (female)

Day 29-35: Probably pregnant.
Day 36: Separated from male.

Animal Nos 169-172 (females)

No adverse clinical signs.

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Clinical signs - Individual Observations

Parental animals (F0-generation)

Group 4: 22% BioProtein

Animal No 73 (male)

No adverse clinical signs.

Animal No 74 (male)

Day 64-79: Blind on left eye.

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Clinical signs - Individual Observations

Offspring males after weaning (F1-generation)

Group 1C: 0% BioProtein

Animal Nos 201-220

No adverse clinical signs.

Group 1B: 16.5% BioProtein

Animal Nos 221-235

No adverse clinical signs.

Animal No 236

Day 91-105: Scratch wound at shoulder region.

Animal Nos 237-240

No adverse clinical signs.

Group 4C: 0% BioProtein

Animal Nos 241-260

No adverse clinical signs.

Group 4B: 16.5% BioProtein

Animal Nos 261-269

No adverse clinical signs.

Animal No 270

Day 53: Distorted position of incisors, unable to eat, terminated.

Animal Nos 271-280

No adverse clinical signs.

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental animals

Individual values - Premating and mating dosing period

Males

GROUP	ANIMAL NO	ON ARRIVAL	DAY 1	DAY 8	DAY 15	DAY 22	DAY 29	DAY 36	DAY 43	DAY 50	DAY 57	DAY 64	DAY 71	DAY 78	BODY WT GAIN 1-78	DAY 85	DAY 92
1	1	126	154	200	237	267	285	284	336	347	361	375	377	382	228	387	395
	2	120	154	199	237	266	261	277	328	342	359	374	381	390	236	406	412
	3	125	154	184	218	227	251	255	298	321	330	335	353	340	186	355	362
	4	125	170	173	228	267	285	316	356	373	386	410	417	417	247		
	5	130	174	200	255	263	315	314	335	377	397	411	421	431	257	435	441
	6	132	170	205	243	273	291	311	338	353	373	385	386	402	232	407	422
	7	142	161	195	220	244	261	274	295	305	323	334	341	344	183		
	8	132	160	195	225	246	271	285	301	311	330	340	345	346	186		
	9	131	166	184	246	264	247	314	357	371	390	409	408	423	257		
	10	132	165	202	235	265	272	305	322	335	347	360	371	373	208		
	11	135	165	205	239	224	276	287	324	335	343	353	369	374	209		
	12	133	162	156	229	270	297	317	341	378	401	416	428	434	272		
	13	125	151	189	212	231	265	276	293	309	323	333	342	344	193		
	14	122	157	196	233	265	287	307	325	333	349	362	364	372	215		
	15	128	161	196	234	260	250	292	316	331	345	352	374	373	212		
	16	129	155	197	237	268	268	315	336	350	354	363	380	380	225		
	17	135	166	205	238	258	274	297	313	326	341	358	363	357	191		
	18	128	159	202	238	268	254	288	317	333	347	359	367	360	201		
	19	132	163	207	245	268	282	297	325	349	365	366	385	390	227		
	20	130	163	201	233	244	259	278	299	313	326	341	351	353	190		
	21	122	155	190	219	252	256	279	307	329	351	356	365	373	218		
	22	129	154	187	220	247	263	287	301	311	330	349	358	355	201		
	23	126	163	202	238	264	244	282	305	321	333	348	354	359	196		
	24	124	155	182	227	237	267	292	305	329	346	357	367	364	209		

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental animals

Individual values - Premating and mating dosing period

Males

GROUP	ANIMAL NO	ON ARRIVAL	DAY 1	DAY 8	DAY 15	DAY 22	DAY 29	DAY 36	DAY 43	DAY 50	DAY 57	DAY 64	DAY 71	DAY 78	BODY WT GAIN 1-78	DAY 85	DAY 92
2	25	124	146	133	153	195	210	217	227	233	241	252	264	270	124		
	26	134	158	178	223	244	260	275	305	311	333	345	347	372	214		
	27	119	148	185	219	244	260	285	305	322	337	352	356	374	226		
	28	137	173	201	238	258	265	263	304	320	344	352	358	365	192		
	29	136	168	200	241	254	240	283	312	324	335	351	368	337	169		
	30	138	178	190	266	299	319	352	379	408	418	441	452	454	276		
	31	126	159	203	244	269	264	312	335	351	371	385	391	397	238		
	32	127	164	207	243	256	278	259	306	341	354	362	380	382	218		
	33	114	150	190	233	263	268	252	315	329	354	366	375	383	233		
	34	132	161	203	238	268	285	299	329	339	352	368	375	382	221		
	35	134	177	205	254	290	314	309	359	372	386	399	401	406	229		
	36	125	160	178	237	274	302	331	351	364	380	399	412	420	260		
	37	124	154	148	220	249	250	268	298	322	338	354	372	375	221		
	38	117	148	188	222	246	272	290	315	330	341	353	358	373	225		
	39	124	157	159	224	262	281	306	326	338	354	371	382	382	225		
	40	134	165	171	233	268	278	286	316	357	380	391	399	408	243		
	41	126	154	190	228	255	263	271	307	319	338	349	365	356	202		
	42	124	159	149	210	239	259	264	290	320	338	358	364	368	209		
	43	127	165	194	243	276	305	334	350	364	379	389	402	411	246		
	44	139	171	177	246	282	299	297	353	378	397	408	414	427	256		
	45	130	161	165	229	260	272	309	326	338	350	364	365	370	209		
	46	125	156	158	221	251	272	278	307	323	339	345	349	358	202		
	47	125	165	150	224	262	274	313	333	353	377	394	394	403	238		
	48	137	165	172	233	263	285	310	332	346	355	360	381	388	223		

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental animals

Individual values - Premating and mating dosing period

Males

GROUP	ANIMAL NO	ON ARRIVAL	DAY 1	DAY 8	DAY 15	DAY 22	DAY 29	DAY 36	DAY 43	DAY 50	DAY 57	DAY 64	DAY 71	DAY 78	BODY WT GAIN 1-78	DAY 85	DAY 92
3	49	133	171	196	251	283	300	338	352	368	378	398	406	415	244		
	50	126	159	203	246	256	262	311	337	362	373	382	381	395	236		
	51	123	160	203	236	246	250	295	314	332	343	357	363	376	216		
	52	133	165	165	223	247	224	284	315	337	353	373	371	385	220		
	53	115	151	198	246	271	287	333	349	369	388	408	417	428	277		
	54	130	163	180	217	240	204	266	291	305	317	331	335	335	172		
	55	136	166	209	250	268	259	325	352	372	387	409	385	425	259		
	56	143	174	212	251	277	268	331	354	367	385	393	407	408	234		
	57	127	166	147	226	254	223	292	324	351	367	386	395	411	245		
	58	127	155	179	225	258	237	290	313	329	351	370	374	381	226		
	59	125	158	200	248	267	285	324	352	370	378	396	393	420	262		
	60	130	158	195	229	252	271	287	307	325	342	354	341	373	215		
	61	131	165	174	223	273	244	265	315	346	366	385	389	402	237	411	420
	62	127	161	202	240	273	279	327	348	370	395	406	420	425	264	438	451
	63	133	166	209	248	275	294	326	347	367	384	399	409	382	216		
	64	141	168	195	242	269	285	291	327	364	379	391	396	401	233		
	65	137	166	197	244	263	269	282	318	343	360	370	380	389	223		
	66	129	159	185	230	253	269	299	325	339	363	373	381	398	239	406	415
	67	130	163	191	219	257	268	272	322	345	359	376	386	397	234	408	413
	68	125	164	163	243	286	290	293	339	358	373	393	400	408	244	422	
	69	131	163	208	245	275	291	319	335	354	374	382	382	377	214		
	70	128	164	204	244	280	292	329	348	362	385	399	397	410	246		
	71	136	167	177	235	268	283	314	346	371	383	405	411	424	257		
	72	122	152	163	223	255	234	294	323	351	367	383	392	407	255		

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental animals

Individual values - Premating and mating dosing period

Males

GROUP	ANIMAL NO	ON ARRIVAL	DAY 1	DAY 8	DAY 15	DAY 22	DAY 29	DAY 36	DAY 43	DAY 50	DAY 57	DAY 64	DAY 71	DAY 78	BODY WT GAIN 1-78	DAY 85	DAY 92
4	73	130	161	189	218	238	253	272	286	297	309	324	330	338	177		
	74	132	166	152	215	247	214	257	296	323	311	333	355	361	195		
	75	130	160	154	222	258	271	291	328	350	368	379	379	385	225		
	76	129	153	186	224	257	269	312	334	353	364	378	395	400	247		
	77	132	169	212	208	229	249	269	316	334	349	361	390	393	224		
	78	132	173	203	243	284	297	320	353	372	382	383	406	403	230		
	79	129	160	179	193	236	223	254	285	301	316	329	339	350	190		
	80	132	164	199	239	248	254	290	333	351	369	375	395	393	229		
	81	131	163	169	218	245	247	285	309	327	349	360	376	388	225		
	82	126	156	188	226	255	266	303	317	334	351	361	367	371	215		
	83	139	164	200	232	249	248	265	304	326	348	362	375	375	211		
	84	128	161	188	234	266	286	322	346	357	372	386	392	392	231		
	85	133	163	196	235	259	266	300	325	352	371	381	386	403	240		
	86	131	166	197	230	266	282	308	329	344	359	363	382	383	217		
	87	123	155	163	215	239	253	239	288	305	324	333	344	348	193		
	88	127	163	200	236	265	263	306	337	351	365	383	391	402	239		
	89	129	157	184	223	257	248	275	310	333	344	355	373	374	217		
	90	138	173	203	223	253	255	315	352	368	381	384	400	418	245		
	91	140	170	183	175	242	236	261	314	339	356	370	388	392	222		
	92	137	169	202	235	267	255	260	313	340	357	368	379	384	215		
	93	127	160	194	237	264	269	288	321	337	349	357	369	374	214		
	94	124	156	192	225	265	247	297	332	355	373	386	404	408	252		
	95	133	165	209	243	277	272	315	348	365	383	398	408	413	248		
	96	125	154	193	232	259	265	296	319	333	342	353	364	362	208		

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental animals

Individual values - Premating period

Females

GROUP	ANIMAL NO	ON ARRIVAL	DAY 1	DAY 8	DAY 15	BODY WT GAIN 1-15
1	101	180	188	191	197	9
	102	174	180	181	187	7
	103	186	192	200	202	10
	104	166	177	192	192	15
	105	170	172	189	186	14
	106	166	164	190	194	30
	107	176	185	187	189	4
	108	170	177	187	175	-2
	109	184	195	197	201	6
	110	168	178	193	203	25
	111	181	194	200	199	5
	112	175	181	184	183	2
	113	177	190	190	200	10
	114	178	183	183	187	4
	115	171	191	190	191	0
	116	171	185	179	192	7
	117	159	156	179	190	34
	118	168	165	190	192	27
	119	188	200	205	211	11
	120	174	191	200	208	17
	121	169	185	197	191	6
	122	167	178	187	180	2
	123	178	194	211	221	27
	124	185	203	210	210	7

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental animals

Individual values - Premating period

Females

GROUP	ANIMAL NO	ON ARRIVAL	DAY 1	DAY 8	DAY 15	BODY WT GAIN 1-15
2	125	177	191	202	213	22
	126	157	166	169	173	7
	127	171	170	181	185	15
	128	171	182	187	200	18
	129	170	186	192	193	7
	130	177	196	218	231	35
	131	173	201	193	195	-6
	132	193	182	192	196	14
	133	169	178	188	200	22
	134	165	175	185	189	14
	135	184	185	188	195	10
	136	174	185	200	213	28
	137	173	187	189	203	16
	138	172	181	194	202	21
	139	158	171	181	177	6
	140	171	183	197	188	5
	141	175	186	200	205	19
	142	162	171	189	190	19
	143	182	185	199	193	8
	144	175	183	207	198	15
	145	185	191	199	201	10
	146	173	179	194	201	22
	147	174	186	196	196	10
	148	173	184	189	195	11

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) : Parental animals

Individual values - Premating period

Females

GROUP	ANIMAL NO	ON ARRIVAL	DAY 1	DAY 8	DAY 15	BODY WT GAIN 1-15
3	149	176	186	192	191	5
	150	184	194	204	209	15
	151	181	186	194	202	16
	152	167	172	185	194	22
	153	174	188	183	197	9
	154	177	190	185	191	1
	155	180	185	185	194	9
	156	188	193	190	193	0
	157	175	187	198	196	9
	158	176	188	196	197	9
	159	170	179	188	185	6
	160	182	199	201	208	9
	161	171	177	181	192	15
	162	164	174	189	197	23
	163	172	184	179	183	-1
	164	172	184	186	195	11
	165	180	185	189	199	14
	166	174	185	190	203	18
	167	191	204	209	231	27
	168	174	182	191	207	25
	169	184	196	198	211	15
	170	188	196	197	212	16
	171	185	200	204	207	7
	172	170	180	184	184	4

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental animals

Individual values - Premating period

Females

GROUP	ANIMAL NO	ON ARRIVAL	DAY 1	DAY 8	DAY 15	BODY WT GAIN 1-15
4	173	168	186	183	178	-8
	174	163	180	190	193	13
	175	180	194	200	201	7
	176	179	189	200	212	23
	177	175	192	202	209	17
	178	169	181	191	202	21
	179	160	175	187	194	19
	180	181	197	208	210	13
	181	178	187	197	208	21
	182	172	186	190	198	12
	183	169	183	189	200	17
	184	170	183	200	212	29
	185	173	186	194	208	22
	186	171	188	186	199	11
	187	172	185	197	199	14
	188	175	180	191	200	20
	189	173	185	201	204	19
	190	162	173	182	180	7
	191	166	184	205	207	23
	192	186	203	211	208	5
	193	168	179	192	194	15
	194	161	177	183	183	6
	195	176	176	165	174	-2
	196	163	175	164	175	0

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental females

Individual values - During gestation

GROUP	ANIMAL NO	GD 0	GD 7	GD 14	GD 20	BW GAIN GD 0-20
1	101	204	226	254	320	116
	102	196	217	234	299	103
	103	230	249	290	332	102
	104	201	234	271	336	135
	105	196	212	241	302	106
	106	202	231	262	327	125
	107	198	233	261	323	125
	108	191	218	245	315	124
	109	211	236	256	332	121
	110	213	242	278	345	132
	111	213	237	272	333	120
	112	199	227	263	326	127
	113	205	224	248	301	96
	114	192	211	240	301	109
	115	199	215	246	303	104
	116	207	225	258	317	110
	117	197	228	260	304	107
	118	207	217	241	293	86
	119	222	251	285	349	127
	120	213	246	278	350	137
	121	198	229	259	321	123
	122	195	230	264	326	131
	123	223	248	280	328	105
	124	218	244	276	337	119

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental females

Individual values - During gestation

GROUP	ANIMAL NO		GD 0	GD 7	GD 14	GD 20	BW GAIN GD 0-20
2	125		220	249	283	333	113
	126	¤	178	202	207	207	29
	127		187	200	242	304	117
	128		200	219	247	289	89
	129		203	233	260	307	104
	130		243#	261	305	368	125
	131		204	236	269	323	119
	132		202	223	258	325	123
	133		203	216	245	296	93
	134		191	204	255	315	124
	135		199	232	262	327	128
	136		214	232	280	355	141
	137		210	246	280	352	142
	138		205	228	253	301	96
	139		196	212	240	298	102
	140		198	229	263	321	123
	141		215	248	278##	318	103
	142		198	233	266	331	133
	143	¤	207	235	248	247	40
	144		212	242	279	337	125
	145		218	251	300	354	136
	146		193	227	256	320	127
	147		203	241	272	331	128
	148		202	230	265	325	123

¤ = not pregnant - all results excluded from statistical analysis
= weighed GD 1 - result excluded from statistical analysis
= weighed GD 15 - result excluded from statistical analysis

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental females

Individual values - During gestation

GROUP	ANIMAL NO		GD 0	GD 7	GD 14	GD 20	BW GAIN GD 0-20
3	149		201	.	252	313	112
	150		218	239	268	329	111
	151		208	233	259	313	105
	152		202	222	253	301	99
	153		204	236	260	321	117
	154		213	252	294	312	99
	155		195	225	243	308	113
	156		190	217	247	288	98
	157		199	220	263	323	124
	158		211	243	271	322	111
	159		201	227	258	305	104
	160		222	247	272	355	133
	161		193	218	249	298	105
	162		202	225	263	316	114
	163		192	218	246	280	88
	164		198	231	257	313	115
	165		209	244	271	337	128
	166		210	227	265	330	120
	167		235	253	306	381	146
	168	¤¤	.	.	.	.	.
	169		243	234	291	362	119
	170		215	240	279	348	133
	171		219	259	304	365	146
	172		197	220	247	319	122

¤¤ = conception not noticed

. = not recorded in error

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental females

Individual values - During gestation

GROUP	ANIMAL NO	GD 0	GD 7	GD 14	GD 20	BW GAIN GD 0-20
4	173	203	233	264	321	118
	174	198	225	260	314	116
	175	212	237	270	335	123
	176	218	224	263	335	117
	177	215	244	269	296	81
	178	206	226	259	331	125
	179	193	224	251	302	109
	180	217	245	266	302	85
	181	213	239	286	343	130
	182	203	227	254	313	110
	183	202	227	253	321	119
	184	212	237	268	334	122
	185	212	244	289	338	126
	186	209	238	275	343	134
	187	204	222	247	299	95
	188	211	235	266	320	109
	189	214	233	284	328	114
	190	189	214	244	289	100
	191	217	249	293	341	124
	192	221	259	296	368	147
	193	200	237	277	337	137
	194	193	224	244	313	120
	195	187	208	239	296	109
	196	196	234	238	332	136

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental females

Individual values - During lactation

GROUP	ANIMAL NO	LD 1	LD 4	LD 7	LD 14	LD 21	BW GAIN LD 1-21
1	101	213	239	255	246	263	50
	102	234	246	259	233	273	39
	103	276	277	280	270	290	14
	104	262	255	285	288	269	7
	105	224	238	257	261	251	27
	106	230	212	252	252	263	33
	107	242	252	264	270	275	33
	108	234	247	253	221	218	-16
	109	245	260	272	274	257	12
	110	257	262	273	263	258	1
	111	243	262	266	258	266	23
	112	247	241	257	249	268	21
	113	233	244	255	226	242	9
	114	202	220	244	248	250	48
	115	219	229	238	223	245	26
	116	241	246	258	279	265	24
	117	251	246	263	262	276	25
	118	235	218	242	222	216	-19
	119	251	268	297	288	281	30
	120	268	282	268	294	275	7
	121	254	251	270	283	269	15
	122	242	251	254	249	277	35
	123	275	264	302	300	276	1
	124	246	235	270	256	270	24

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental females

Individual values - During lactation

GROUP	ANIMAL NO		LD 1	LD 4	LD 7	LD 14	LD 21	BW GAIN LD 1-21
2	125		277	289	297	280	297	20
	126	¤	.	.	.	.	.	.
	127		221	237	242	254	256	35
	128		244	250	261	274	263	19
	129		237	244	274	270	245	8
	130		283	277	311	308	310	27
	131		246	261	275	284	279	33
	132		234	250	255	258	211	-23
	133		224	247	268	265	254	30
	134		235	252	241	257	264	29
	135		247	240	277	290	275	28
	136		250	257	258	273	269	19
	137		248	260	286	269	281	33
	138		248	248	275	268	269	21
	139		193	215	239	254	253	60
	140		235	255	270	278	269	34
	141		238	250	279	292	293	55
	142		255	272	273	271	280	25
	143	¤	.	.	.	.	.	.
	144		246	252	267	271	280	34
	145		283	266	310	312	273	-10
	146		235	236	245	236	262	27
	147		239	256	280	270	284	45
	148		238	254	269	251	284	46

¤ = not pregnant - all results excluded from statistical analysis
. = not recorded in error

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental females

Individual values - During lactation

GROUP	ANIMAL NO	LD 1	LD 4	LD 7	LD 14	LD 21	BW GAIN LD 1-21
3	149	219	237	246	246	266	47
	150	275	269	271	289	276	1
	151	261	264	270	248	260	-1
	152	249	261	263	265	262	13
	153	242	255	275	240	281	39
	154	234	273	240	309	293	59
	155	233	252	278	281	259	26
	156	242	235	246	271	268	26
	157	242	237	263	278	271	29
	158	242	255	280	300	287	45
	159	220	241	261	277	270	50
	160	250	249	274	290	283	33
	161	236	241	249	225	231	-5
	162	237	258	261	254	254	17
	163	245	238	247	268	256	11
	164	245	244	265	275	265	20
	165	254	252	270	290	282	28
	166	227	236	260	233	256	29
	167	278	302	318	290	296	18
	168	253	266	281	276	274	21
	169	289	290	307	319	300	11
	170	244	271	273	291	272	28
	171	273	283	290	318	302	29
	172	236	246	265	266	257	21

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight and body weight gain (g) - Parental females

Individual values - During lactation

GROUP	ANIMAL NO	LD 1	LD 4	LD 7	LD 14	LD 21	BW GAIN LD 1-21
4	173	249	252	269	266	260	11
	174	227	224	251	240	253	26
	175	253	259	274	285	269	16
	176	268	248	277	260	280	12
	177	252	262	279	236	278	26
	178	239	245	264	240	275	36
	179	235	237	254	231	250	15
	180	243	243	252	268	273	30
	181	249	254	250	273	255	6
	182	248	256	265	205	256	8
	183	244	255	270	281	263	19
	184	257	266	270	240	265	8
	185	267	269	275+	259	256	-11
	186	254	250	265	294	283	29
	187	237	239	245	252	248	11
	188	262	256	265	282	284	22
	189	267	244	254	258	267	0
	190	231	241	256	247	257	26
	191	278	282	292	299	277	-1
	192	297	271	285	311	230	-67
	193	254	240	264	245	250	-4
	194	237	228	241	276	245	8
	195	208	219	229	221	241	33
	196	233	243	256	270	213	-20

+ = erroneously weighed LD 8 - result excluded from statistical analysis

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g) - Offspring - Preweaning period

Individual litter means

GROUP	ANIMAL NO	PND 1	PND 4	PND 7	PND 14
1	101	5.2	7.2	12.3	20.0
	102	6.4	10.5	15.7	22.4
	103	5.9	9.5	14.6	28.2
	104	6.1	9.4	14.1	25.8
	105	5.9	7.2	9.7	24.2
	106	5.8	7.4	10.6	22.4
	107	6.4	8.4	11.5	18.0
	108	6.5	11.0	17.5	29.5
	109	6.2	8.6	13.2	25.4
	110	6.4	9.7	14.9	27.8
	111	6.0	9.7	15.6	31.1
	112	5.8	7.9	12.3	25.5
	113	5.7	9.3	14.3	19.3
	114	4.0	6.2	12.7	30.1
	115	6.9	10.1	15.1	28.0
	116	5.5	7.8	11.7	25.2
	117	6.3	10.1	15.9	26.4
	118	6.6	9.2	14.8	28.2
	119	5.7	8.8	14.4	22.7
	120	6.0	9.2	14.2	22.8
	121	5.8	9.7	15.3	27.3
	122	5.4	7.5	11.4	18.9
	123	6.9	9.2	14.6	28.0
	124	6.1	8.0	11.8	18.8

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g) - Offspring - Preweaning period

Individual litter means

GROUP	ANIMAL NO		PND 1	PND 4	PND 7	PND 14
2	125		6.1	10.2	16.3	25.0
	126	¤	.	.	.	.
	127		5.8	8.0	11.9	22.7
	128		7.1	11.3	17.1	28.5
	129		6.3	8.7	14.0	28.3
	130		6.4	10.3	16.3	33.8
	131		6.6	8.6	11.9	21.6
	132		5.6	8.1	11.8	19.0
	133		7.1	10.9	16.1	28.5
	134		6.5	9.9	13.4	26.6
	135		5.5	7.2	10.9	26.5
	136		6.3	8.7	11.3	25.3
	137		5.7	7.0	9.6	18.6
	138		7.0	10.8	16.4	30.4
	139		4.5	6.7	11.3	25.4
	140		6.0	8.9	13.2	26.4
	141		5.3	8.8	14.5	29.6
	142		6.1	9.8	14.1	23.3
	143	¤	.	.	.	.
	144		5.7	6.4	9.6	23.5
	145		7.9	11.7	17.3	33.5
	146		6.3	9.0	13.0	24.1
	147		5.6	6.8	9.9	22.4
	148		5.3	7.8	13.2	17.1

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g) - Offspring - Preweaning period

Individual litter means

GROUP	ANIMAL NO	PND 1	PND 4	PND 7	PND 14
3	149	5.7	7.4	11.2	25.0
	150	5.9	7.1	10.4	21.5
	151	6.3	10.4	16.4	25.2
	152	6.3	10.4	15.5	25.9
	153	5.9	8.2	12.8	15.5
	154	4.9	5.7	7.9	19.8
	155	5.7	8.1	12.8	27.0
	156	5.9	8.4	12.2	29.5
	157	6.6	9.2	14.3	28.1
	158	5.1	6.2	9.4	23.3
	159	6.5	9.4	14.3	29.7
	160	6.2	9.1	14.0	24.3
	161	7.1	11.0	15.2	24.5
	162	6.4	10.1	16.1	27.2
	163	6.2	9.2	12.6	27.4
	164	5.7	7.9	11.5	23.6
	165	5.1	6.2	9.9	23.1
	166	5.8	7.6	10.9	14.0
	167	6.4	9.9	13.5	22.4
	168	6.2	9.4	14.4	24.1
	169	6.3	10.8	15.9	.
	170	5.8	7.2	10.8	24.1
	171	6.4	8.9	12.9	23.7
	172	5.8	7.7	13.0	25.2

. = not recorded in error

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g) - Offspring - Preweaning period

Individual litter means

GROUP	ANIMAL NO	PND 1	PND 4	PND 7	PND 14
4	173	6.3	9.5	14.8	28.3
	174	5.1	7.4	10.5	14.9
	175	5.6	7.5	10.9	22.7
	176	6.2	7.9	12.1	17.0
	177	8.0	12.9	20.0	29.7
	178	5.7	8.1	12.7	15.6
	179	6.8	10.0	14.8	25.8
	180	6.8	9.3	13.6	28.3
	181	6.7	10.0	12.8	24.3
	182	6.0	8.9	13.7	17.3
	183	5.3	8.5	13.0	24.3
	184	6.1	9.8	14.5	19.9
	185	5.7	8.4	13.5	22.0
	186	5.4	7.5	11.0+	24.5
	187	5.9	8.8	12.5	21.9
	188	6.9	10.7	14.5	29.0
	189	6.4	9.0	13.3	23.0
	190	6.3	10.0	14.9	25.5
	191	7.2	11.1	16.2	28.6
	192	5.9	8.3	12.5	24.5
	193	6.5	9.7	14.0	17.2
	194	5.8	8.2	12.4	25.9
	195	5.1	7.3	11.0	16.4
	196	5.8	7.9	11.2	20.3

+ = erroneously weighed LD 8 - results excluded from statistical analysis

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=1

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
101	MALE	34		
101	MALE	36		
101	MALE	36		
101	MALE	35		
101	MALE	36	63	0.175
101	FEMALE	35		
101	FEMALE	32	65	0.203
101	FEMALE	34		
101	FEMALE	33	56	0.170
101	FEMALE	31		
101	FEMALE	35		
101	FEMALE	34	61	0.179
101	FEMALE	34		
102	MALE	40		
102	MALE	37	53	0.143
102	MALE	39	77	0.197
102	FEMALE	39		
102	FEMALE	37		
102	FEMALE	39		
102	FEMALE	40	85	0.213
102	FEMALE	38		
102	FEMALE	37		
102	FEMALE	38	45	0.118
103	MALE	42	73	0.174
103	MALE	43	88	0.205
103	MALE	41		
103	MALE	46		
103	FEMALE	42		
103	FEMALE	42		
103	FEMALE	42		
103	FEMALE	44		
103	FEMALE	46		
103	FEMALE	48	78	0.163
103	FEMALE	45	84	0.187
104	MALE	39	50	0.128
104	MALE	44		
104	MALE	40	66	0.165
104	MALE	43		
104	MALE	35		
104	FEMALE	44		
104	FEMALE	38		

= = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=1
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
104	FEMALE	37	55	0.149
104	FEMALE	38		
104	FEMALE	42		
104	FEMALE	39	54	0.138
105	MALE	46		
105	MALE	41		
105	MALE	35		
105	MALE	38		
105	FEMALE	44	96	0.218
105	FEMALE	44	61	0.139
105	FEMALE	40	93	0.233
105	FEMALE	39	79	0.203
106	MALE	37		
106	MALE	35		
106	MALE	35		
106	MALE	33		
106	FEMALE	35	53	0.151
106	FEMALE	41		
106	FEMALE	43	57	1.321
106	FEMALE	39	74	0.190
106	FEMALE	35		
106	FEMALE	37	63	0.170
107	MALE	30		
107	MALE	31		
107	MALE	31		
107	MALE	33		
107	MALE	35	57	0.163
107	MALE	31	52	0.168
107	FEMALE	31	49	0.158
107	FEMALE	31		
107	FEMALE	32	41	0.128
107	FEMALE	36		
107	FEMALE	32		
107	FEMALE	30		
108	MALE	40	69	0.173
108	MALE	41	62	0.151
108	MALE	38		
108	FEMALE	39	73	0.187
108	FEMALE	39	82	0.210
108	FEMALE	36		

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=1
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
108	FEMALE	40		
108	FEMALE	38		
108	FEMALE	37		
109	MALE	40	49	0.123
109	MALE	37	47	0.127
109	MALE	39		
109	MALE	37		
109	MALE	42		
109	MALE	40		
109	FEMALE	40	52	0.130
109	FEMALE	43		
109	FEMALE	28		
109	FEMALE	35		
109	FEMALE	43	82	0.191
109	FEMALE	41		
109	FEMALE	38		
110	MALE	43		
110	MALE	43		
110	MALE	45		
110	MALE	42		
110	MALE	42	58	0.138
110	MALE	42	71	0.169
110	FEMALE	42		
110	FEMALE	43	81	0.188
110	FEMALE	44		
110	FEMALE	39		
110	FEMALE	42		
110	FEMALE	41		
110	FEMALE	40	51	0.128
111	MALE	51		
111	MALE	49		
111	MALE	41	100	0.244
111	MALE	56		
111	MALE	49	79	0.161
111	MALE	49		
111	FEMALE	47		
111	FEMALE	50	116	0.232
111	FEMALE	47		
111	FEMALE	48		
111	FEMALE	49	62	0.127

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=1
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
112	MALE	43	87	0.202
112	MALE	42	62	0.148
112	MALE	40		
112	FEMALE	38		
112	FEMALE	42		
112	FEMALE	40		
112	FEMALE	33		
112	FEMALE	39		
112	FEMALE	39	75	0.192
112	FEMALE	42		
112	FEMALE	36	49	0.136
113	MALE	34		
113	MALE	35		
113	MALE	33		
113	MALE	36		
113	MALE	35	67	0.191
113	FEMALE	34		
113	FEMALE	33		
113	FEMALE	34	44	0.129
113	FEMALE	35	74	0.211
113	FEMALE	31		
113	FEMALE	33	67	0.203
114	MALE	46		
114	MALE	51	106	0.208
114	MALE	54	95	0.176
114	MALE	24		
114	FEMALE	45		
114	FEMALE	50	126	0.252
114	FEMALE	56	146	0.261
115	MALE	44		
115	MALE	44	116	0.264
115	MALE	44	79	0.180
115	FEMALE	43		
115	FEMALE	42		
115	FEMALE	44		
115	FEMALE	42	84	0.200
115	FEMALE	42	115	0.274
115	FEMALE	42		
115	FEMALE	44		
116	MALE	38		

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=1
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
116	MALE	36		
116	MALE	37		
116	MALE	35		
116	MALE	39		
116	MALE	38		
116	MALE	40	69	0.173
116	MALE	36	66	0.183
116	MALE	37		
116	FEMALE	36	55	0.153
116	FEMALE	37		
116	FEMALE	36	71	0.197
116	FEMALE	38		
117	MALE	47		
117	MALE	47		
117	MALE	47		
117	MALE	47		
117	MALE	44	60	0.136
117	MALE	44	73	0.166
117	FEMALE	42	72	0.171
117	FEMALE	45		
117	FEMALE	43	81	0.188
118	MALE	42	50	0.119
118	MALE	40	77	0.193
118	MALE	41		
118	MALE	41		
118	MALE	41		
118	MALE	40		
118	FEMALE	39		
118	FEMALE	40		
118	FEMALE	40		
118	FEMALE	39	53	0.136
118	FEMALE	38	57	0.150
119	MALE	34		
119	MALE	41		
119	MALE	34		
119	MALE	42		
119	MALE	35		
119	MALE	41		
119	MALE	39		
119	MALE	40		

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=1
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
119	MALE	40	26	0.065
119	MALE	41	56	0.137
119	FEMALE	42	74	0.176
119	FEMALE	39	55	0.141
119	FEMALE	41		
119	FEMALE	39		
120	MALE	36		
120	MALE	38		
120	MALE	37		
120	MALE	25		
120	FEMALE	40		
120	FEMALE	39		
120	FEMALE	37		
120	FEMALE	37	64	0.173
120	FEMALE	35	46	0.131
120	FEMALE	35	63	0.180
120	FEMALE	36		
120	FEMALE	37	37	0.100
120	FEMALE	35		
121	MALE	42		
121	MALE	41		
121	MALE	43		
121	MALE	41		
121	MALE	43	51	0.119
121	MALE	41	90	0.220
121	MALE	43		
121	MALE	41		
121	MALE	41		
121	FEMALE	44	104	0.236
121	FEMALE	40	69	0.173
121	FEMALE	43		
122	MALE	31		
122	MALE	31	40	0.129
122	MALE	30	26	0.087
122	MALE	33		
122	FEMALE	30		
122	FEMALE	28		
122	FEMALE	31		
122	FEMALE	31		
122	FEMALE	28		

[a] = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=1
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
122	FEMALE	28		
122	FEMALE	29		
122	FEMALE	29	36	0.124
122	FEMALE	31		
122	FEMALE	30	41	0.137
123	MALE	45	49	0.109
123	FEMALE	44		
123	FEMALE	43	61	0.142
123	FEMALE	45		
123	FEMALE	44		
123	FEMALE	45		
123	FEMALE	45		
123	FEMALE	44	73	0.166
123	FEMALE	45		
123	FEMALE	43	67	0.156
123	FEMALE	45		
124	MALE	33		
124	MALE	34		
124	MALE	32	56	0.175
124	MALE	34		
124	MALE	33	49	0.148
124	MALE	35		
124	FEMALE	33		
124	FEMALE	35		
124	FEMALE	32	54	0.169
124	FEMALE	35		
124	FEMALE	34	41	0.121
124	FEMALE	33		

GROUP=2

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
125	MALE	42	69	0.164
125	MALE	43		
125	MALE	36		
125	MALE	43		

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=2
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
125	MALE	46	103	0.224
125	FEMALE	43	57	0.133
125	FEMALE	40		
125	FEMALE	40	63	0.158
125	FEMALE	45		
125	FEMALE	43		
126	¤			
127	MALE	43	65	0.151
127	MALE	41	98	0.239
127	MALE	36		
127	MALE	36		
127	MALE	35		
127	FEMALE	38		
127	FEMALE	42		
127	FEMALE	35	53	0.151
127	FEMALE	37		
127	FEMALE	41		
127	FEMALE	41	69	0.168
128	MALE	49	80	0.163
128	MALE	50		
128	MALE	45	79	0.176
128	MALE	47		
128	MALE	44		
128	MALE	51		
128	FEMALE	46	94	0.204
128	FEMALE	43		
128	FEMALE	49	83	0.169
129	MALE	47	94	0.200
129	MALE	48	73	0.152
129	MALE	46		
129	MALE	46		
129	FEMALE	46		
129	FEMALE	48	112	0.233
129	FEMALE	46	146	0.317
129	FEMALE	47		
129	FEMALE	45		
129	FEMALE	45		
129	FEMALE	47		
130	MALE	58	68	0.117
130	MALE	52	84	0.162

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=2
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
130	FEMALE	55		
130	FEMALE	57		
130	FEMALE	54		
130	FEMALE	54		
130	FEMALE	53		
130	FEMALE	59	73	0.124
130	FEMALE	53	95	0.179
131	MALE	33		
131	MALE	31		
131	MALE	39	68	0.174
131	MALE	34		
131	FEMALE	34	53	0.156
131	FEMALE	40	82	0.205
131	FEMALE	34	54	0.159
131	FEMALE	31		
131	FEMALE	35		
131	FEMALE	35		
131	FEMALE	32		
132	MALE	28	64	0.229
132	MALE	28		
132	MALE	27		
132	MALE	29		
132	MALE	30	62	0.207
132	MALE	30		
132	MALE	30		
132	MALE	28		
132	FEMALE	25	42	0.168
132	FEMALE	31	58	0.187
132	FEMALE	24		
132	FEMALE	27		
132	FEMALE	29		
133	MALE	50	79	0.158
133	MALE	52	78	0.150
133	MALE	49		
133	MALE	48		
133	MALE	50		
133	MALE	49		
133	FEMALE	51	114	0.224
133	FEMALE	48		
133	FEMALE	46		

= not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=2
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
133	FEMALE	48		
134	MALE	42	58	0.138
134	MALE	41		
134	MALE	40		
134	MALE	42		
134	MALE	41		
134	MALE	44		
134	MALE	44	56	0.127
134	FEMALE	40	64	0.160
134	FEMALE	37	54	0.146
134	FEMALE	45		
134	FEMALE	39		
135	MALE	43	55	0.128
135	MALE	42	58	0.138
135	MALE	47		
135	MALE	41		
135	MALE	43		
135	FEMALE	42		
135	FEMALE	36		
135	FEMALE	45	76	0.169
135	FEMALE	43	86	0.200
135	FEMALE	44		
135	FEMALE	42		
136	MALE	37		
136	MALE	36	53	0.147
136	MALE	34		
136	MALE	38		
136	MALE	38	95	0.250
136	FEMALE	38	108	0.284
136	FEMALE	38	66	0.174
136	FEMALE	40		
136	FEMALE	41		
136	FEMALE	40		
136	FEMALE	37		
137	MALE	32	46	0.144
137	MALE	32		
137	MALE	34	69	0.203
137	MALE	31		
137	MALE	24		
137	FEMALE	28		

p = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=2
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
137	FEMALE	27		
137	FEMALE	32		
137	FEMALE	30	74	0.247
137	FEMALE	25		
137	FEMALE	27		
137	FEMALE	27		
137	FEMALE	33	46	0.139
137	FEMALE	23		
138	MALE	50		
138	MALE	50	130	0.260
138	MALE	51	86	0.169
138	MALE	52		
138	FEMALE	49		
138	FEMALE	49		
138	FEMALE	50	74	0.148
138	FEMALE	51	79	0.155
139	MALE	43		
139	MALE	39	50	0.128
139	MALE	41	71	0.173
139	MALE	37		
139	MALE	41		
139	MALE	40		
139	FEMALE	38		
139	FEMALE	37		
139	FEMALE	39		
139	FEMALE	39	73	0.187
139	FEMALE	38	44	0.116
140	MALE	41		
140	MALE	43	83	0.193
140	MALE	46		
140	MALE	49		
140	MALE	40	73	0.183
140	FEMALE	40	77	0.193
140	FEMALE	44		
140	FEMALE	46	67	0.146
140	FEMALE	42		
140	FEMALE	42		
141	MALE	50	80	0.160
141	MALE	48		
141	MALE	49	88	0.180

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=2
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
141	MALE	46		
141	MALE	47		
141	FEMALE	45		
141	FEMALE	44		
141	FEMALE	50	83	0.166
141	FEMALE	46	81	0.176
141	FEMALE	48		
142	MALE	37	56	0.151
142	MALE	42		
142	MALE	38	58	0.153
142	FEMALE	39	71	0.182
142	FEMALE	36		
142	FEMALE	35		
142	FEMALE	37		
142	FEMALE	38		
142	FEMALE	37		
142	FEMALE	36		
142	FEMALE	39	62	0.159
143	#			
144	MALE	34		
144	MALE	44	79	0.180
144	MALE	36	55	0.153
144	MALE	35		
144	MALE	36		
144	MALE	37		
144	FEMALE	36		
144	FEMALE	39		
144	FEMALE	35		
144	FEMALE	43	93	0.216
144	FEMALE	41	63	0.154
145	MALE	53	90	0.170
145	MALE	54		
145	MALE	54	109	0.202
145	MALE	51		
145	MALE	52		
145	MALE	52		
145	FEMALE	54	106	0.196
145	FEMALE	53		
145	FEMALE	50	123	0.246
146	MALE	37		

= not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=2
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
146	MALE	37		
146	MALE	38	69	0.182
146	MALE	39		
146	MALE	39	73	0.187
146	MALE	37		
146	FEMALE	35		
146	FEMALE	35	58	0.166
146	FEMALE	38		
146	FEMALE	39		
146	FEMALE	35		
146	FEMALE	38	92	0.242
147	MALE	41	70	0.171
147	MALE	36	60	0.167
147	MALE	38		
147	MALE	33		
147	MALE	38		
147	FEMALE	38		
147	FEMALE	37		
147	FEMALE	34		
147	FEMALE	39	56	0.144
147	FEMALE	36		
147	FEMALE	35	82	0.234
148	MALE	28		
148	MALE	27	42	0.156
148	MALE	30	42	0.140
148	MALE	30		
148	MALE	30		
148	MALE	32		
148	MALE	28		
148	MALE	31		
148	FEMALE	31		
148	FEMALE	29		
148	FEMALE	29		
148	FEMALE	30	52	0.173
148	FEMALE	30	55	0.183

[a] = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=3

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
149	MALE	39	64	0.164
149	MALE	40	61	0.153
149	MALE	41		
149	MALE	40		
149	FEMALE	42	64	0.152
149	FEMALE	42		
149	FEMALE	42		
149	FEMALE	36		
149	FEMALE	34		
149	FEMALE	46		
149	FEMALE	43	69	0.160
149	FEMALE	38		
150	MALE	32	49	0.153
150	MALE	32	88	0.275
150	MALE	31		
150	MALE	32		
150	MALE	37		
150	FEMALE	36		
150	FEMALE	37	69	0.186
150	FEMALE	36	71	0.197
150	FEMALE	32		
150	FEMALE	33		
151	MALE	43	39	0.091
151	MALE	44	157	0.357
151	MALE	41		
151	FEMALE	42		
151	FEMALE	43		
151	FEMALE	41		
151	FEMALE	43		
151	FEMALE	41	132	0.322
151	FEMALE	41	82	0.200
152	MALE	43	64	0.149
152	MALE	39	59	0.151
152	MALE	45		
152	MALE	41		
152	MALE	42		
152	FEMALE	38	63	0.166
152	FEMALE	43		
152	FEMALE	46	98	0.213
152	FEMALE	40		
152	FEMALE	41		

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=3
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
153	MALE	28	50	0.179
153	MALE	26		
153	MALE	26		
153	MALE	27	35	0.130
153	MALE	27		
153	MALE	28		
153	FEMALE	30		
153	FEMALE	28		
153	FEMALE	30		
153	FEMALE	27	43	0.159
153	FEMALE	27		
153	FEMALE	30	40	0.133
154	MALE	34		
154	MALE	32	32	0.100
154	MALE	29	41	0.141
154	MALE	33		
154	MALE	29		
154	MALE	36		
154	MALE	35		
154	FEMALE	31		
154	FEMALE	29		
154	FEMALE	31		
154	FEMALE	34		
154	FEMALE	32	31	0.097
154	FEMALE	31	32	0.103
154	FEMALE	26		
155	MALE	44	61	0.139
155	MALE	42		
155	MALE	41	93	0.227
155	MALE	42		
155	MALE	41		
155	MALE	44		
155	FEMALE	41	127	0.310
155	FEMALE	40		
155	FEMALE	42	69	0.164
155	FEMALE	44		
155	FEMALE	43		
155	FEMALE	43		
156	MALE	52	70	0.135
156	MALE	49	112	0.229

= not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=3
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
156	FEMALE	48	88	0.183
156	FEMALE	47	76	0.162
156	FEMALE	46		
156	FEMALE	50		
157	MALE	45	59	0.131
157	MALE	44		
157	MALE	46		
157	MALE	43	68	0.158
157	FEMALE	46		
157	FEMALE	46		
157	FEMALE	47		
157	FEMALE	44	101	0.230
157	FEMALE	48	56	0.117
157	FEMALE	42		
157	FEMALE	23		
158	MALE	33	43	0.130
158	MALE	35	41	0.117
158	MALE	38		
158	MALE	37		
158	MALE	38		
158	MALE	41		
158	FEMALE	40		
158	FEMALE	33		
158	FEMALE	32		
158	FEMALE	34	49	0.144
158	FEMALE	33	39	0.118
158	FEMALE	34		
158	FEMALE	33		
158	FEMALE	24		
159	MALE	52	75	0.144
159	MALE	48	72	0.150
159	MALE	48		
159	FEMALE	49		
159	FEMALE	44	80	0.182
159	FEMALE	45		
159	FEMALE	44		
159	FEMALE	46		
159	FEMALE	48	93	0.194
159	FEMALE	38		
160	MALE	41		

= not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=3
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
160	MALE	39	58	0.149
160	MALE	43	52	0.121
160	MALE	41		
160	MALE	37		
160	MALE	38		
160	FEMALE	43		
160	FEMALE	39		
160	FEMALE	39	80	0.205
160	FEMALE	41		
160	FEMALE	41		
160	FEMALE	37		
160	FEMALE	41	78	0.190
160	FEMALE	37		
161	MALE	42		
161	MALE	40	41	0.103
161	MALE	36	44	0.122
161	FEMALE	42	77	0.183
161	FEMALE	40		
161	FEMALE	44		
161	FEMALE	41		
161	FEMALE	41	65	0.159
161	FEMALE	37		
161	FEMALE	39		
162	MALE	45		
162	MALE	43		
162	MALE	46	129	0.280
162	MALE	45		
162	MALE	46	73	0.159
162	MALE	44	75	0.170
162	MALE	44		
162	FEMALE	44	76	0.173
162	FEMALE	43		
162	FEMALE	43		
163	MALE	48	54	0.113
163	MALE	51	93	0.182
163	FEMALE	45		
163	FEMALE	46	68	0.148
163	FEMALE	46		
163	FEMALE	40	53	0.133
164	MALE	32	73	0.228

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=3
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
164	MALE	44	96	0.218
164	MALE	40		
164	MALE	38		
164	MALE	34		
164	MALE	35		
164	MALE	37		
164	FEMALE	43	94	0.219
164	FEMALE	39		
164	FEMALE	42	69	0.164
164	FEMALE	34		
165	MALE	36	56	0.156
165	MALE	31		
165	MALE	34	69	0.203
165	MALE	34		
165	MALE	36		
165	MALE	36		
165	FEMALE	39	51	0.131
165	FEMALE	37	69	0.186
165	FEMALE	35		
165	FEMALE	38		
165	FEMALE	36		
166	MALE	29	93	0.321
166	MALE	24		
166	MALE	27	43	0.159
166	FEMALE	30	44	0.147
166	FEMALE	26		
166	FEMALE	24		
166	FEMALE	23		
166	FEMALE	27		
166	FEMALE	30		
166	FEMALE	30	56	0.187
166	FEMALE	25		
167	MALE	35		
167	MALE	38		
167	MALE	36		
167	MALE	36		
167	MALE	37	67	0.181
167	MALE	32	88	0.275
167	FEMALE	35	70	0.200
167	FEMALE	32		

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=3
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
167	FEMALE	36		
167	FEMALE	39		
167	FEMALE	37		
167	FEMALE	33	49	0.148
167	FEMALE	35		
168	MALE	38		
168	MALE	42		
168	MALE	41	54	0.132
168	MALE	42		
168	MALE	42		
168	MALE	41	64	0.156
168	MALE	38		
168	FEMALE	43	62	0.144
168	FEMALE	36		
168	FEMALE	38		
168	FEMALE	41	56	0.137
169	MALE	59	92	0.156
169	MALE	55	66	0.120
169	MALE	57		
169	FEMALE	56	60	0.107
169	FEMALE	52	92	0.177
169	FEMALE	51		
169	FEMALE	55		
169	FEMALE	61		
169	FEMALE	47		
170	MALE	37		
170	MALE	33		
170	MALE	24		
170	MALE	38		
170	MALE	38	42	0.111
170	MALE	36	41	0.114
170	MALE	36		
170	MALE	37		
170	FEMALE	41		
170	FEMALE	38		
170	FEMALE	36		
170	FEMALE	37		
170	FEMALE	32	42	0.131
170	FEMALE	42	53	0.126
170	FEMALE	38		

P = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=3
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
171	MALE	36		
171	MALE	43		
171	MALE	45		
171	MALE	43	34	0.079
171	MALE	44		
171	MALE	45		
171	MALE	43		
171	MALE	37		
171	MALE	42	55	0.131
171	FEMALE	41	63	0.154
171	FEMALE	41	54	0.132
172	MALE	43		
172	MALE	41	71	0.173
172	MALE	40	64	0.160
172	MALE	42		
172	FEMALE	42		
172	FEMALE	39		
172	FEMALE	36	79	0.219
172	FEMALE	39	77	0.197
172	FEMALE	42		
172	FEMALE	42		
172	FEMALE	37		

GROUP=4

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
173	MALE	38	57	0.150
173	MALE	43	68	0.158
173	MALE	41		
173	FEMALE	41		
173	FEMALE	39		
173	FEMALE	40	50	0.125
173	FEMALE	45		
173	FEMALE	41	39	0.095
173	FEMALE	42		
173	FEMALE	43		

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=4
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
173	FEMALE	42		
173	FEMALE	40		
174	MALE	23		
174	MALE	24		
174	MALE	24		
174	MALE	23		
174	MALE	21	24	0.114
174	FEMALE	24		
174	FEMALE	24	58	0.242
174	FEMALE	24	32	0.133
174	FEMALE	23		
174	FEMALE	23	56	0.243
174	FEMALE	24		
174	FEMALE	24		
174	FEMALE	23		
175	MALE	29		
175	MALE	34		
175	MALE	29		
175	MALE	34	29	0.085
175	MALE	32		
175	MALE	21		
175	MALE	36		
175	MALE	35	86	0.246
175	MALE	33		
175	FEMALE	32		
175	FEMALE	32	51	0.159
175	FEMALE	36	38	0.106
175	FEMALE	31		
175	FEMALE	32		
176	MALE	26		
176	MALE	27		
176	MALE	28		
176	MALE	27		
176	MALE	27	49	0.181
176	MALE	26		
176	MALE	30		
176	MALE	30	29	0.097
176	FEMALE	29		
176	FEMALE	29	38	0.131
176	FEMALE	28	45	0.161

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=4
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
176	FEMALE	29		
177	MALE	51	70	0.137
177	FEMALE	50	75	0.150
177	FEMALE	52	52	0.100
177	FEMALE	50	119	0.238
178	MALE	25		
178	MALE	24		
178	MALE	27		
178	MALE	26		
178	MALE	26		
178	MALE	28	55	0.196
178	MALE	25		
178	MALE	27	48	0.178
178	FEMALE	26	45	0.173
178	FEMALE	26	50	0.192
178	FEMALE	24		
178	FEMALE	26		
179	MALE	46	123	0.267
179	MALE	42	77	0.183
179	MALE	43		
179	MALE	44		
179	MALE	42		
179	FEMALE	41		
179	FEMALE	40	82	0.205
179	FEMALE	44		
179	FEMALE	40	75	0.188
180	MALE	49	97	0.198
180	MALE	40	58	0.145
180	MALE	43		
180	MALE	45		
180	MALE	45		
180	MALE	44		
180	FEMALE	45		
180	FEMALE	40		
180	FEMALE	48	50	0.104
180	FEMALE	39	70	0.179
181	MALE	37	62	0.168
181	MALE	36	57	0.158
181	MALE	36		
181	MALE	35		

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=4
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
181	MALE	37		
181	FEMALE	36		
181	FEMALE	37		
181	FEMALE	38		
181	FEMALE	37	73	0.197
181	FEMALE	37	83	0.224
181	FEMALE	38		
181	FEMALE	40		
181	FEMALE	38		
182	MALE	25		
182	MALE	24		
182	MALE	27		
182	MALE	23		
182	MALE	25	43	0.172
182	FEMALE	27	60	0.222
182	FEMALE	26		
182	FEMALE	24	31	0.129
182	FEMALE	24		
182	FEMALE	29		
182	FEMALE	26		
182	FEMALE	26	53	0.204
183	MALE	36	95	0.264
183	MALE	34		
183	MALE	37		
183	MALE	35	101	0.289
183	MALE	38		
183	FEMALE	35	79	0.226
183	FEMALE	36	69	0.192
183	FEMALE	35		
183	FEMALE	35		
183	FEMALE	36		
183	FEMALE	37		
183	FEMALE	35		
184	MALE	30		
184	MALE	30		
184	MALE	30		
184	MALE	31		
184	MALE	30	79	0.263
184	FEMALE	30		
184	FEMALE	27	40	0.148

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=4
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
184	FEMALE	30		
184	FEMALE	30		
184	FEMALE	30		
184	FEMALE	29	46	0.159
184	FEMALE	29		
184	FEMALE	28	50	0.179
185	MALE	30		
185	MALE	35		
185	MALE	32		
185	MALE	35		
185	MALE	32	56	0.175
185	FEMALE	23	35	0.152
185	FEMALE	35	65	0.186
185	FEMALE	36	74	0.206
185	FEMALE	32		
185	FEMALE	35		
185	FEMALE	36		
185	FEMALE	32		
186	MALE	34		
186	MALE	38	68	0.179
186	MALE	38	80	0.211
186	MALE	36		
186	MALE	35		
186	MALE	35		
186	MALE	36		
186	FEMALE	37		
186	FEMALE	39	75	0.192
186	FEMALE	33	57	0.173
186	FEMALE	34		
186	FEMALE	32		
186	FEMALE	35		
186	FEMALE	36		
187	MALE	33		
187	MALE	32		
187	MALE	37		
187	MALE	35		
187	MALE	33	47	0.142
187	FEMALE	33		
187	FEMALE	34	53	0.156
187	FEMALE	35	57	0.163

n = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=4
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
187	FEMALE	34		
187	FEMALE	34		
187	FEMALE	32	58	0.181
188	MALE	50	85	0.170
188	MALE	51		
188	MALE	51	89	0.175
188	FEMALE	49		
188	FEMALE	47	84	0.179
188	FEMALE	49	103	0.210
189	MALE	35		
189	MALE	38		
189	MALE	36		
189	MALE	35		
189	MALE	38	66	0.174
189	FEMALE	39		
189	FEMALE	38	53	0.139
189	FEMALE	36		
189	FEMALE	32		
189	FEMALE	36	58	0.161
189	FEMALE	36	67	0.186
189	FEMALE	37		
190	MALE	45		
190	MALE	40		
190	MALE	43		
190	MALE	44		
190	MALE	43	62	0.144
190	MALE	44	92	0.209
190	FEMALE	41	60	0.146
190	FEMALE	37	84	0.227
191	MALE	43	78	0.181
191	MALE	43		
191	MALE	41	82	0.200
191	MALE	45		
191	FEMALE	43	28	0.065
191	FEMALE	42		
191	FEMALE	44		
191	FEMALE	43		
191	FEMALE	42		
191	FEMALE	40		
191	FEMALE	45	82	0.182

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=4
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
192	MALE	30	52	0.173
192	MALE	31	45	0.145
192	MALE	31		
192	MALE	31		
192	MALE	31		
192	MALE	31		
192	MALE	29		
192	MALE	28		
192	MALE	30		
192	MALE	30		
192	MALE	33		
192	FEMALE	33	61	0.185
192	FEMALE	28	60	0.214
192	FEMALE	26		
193	MALE	28	42	0.150
193	MALE	29	53	0.183
193	FEMALE	27		
193	FEMALE	26		
193	FEMALE	27	54	0.200
193	FEMALE	27	65	0.241
193	FEMALE	27		
193	FEMALE	27		
193	FEMALE	28		
193	FEMALE	28		
193	FEMALE	28		
193	FEMALE	27		
193	FEMALE	26		
194	MALE	39		
194	MALE	39	71	0.182
194	MALE	38	48	0.126
194	MALE	40		
194	FEMALE	40		
194	FEMALE	36	83	0.231
194	FEMALE	39		
194	FEMALE	38	72	0.189
194	FEMALE	42		
194	FEMALE	40		
195	MALE	26		
195	MALE	25		
195	MALE	28		

= not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Sex, individual body weight (g), absolute (mg) and relative (% of b.wt.) of mesenteric lymph nodes - Offspring at weaning

Individual values

GROUP=4
(continued)

DAM NO	FETAL SEX	BODY WEIGHT DAY 21	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
195	MALE	27		
195	MALE	26	40	0.154
195	MALE	26		
195	MALE	25		
195	MALE	24		
195	FEMALE	24	36	0.150
195	FEMALE	25		
195	FEMALE	26		
195	FEMALE	24	48	0.200
195	FEMALE	25	45	0.180
195	FEMALE	24		
196	MALE	28		
196	MALE	26	45	0.173
196	MALE	27	38	0.141
196	MALE	22		
196	MALE	26		
196	MALE	25		
196	FEMALE	28		
196	FEMALE	26		
196	FEMALE	27	49	0.181
196	FEMALE	24		
196	FEMALE	23		
196	FEMALE	27		
196	FEMALE	27		
196	FEMALE	26	51	0.196

= not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g) and body weight gain (g) - Selected offspring

Individual values - From weaning to day 105

Males

		BODY WEIGHT					BODY WT	BODY WEIGHT								BODY WT
	ANIMAL	DAY	DAY	DAY	DAY	DAY	GAIN	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	GAIN
GROUP	NO	21	28	35	42	49	21-49	56	63	70	77	84	91	98	105	21-105
1C	201	34	61	103	148	190	156									
	202	36	65	111	157	200	164	247	265	293	330	330	360	373	384	348
	203	30	56	95	139	170	140									
	204	31	55	90	134	165	134	192	227	251	263	272	294	301	314	283
	205	34	61	111	154	199	165									
	206	35	58	105	144	186	151	230	266	292	311	331	354	361	373	338
	207	38	60	113	157	201	163									
	208	36	57	102	143	188	152	217	266	293	309	322	342	344	358	322
	209	47	73	120	173	220	173									
	210	47	72	116	169	215	168	268	309	334	354	361	389	398	411	364
	211	46	78	122	168	217	171									
	212	41	71	114	158	203	162	252	287	310	336	364	375	387	400	359
	213	37	62	109	153	198	161									
	214	35	63	109	153	200	165	249	288	314	343	363	385	393	401	366
	215	36	66	113	161	205	169									
	216	38	65	112	163	207	169	248	285	290	328	353	367	370	351	313
	217	42	63	114	155	191	149									
	218	41	63	113	159	194	153	238	268	277	294	315	328	332	336	295
	219	43	73	127	176	214	171									
	220	43	70	114	159	204	161	243	277	309	323	348	360	376	366	323
1B	221	36	62	105	145	175	139									
	222	35	63	108	147	178	143	228	260	286	304	320	332	337	357	322
	223	31	56	97	138	165	134									
	224	33	60	102	148	179	146	219	258	303	319	330	355	356	373	340
	225	33	60	91	142	164	131									
	226	36	70	106	163	191	155	252	296	326	341	352	376	385	397	361
	227	37	64	103	148	189	152									
	228	35	65	105	149	188	153	219	268	292	315	333	351	359	370	335
	229	47	77	104	163	209	162									
	230	47	77	107	166	208	161	257	293	310	326	341	359	360	374	327
	231	35	56	109	153	202	167									
	232	38	55	102	141	185	147	233	274	298	325	349	361	375	384	346
	233	35	60	101	137	181	146									
	234	33	61	100	135	183	150	219	258	286	314	335	345	362	360	327
	235	37	68	108	151	196	159									
	236	25	50	79	114	145	120	180	206	225	250	267	279	291	299	274
	237	43	69	109	154	200	157									
	238	41	73	114	154	182	141	221	253	270	287	301	309	316	322	281
	239	45	78	126	175	227	182									
	240	42	75	120	165	217	175	250	282	314	308	346	362	379	356	314

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g) and body weight gain (g) - Selected offspring

Individual values - From weaning to day 105

Males

GROUP	ANIMAL NO		BODY WEIGHT DAY 21	DAY 28	DAY 35	DAY 42	DAY 49	BODY WT GAIN 21-49	BODY WEIGHT DAY 56	DAY 63	DAY 70	DAY 77	DAY 84	DAY 91	DAY 98	DAY 105	BODY WT GAIN 21-105
4C	241		26	50	84	123	156	130									
	242		27	49	82	120	152	125	195	236	268	279	291	317	321	334	307
	243		25	52	94	136	171	146									
	244		24	41	75	121	151	127	201	237	258	288	301	324	318	343	319
	245		25	51	91	134	173	148									
	246		24	53	90	134	173	149	211	240	265	279	283	312	329	335	311
	247		26	54	91	126	163	137									
	248		25	55	94	134	172	147	213	247	271	287	306	323	321	341	316
	249		23	46	81	117	159	136									
	250		24	49	87	127	172	148	211	242	260	283	297	311	318	326	302
	251		30	62	104	145	184	154									
	252		30	65	114	157	202	172	243	279	298	323	344	359	368	380	350
	253		30	58	96	135	172	142									
	254		35	68	113	156	200	165	250	283	306	337	357	376	386	378	343
	255		33	62	103	138	175	142									
	256		32	64	108	144	183	151	216	250	270	291	309	318	330	324	292
	257		35	58	105	149	192	157									
	258		38	67	119	169	217	179	266	301	327	340	372	383	395	409	371
	259		45	79	125	182	231	186									
	260		40	67	107	161	206	166	248	289	293	325	349	364	372	383	343
4B	261		28	52	89	129	163	135									
	262		27	48	81	118	150	123	187	220	249	267	281	297	301	310	283
	263		27	53	90	135	163	136									
	264		26	50	91	142	171	145	228	266	287	302	318	335	342	351	325
	265		27	58	101	148	186	159									
	266		23	47	83	125	160	137	186	226	252	265	268	294	300	312	289
	267		28	60	104	153	195	167									
	268		27	58	96	138	178	151	222	259	275	301	302	329	344	357	330
	269		24	45	81	121	158	134									
	270	@	23	42	71	91	101	78									
	271		30	63	109	161	207	177									
	272		31	66	113	168	216	185	255	293	314	343	358	373	385	385	354
	273		32	66	109	153	186	154									
	274		35	68	112	155	190	155	230	255	277	300	318	333	338	347	312
	275		37	68	113	157	195	158									
	276		35	64	103	145	183	148	219	241	267	289	308	320	328	337	302
	277		36	65	109	148	184	148									
	278		35	63	104	148	184	149	218	264	294	324	340	352	352	367	332
	279		43	74	117	172	219	176									
	280		44	74	118	173	222	178	274	314		368	395	408	404	422	378

@ = weight loss not related to treatment - all results excluded from statistical analysis

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental animals

Values per animal per cage - Premating period

Males

GROUP	ANIMAL NO	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8	WEEK 9	WEEK 10	TOTAL 1-10
1	1	145	192	194	224	248	282	205	199	211	224	2124
	2	135	192	204	190	235	274	251	256	267	322	2326
	3	216	209	230	201	250	286	321	274	239	361	2587
	4	205	203	227	186	243	278	286	288	291	301	2508
	5	229	225	218	204	226	267	293	283	278	315	2538
	6	218	215	216	206	220	347	305	262	233	326	2548
	7	162	147	219	121	222	.	210	192	198	226	.
	8	192	165	184	205	176	238	184	181	176	214	1915
	9	206	241	223	193	236	284	278	276	252	278	2467
	10	183	206	217	183	241	267	307	245	266	288	2403
	11	241	238	212	193	225	253	274	229	248	289	2402
	12	174	226	205	191	243	278	346	283	314	325	2585
	13	182	151	216	242	244	255	232	182	225	251	2180
	14	184	147	251	223	213	220	308	200	198	265	2209
	15	211	211	222	183	246	250	263	196	251	236	2269
	16	167	221	246	202	231	247	238	231	233	272	2288
	17	183	163	180	168	196	196	222	273	292	258	2131
	18	229	210	209	216	233	261	263	207	255	233	2316
	19	227	213	217	204	254	288	260	327	295	269	2554
	20	198	226	205	221	217	259	300	231	236	258	2351
	21	209	185	182	225	235	261	292	237	269	310	2405
	22	219	193	155	187	200	227	289	223	235	291	2219
	23	190	227	209	168	207	266	266	240	201	233	2207
	24	205	227	219	194	252	274	286	227	260	225	2369

. = not recorded in error

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental animals

Values per animal per cage - Premating period

Males

GROUP	ANIMAL NO	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8	WEEK 9	WEEK 10	TOTAL 1-10
2	25	214	234	203	169	226	188	321	186	215	158	2114
	26	169	202	181	242	231	221	297	208	227	221	2199
	27	202	216	204	198	231	247	324	296	289	206	2413
	28	179	241	215	190	223	259	316	243	253	232	2351
	29	187	211	195	194	239	270	346	233	237	234	2346
	30	210	247	198	190	235	300	351	293	293	271	2588
	31	179	210	187	183	233	280	345	266	261	232	2376
	32	206	214	207	220	244	292	353	290	294	.	.
	33	119	208	196	212	224	266	237	255	240	283	2240
	34	176	186	214	187	237	245	245	211	256	222	2179
	35	186	214	185	193	232	254	251	245	217	206	2183
	36	150	242	199	197	227	213	288	169	225	212	2122
	37	183	243	220	186	217	297	361	291	272	271	2541
	38	126	183	202	212	239	254	303	249	263	224	2255
	39	138	196	177	210	247	250	314	292	264	224	2312
	40	176	232	199	208	230	272	347	246	259	205	2374
	41	162	196	224	223	214	234	308	227	261	212	2261
	42	150	210	220	220	237	259	327	358	265	255	2501
	43	153	168	169	158	210	204	265	175	189	196	1887
	44	115	193	208	210	248	278	243	242	209	189	2135
	45	119	189	219	196	250	245	274	228	272	213	2205
	46	117	192	211	191	244	257	262	199	240	206	2119
	47	100	189	192	188	217	245	275	222	212	214	2054
	48	116	175	201	178	229	247	223	195	256	207	2027

. = not recorded in error

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental animals

Values per animal per cage - Premating period

Males

GROUP	ANIMAL NO	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8	WEEK 9	WEEK 10	TOTAL 1-10
3	49	176	223	212	166	212	229	293	278	296	351	2436
	50	148	217	191	160	220	267	364	342	336	261	2506
	51	162	179	181	133	237	267	281	276	272	232	2220
	52	159	206	175	142	203	296	305	286	288	261	2321
	53	174	178	181	166	209	256	235	220	211	241	2071
	54	177	182	187	138	195	253	208	273	227	240	2080
	55	164	201	196	151	206	252	347	302	282	251	2352
	56	156	175	202	133	197	213	189	197	177	244	1883
	57	157	222	203	152	230	300	324	277	246	223	2334
	58	175	183	195	143	211	229	279	272	266	275	2228
	59	193	186	179	139	232	261	211	238	274	251	2164
	60	150	188	208	183	223	283	294	289	268	252	2338
	61	188	209	239	151	227	288	304	293	271	229	2399
	62	124	176	185	173	197	223	218	200	221	223	1940
	63	185	164	161	149	213	246	204	202	187	248	1959
	64	172	208	195	152	203	260	327	250	249	249	2265
	65	157	219	200	182	217	272	324	217	278	247	2313
	66	182	190	185	170	241	257	293	205	269	252	2244
	67	217	205	186	186	242	282	303	289	254	287	2451
	68	177	193	204	170	227	282	283	277	273	234	2320
	69	189	205	158	166	211	229	240	196	221	231	2046
	70	148	176	187	180	220	258	269	275	191	224	2128
	71	174	201	207	206	215	266	355	270	271	285	2450
	72	169	167	214	184	241	289	354	317	308	295	2538

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental animals

Values per animal per cage - Premating period

Males

GROUP	ANIMAL NO	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8	WEEK 9	WEEK 10	TOTAL 1-10
4	73	146	160	172	178	205	201	219	164	179	214	1838
	74	152	229	205	136	251	.	408	326	252	285	.
	75	176	201	168	150	226	297	279	243	217	248	2205
	76	140	160	167	139	208	221	214	168	210	270	1897
	77	175	194	.	170	196	272	281	239	236	251	.
	78	160	192	203	161	209	250	294	250	236	269	2224
	79	156	195	201	156	227	228	239	239	226	238	2105
	80	151	195	181	145	217	295	277	166	236	237	2100
	81	173	205	204	158	244	220	238	224	225	205	2096
	82	177	192	174	149	222	215	225	215	215	219	2003
	83	163	177	200	178	213	254	284	247	216	191	2123
	84	148	173	200	183	213	206	236	208	216	230	2013
	85	169	167	173	145	222	261	218	240	208	207	2010
	86	167	168	165	149	171	209	214	209	217	204	1873
	87	136	151	179	175	236	275	269	268	226	225	2140
	88	168	204	192	166	239	225	265	259	226	208	2152
	89	163	195	193	139	211	279	272	271	256	227	2206
	90	187	200	201	161	237	278	266	248	247	238	2263
	91	167	160	208	179	243	309	328	288	265	243	2390
	92	139	208	218	179	225	266	311	348	274	244	2412
	93	166	159	164	153	214	284	274	241	244	219	2118
	94	140	177	158	140	213	247	208	252	244	253	2032
	95	158	197	168	148	205	232	229	242	264	244	2087
	96	144	146	159	138	205	195	235	220	233	211	1886

= not recorded in error

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental animals

Values per animal per cage - Premating period

Females

GROUP	ANIMAL NO	WEEK 1	WEEK 2	TOTAL 1-2
1	149	167.5	165.5	333.0
	150	153.5	149.0	302.5
	151	157.0	143.0	300.0
	152	150.0	141.0	291.0
	153	156.5	153.0	309.5
	154	156.5	154.5	311.0
	155	165.5	146.0	311.5
	156	152.5	146.5	299.0
	157	151.0	161.5	312.5
	158	152.0	162.5	314.5
	159	137.5	133.5	271.0
	160	148.5	152.5	301.0
2	161	154.5	135.0	289.5
	162	145.5	132.0	277.5
	163	158.5	135.5	294.0
	164	155.0	137.0	292.0
	165	139.5	133.5	273.0
	166	164.0	118.5	282.5
	167	152.0	184.0	336.0
	168	153.5	139.5	293.0
	169	157.0	155.5	312.5
	170	152.5	138.5	291.0
	171	157.5	139.5	297.0
	172	158.5	147.0	305.5

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental animals

Values per animal per cage - Premating period

Females

GROUP	ANIMAL NO	WEEK 1	WEEK 2	TOTAL 1-2
3	173	129.0	124.5	253.5
	174	140.5	125.0	265.5
	175	140.5	133.5	274.0
	176	157.0	141.5	298.5
	177	149.0	131.0	280.0
	178	162.0	131.5	293.5
	179	141.0	134.0	275.0
	180	135.5	134.5	270.0
	181	155.0	133.5	288.5
	182	141.0	163.0	304.0
	183	140.5	150.5	291.0
	184	139.0	117.5	256.5
4	185	142.5	134.0	276.5
	186	146.0	131.0	277.0
	187	149.5	134.0	283.5
	188	155.0	129.0	284.0
	189	155.0	132.5	287.5
	190	137.0	141.0	278.0
	191	158.0	148.0	306.0
	192	147.0	130.5	277.5
	193	156.5	121.0	277.5
	194	155.5	125.0	280.5
	195	167.5	115.0	282.5
	196	162.5	132.0	294.5

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental females

Individual values - During gestation

GROUP	ANIMAL NO	GD 0-7	GD 7-14	GD 14-20	TOTAL GD 0-20
1	101	247	230	184	661
	102	211	172	260	643
	103	212	188	193	593
	104	234	231	206	671
	105	126	181	162	469
	106	194	212	252	658
	107	208	235	243	686
	108	186	186	206	578
	109	190	173	176	539
	110	203	202	172	577
	111	205	172	153	530
	112	252	225	262	739
	113	172	172	148	492
	114	173	180	171	524
	115	181	201	237	619
	116	194	214	256	664
	117	189	211	185	585
	118	360	69	156	585
	119	162	181	159	502
	120	196	189	194	579
	121	226	199	187	612
	122	191	220	198	609
	123	226	212	165	603
	124	173	255	187	615

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental females

Individual values - During gestation

GROUP	ANIMAL NO		GD 0-7	GD 7-14	GD 14-20	TOTAL GD 0-20
2	125		234	205	189	628
	126	¤	222	215	172	609
	127		197	202	187	586
	128		192	215	172	579
	129		198	178	148	524
	130		183¤¤¤	296	193	672
	131		280	200	219	699
	132		256	247	212	715
	133		179	159	136	474
	134		175	221	184	580
	135		236	217	259	712
	136		206	211	183	600
	137		232	250	190	672
	138		218	203	176	597
	139		150	185	130	465
	140		173	162	139	474
	141		256	277	144	677
	142		276	218	223	717
	143	¤	211	8	200	419
	144		222	192	211	625
	145		239	288	206	733
	146		197	234	199	630
	147		233	207	195	635
	148		230	179	155	564

¤ = not pregnant, all results excluded from statistical analysis

¤¤¤ = only GD 1-7, result excluded from statistical analysis

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental females

Individual values - During gestation

GROUP	ANIMAL NO		GD 0-7	GD 7-14	GD 14-20	TOTAL GD 0-20
3	149		173	236	206	615
	150		190	193	199	582
	151		213	212	194	619
	152		206	201	217	624
	153		199	167	176	542
	154		211	242	197	650
	155		151	172	151	474
	156		257	255	187	699
	157		223	184	168	575
	158		233	276	169	678
	159		166	187	159	512
	160		156	194	193	543
	161		221	193	192	606
	162		202	209	169	580
	163		200	205	187	592
	164		221	200	149	570
	165		307	187	206	700
	166		212	227	174	613
	167		176	212	197	585
	168	¤¤				
	169		218	231	274	723
	170		238	251	211	700
	171		242	283	188	713
	172		170	190	183	543

¤¤ = conception not noticed

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental females

Individual values - During gestation

GROUP	ANIMAL NO	GD 0-7	GD 7-14	GD 14-20	TOTAL GD 0-20
4	173	209	231	198	638
	174	125	229	162	516
	175	250	261	204	715
	176	186	193	202	581
	177	203	222	168	593
	178	169	223	245	637
	179	220	176	167	563
	180	215	220	259	694
	181	187	219	215	621
	182	171	195	197	563
	183	162	181	176	519
	184	190	209	213	612
	185	169	264	181	614
	186	250	219	223	692
	187	176	227	231	634
	188	195	189	249	633
	189	176	247	176	599
	190	177	187	179	543
	191	293	248	175	716
	192	206	198	172	576
	193	180	251	218	649
	194	269	164	205	638
	195	188	203	181	572
	196	347	173	242	762

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental females

Individual values - During lactation

GROUP	ANIMAL NO	LD 1-4	LD 4-7	LD 7-14	LD 14-21	TOTAL LD 1-21
1	101	121	239	311	540	1211
	102	122	152	224	539	1037
	103	193	220	484	730	1627
	104	90	174	473	584	1321
	105	152	113	322	524	1111
	106	168	155	368	520	1211
	107	118	210	367	649	1344
	108	131	175	348	512	1166
	109	207	213	453	551	1424
	110	113	191	469	543	1316
	111	126	188	455	549	1318
	112	139	171	438	597	1345
	113	90	135	245	467	937
	114	130	237	450	557	1374
	115	165	171	374	538	1248
	116	174	206	447	701	1528
	117	93	196	293	531	1113
	118	108	156	438	518	1220
	119	114	204	349	621	1288
	120	163	155	382	498	1198
	121	127	176	405	547	1255
	122	115	182	326	585	1208
	123	88	166	413	590	1257
	124	146	147	299	550	1142

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental females

Individual values - During lactation

GROUP	ANIMAL NO		LD 1-4	LD 4-7	LD 7-14	LD 14-21	TOTAL LD 1-21
2	125		106	197	359	526	1188
	126	¤	.	.	.	.	.
	127		85	165	352	580	1182
	128		111	154	358	543	1166
	129		173	147	453	557	1330
	130		158	199	.	654	.
	131		138	141	341	487	1107
	132		136	199	396	399	1130
	133		131	164	364	524	1183
	134		126	120	438	530	1214
	135		129	180	518	632	1459
	136		124	138	421	524	1207
	137		136	237	403	521	1297
	138		84	186	393	495	1158
	139		104	140	395	565	1204
	140		124	162	379	494	1159
	141		170	209	418	633	1430
	142		173	159	369	484	1185
	143	¤	.	.	.	.	.
	144		191	250	477	607	1525
	145		125	179	476	636	1416
	146		88	160	469	552	1269
	147		131	220	419	569	1339
	148		86	169	246	490	991

¤ = not pregnant, all results excluded from statistical analysis
. = not recorded in error

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental females

Individual values - During lactation

GROUP	ANIMAL NO	LD 1-4	LD 4-7	LD 7-14	LD 14-21	TOTAL LD 1-21
3	149	220	181	456	546	1403
	150	79	168	401	430	1078
	151	103	174	258	466	1001
	152	171	147	329	501	1148
	153	121	180	212	508	1021
	154	119	143	500	542	1304
	155	140	161	445	508	1254
	156	184	224	484	460	1352
	157	100	159	457	718	1434
	158	154	208	561	563	1486
	159	177	193	472	534	1376
	160	115	183	450	600	1348
	161	151	142	304	498	1095
	162	145	161	346	496	1148
	163	128	182	528	430	1268
	164	128	157	419	529	1233
	165	89	184	497	498	1268
	166	118	255	226	461	1060
	167	162	147	334	538	1181
	168	209	132	303	528	1172
	169	186	320	579	673	1758
	170	159	208	458	596	1421
	171	194	219	453	581	1447
	172	151	181	379	551	1262

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Parental females

Individual values - During lactation

GROUP	ANIMAL NO	LD 1-4	LD 4-7	LD 7-14	LD 14-21	TOTAL LD 1-21
4	173	142	189	497	542	1370
	174	123	116	253	405	897
	175	139	190	488	563	1380
	176	.	220	235	515	.
	177	193	141	205	350	889
	178	132	214	247	515	1108
	179	98	164	334	500	1096
	180	151	208	496	496	1351
	181	88	188	503	565	1344
	182	135	201	347	483	1166
	183	129	152	407	498	1186
	184	110	167	191	474	942
	185	127	153+	312	475	1067
	186	120	214	516	523	1373
	187	154	158	328	451	1091
	188	78	163	356	482	1079
	189	.	161	381	529	.
	190	142	118	311	646	1217
	191	115	177	425	472	1189
	192	98	153	451	351	1053
	193	166	141	253	444	1004
	194	149	179	464	499	1291
	195	143	199	268	495	1105
	196	98	185	495	465	1243

+ = erroneously weighed LD 8 - result excluded from statistical analysis
. = not recorded in error

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Food consumption (g) - Selected offspring

Values per animal - From weaning to week 15

Males

GROUP	ANIMAL NO	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8	WEEK 9	WEEK 10	WEEK 11	WEEK 12	WEEK 13	WEEK 14	WEEK 15	TOTAL 4-15
1C	201	59.5	111.5	145.0	225.5	401	373	332	432	394	372	460	597	3902.5
	202	64.0	110.5	161.0	248.0	419	441	394	460	447	476	452	644	4316.5
	203	56.0	109.0	133.0	153.5	250	326	306	309	291	274	280	516	3003.5
	204	52.5	108.0	133.5	151.0	223	244	255	269	297	281	234	401	2649.0
	205	57.5	106.0	148.5	182.0	186	230	256	268	284	224	236	437	2615.0
	206	66.5	109.0	160.0	213.0	380	458	434	461	534	462	485	655	4417.5
	207	56.5	122.0	221.0	282.5	441	565	543	553	594	606	438	503	4925.0
	208	57.0	103.0	222.0	222.0	476	493	289	461	463	324	420	356	3886.0
	209	60.0	128.5	190.0	213.5	304	498	372	328	525	357	352	408	3736.0
	210	70.0	137.0	156.5	177.5	280	380	398	373	356	355	397	212	3292.0
1B	211	54.0	102.0	135.5	156.0	184	309	302	335	309	289	332	480	2987.5
	212	62.0	103.5	194.5	193.5	477	443	438	455	350	346	357	533	3952.5
	213	65.0	96.5	160.5	176.0	218	326	288	336	282	316	315	499	3078.0
	214	65.5	102.0	133.0	157.0	211	254	251	263	278	309	284	371	2678.5
	215	64.0	85.5	141.0	330.5	252	166	250	217	276	230	257	457	2726.0
	216	52.0	107.5	134.0	228.5	330	338	398	294	426	339	383	584	3614.0
	217	64.5	98.5	110.0	232.0	268	322	435	426	400	314	390	364	3424.0
	218	61.5	105.5	143.0	191.0	170	226	250	206	287	262	232	524	2658.0
	219	64.0	112.0	140.5	163.5	234	239	219	222	241	216	222	382	2455.0
	220	70.0	120.5	146.0	175.5	199	254	292	243	304	333	324	190	2651.0
4C	221	167.5	106.0	134.5	181.0	318	249	352	320	389	341	293	549	3400.0
	222	186.5	114.0	148.5	193.0	321	312	375	328	367	387	304	613	3649.0
	223	165.5	108.5	158.0	211.0	309	303	330	348	405	315	361	613	3627.0
	224	59.5	92.5	132.5	137.5	209	215	203	193	339	285	265	551	2682.0
	225	47.0	99.5	129.0	204.5	458	488	409	435	437	408	408	610	4133.0
	226	68.0	110.0	166.5	215.0	389	354	432	313	316	368	362	544	3637.5
	227	69.0	104.0	162.0	209.0	190	241	280	290	326	321	316	.	..
	228	64.0	113.0	190.5	246.5	250	288	266	263	249	253	275	384	2842.0
	229	58.5	119.0	213.5	260.5	343	344	434	331	487	378	348	500	3816.5
	230	66.5	105.5	154.5	172.0	214	242	216	261	269	236	243	424	2603.5
4B	231	53.5	89.5	139.5	157.0	235	308	281	307	272	238	223	438	2741.5
	232	61.5	89.0	164.5	211.0	222	236	213	222	251	272	249	492	2683.0
	233	67.5	96.5	139.5	201.5	308	408	318	351	340	412	410	554	3606.0
	234	61.5	101.5	158.0	179.5	288	339	327	318	293	331	315	496	3207.5
	235 @	59.5	88.0	118.0	208.0	.	.	.	.	.	.	.	.	.
	236	65.0	101.5	181.5	218.0	277	291	335	297	305	222	335	393	3021.0
	237	67.0	112.5	169.0	197.0	244	221	234	269	337	312	341	475	2978.5
	238	63.5	104.5	140.0	182.5	200	194	193	247	458	238	209	413	2642.5
	239	67.0	122.5	204.0	224.5	350	415	442	473	500	404	399	547	4148.0
	240	61.5	99.0	167.0	184.0	268	263	361	300	431	291	265	511	3201.5

@ = low food consumption not related to treatment - all results excluded from statistical analysis

. = not recorded in error

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Reproduction parameters

Individual values

GROUP	ANIMAL NO	MATING PERIOD (DAYS)	GESTATION PERIOD (DAYS)	NO OF IMPLANTATIONS	POST-IMPLANTATION - LOSS, %
1	101	1	21	12	0.0
	102	1	21	13	23.1
	103	17	21	13	15.4
	104	4	21	12	0.0
	105	2	21	11	9.1
	106	2	21	14	0.0
	107	1	21	14	14.3
	108	2	22	13	30.8
	109	3	22	14	7.1
	110	2	22	15	13.3
	111	2	22	14	14.3
	112	3	21	15	0.0
	113	1	21	12	0.0
	114	3	22	17	0.0
	115	2	22	11	9.1
	116	1	21	13	0.0
	117	1	21	11	18.2
	118	6	22	12	8.3
	119	3	22	16	12.5
	120	2	21	14	0.0
	121	2	21	13	0.0
	122	4	21	16	6.3
	123	3	22	12	8.3
	124	4	21	14	14.3

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Reproduction parameters

Individual values

GROUP	ANIMAL NO		MATING PERIOD (DAYS)	GESTATION PERIOD (DAYS)	NO OF IMPLANTATIONS	POST-IMPLANTATION - LOSS, %
2	125		1	21	13	23.1
	126	¤	.	.	.	.
	127		2	21	13	15.4
	128		1	22	12	25.0
	129		3	22	14	14.3
	130		3	21	15	40.0
	131		2	21	13	15.4
	132		1	21	13	0.0
	133		1	22	11	9.1
	134		2	22	10	30.0
	135		3	22	16	0.0
	136		2	22	17	17.6
	137		3	21	16	0.0
	138		2	22	14	42.9
	139		4	22	15	0.0
	140		2	21	11	0.0
	141		4	22	13	23.1
	142		2	21	13	0.0
	143	¤	.	.	.	.
	144		3	21	16	0.0
	145		4	23	12	25.0
	146		2	21	13	7.7
	147		3	21	13	0.0
	148		1	21	14	7.1

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Reproduction parameters

Individual values

GROUP	ANIMAL NO	MATING PERIOD (DAYS)	GESTATION PERIOD (DAYS)	NO OF IMPLANTATIONS	POST-IMPLANTATION - LOSS$_1$ %
3	149	3	21	14	0.0
	150	1	22	11	0.0
	151	1	21	9	0.0
	152	2	21	11	9.1
	153	1	21	11	0.0
	154	4	21	17	5.9
	155	3	21	14	14.3
	156	4	21	9	0.0
	157	3	22	12	8.3
	158	4	21	16	6.3
	159	3	22	14	28.6
	160	3	22	15	0.0
	161	1	22	11	9.1
	162	2	21	10	0.0
	163	4	21	16	56.3
	164	4	21	12	0.0
	165	3	21	16	6.3
	166	1	21	16	0.0
	167	1	22	15	6.7
	168	21 ¤¤	.	12	8.3
	169	8 ++	22	11	18.2
	170	4	21	15	0.0
	171	3	22	16	6.3
	172	3	21	13	7.7

¤¤ = conception not noticed - result excluded from statistical analysis
++ = male removed 2 days - result excluded from statistical analysis

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Reproduction parameters

Individual values

GROUP	ANIMAL NO	MATING PERIOD (DAYS)	GESTATION PERIOD (DAYS)	NO OF IMPLANTATIONS	POST-IMPLANTATION - LOSS, %
4	173	4	22	12	0.0
	174	2	21	14	7.1
	175	4	21	15	6.7
	176	1	21	13	7.7
	177	4	22	5	20.0
	178	1	21	14	0.0
	179	2	22	13	30.8
	180	4	22	12	16.7
	181	2	22	15	13.3
	182	1	21	12	0.0
	183	3	21	12	0.0
	184	2	21	15	13.3
	185	2	21	13	0.0
	186	4	21	15	0.0
	187	2	21	12	8.3
	188	1	22	12	50.0
	189	2	21	12	0.0
	190	2	21	9	0.0
	191	2	22	14	21.4
	192	3	21	14	0.0
	193	2	21	13	0.0
	194	3	21	15	26.7
	195	1	21	13	30.8
	196	3	21	16	6.3

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Litter size (LS including dead pups), number of live and dead pups, number of surviving pups (S)

Individual values

GROUP	ANIMAL NO	LS DAY 0-1	LS MALE DAY 0-1	LS FEMALE DAY 0-1	LIVE DAY 0-1	DEAD DAY 0-1	S DAY 1	S DAY 4	S DAY 7	S DAY 14	S DAY 21
1	101	13	5	8	13	0	13	13	13	13	13
	102	10	3	7	10	0	10	10	10	10	10
	103	11	4	7	11	0	11	11	11	11	11
	104	12	5	7	12	0	12	12	12	12	12
	105	11	6	5	11	0	10	10	10	8	8
	106	14	7	7	14	0	14	14	13	10	10
	107	12	6	6	12	0	12	12	12	12	12
	108	9	3	6	9	0	9	9	9	9	9
	109	14	7	7	13	1	13	13	13	13	13
	110	13	6	7	13	0	13	13	13	13	13
	111	12	6	6	12	0	12	12	12	11	11
	112	15	4	11	15	0	15	12	12	12	11
	113	12	6	6	12	0	12	12	12	12	12
	114	17	11	6	17	0	11	8	7	7	7
	115	10	3	7	10	0	10	10	10	10	10
	116	13	9	4	13	0	13	13	13	13	13
	117	9	6	3	9	0	9	9	9	9	9
	118	11	6	5	11	0	11	11	11	11	11
	119	14	10	4	14	0	14	14	14	14	14
	120	14	5	9	14	0	14	14	13	13	13
	121	13	10	3	13	0	13	13	12	12	12
	122	15	5	10	15	0	15	15	14	14	14
	123	11	1	10	11	0	11	11	11	11	11
	124	13	7	6	12	1	12	12	12	12	12

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Litter size (LS including dead pups), number of live and dead pups, number of surviving pups (S)

Individual values

GROUP	ANIMAL NO		LS DAY 0-1	LS MALE DAY 0-1	LS FEMALE DAY 0-1	LIVE DAY 0-1	DEAD DAY 0-1	S DAY 1	S DAY 4	S DAY 7	S DAY 14	S DAY 21
2	125		10	5	5	10	0	10	10	10	10	10
	126	¤	.	.	.	.	.	.	.	.	.	.
	127		11	5	6	11	0	11	11	11	11	11
	128		9	6	3	9	0	9	9	9	9	9
	129		12	5	7	12	0	12	12	11	11	11
	130		9	2	7	9	0	9	9	9	9	9
	131		11	4	7	11	0	11	11	11	11	11
	132		13	8	5	13	0	13	13	13	13	13
	133		10	6	4	10	0	10	10	10	10	10
	134		11	7	4	11	0	11	11	11	11	11
	135		16	8	8	16	0	16	13	13	11	11
	136		14	6	8	14	0	14	14	14	12	11
	137		16	7	9	16	0	16	16	16	14	14
	138		8	4	4	8	0	8	8	8	8	8
	139		16	10	6	16	0	13	13	11	11	11
	140		11	6	5	11	0	11	11	11	10	10
	141		10	5	5	10	0	10	10	10	10	10
	142		13	5	8	13	0	13	12	12	12	11
	143	¤	.	.	.	.	.	.	.	.	.	.
	144		16	8	8	16	0	16	14	13	11	11
	145		9	6	3	9	0	9	9	9	9	9
	146		12	6	6	12	0	12	12	12	12	12
	147		13	6	7	13	0	13	11	11	11	11
	148		13	8	5	13	0	13	13	13	13	13

¤ = not pregnant

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Litter size (LS including dead pups), number of live and dead pups, number of surviving pups (S)

Individual values

GROUP	ANIMAL NO		LS DAY 0-1	LS MALE DAY 0-1	LS FEMALE DAY 0-1	LIVE DAY 0-1	DEAD DAY 0-1	S DAY 1	S DAY 4	S DAY 7	S DAY 14	S DAY 21
3	149		14	5	9	14	0	14	12	12	12	12
	150		11	6	5	11	0	11	11	11	10	10
	151		9	3	6	9	0	9	9	9	9	9
	152		10	5	5	10	0	10	10	10	10	10
	153		12	6	6	12	0	12	12	12	12	12
	154		16	7	9	16	0	16	14	14	14	14
	155		12	6	6	12	0	12	12	12	12	12
	156		9	3	6	9	0	9	7	7	6	6
	157		11	4	7	11	0	11	11	11	11	11
	158		15	6	9	15	0	15	15	14	14	14
	159		10	3	7	10	0	10	10	10	10	10
	160		15	6	9	15	0	15	15	14	14	14
	161		10	3	7	10	0	10	10	10	10	10
	162		10	7	3	10	0	10	10	10	10	10
	163		7	2	5	7	0	7	7	7	6	6
	164		12	7	5	12	0	12	11	11	11	11
	165		15	9	6	15	0	15	14	14	11	11
	166		16	8	8	16	0	16	16	14	11	11
	167		14	8	6	14	0	14	14	14	13	13
	168	¤¤	11	7	4	11	0	11	11	11	11	11
	169		10	4	6	10	0	9	9	9	9	9
	170		15	8	7	15	0	15	15	15	15	15
	171		15	11	4	15	0	14	12	11	11	11
	172		12	5	7	12	0	12	11	11	11	11

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Litter size (LS including dead pups), number of live and dead pups, number of surviving pups (S)

Individual values

GROUP	ANIMAL NO		LS DAY 0-1	LS MALE DAY 0-1	LS FEMALE DAY 0-1	LIVE DAY 0-1	DEAD DAY 0-1	S DAY 1	S DAY 4	S DAY 7	S DAY 14	S DAY 21
4	173		12	3	9	12	0	12	12	12	12	12
	174		13	5	8	13	0	13	13	13	13	13
	175		15	9	6	15	0	15	15	15	14	14
	176		12	8	4	12	0	12	12	12	12	12
	177		4	1	3	4	0	4	4	4	4	4
	178		14	9	5	14	0	14	14	13	13	12
	179		10	6	4	9	1	9	9	9	9	9
	180		10	6	4	10	0	10	10	10	10	10
	181		13	5	8	13	0	13	13	13	13	13
	182	++	12	.	.	12	0	12	12	12	12	12
	183		13	5	8	13	0	13	12	12	12	12
	184		13	5	8	13	0	13	13	13	13	13
	185		13	6	7	13	0	13	12	12	12	12
	186		15	9	6	15	0	15	15	15+	14	14
	187		11	5	6	11	0	11	11	11	11	11
	188		7	3	4	6	1	6	6	6	6	6
	189		12	5	7	12	0	12	12	12	12	12
	190		9	6	3	9	0	9	8	8	8	8
	191		11	4	7	11	0	11	11	11	11	11
	192		14	11	3	14	0	14	14	14	14	14
	193		13	2	11	13	0	13	13	13	13	13
	194		11	5	6	11	0	11	10	10	10	10
	195		13	8	5	13	0	13	13	13	13	13
	196		15	7	8	15	0	15	15	15	14	14

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes

Individual values

F0-generation - Males

GROUP	ANIMAL NO	BODY WEIGHT	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
1	1	398	351	0.0882
	2	420	450	0.1071
	3	372	221	0.0594
	4	426	419	0.0984
	5	456	384	0.0842
	6	435	359	0.0825
	7	350	272	0.0777
	8	354	254	0.0718
	9	426	259	0.0608
	10	378	224	0.0593
	11	374	325	0.0869
	12	438	356	0.0813
	13	353	197	0.0558
	14	370	396	0.1070
	15	375	324	0.0864
	16	394	332	0.0843
	17	375	276	0.0736
	18	364	262	0.0720
	19	395	274	0.0694
	20	358	252	0.0704
	21	384	845	0.2201
	22	361	184	0.0510
	23	362	299	0.0826
	24	367	253	0.0689

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes

Individual values

F0-generation - Males

GROUP	ANIMAL NO	BODY WEIGHT	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
2	25	279	258	0.0925
	26	375	380	0.1013
	27	372	466	0.1253
	28	369	358	0.0970
	29	355	499	0.1406
	30	456	623	0.1366
	31	401	255	0.0636
	32	387	368	0.0951
	33	385	339	0.0881
	34	386	299	0.0775
	35	410	556	0.1356
	36	425	347	0.0816
	37	378	333	0.0881
	38	371	456	0.1229
	39	382	341	0.0893
	40	414	279	0.0674
	41	363	360	0.0992
	42	378	396	0.1048
	43	420	468	0.1114
	44	432	343	0.0794
	45	380	299	0.0787
	46	370	285	0.0770
	47	417	305	0.0731
	48	391	377	0.0964

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes

Individual values

F0-generation - Males

GROUP	ANIMAL NO	BODY WEIGHT	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
3	49	415	363	0.0875
	50	392	435	0.1110
	51	376	354	0.0941
	52	385	224	0.0582
	53	442	444	0.1005
	54	337	323	0.0958
	55	433	367	0.0848
	56	411	379	0.0922
	57	412	451	0.1095
	58	387	392	0.1013
	59	426	477	0.1120
	60	378	265	0.0701
	61	428	513	0.1199
	62	455	291	0.0640
	63	379	311	0.0821
	64	406	310	0.0764
	65	399	338	0.0847
	66	423	338	0.0799
	67	421	444	0.1055
	68	436	399	0.0915
	69	369	555	0.1504
	70	412	380	0.0922
	71	424	685	0.1616
	72	419	359	0.0857

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes

Individual values

FO-generation - Males

GROUP	ANIMAL NO	BODY WEIGHT	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
4	73	339	456	0.1345
	74	375	337	0.0899
	75	388	452	0.1165
	76	406	360	0.0887
	77	401	512	0.1277
	78	415	373	0.0899
	79	356	454	0.1275
	80	394	414	0.1051
	81	396	286	0.0722
	82	380	386	0.1016
	83	377	334	0.0886
	84	399	384	0.0962
	85	406	234	0.0576
	86	384	366	0.0953
	87	352	306	0.0869
	88	413	432	0.1046
	89	379	333	0.0879
	90	427	493	0.1155
	91	402	315	0.0784
	92	386	401	0.1039
	93	382	364	0.0953
	94	418	419	0.1002
	95	421	767	0.1822
	96	368	572	0.1554

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes

Individual values

F0-generation - Females

GROUP	ANIMAL NO	BODY WEIGHT	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
1	101	263	401	0.1525
	102	273	266	0.0974
	103	290	482	0.1662
	104	269	377	0.1401
	105	251	309	0.1231
	106	263	257	0.0977
	107	275	227	0.0825
	108	218	196	0.0899
	109	257	362	0.1409
	110	258	372	0.1442
	111	266	323	0.1214
	112	268	436	0.1627
	113	242	225	0.0930
	114	250	397	0.1588
	115	245	405	0.1653
	116	265	478	0.1804
	117	276	441	0.1598
	118	216	395	0.1829
	119	281	372	0.1324
	120	275	301	0.1095
	121	269	233	0.0866
	122	277	508	0.1834
	123	276	369	0.1337
	124	270	520	0.1926

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes

Individual values

FO-generation - Females

GROUP	ANIMAL NO	BODY WEIGHT	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
2	125	297	287	0.0966
	126	220	601	0.2732
	127	256	412	0.1609
	128	263	352	0.1338
	129	245	357	0.1457
	130	310	612	0.1974
	131	279	347	0.1244
	132	211	167	0.0791
	133	254	529	0.2083
	134	264	479	0.1814
	135	275	370	0.1345
	136	269	432	0.1606
	137	281	399	0.1420
	138	269	397	0.1476
	139	253	333	0.1316
	140	269	413	0.1535
	141	293	543	0.1853
	142	280	430	0.1536
	143	256	737	0.2879
	144	280	490	0.1750
	145	273	610	0.2234
	146	262	306	0.1168
	147	284	480	0.1690
	148	284	365	0.1285

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes

Individual values

F0-generation - Females

GROUP	ANIMAL NO	BODY WEIGHT	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
3	149	266	508	0.1910
	150	276	342	0.1239
	151	260	611	0.2350
	152	262	539	0.2057
	153	281	373	0.1327
	154	293	452	0.1543
	155	259	694	0.2680
	156	268	590	0.2201
	157	271	517	0.1908
	158	287	508	0.1770
	159	270	663	0.2456
	160	283	462	0.1633
	161	231	312	0.1351
	162	254	379	0.1492
	163	256	749	0.2926
	164	265	734	0.2770
	165	282	485	0.1720
	166	256	496	0.1938
	167	296	818	0.2764
	168	274	299	0.1091
	169	300	652	0.2173
	170	272	532	0.1956
	171	302	611	0.2023
	172	257	625	0.2432

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes

Individual values

F0-generation - Females

GROUP	ANIMAL NO	BODY WEIGHT	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
4	173	260	411	0.1581
	174	253	578	0.2285
	175	269	680	0.2528
	176	280	360	0.1286
	177	278	437	0.1572
	178	275	551	0.2004
	179	250	623	0.2492
	180	273	550	0.2015
	181	255	353	0.1384
	182	256	481	0.1879
	183	263	584	0.2221
	184	265	418	0.1577
	185	256	380	0.1484
	186	283	484	0.1710
	187	248	439	0.1770
	188	284	760	0.2676
	189	267	273	0.1022
	190	257	474	0.1844
	191	277	790	0.2852
	192	230	329	0.1430
	193	250	498	0.1992
	194	245	613	0.2502
	195	241	403	0.1672
	196	213	430	0.2019

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes - Selected offspring

Individual values

WEEK	GROUP	ANIMAL NO	BODY WEIGHT	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
4	1B	221	175	410	0.234
		223	165	383	0.232
		225	164	378	0.230
		227	189	524	0.277
		229	209	555	0.266
		231	202	712	0.352
		233	181	386	0.213
		235	196	553	0.282
		237	200	459	0.230
		239	227	576	0.254
4	1C	201	190	514	0.271
		203	170	403	0.237
		205	199	432	0.217
		207	201	413	0.205
		209	220	589	0.268
		211	217	332	0.153
		213	198	480	0.242
		215	205	487	0.238
		217	191	434	0.227
		219	214	431	0.201
4	4B	261	163	273	0.167
		263	163	402	0.247
		265	186	469	0.252
		267	195	425	0.218
		269	158	296	0.187
		271	207	482	0.233
		273	186	399	0.215
		275	195	517	0.265
		277	184	504	0.274
		279	219	616	0.281
4	4C	241	156	396	0.254
		243	171	353	0.206
		245	173	283	0.164
		247	163	385	0.236
		249	159	332	0.209
		251	184	309	0.168
		253	172	229	0.133
		255	175	493	0.282
		257	192	428	0.223
		259	231	644	0.279

BioProtein

ONE-GENERATION REPRODUCTION TOXICITY STUDY IN THE RAT

Body weight (g), absolute (mg) and relative (% of b.wt.) weight of mesenteric lymph nodes - Selected offspring

Individual values

WEEK	GROUP	ANIMAL NO	BODY WEIGHT	MESENTERIC LYMPH NODE, ABSOLUTE	MESENTERIC LYMPH NODE, RELATIVE
12	1B	222	360	591	0.164
		224	375	410	0.109
		226	400	560	0.140
		228	369	478	0.130
		230	370	524	0.142
		232	384	727	0.189
		234	360	685	0.190
		236	299	635	0.212
		238	322	697	0.216
		240	356	435	0.122
12	1C	202	388	430	0.111
		204	315	711	0.226
		206	373	647	0.173
		208	363	593	0.163
		210	413	732	0.177
		212	400	558	0.140
		214	401	649	0.162
		216	351	491	0.140
		218	336	280	0.083
		220	366	341	0.093
12	4B	262	311	439	0.141
		264	351	762	0.217
		266	313	467	0.149
		268	353	460	0.130
		272	385	513	0.133
		274	347	557	0.161
		276	337	563	0.167
		278	367	604	0.165
		280	442	711	0.161
12	4C	242	336	344	0.102
		244	344	577	0.168
		246	338	493	0.146
		248	346	541	0.156
		250	326	612	0.188
		252	380	568	0.149
		254	378	503	0.133
		256	324	344	0.106
		258	409	937	0.229
		260	383	409	0.107

PATHOLOGY REPORT PAGE : I

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

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TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        :                (b) (4)                 PathData® System V5.1b
-------------------------------------------------------------------------------
EXPLANATION OF CODES AND SYMBOLS
-------------------------------------------------------------------------------


CODES AND SYMBOLS USED AT ANIMAL  LEVEL:
----------------------------------------
M       = Male animal
F       = Female animal
K0      = Terminal sacrifice group
+       = Intercurrent death/sacrificed moribund
+3      = Accidental death


CODES AND SYMBOLS USED AT ORGAN  LEVEL:
---------------------------------------
G       = Gross observation checked off histologically
*       = Comment in text of individual animal data
0       = Tissue not present for histologic examination
'       = Histologic examination not required
+       = Organ examined, findings present
-       = Organ examined, no pathologic findings noted (AOFT only)
(       = Only one of paired organs examined/present


CODES AND SYMBOLS USED AT FINDING  LEVEL:
-----------------------------------------
GRADE 1 = Minimal / very few / very small
GRADE 2 = Slight / few / small
GRADE 3 = Moderate / moderate number / moderate size
GRADE 4 = Marked / many / large
(       = Finding unilateral in paired organs
*       = Comment in text of individual animal data
```

PATHOLOGY REPORT PAGE : 2/ 126
SUMMARY TABLES

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

NUMBER OF ANIMALS WITH NECROPSY FINDINGS BY ORGAN/GROUP/SEX
STATUS AT NECROPSY: K0, INCL. DEATHS
BioProtein

DOSE GROUP:	1		2		3		4	
SEX:	M	F	M	F	M	F	M	F
ORGAN/FINDING ANIM.EXAM.:	24	24	24	24	24	24	24	24
SEMINAL VESICLE :								
- DIMINISHED :	-	-	-	-	1	-	-	-
MESENTERIC LYMPH N. :								
- REDDENED :	-	-	-	-	-	-	1	-
THYMUS :								
- HEMORRHAGES :	1	-	-	-	1	-	-	-
KIDNEYS :								
- FOCI, PALE :	-	-	-	-	1	-	-	-

PATHOLOGY REPORT PAGE : 3/ 126
SUMMARY TABLES

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX
STATUS AT NECROPSY: K0, INCL. DEATHS
BioProtein

DOSE GROUP:	1		2		3		4	
SEX :	M	F	M	F	M	F	M	F
NO.ANIMALS:	24	24	24	24	24	24	24	24
OVARIES :	-	24	-	-	-	-	-	24
- Microabcess luteal :	-	-	-	-	-	-	-	1
Grade 1:	-	-	-	-	-	-	-	1
COAGULATING GLANDS :	24	-	-	-	-	-	24	-
- Hyperplasia reactive:	-	-	-	-	-	-	1	-
Grade 1:	-	-	-	-	-	-	1	-
PROSTATE GLAND :	24	-	-	-	-	-	24	-
- Lymphoid cells focal:	6	-	-	-	-	-	2	-
Grade 1:	6	-	-	-	-	-	2	-
EPIDIDYMIDES :	24	-	-	-	-	-	24	-
- Vacuolation epithel :	2	-	-	-	-	-	1	-
Grade 1:	2	-	-	-	-	-	1	-
MESENTERIC LYMPH N. :	24	24	-	-	-	-	24	24
- Granuloma formation :	2	2	-	-	-	-	12	6
Grade 1:	2	2	-	-	-	-	4	3
Grade 2:	-	-	-	-	-	-	4	3
Grade 3:	-	-	-	-	-	-	4	-
- Inflamm chronic/foc :	-	-	-	-	-	-	1	-
Grade 2:	-	-	-	-	-	-	1	-
- Histiocytosis diff :	24	24	-	-	-	-	24	24
Grade 1:	14	-	-	-	-	-	2	-
Grade 2:	10	18	-	-	-	-	19	17
Grade 3:	-	6	-	-	-	-	3	7
- Hemorrhage sinusoid :	2	17	-	-	-	-	5	15
Grade 1:	2	10	-	-	-	-	5	9
Grade 2:	-	7	-	-	-	-	-	6
- Dilatation sinusoid :	2	-	-	-	-	-	1	3
Grade 1:	2	-	-	-	-	-	1	2
Grade 2:	-	-	-	-	-	-	-	1

PATHOLOGY REPORT PAGE : 4/ 126
SUMMARY TABLES

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

NUMBER OF ANIMALS WITH MICROSCOPIC FINDINGS BY ORGAN/GROUP/SEX
STATUS AT NECROPSY: K0, INCL. DEATHS
BioProtein

DOSE GROUP:	1		2		3		4	
SEX :	M	F	M	F	M	F	M	F
NO.ANIMALS:	24	24	24	24	24	24	24	24
THYMUS :	2	-	-	-	1	-	-	-
- Hemorrhage interstit:	2	-	-	-	1	-	-	-
Grade 1:	2	-	-	-	-	-	-	-
Grade 2:	-	-	-	-	1	-	-	-
KIDNEYS :	-	-	-	-	1	-	-	-
- Vacuolation tubular :	-	-	-	-	1	-	-	-
Grade 4:	-	-	-	-	1	-	-	-

PATHOLOGY REPORT PAGE : 5/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 1, 0%

ANIMAL NUMBER :	1	2	3	4	5	6	7	8	9	10
	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0
SEMINAL VESICLE	-	-	-	-	-	-	-	-	-	-
COAGULATING GLANDS	-	-	-	-	-	-	-	-	-	-
PROSTATE GLAND	+	-	+	-	+	+	-	-	-	-
- Lymphoid cells focal	1.	.	1.	.	1.	1.	.	.	.	.
EPIDIDYMIDES	-	-	-	-	-	-	-	-	-	-
TESTES	-	-	-	-	-	-	-	-	-	-
MESENTERIC LYMPH N.	+	+	+	+	+	+	+	+	+	+
- Granuloma formation	.	.	.	.	.	1.	.	.	.	.
- Histiocytosis diff	1.	1.	1.	2.	1.	1.	1.	2.	2.	2.
- Hemorrhage sinusoid	.	.	.	.	.	.	1.	.	.	.
- Dilatation sinusoid	.	.	.	.	.	1.	.	.	.	.
PITUITARY DISTALIS	-	-	-	-	-	-	-	-	-	-
PITUITARY INTER/NERV	-	-	-	-	-	-	-	-	-	-
THYMUS	'	'	'	'	+	+G	'	'	'	'
- Hemorrhage interstit					1.	1.				

PATHOLOGY REPORT PAGE : 6/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 1, 0%

ANIMAL NUMBER :	11 MK0	12 MK0	13 MK0	14 MK0	15 MK0	16 MK0	17 MK0	18 MK0	19 MK0	20 MK0
SEMINAL VESICLE	-	-	-	-	-	-	-	-	-	-
COAGULATING GLANDS	-	-	-	-	-	-	-	-	-	-
PROSTATE GLAND	-	+	-	-	-	-	+	-	-	-
- Lymphoid cells focal	.	1.	.	.	.	.	1.	.	.	.
EPIDIDYMIDES	+	-	-	-	-	-	+	-	-	-
- Vacuolation epithel	1.	.	.	.	.	.	1.	.	.	.
TESTES	-	-	-	-	-	-	-	-	-	-
MESENTERIC LYMPH N.	+	+	+	+	+	+	+	+	+	+
- Histiocytosis diff	1.	2.	2.	2.	2.	1.	2.	1.	1.	2.
- Dilatation sinusoid	.	.	.	.	.	.	1.	.	.	.
PITUITARY DISTALIS	-	-	-	-	-	-	-	-	-	-
PITUITARY INTER/NERV	-	-	-	-	-	-	-	-	-	0

PATHOLOGY REPORT PAGE : 7/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 1, 0%

ANIMAL NUMBER :	21 MK0	22 MK0	23 MK0	24 MK0
SEMINAL VESICLE	-	-	-	-
COAGULATING GLANDS	-	-	-	-
PROSTATE GLAND	-	-	-	-
EPIDIDYMIDES	-	-	-	-
TESTES	-	-	-	-
MESENTERIC LYMPH N.	+	+	+	+
- Granuloma formation	1.	.	.	.
- Histiocytosis diff	1.	1.	1.	1.
- Hemorrhage sinusoid	.	.	1.	.
PITUITARY DISTALIS	-	-	-	-
PITUITARY INTER/NERV	-	-	-	-

PATHOLOGY REPORT PAGE : 8/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 1, 0%

ANIMAL NUMBER :	101 FK0	102 FK0	103 FK0	104 FK0	105 FK0	106 FK0	107 FK0	108 FK0	109 FK0	110 FK0
OVARIES	-	-	-	-	-	-	-	-	-	-
UTERUS	-	-	-	-	-	-	-	-	-	-
IMPLANTATION SITE	-	-	-	-	-	-	-	-	-	-
CERVIX	-	-	-	-	-	-	-	-	-	-
VAGINA	-	-	-	-	-	-	-	-	-	-
MESENTERIC LYMPH N.	+	+	+	+	+	+	+	+	+	+
- Histiocytosis diff	2.	2.	2.	2.	3.	2.	2.	3.	2.	2.
- Hemorrhage sinusoid	2.	.	2.	2.	2.	1.	2.	.	1.	2.
PITUITARY DISTALIS	-	-	-	-	-	-	-	-	-	-
PITUITARY INTER/NERV	-	-	-	-	-	-	-	-	-	-

PATHOLOGY REPORT PAGE : 9/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 1, 0%

ANIMAL NUMBER :	111 FK0	112 FK0	113 FK0	114 FK0	115 FK0	116 FK0	117 FK0	118 FK0	119 FK0	120 FK0
OVARIES	-	-	-	-	-	-	-	-	-	-
UTERUS	-	-	-	-	-	-	-	-	-	-
IMPLANTATION SITE	-	-	-	-	-	-	-	-	-	-
CERVIX	-	-	-	-	-	-	-	-	-	-
VAGINA	-	-	-	-	-	-	-	-	-	-
MESENTERIC LYMPH N.	+	+	+	+	+	+	+	+	+	+
- Granuloma formation	1.	.	.	.	.	.	.	.	.	.
- Histiocytosis diff	3.	3.	2.	2.	2.	2.	3.	2.	2.	3.
- Hemorrhage sinusoid	.	1.	.	1.	.	.	2.	1.	1.	.
PITUITARY DISTALIS	-	-	-	-	-	-	-	-	-	-
PITUITARY INTER/NERV	0	-	-	-	-	-	-	-	-	-

PATHOLOGY REPORT PAGE : 10/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 1, 0%

ANIMAL NUMBER :	121 FK0	122 FK0	123 FK0	124 FK0
OVARIES	-	-	-	-
UTERUS	-	-	-	-
IMPLANTATION SITE	-	-	-	-
CERVIX	-	-	-	-
VAGINA	-	-	-	-
MESENTERIC LYMPH N.	+	+	+	+
- Granuloma formation	.	.	.	1.
- Histiocytosis diff	2.	2.	2.	2.
- Hemorrhage sinusoid	1.	1.	1.	1.
PITUITARY DISTALIS	-	-	-	-
PITUITARY INTER/NERV	-	-	-	-

PATHOLOGY REPORT PAGE : 11/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 3, 11.0%

ANIMAL NUMBER :

	59	60	61	62	63	64	65	66	67	68
	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0	MK0
THYMUS	'	'	'	'	'	'	'	'	+G	'
- Hemorrhage interstit									2.	

PATHOLOGY REPORT PAGE : 12/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 3, 11.0%

ANIMAL NUMBER :	69	70	71	72
	MK0	MK0	MK0	MK0
SEMINAL VESICLE	-G	'	'	'
KIDNEYS	+G	'	'	'
- Vacuolation tubular	(4.			

PATHOLOGY REPORT PAGE : 13/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 4, 22.0%

ANIMAL NUMBER :	73 MK0	74 MK0	75 MK0	76 MK0	77 MK0	78 MK0	79 MK0	80 MK0	81 MK0	82 MK0
SEMINAL VESICLE	-	-	-	-	-	-	-	-	-	-
COAGULATING GLANDS	-	-	-	-	-	-	-	-	-	-
PROSTATE GLAND	-	-	-	-	-	-	-	-	-	-
EPIDIDYMIDES	-	+	-	-	-	-	-	-	-	-*
- Vacuolation epithel	.	(1.	.	.	.	.	.	.	.	.
TESTES	-	-	-	-	-	-	-	-	-	-
MESENTERIC LYMPH N.	+	+	+	+	+G	+	+	+	+	+
- Granuloma formation	2.	1.	3.	.	.	3.	3.	.	.	2.
- Inflamm chronic/foc	.	.	.	.	.	.	.	.	2.	.
- Histiocytosis diff	1.	2.	2.	2.	2.	2.	2.	3.	3.	2.
- Hemorrhage sinusoid	.	.	.	.	.	.	.	1.	.	.
PITUITARY DISTALIS	-	-	-	-	-	-	-	-	-	-
PITUITARY INTER/NERV	-	-	-	-	-	-	-	-	-	-

PATHOLOGY REPORT PAGE : 14/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 4, 22.0%

ANIMAL NUMBER :	83 MK0	84 MK0	85 MK0	86 MK0+	87 MK0	88 MK0	89 MK0	90 MK0	91 MK0	92 MK0
SEMINAL VESICLE	-	-	-	-	-	-	-	-	-	-
COAGULATING GLANDS	+	-	-	-	-	-	-	-	-	-
- Hyperplasia reactive	1*	.	.	.	.	.	.	.	.	.
PROSTATE GLAND	-	-	-	-	-	-	-	-	-	-
EPIDIDYMIDES	-	-	-	-	-	-	-	-	-	-
TESTES	-	-	-	-	-	-	-	-	-	-
MESENTERIC LYMPH N.	+	+	+	+	+	+	+	+	+	+
- Granuloma formation	1.	.	2.	.	.	.	3.	.	.	1.
- Histiocytosis diff	2.	2.	2.	3.	2.	2.	2.	1.	2.	2.
- Hemorrhage sinusoid	.	.	.	.	.	.	1.	.	.	.
- Dilatation sinusoid	.	.	.	.	.	1.	.	.	.	.
PITUITARY DISTALIS	-	-	-	-	-	-	-	-	-	-
PITUITARY INTER/NERV	-	-	-	-	-	-	-	-	-	-

PATHOLOGY REPORT PAGE : 15/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 4, 22.0%

ANIMAL NUMBER :	93 MK0	94 MK0	95 MK0	96 MK0
SEMINAL VESICLE	-	-	-	-
COAGULATING GLANDS	-	-	-	-
PROSTATE GLAND	-	-	+	+
- Lymphoid cells focal	.	.	1.	1.
EPIDIDYMIDES	-	-	-	-
TESTES	-	-	-	-
MESENTERIC LYMPH N.	+	+	+	+
- Granuloma formation	.	1.	.	2.
- Histiocytosis diff	2.	2.	2.	2.
- Hemorrhage sinusoid	1.	1.	1.	.
PITUITARY DISTALIS	-	-	-	-
PITUITARY INTER/NERV	-	-	-	-

PATHOLOGY REPORT PAGE : 16/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 4, 22.0%

ANIMAL NUMBER :	173 FK0	174 FK0	175 FK0	176 FK0	177 FK0	178 FK0	179 FK0	180 FK0	181 FK0	182 FK0
OVARIES	-	-	-	-	-	-	-	-	-	+
- Microabcess luteal	.	.	.	.	.	.	.	.	.	1.
UTERUS	-	-	-	-	-	-	-	-	-	-
IMPLANTATION SITE	-	-	-	-	-	-	-	-	-	-
CERVIX	-	-	-	-	-	-	-	-	-	-
VAGINA	-	-	-	-	-	-	-	-	-	-
MESENTERIC LYMPH N.	+	+	+	+	+	+	+	+	+	+
- Granuloma formation	.	.	.	2.	.	.	.	.	.	2.
- Histiocytosis diff	3.	2.	2.	3.	2.	3.	3.	2.	2.	2.
- Hemorrhage sinusoid	.	1.	.	2.	1.	1.	2.	1.	1.	2.
- Dilatation sinusoid	.	.	2.	.	.	.	.	.	.	.
PITUITARY DISTALIS	-	-	-	-	-	-	-	-	-	-
PITUITARY INTER/NERV	-	-	-	-	0	-	-	-	-	-

PATHOLOGY REPORT PAGE : 17/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 4, 22.0%

ANIMAL NUMBER :	183 FK0	184 FK0	185 FK0	186 FK0	187 FK0	188 FK0	189 FK0	190 FK0	191 FK0	192 FK0
OVARIES	-	-	-	-	-	-	-	-	-	-
UTERUS	-	-	-	-	-	-	-	-	-	-
IMPLANTATION SITE	-	-	-	-	-	-	-	-	-	-
CERVIX	-	-	-	-	-	-	-	-	-	-
VAGINA	-	-	-	-	-	-	-	-	-	-
MESENTERIC LYMPH N.	+	+	+	+	+	+	+	+	+	+
- Granuloma formation	1.	.	.	.	1.	2.	.	.	.	1.
- Histiocytosis diff	2.	2.	2.	2.	2.	2.	2.	3.	2.	3.
- Hemorrhage sinusoid	1.	.	.	1.	.	.	2.	1.	.	.
- Dilatation sinusoid	.	.	.	.	.	1.	1.	.	.	.
PITUITARY DISTALIS	-	-	-	-	-	-	-	-	-	-
PITUITARY INTER/NERV	-	-	-	-	-	-	-	-	-	-

PATHOLOGY REPORT PAGE : 18/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TABLE OF INDIVIDUAL MICROSCOPIC FINDINGS (AOFT)
DOSE GROUP : 4, 22.0%

ANIMAL NUMBER :	193 FK0	194 FK0	195 FK0	196 FK0
OVARIES	(-	-	-	-
UTERUS	-	-	-	-
IMPLANTATION SITE	-	-	-	-
CERVIX	-	-	-	-
VAGINA	-	-	-	-
MESENTERIC LYMPH N.	+	+	+	+
- Histiocytosis diff	2.	2.	2.	3.
- Hemorrhage sinusoid	1.	2.	2.	.
PITUITARY DISTALIS	-	-	-	-
PITUITARY INTER/NERV	-	-	-	-

PATHOLOGY REPORT PAGE : 19/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

ANIMAL HEADING DATA
DOSE GROUP : 1, 0%

ANIMAL NUMBER	SEX M/F	DEFINED AND FINAL STATE OF NECROPSY		TEST DAYS	FIRST AND LAST DAY UNDER TEST		DATE OF NECROPSY
1	M	K0	K0	93	12-JUN-00	12-SEP-00	12-SEP-00
2	M	K0	K0	93	12-JUN-00	12-SEP-00	12-SEP-00
3	M	K0	K0	93	12-JUN-00	12-SEP-00	12-SEP-00
4	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
5	M	K0	K0	93	12-JUN-00	12-SEP-00	12-SEP-00
6	M	K0	K0	93	12-JUN-00	12-SEP-00	12-SEP-00
7	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
8	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
9	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
10	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
11	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
12	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
13	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
14	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
15	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
16	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
17	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
18	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
19	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
20	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
21	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
22	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
23	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
24	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
101	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
102	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
103	F	K0	K0	74	07-AUG-00	19-OCT-00	19-OCT-00
104	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
105	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
106	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
107	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
108	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
109	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
110	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
111	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
112	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
113	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
114	F	K0	K0	65	07-AUG-00	10-OCT-00	10-OCT-00
115	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
116	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00

PATHOLOGY REPORT PAGE : 20/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

ANIMAL HEADING DATA
DOSE GROUP : 1, 0%

ANIMAL NUMBER	SEX M/F	DEFINED AND FINAL STATE OF NECROPSY		TEST DAYS	FIRST AND LAST DAY UNDER TEST		DATE OF NECROPSY
117	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
118	F	K0	K0	64	07-AUG-00	09-OCT-00	09-OCT-00
119	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
120	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
121	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
122	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
123	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
124	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00

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PATHOLOGY REPORT                                        PAGE       :   21/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : [redacted] (b) (4)                     PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  1, 0%                                                   MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    93                        * ANIMAL NO. :          1
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  PROSTATE GLAND:
   -Focal interstitial accumulation of lymphoid cells, grade 1
  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    93                        * ANIMAL NO. :          2
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


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PATHOLOGY REPORT                                        PAGE       :   22/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  1, 0%                                                   MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: KO
  DAYS ON TEST      :    93                        * ANIMAL NO. :          3
-------------------------------------------------------------------------------

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  PROSTATE GLAND:
   -Focal interstitial accumulation of lymphoid cells, grade 1
  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: KO
  DAYS ON TEST      :    79                        * ANIMAL NO. :          4
-------------------------------------------------------------------------------

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


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PATHOLOGY REPORT                                        PAGE       :  23/ 126
INDIVIDUAL ANIMAL DATA
------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  1, 0%                                                   MALE
------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    93                        * ANIMAL NO. :         5
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  PROSTATE GLAND:
   -Focal interstitial accumulation of lymphoid cells, grade 1
  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 1
  THYMUS:
   -Focal interstitial hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    93                        * ANIMAL NO. :         6
..............................................................................

* NECROPSY FINDINGS

  THYMUS:
    01: HEMORRHAGE.
  NO OTHER NECROPSY OBSERVATIONS NOTED


* MICROSCOPIC FINDINGS

  PROSTATE GLAND:
   -Focal interstitial accumulation of lymphoid cells, grade 1
  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 1
```

```
PATHOLOGY REPORT                                        PAGE       :   24/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  1, 0%                                                   MALE
-------------------------------------------------------------------------------

                                          CONT./FF. ANIMAL NO. :         6
.....

   -Diffuse cortical/medullar histiocytosis, grade 1
   -Focal sinusoidal dilatation, grade 1
  THYMUS:
   -Focal interstitial hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: KO
  DAYS ON TEST     :    81                      * ANIMAL NO. :         7
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 1
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
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PATHOLOGY REPORT                                        PAGE       :   25/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  1, 0%                                                   MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    81                         * ANIMAL NO. :          8
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    79                         * ANIMAL NO. :          9
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


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PATHOLOGY REPORT                                        PAGE       :   26/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  1, 0%                                                   MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    81                         * ANIMAL NO. :         10
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    79                         * ANIMAL NO. :         11
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  EPIDIDYMIDES:
   -Focal cytoplasmic vacuolation in the duct epithelium,
    unilateral, grade 1
  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
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```
PATHOLOGY REPORT                                        PAGE       :   27/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : [redacted] (b) (4)                     PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  1, 0%                                                   MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    79                       * ANIMAL NO. :         12
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  PROSTATE GLAND:
   -Focal interstitial accumulation of lymphoid cells, grade 1
  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    81                       * ANIMAL NO. :         13
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


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```
PATHOLOGY REPORT                                        PAGE       :   28/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  1, 0%                                                    MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                        * ANIMAL NO. :         14
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                        * ANIMAL NO. :         15
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


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PATHOLOGY REPORT                                        PAGE       :   29/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  1, 0%                                                    MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    81                        * ANIMAL NO. :         16
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    81                        * ANIMAL NO. :         17
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  PROSTATE GLAND:
   -Focal interstitial accumulation of lymphoid cells, grade 1
  EPIDIDYMIDES:
   -Focal cytoplasmic vacuolation in the duct epithelium, bilateral,
    grade 1
  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal dilatation, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


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PATHOLOGY REPORT                                        PAGE       :   30/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  1, 0%                                                    MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                        * ANIMAL NO. :         18
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                        * ANIMAL NO. :         19
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   31/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        :                                        PathData® System V5.1b
                 (b) (4)
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  1, 0%                                                   MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: KO
  DAYS ON TEST      :    80                        * ANIMAL NO. :         20
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  PITUITARY GLAND (PARS INTERMEDIA & NERVOSA):
    Tissue not present for histologic examination
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: KO
  DAYS ON TEST      :    80                        * ANIMAL NO. :         21
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 1
   -Diffuse cortical/medullar histiocytosis, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 32/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 1, 0% MALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 80 * ANIMAL NO. : 22

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

MESENTERIC LYMPH NODE:
-Diffuse cortical/medullar histiocytosis, grade 1
ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

* STATE AT NECROPSY: K0
DAYS ON TEST : 80 * ANIMAL NO. : 23

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

MESENTERIC LYMPH NODE:
-Diffuse cortical/medullar histiocytosis, grade 1
-Focal sinusoidal hemorrhage, grade 1
ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

PATHOLOGY REPORT PAGE : 33/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 1, 0% MALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 80 * ANIMAL NO. : 24

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

MESENTERIC LYMPH NODE:
-Diffuse cortical/medullar histiocytosis, grade 1
ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

```
PATHOLOGY REPORT                                        PAGE       :   34/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  1, 0%                                                  FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    58                        * ANIMAL NO. :       101
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    58                        * ANIMAL NO. :       102
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


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PATHOLOGY REPORT                                        PAGE       :  35/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : [redacted] (b)(4)                      PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  1, 0%                                                 FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: KO
  DAYS ON TEST      :    74                         * ANIMAL NO. :       103
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: KO
  DAYS ON TEST      :    61                         * ANIMAL NO. :       104
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   36/ 126
INDIVIDUAL ANIMAL DATA
------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  1, 0%                                                  FEMALE
------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    59                        * ANIMAL NO. :       105
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 3
   -Focal sinusoidal hemorrhage, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    59                        * ANIMAL NO. :       106
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


------------------------------------------------------------------------------
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```
PATHOLOGY REPORT                                        PAGE       :  37/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       :                                         PathData® System V5.1b
                                (b) (4)
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  1, 0%                                                  FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    58                        * ANIMAL NO. :       107
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    60                        * ANIMAL NO. :       108
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 3
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
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```
PATHOLOGY REPORT                                        PAGE       :   38/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  1, 0%                                                  FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    61                         * ANIMAL NO. :        109
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    60                         * ANIMAL NO. :        110
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   39/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  1, 0%                                                  FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    60                        * ANIMAL NO. :       111
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 1
   -Diffuse cortical/medullar histiocytosis, grade 3
  PITUITARY GLAND (PARS INTERMEDIA & NERVOSA):
    Tissue not present for histologic examination
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    60                        * ANIMAL NO. :       112
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 3
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   40/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  1, 0%                                                  FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    58                         * ANIMAL NO. :        113
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    65                         * ANIMAL NO. :        114
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 41/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 1, 0% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 60 * ANIMAL NO. : 115

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

MESENTERIC LYMPH NODE:
-Diffuse cortical/medullar histiocytosis, grade 2
ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

* STATE AT NECROPSY: K0
DAYS ON TEST : 58 * ANIMAL NO. : 116

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

MESENTERIC LYMPH NODE:
-Diffuse cortical/medullar histiocytosis, grade 2
ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

```
PATHOLOGY REPORT                                        PAGE       :   42/ 126
INDIVIDUAL ANIMAL DATA
------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  1, 0%                                                  FEMALE
------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    58                       * ANIMAL NO. :       117
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 3
   -Focal sinusoidal hemorrhage, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    64                       * ANIMAL NO. :       118
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  43/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : [redacted] (b) (4)                      PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    : 1, 0%                                                   FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    61                        * ANIMAL NO. :       119
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    59                        * ANIMAL NO. :       120
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 3
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                    PAGE       :  44/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                          PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL    DATE       : 08-OCT-01
SPONSOR       : (b) (4)                             PathData® System V5.1b
-------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  1, 0%                                              FEMALE
-------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    59                    * ANIMAL NO. :       121
.........................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    61                    * ANIMAL NO. :       122
.........................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   45/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : [redacted] (b) (4)                     PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  1, 0%                                                  FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    61                         * ANIMAL NO. :        123
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    61                         * ANIMAL NO. :        124
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 1
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 46/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

ANIMAL HEADING DATA
DOSE GROUP : 2, 5.5%

ANIMAL NUMBER	SEX M/F	DEFINED AND FINAL STATE OF NECROPSY		TEST DAYS	FIRST AND LAST DAY UNDER TEST		DATE OF NECROPSY
25	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
26	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
27	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
28	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
29	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
30	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
31	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
32	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
33	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
34	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
35	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
36	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
37	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
38	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
39	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
40	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
41	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
42	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
43	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
44	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
45	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
46	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
47	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
48	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
125	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
126	F	K0	K0	64	07-AUG-00	09-OCT-00	09-OCT-00
127	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
128	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
129	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
130	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
131	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
132	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
133	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
134	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
135	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
136	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
137	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
138	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
139	F	K0	K0	62	07-AUG-00	07-OCT-00	07-OCT-00
140	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00

PATHOLOGY REPORT PAGE : 47/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

ANIMAL HEADING DATA
DOSE GROUP : 2, 5.5%

ANIMAL NUMBER	SEX M/F	DEFINED AND FINAL STATE OF NECROPSY		TEST DAYS	FIRST AND LAST DAY UNDER TEST		DATE OF NECROPSY
141	F	K0	K0	62	07-AUG-00	07-OCT-00	07-OCT-00
142	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
143	F	K0	K0	64	07-AUG-00	09-OCT-00	09-OCT-00
144	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
145	F	K0	K0	63	07-AUG-00	08-OCT-00	08-OCT-00
146	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
147	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
148	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00

PATHOLOGY REPORT PAGE : 48/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 2, 5.5% MALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 80 * ANIMAL NO. : 25

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 79 * ANIMAL NO. : 26

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT PAGE : 49/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 2, 5.5% MALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 80 * ANIMAL NO. : 27

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 80 * ANIMAL NO. : 28

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

```
PATHOLOGY REPORT                                        PAGE       :   50/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                 MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    79                        * ANIMAL NO. :         29
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    79                        * ANIMAL NO. :         30
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   51/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                 MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                         * ANIMAL NO. :         31
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                         * ANIMAL NO. :         32
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   52/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                  MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                         * ANIMAL NO. :         33
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    81                         * ANIMAL NO. :         34
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   53/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    : 2, 5.5%                                                  MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                        * ANIMAL NO. :         35
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    81                        * ANIMAL NO. :         36
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 54/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 2, 5.5% MALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 79 * ANIMAL NO. : 37

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 79 * ANIMAL NO. : 38

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

```
PATHOLOGY REPORT                                        PAGE       :   55/ 126
INDIVIDUAL ANIMAL DATA
------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  2, 5.5%                                                  MALE
------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                        * ANIMAL NO. :        39
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                        * ANIMAL NO. :        40
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   56/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                 MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                        * ANIMAL NO. :         41
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                        * ANIMAL NO. :         42
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   57/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        :                                        PathData® System V5.1b
                 (b) (4)
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                  MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    81                         * ANIMAL NO. :         43
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                         * ANIMAL NO. :         44
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   58/ 126
INDIVIDUAL ANIMAL DATA
--------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
--------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  2, 5.5%                                                  MALE
--------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    80                        * ANIMAL NO. :         45
................................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


--------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    80                        * ANIMAL NO. :         46
................................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


--------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   59/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                 MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                        * ANIMAL NO. :         47
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    81                        * ANIMAL NO. :         48
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   60/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        :                                        PathData® System V5.1b
                                (b) (4)
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    58                         * ANIMAL NO. :        125
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    64                         * ANIMAL NO. :        126
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 61/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 2, 5.5% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 59 * ANIMAL NO. : 127

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 59 * ANIMAL NO. : 128

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

```
PATHOLOGY REPORT                                        PAGE       :   62/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    61                        * ANIMAL NO. :       129
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    61                        * ANIMAL NO. :       130
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   63/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    59                       * ANIMAL NO. :       131
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    58                       * ANIMAL NO. :       132
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   64/ 126
INDIVIDUAL ANIMAL DATA
------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                FEMALE
------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    59                       * ANIMAL NO. :       133
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    60                       * ANIMAL NO. :       134
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 65/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 2, 5.5% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 61 * ANIMAL NO. : 135

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 60 * ANIMAL NO. : 136

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

```
PATHOLOGY REPORT                                    PAGE       :   66/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                         PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL   DATE       : 08-OCT-01
SPONSOR        :                (b) (4)            PathData® System V5.1b
-------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     : 2, 5.5%                                          FEMALE
-------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    60                     * ANIMAL NO. :       137
.........................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    60                     * ANIMAL NO. :       138
.........................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   67/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    62                       * ANIMAL NO. :       139
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    59                       * ANIMAL NO. :       140
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 68/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 2, 5.5% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 62 * ANIMAL NO. : 141

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 59 * ANIMAL NO. : 142

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT PAGE : 69/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 2, 5.5% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 64 * ANIMAL NO. : 143

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 60 * ANIMAL NO. : 144

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

```
PATHOLOGY REPORT                                        PAGE       :   70/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    63                       * ANIMAL NO. :       145
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    59                       * ANIMAL NO. :       146
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   71/ 126
INDIVIDUAL ANIMAL DATA
------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  2, 5.5%                                                FEMALE
------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    60                         * ANIMAL NO. :       147
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    58                         * ANIMAL NO. :       148
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 72/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

ANIMAL HEADING DATA
DOSE GROUP : 3, 11.0%

ANIMAL NUMBER	SEX M/F	DEFINED AND FINAL STATE OF NECROPSY		TEST DAYS	FIRST AND LAST DAY UNDER TEST		DATE OF NECROPSY
49	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
50	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
51	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
52	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
53	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
54	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
55	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
56	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
57	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
58	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
59	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
60	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
61	M	K0	K0	93	12-JUN-00	12-SEP-00	12-SEP-00
62	M	K0	K0	93	12-JUN-00	12-SEP-00	12-SEP-00
63	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
64	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
65	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
66	M	K0	K0	93	12-JUN-00	12-SEP-00	12-SEP-00
67	M	K0	K0	93	12-JUN-00	12-SEP-00	12-SEP-00
68	M	K0	K0	93	12-JUN-00	12-SEP-00	12-SEP-00
69	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
70	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
71	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
72	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
149	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
150	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
151	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
152	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
153	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
154	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
155	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
156	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
157	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
158	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
159	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
160	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
161	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
162	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
163	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
164	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00

PATHOLOGY REPORT PAGE : 73/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

ANIMAL HEADING DATA
DOSE GROUP : 3, 11.0%

ANIMAL NUMBER	SEX M/F	DEFINED AND FINAL STATE OF NECROPSY		TEST DAYS	FIRST AND LAST DAY UNDER TEST		DATE OF NECROPSY
165	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
166	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
167	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
168	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
169	F	K0	K0	66	07-AUG-00	11-OCT-00	11-OCT-00
170	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
171	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
172	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00

```
PATHOLOGY REPORT                                        PAGE       :   74/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  3, 11.0%                                                MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                       * ANIMAL NO. :         49
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                       * ANIMAL NO. :         50
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   75/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        :                (b) (4)                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  3, 11.0%                                                MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                        * ANIMAL NO. :         51
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                        * ANIMAL NO. :         52
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 76/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 3, 11.0% MALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 81 * ANIMAL NO. : 53

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 79 * ANIMAL NO. : 54

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

```
PATHOLOGY REPORT                                        PAGE       :   77/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  3, 11.0%                                                MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    81                       * ANIMAL NO. :         55
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    79                       * ANIMAL NO. :         56
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 78/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 3, 11.0% MALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 79 * ANIMAL NO. : 57

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 79 * ANIMAL NO. : 58

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

```
PATHOLOGY REPORT                                        PAGE       :   79/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  3, 11.0%                                                 MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                        * ANIMAL NO. :         59
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                        * ANIMAL NO. :         60
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   80/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  3, 11.0%                                                 MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    93                        * ANIMAL NO. :         61
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    93                        * ANIMAL NO. :         62
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   81/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  3, 11.0%                                                MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    79                       * ANIMAL NO. :         63
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                       * ANIMAL NO. :         64
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   82/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  3, 11.0%                                                 MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    80                        * ANIMAL NO. :        65
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    93                        * ANIMAL NO. :        66
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   83/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  3, 11.0%                                                MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    93                        * ANIMAL NO. :         67
.......................................................................

* NECROPSY FINDINGS

  THYMUS:
    01: HEMORRHAGE.
  NO OTHER NECROPSY OBSERVATIONS NOTED


* MICROSCOPIC FINDINGS

  THYMUS:
   -Focal interstitial hemorrhage, grade 2


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    93                        * ANIMAL NO. :         68
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   84/ 126
INDIVIDUAL ANIMAL DATA
------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  3, 11.0%                                                MALE
------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    79                       * ANIMAL NO. :         69
..............................................................................

* NECROPSY FINDINGS

  SEMINAL VESICLE:
    01: DIMINISHED.
  KIDNEYS:
    01: LEFT SIDE: FOCI, PALE, SEVERAL, UP TO 0.5 MM IN DIAMETER.
  NO OTHER NECROPSY OBSERVATIONS NOTED


* MICROSCOPIC FINDINGS

  SEMINAL VESICLE:
    Organ examined, no pathologic findings noted
  KIDNEYS:
   -Diffuse cortical cytoplasmic vacuolation of tubules, unilateral,
    grade 4


------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                       * ANIMAL NO. :         70
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   85/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  3, 11.0%                                                 MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    80                        * ANIMAL NO. :         71
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    80                        * ANIMAL NO. :         72
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   86/ 126
INDIVIDUAL ANIMAL DATA
------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  3, 11.0%                                              FEMALE
------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    60                       * ANIMAL NO. :       149
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    59                       * ANIMAL NO. :       150
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 87/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 3, 11.0% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 58 * ANIMAL NO. : 151

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 59 * ANIMAL NO. : 152

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT PAGE : 88/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 3, 11.0% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 58 * ANIMAL NO. : 153

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 61 * ANIMAL NO. : 154

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT PAGE : 89/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 3, 11.0% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 60 * ANIMAL NO. : 155

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 61 * ANIMAL NO. : 156

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

```
PATHOLOGY REPORT                                    PAGE       :   90/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                          PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL    DATE       : 08-OCT-01
SPONSOR       : [redacted] (b) (4)                  PathData® System V5.1b
-------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  3, 11.0%                                          FEMALE
-------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    61                    * ANIMAL NO. :       157
.........................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    61                    * ANIMAL NO. :       158
.........................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

-------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   91/ 126
INDIVIDUAL ANIMAL DATA
------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  3, 11.0%                                              FEMALE
------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    61                       * ANIMAL NO. :        159
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    61                       * ANIMAL NO. :        160
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.

------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :   92/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  3, 11.0%                                              FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    59                       * ANIMAL NO. :       161
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    59                       * ANIMAL NO. :       162
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 93/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 3, 11.0% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 61 * ANIMAL NO. : 163

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 61 * ANIMAL NO. : 164

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT PAGE : 94/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 3, 11.0% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 60 * ANIMAL NO. : 165

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 58 * ANIMAL NO. : 166

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

```
PATHOLOGY REPORT                                        PAGE       :   95/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  3, 11.0%                                              FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    59                       * ANIMAL NO. :       167
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    59                       * ANIMAL NO. :       168
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  NO EXAMINATION REQUIRED.


-------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 96/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 3, 11.0% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 66 * ANIMAL NO. : 169

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 61 * ANIMAL NO. : 170

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT PAGE : 97/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 3, 11.0% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 61 * ANIMAL NO. : 171

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

* STATE AT NECROPSY: K0
DAYS ON TEST : 60 * ANIMAL NO. : 172

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

NO EXAMINATION REQUIRED.

PATHOLOGY REPORT PAGE : 98/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

ANIMAL HEADING DATA
DOSE GROUP : 4, 22.0%

ANIMAL NUMBER	SEX M/F	DEFINED STATE OF NECROPSY	FINAL STATE OF NECROPSY	TEST DAYS	FIRST DAY UNDER TEST	LAST DAY UNDER TEST	DATE OF NECROPSY
73	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
74	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
75	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
76	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
77	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
78	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
79	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
80	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
81	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
82	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
83	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
84	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
85	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
86	M	K0	+3	79	12-JUN-00	29-AUG-00	29-AUG-00
87	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
88	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
89	M	K0	K0	79	12-JUN-00	29-AUG-00	29-AUG-00
90	M	K0	K0	80	12-JUN-00	30-AUG-00	30-AUG-00
91	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
92	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
93	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
94	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
95	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
96	M	K0	K0	81	12-JUN-00	31-AUG-00	31-AUG-00
173	F	K0	K0	62	07-AUG-00	07-OCT-00	07-OCT-00
174	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
175	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
176	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
177	F	K0	K0	62	07-AUG-00	07-OCT-00	07-OCT-00
178	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
179	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
180	F	K0	K0	62	07-AUG-00	07-OCT-00	07-OCT-00
181	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
182	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
183	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
184	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
185	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
186	F	K0	K0	61	07-AUG-00	06-OCT-00	06-OCT-00
187	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
188	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00

PATHOLOGY REPORT PAGE : 99/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

ANIMAL HEADING DATA
DOSE GROUP : 4, 22.0%

ANIMAL NUMBER	SEX M/F	DEFINED AND FINAL STATE OF NECROPSY		TEST DAYS	FIRST AND LAST DAY UNDER TEST		DATE OF NECROPSY
189	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
190	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
191	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
192	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
193	F	K0	K0	59	07-AUG-00	04-OCT-00	04-OCT-00
194	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00
195	F	K0	K0	58	07-AUG-00	03-OCT-00	03-OCT-00
196	F	K0	K0	60	07-AUG-00	05-OCT-00	05-OCT-00

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PATHOLOGY REPORT                                        PAGE       :  100/ 126
INDIVIDUAL ANIMAL DATA
------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                 MALE
------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                        * ANIMAL NO. :         73
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 2
   -Diffuse cortical/medullar histiocytosis, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    80                        * ANIMAL NO. :         74
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  EPIDIDYMIDES:
   -Focal cytoplasmic vacuolation in the duct epithelium,
    unilateral, grade 1
  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 1
   -Diffuse cortical/medullar histiocytosis, grade 2
```

```
PATHOLOGY REPORT                                        PAGE       :  101/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                 MALE
-------------------------------------------------------------------------------

                                          CONT./FF. ANIMAL NO. :         74
.......................................................................

  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                      * ANIMAL NO. :         75
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 3
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    80                      * ANIMAL NO. :         76
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.
```

```
PATHOLOGY REPORT                                        PAGE       :  102/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                 MALE
-------------------------------------------------------------------------------

                                          CONT./FF. ANIMAL NO. :         76
.......................................................................

* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                    * ANIMAL NO. :         77
-------------------------------------------------------------------------------

* NECROPSY FINDINGS

  MESENTERIC LYMPH NODE:
    01: REDDENED.
  NO OTHER NECROPSY OBSERVATIONS NOTED


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  103/ 126
INDIVIDUAL ANIMAL DATA
------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                 MALE
------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    80                        * ANIMAL NO. :        78
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 3
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    80                        * ANIMAL NO. :        79
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 3
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  104/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  4, 22.0%                                                MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    79                        * ANIMAL NO. :         80
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 3
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                        * ANIMAL NO. :         81
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal chronic active inflammation in the lymphoid follicle,
    grade 2
   -Diffuse cortical/medullar histiocytosis, grade 3
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                    PAGE       :  105/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                         PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL   DATE       : 08-OCT-01
SPONSOR        : NORFERM DANMARK A/S                PathData® System V5.1b
-------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  4, 22.0%                                          MALE
-------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    80                     * ANIMAL NO. :        82
.........................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  EPIDIDYMIDES:
    Minimal focal unilateral accumulation of mononuclear cells
    in the adipose tissue around the capsule.
  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 2
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    79                     * ANIMAL NO. :        83
.........................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  COAGULATING GLANDS:
   -Focal reactive hyperplasia in the acini, bilateral, grade 1
    associated with inflammatory debri in the lumen.
  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 1
```

```
PATHOLOGY REPORT                                        PAGE       :  106/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                 MALE
-------------------------------------------------------------------------------

                                          CONT./FF. ANIMAL NO. :        83
.....................................................................

   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    81                      * ANIMAL NO. :        84
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    80                      * ANIMAL NO. :        85
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.
```

```
PATHOLOGY REPORT                                        PAGE       :  107/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                 MALE
-------------------------------------------------------------------------------

                                          CONT./FF. ANIMAL NO. :         85
.......................................................................

* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 2
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0/+3
  DAYS ON TEST     :    79                      * ANIMAL NO. :         86
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 3
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  108/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  4, 22.0%                                                MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    79                       * ANIMAL NO. :         87
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    81                       * ANIMAL NO. :         88
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal dilatation, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  109/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                 MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    79                        * ANIMAL NO. :         89
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 3
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    80                        * ANIMAL NO. :         90
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  110/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b)(4)                                  PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                 MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    81                        * ANIMAL NO. :         91
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    81                        * ANIMAL NO. :         92
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 1
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  111/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                 MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    81                        * ANIMAL NO. :        93
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    81                        * ANIMAL NO. :        94
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 1
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  112/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                 MALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: KO
  DAYS ON TEST     :    81                        * ANIMAL NO. :        95
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  PROSTATE GLAND:
   -Focal interstitial accumulation of lymphoid cells, grade 1
  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: KO
  DAYS ON TEST     :    81                        * ANIMAL NO. :        96
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  PROSTATE GLAND:
   -Focal interstitial accumulation of lymphoid cells, grade 1
  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 2
   -Diffuse cortical/medullar histiocytosis, grade 2
```

PATHOLOGY REPORT PAGE : 113/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 4, 22.0% MALE

CONT./FF. ANIMAL NO. : 96

ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

```
PATHOLOGY REPORT                                        PAGE       :  114/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                              FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    62                        * ANIMAL NO. :       173
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 3
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    59                        * ANIMAL NO. :       174
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  115/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    61                        * ANIMAL NO. :       175
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal dilatation, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    58                        * ANIMAL NO. :       176
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 2
   -Diffuse cortical/medullar histiocytosis, grade 3
   -Focal sinusoidal hemorrhage, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  116/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                              FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    62                        * ANIMAL NO. :       177
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  PITUITARY GLAND (PARS INTERMEDIA & NERVOSA):
    Tissue not present for histologic examination
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    58                        * ANIMAL NO. :       178
.......................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 3
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  117/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       :                                         PathData® System V5.1b
                                    (b) (4)
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    60                        * ANIMAL NO. :       179
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 3
   -Focal sinusoidal hemorrhage, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    62                        * ANIMAL NO. :       180
...............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  118/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                              FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    60                        * ANIMAL NO. :       181
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    58                        * ANIMAL NO. :       182
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  OVARIES:
   -Focal luteal microabcess (accumulation of neutrophils),
    bilateral, grade 1
  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 2
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 2
```

```
PATHOLOGY REPORT                                        PAGE       :  119/ 126
INDIVIDUAL ANIMAL DATA
------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                              FEMALE
------------------------------------------------------------------------------

                                          CONT./FF. ANIMAL NO. :       182
..............................................................................

  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    60                      * ANIMAL NO. :       183
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 1
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    59                      * ANIMAL NO. :       184
..............................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.
```

```
PATHOLOGY REPORT                                        PAGE       :  120/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                                FEMALE
-------------------------------------------------------------------------------

                                          CONT./FF. ANIMAL NO. :       184
.....

* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    59                      * ANIMAL NO. :       185
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  121/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : [redacted] (b) (4)                      PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                              FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    61                        * ANIMAL NO. :       186
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    59                        * ANIMAL NO. :       187
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 1
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  122/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                              FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    59                        * ANIMAL NO. :       188
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 2
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal dilatation, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    59                        * ANIMAL NO. :       189
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 2
   -Focal sinusoidal dilatation, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  123/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : (b) (4)                                 PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                              FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    59                        * ANIMAL NO. :       190
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 3
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    60                        * ANIMAL NO. :       191
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  124/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE  : BioProtein                              PATHOL. NO.: 25995 ZDT
TEST SYSTEM   : RAT, ONE-GENERATION REPRO., ORAL        DATE       : 08-OCT-01
SPONSOR       : [redacted] (b) (4)                      PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP    :  4, 22.0%                                              FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    60                        * ANIMAL NO. :       192
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Focal/multifocal cortical/medullar granuloma formation,
    grade 1
   -Diffuse cortical/medullar histiocytosis, grade 3
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST     :    59                        * ANIMAL NO. :       193
.....

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  OVARIES:
    Only one of paired organs examined/present
  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 1
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

```
PATHOLOGY REPORT                                        PAGE       :  125/ 126
INDIVIDUAL ANIMAL DATA
-------------------------------------------------------------------------------
TEST ARTICLE   : BioProtein                             PATHOL. NO.: 25995 ZDT
TEST SYSTEM    : RAT, ONE-GENERATION REPRO., ORAL       DATE       : 08-OCT-01
SPONSOR        : (b) (4)                                PathData® System V5.1b
-------------------------------------------------------------------------------
TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP     :  4, 22.0%                                              FEMALE
-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    60                       * ANIMAL NO. :        194
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------

* STATE AT NECROPSY: K0
  DAYS ON TEST      :    58                       * ANIMAL NO. :        195
.....................................................................

* NECROPSY FINDINGS

  NO NECROPSY OBSERVATIONS NOTED.


* MICROSCOPIC FINDINGS

  MESENTERIC LYMPH NODE:
   -Diffuse cortical/medullar histiocytosis, grade 2
   -Focal sinusoidal hemorrhage, grade 2
  ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.


-------------------------------------------------------------------------------
```

PATHOLOGY REPORT PAGE : 126/ 126
INDIVIDUAL ANIMAL DATA

TEST ARTICLE : BioProtein PATHOL. NO.: 25995 ZDT
TEST SYSTEM : RAT, ONE-GENERATION REPRO., ORAL DATE : 08-OCT-01
SPONSOR : (b) (4) PathData® System V5.1b

TEXT OF GROSS AND MICROSCOPIC FINDINGS
DOSE GROUP : 4, 22.0% FEMALE

* STATE AT NECROPSY: K0
DAYS ON TEST : 60 * ANIMAL NO. : 196

* NECROPSY FINDINGS

NO NECROPSY OBSERVATIONS NOTED.

* MICROSCOPIC FINDINGS

MESENTERIC LYMPH NODE:
-Diffuse cortical/medullar histiocytosis, grade 3
ALL OTHER PROTOCOL TISSUES WITHOUT PATHOLOGIC FINDINGS.

(b) (4)

(b) (4)

TEST REPORT

SPONSOR: (b) (4)

BIOPROTEIN

EIGHT-WEEK LYMPH NODE TOXICITY STUDY IN THE RAT

AUTHOR: (b) (4)

(b) (4)

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The part of the investigation performed by (b) (4) described in this report "BIOPROTEIN - Eight-Week Lymph Node Toxicity Study in the Rat" was carried out under my supervision and responsibility and in accordance with the OECD Principles of Good Laboratory Practice (as revised in 1997) which are essentially in conformity with:

EEC Principles of Good Laboratory Practice, Directive 87/18/EEC,
United States Food and Drug Administration, Title 21, CFR, Part 58, and
Japanese Ministry of Health and Welfare, PAB Notification No. 424.

The report is a complete and accurate account of the methods employed and the data obtained.

(b) (4)

20 September 1999

(b) (4)

Study Director

(b) (4)

QUALITY ASSURANCE STATEMENT

The Quality system at [redacted] complies with the OECD Principles of Good Laboratory Practice (as revised in 1997) and the European Standard EN45001.

The part of this study "BIOPROTEIN - Eight-Week Lymph Node Toxicity Study in the Rat" performed by [redacted] has been inspected by the Quality Assurance Unit in compliance with the principles of Good Laboratory Practice. Inspection reports have been communicated to the Study Director and to the management of [redacted]

Protocol reviewed on	27 October 1998	
Study-based inspections:	Performed on	Reported on
	18 November 1998	18 November 1998
	13 January 1998	13 January 1998
	Audited on	Reported on
Report	27 May 1998	27 May 1998
	20 September 1999	No report

The part of the report relating to the work done by [redacted] has been audited by the Quality Assurance Unit and was found to be an accurate description of the methods and procedures used during the conduct of the study and an accurate reflection of the raw data.

20 September 1999

[redacted]

Head of Quality Assurance

PERSONNEL RESPONSIBLE FOR THE STUDY

(b) (4)

Study Director	[redacted]
Haemathology	
Pathology	
Statistics	
Quality Assurance	
Sponsor Monitor	

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TABLES

APPENDICES

ADDENDUM

SUMMARY

The objective of this study was to assess the possible effects of Bioprotein on the mesenteric lymph nodes, the iliac lymph node, spleen, liver, lungs and the gastrointestinal tract after daily administration in three types of diet to rats for eight weeks.

A total of 30 male SPF Wistar rats was included in the study. The animals were allocated into six groups given Altromin (group 1), Altromin mixed with 15% Bioprotein (group 2), semi-synthetic diet (group 3), semi-synthetic diet mixed with 22% Bioprotein (group 4), synthetic diet (group 5) and synthetic diet mixed with 15% Bioprotein (group 6) for eight weeks, respectively. The different types of diets were offered ad libitum.

For all animals, clinical signs were recorded daily and the body weight and the food consumption were recorded once weekly. At termination of the study, blood samples for haematology were taken from all animals. On day 57, the animals were killed by an i.p. injection of a barbiturate followed by exsanguination. A macroscopic examination was performed. The organ weights of the left iliac lymph node, the mesenteric lymph nodes, the spleen and the liver were recorded, and representative samples from these organs, the lungs and the gastrointestinal tract were collected for histopathological analysis.

No difference in mean body weight and body weight gain between the groups was seen during the study.

No specific relation between food consumption and treatment with Bioprotein or between food consumption and type of diet was seen during the study.

The percentage of neutrofiles and monocytes was higher, and the percentage of lymphocytes was lower for all groups treated with Bioprotein compared to the groups given no Bioprotein in the diet.

The absolute and the relative organ weight of the mesenteric lymph nodes and the spleen of the animals in the groups given Bioprotein in the diet were greater compared to the groups given no Bioprotein in the diet. The relative organ weight of the liver of the animals in groups 5 and 6 was lower compared to the remaining groups, which was considered an incidental finding.

Microscopically, changes in the mesenteric lymph nodes, related to the dosing with Bioprotein, were observed in all three groups treated with Bioprotein. The changes consisted of slightly to moderate sinusoidal dilation. In addition, a slight focal necrosis in the mesenteric lymph node was found in one animal in group 4. Minimal to moderate focal

necrosis and minimal to slight granuloma formation were observed in some of the mesenteric lymph nodes from the animals in group 6. Minimal focal mineralisations in the germinal centre of the mesenteric lymph nodes were seen in some of the animals treated with Bioprotein. No changes, related to the treatment with Bioprotein, were observed in the iliac lymph node, spleen, liver, lung, stomach, intestines or mesenterium.

In conclusion, Bioprotein given continuously in the diet to rats for 8 weeks, at dosages of 15 or 22% Bioprotein in the diet, caused an increase of the number of neutrofiles and monocytes, an increase in the organ weight of the mesenteric lymph nodes and the spleen, and focal necrosis, granuloma formation and increased degree of dilated sinusoids in the mesenteric lymph nodes. A synthetic diet in combination with Bioprotein seemed to aggravate the microscopic findings.

INTRODUCTION

The objective of this study was to assess the possible effects of Bioprotein on the mesenteric lymph nodes, the iliac lymph node, spleen, liver, lungs and the gastrointestinal tract after daily administration in three types of diet to rats for eight weeks.

The rat was selected as the test model because of its proven suitability in toxicology studies.

The dietary route of administration was chosen in order to comply with the intended usage.

The animals arrived on 11 November 1998. Treatment commenced on 18 November 1998. The live animal work was completed on 13 January 1999.

MATERIALS AND METHODS

Test article

The test article Bioprotein (Batch No 127) was supplied by the Sponsor on 21 October 1998. In addition, Bioprotein was supplied on 21 October 1998 as a constituent in the semi-synthetic and synthetic diets specially made for the purpose of this study. At request of the Sponsor, the semi-synthetic and synthetic diets were manufactured (date of manufacture: 22 September 1998) and delivered to (b) (4) The manufacture of semi-synthetic and synthetic diets was subcontracted by the Sponsor.

Semi-synthetic diets

The diets were formulated individually using various "purified" and "natural" ingredients: casein, wheat starch, cellulose, soya oil, wheat, maize and wheat feed together with minerals and vitamins. Casein provided the supplementary protein in the control diet, and Bioprotein provided the supplementary protein in the test diet.

The diets were formulated using analysis of the major ingredients and were calculated to be equal in protein, metabolisable energy, calcium, phosphorus, magnesium, sodium, potassium and limiting amino acids.

Synthetic diets

The diets were formulated individually using various "purified" ingredients: casein, wheat starch, castor sugar, cellulose and soya oil, together with minerals and vitamins. Casein provided all the protein in the control diet and in the other diet the protein was supplied by Bioprotein plus some casein.

Ordinary diets

The basal diet for group 1 and 2 (see paragraph "treatment" on page 11) was Altromin 1321. The diet for group 2 was prepared by adding Bioprotein to Altromin 1321.

All diets were stored at approximately +4°C in the dark.

The rest result relates to the above mentioned test article supplied by the Sponsor.

Animals

The experiment was performed in 30 male SPF Wistar rats of the stock Mol [redacted](b) (4) [redacted](b) (4) At start of the acclimatisation period, the rats were 4 to 5 weeks old and the body weight was in the range of 73 - 88 g. Five animals were available until completion of the acclimatisation period for replacement purposes.

An acclimatisation period of one week was allowed in order to reject animals in poor condition or at extreme weight.

Housing

The study took place in animal room No 18 provided with filtered air at a temperature of 21°C ± 3°C and relative humidity of 55% ± 15%. The relative humidity was below the limit for short periods during the study. This deviation has not affected the outcome of the study. Due to computer failure, no recordings of temperature and relative humidity have been retained for 7 hours on 21 December 1998. The room has been designed to give 10 air changes per hour. The room was illuminated to give a cycle of 12 hours light and 12 hours darkness. Light was on from 0600 h to 1800 h.

The temperature and relative humidity in the animal room were recorded hourly during the study and the records have been retained.

The rats were kept individually in transparent polycarbonate cages (macrolone type III, floor area: 810 cm^2) with a metal grid at the bottom.

Before the animals arrived, the animal room was cleaned and disinfected with Glu-Cid®. During the study, the animal room was cleaned regularly and rinsed with water.

Diet

In the acclimatisation period, all animals received the diets without Bioprotein ad libitum.

Thereafter, the animals received the special Bioprotein containing diets, according to the paragraph "Treatment", ad libitum. The semi-synthetic and synthetic Bioprotein containing diets were prepared by the manufacturer. Admixture of Bioprotein to the Altromin 1321 diet was performed by (b) (4) on 02 November 1998. Analyses for major nutritive components and relevant possible contaminants were performed on the diet by the manufacturer. Certificates of analysis have been retained by the manufacturer.

Drinking water

The animals had free access to bottles with domestic quality drinking water acidified with hydrochloric acid to pH 2.5 in order to prevent microbial growth.

Analyses for relevant possible contaminants were performed regularly. Certificates of analysis have been retained.

Animal randomisation and allocation

On the day of arrival, the animals were allocated to six groups and a group of extra animals using a randomisation scheme.

Animal and cage identification

Each animal was identified by punched earmarks.

Each cage was identified by a colour coded cage card marked with study number (Lab No 30864), cage number, group number and animal numbers.

Treatment

The groups, type of diet, dietary concentrations, total protein content, animal numbers and colour codes were as follows:

Group	Type of diet	Concentration of Bioprotein in the diet (%)	Total protein concentration (%)	Animal No	Colour code
1	Altromin 1321	0	19	1 - 5	White
2		15	25	6 - 10	Blue
3	Semi-synthetic	0	21	11 - 15	Green
4		22	21	16 - 20	Red
5	Synthetic	0	16	21 - 25	Yellow
6		15	16	26 - 30	Blue with black rim

Control of dietary admixture manufacturing

The documentation for correct manufacturing of the semi-synthetic and synthetic diets was the responsibility of (b) (4) All documentation for correct manufacturing will be archived at (b) (4)

Diet samples

Three samples (each of 10 g) were collected from each type of diet at the end of the live phase of the study and stored at -18°C. The samples were sent to the Sponsor for possible later analysis. The result of this analysis will be reported separately by the Sponsor.

Clinical signs

All visible signs of ill health and any behavioural changes were recorded daily. Any deviation from normal was recorded with respect to time of onset, duration and intensity.

Body weight

All animals were weighed on arrival and weekly thereafter, including the first day of dosing. Also the weight at necropsy was recorded.

Food consumption

The consumption of food was recorded weekly for each animal.

Laboratory investigations

Shortly before termination of treatment, blood samples were taken from all animals.

Blood samples were drawn from the orbital venous plexus during CO_2 anaesthesia. For haematology, 750 µl EDTA stabilised blood was taken. In addition, about 2 ml of citrate stabilised blood was taken, plasma was prepared (1270 G, 10 min, +4°C), transferred to Nunc cryotubes, stored at approximately -18°C and sent with dry ice to the Sponsor for possible later analyses. The result of these analyses will be reported separately by the Sponsor.

The parameters, methods and units for the laboratory investigations are stated below:

Haematology

Parameter	Method	Unit
Differential leucocyte count (NEUTRO, LYMPHO, EOS, BASO, MONO)	May-Grünwald Giemsa smear staining	%

Terminal observations

On the day of necropsy, the animals were weighed, examined externally, anaesthetised with an i.p. injection of a barbiturate (Brietal®) and sacrificed by exsanguination. The animals were sacrificed and necropsied in the sequence of one or two animals per group.

Necropsy

A macroscopic examination was performed after opening the cranial, thoracic and abdominal cavities and by observing the appearance of the organs and tissues *in situ*. Any macroscopic change was recorded with details of the location, colour, shape and size in the PathData computer system.

Organ weights

Weights of the following organs were recorded in the PathData computer system:

Iliac lymph node (left)
Mesenteric lymph nodes
Spleen
Liver

Representative specimens from the following organs were collected:

Ililac lymph node (left)
Mesenteric lymph nodes
Spleen
Liver
Stomach
Duodenum
Jejunum
Ileum
Caecum
Colon

Rectum
Mesenterium
Lungs

The tissues were fixed in phosphate buffered neutral 4% formaldehyde.

Processing and microscopic examination

All tissues sampled were trimmed and representative specimens were taken for histological processing, embedded in paraffin and cut at a nominal thickness of about 5 µm, stained with haematoxylin and eosin and examined under the light microscope. Three sections of the mesenteric lymph nodes were examined for each animal.

Statistics

Data were processed to give group mean values and standard deviations where appropriate. Thereafter, each continuous variable was tested for homogeneity of variance with Bartlett's test. If the variance was homogeneous, two-way analysis of variance was carried out for the variable with the factors: type of diet and concentration of Bioprotein. The interaction between these factors was also included in the model. If the variance was heterogeneous, each variable was tested for normality by the Shapiro-Wilk method. In case of normal distribution, possible intergroup differences were identified with Student's t-test. Otherwise, the possible intergroup differences were assessed by Kruskal-Wallis' test. If any significant intergroup differences were detected, the subsequent identification of the groups was carried out with Wilcoxon Rank-Sum test.

The statistical analyses were made with SAS® procedures (version 6.12) described in "SAS/STAT® User's Guide, Version 6, Fourth Edition, Vol. 1+2", 1989, SAS Institute Inc., Cary, North Carolina 27513, USA.

Archives

For a period of 10 years the following material relating to the study will be retained in the archives of [redacted] (b) (4)

Protocol, protocol amendments and correspondence
Test material receipts
Animal records
All original data
Specimens and slides
Final report

At the end of the storage period [redacted] (b) (4) will contact the Sponsor for instructions whether the material should be transferred, retained or destroyed.

RESULTS

The results are summarised in Tables 1 - 4. The individual results are given in Appendices I - VI and Addendum A.

Clinical signs (Appendix I)

No clinical signs related to the treatment were seen during the study.

Body weight (Table 1, Appendix II)

No difference in mean body weight and body weight gain between the groups was seen during the study.

Food consumption (Table 2, Appendix III)

On days 8, 15 and 36, the mean food consumption of the animals in group 3 (semi-synthetic diet, 0% Bioprotein) was lower compared to group 1 (Altromin, 0% Bioprotein), group 4 (semi-synthetic diet, 22% Bioprotein) and group 5 (synthetic diet, 0% Bioprotein). On day 22, the mean food consumption of the animals in group 2 (Altromin, 15% Bioprotein), group 3 (semi-synthetic diet) and group 6 (synthetic diet, 15% Bioprotein) was lower compared to group 1 (Altromin, 0% Bioprotein). In addition, the mean food consumption of the animals in group 3 (semi-synthetic diet) was lower compared to group 4 (semi-synthetic diet, 22% Bioprotein) and group 5 (synthetic diet, 0% Bioprotein). On day 43, the mean food consumption of the animals in group 3 (semi-synthetic diet, 0% Bioprotein) was lower compared to group 1 (Altromin, 0% Bioprotein) and group 5 (synthetic diet, 0% Bioprotein).

The total mean food consumption of the animals in group 3 (semi-synthetic diet, 0% Bioprotein) was lower compared to group 1 (Altromin, 0% Bioprotein) and group 5 (synthetic diet, 0% Bioprotein). All the findings were statistically significant ($p<0.05$).

Haematology (differential count) (Table 3, Appendix IV)

The percentage of neutrofiles and monocytes was higher, and the percentage of lymphocytes was lower for the groups treated with Bioprotein (group 2 (Altromin, 15% Bioprotein), group 4 (semi-synthetic diet, 22% Bioprotein) and group 6 (synthetic diet, 15% Bioprotein)) compared to the respective control groups (group 1 (Altromin, 0% Bioprotein), group 3 (semi-

synthetic diet, 0% Bioprotein) and group 5 (synthetic diet, 0% Bioprotein)) These findings were statistically significant ($p<0.05$).

Organ weights (Table 4, Appendices V-VI)

The absolute and the relative organ weight of the mesenteric lymph nodes and the spleen of the animals in groups 2 (Altromin, 15% Bioprotein), 4 (semi-synthetic diet, 22% Bioprotein) and 6 (synthetic diet, 15% Bioprotein) were greater compared to groups 1 (Altromin, 0% Bioprotein), 3 (semi-synthetic diet, 0% Bioprotein) and 5 (synthetic diet, 0% Bioprotein). The relative organ weight of the liver of the animals in group 5 (synthetic diet, 0% Bioprotein) and 6 (synthetic diet, 15% Bioprotein) was lower compared to the remaining groups. All the findings were statistically significant ($p<0.05$).

Macroscopic findings (Addendum A)

No treatment related findings were observed at necropsy.

Microscopic findings (Addendum A)

Mesenteric lymph nodes

In the mesenteric lymph nodes, changes related to the treatment with Bioprotein, were observed in all three groups treated with Bioprotein (groups 2, 4 and 6). The changes were as follows:

Groups 1 and 2:

In group 1 (Altromin, 0% Bioprotein), one animal had slightly dilated sinusoids and four animals had minimal dilated sinusoids in the mesenteric lymph nodes.

In group 2 (Altromin, 15% Bioprotein), a moderate sinusoidal dilation in the mesenteric lymph nodes was seen in two animals, slight dilation was seen in two animals and minimal dilation was seen in one animal. Minimal focal mineralisations in the germinal centre were observed in the mesenteric lymph nodes of one animal.

Groups 3 and 4:

In group 3 (semi-synthetic diet, 0% Bioprotein), minimal dilation was seen in the mesenteric lymph nodes of all five animals.

Slight focal necrosis in the mesenteric lymph nodes was found in one animal in group 4 (semi-synthetic diet, 22% Bioprotein). In addition, slightly dilated sinusoids was seen in two animals while minimal dilation was seen in three animals. Minimal focal mineralisations in the germinal centre, associated with giant cells, were observed in one animal.

Groups 5 and 6:

Minimal dilation of the sinusoids was seen in all five mesenteric lymph nodes of the animals in group 5 (synthetic diet, 0% Bioprotein).

Minimal to moderate focal necrosis, minimal to slight granuloma formation and a higher degree of dilated sinusoids were observed in some of the mesenteric lymph nodes from the animals in group 6 (synthetic diet, 15% Bioprotein). Focal cortical/paracortical necrosis was present in two animals, one of a moderate degree and one minimal. Minimal to slight granuloma-formation was present in three animals; inconspicuous granulomas, normally seen in mesenteric lymph nodes, and which mostly consisted of a few pigment laden macrophages, were not reported. Moderate dilation was present in one animal, slight dilation in two animals and minimal dilation in the remaining two animals. Minimal focal mineralisations in the germinal centre, associated with giant cells, were observed in two animals.

Iliac lymph node, spleen, liver, lung, stomach, intestines and mesenterium.

No changes related to the treatment with Bioprotein were observed in these organs. The changes observed in these organs included minor degenerative and inflammatory lesion, which are well known incidental alterations for rats of this strain and age.

DISCUSSION

In general, the mean food consumption of the animals in group 3 (semi-synthetic diet, 0% Bioprotein) was lower compared to the other groups throughout the study. However, no specific relation between food consumption and treatment with Bioprotein or between food consumption and type of diet was seen during the study.

The decrease in the number of lymphocytes for the groups treated with Bioprotein might be a secondary effect of an actual increase in neutrofiles and monocytes. As the total number of these cells of interest in the blood samples has not been determined, this hypothesis can not be confirmed.

The decrease in the relative liver weight of the animals in groups 5 (synthetic diet, 0% Bioprotein) and 6 (synthetic diet, 15% Bioprotein) was considered an incidental finding.

However, there was an obvious relation between organ weights of mesenteric lymph nodes and the spleen and treatment with Bioprotein, Bioprotein causing an increased weight of the respective organs.

Whether the apparently milder microscopic reaction to Bioprotein in group 2 (Altromin, 15% Bioprotein) compared to the more severe reaction in group 6 (synthetic diet, 15% Bioprotein) and to a lesser degree to group 4 (semi-synthetic diet, 22% Bioprotein) is real, is difficult to assess on the basis of only five animals per group.

CONCLUSION

Bioprotein given continuously in the diet to rats for 8 weeks at dosages of 15 or 22% Bioprotein in the diet caused an increase of the number of neutrofiles and monocytes, an increase in the organ weight of the mesenteric lymph nodes and the spleen and focal necrosis, granuloma formation and increased degree of dilated sinusoids in the mesenteric lymph nodes. A synthetic diet in combination with Bioprotein seemed to aggravate the microscopic findings.

BIOPROTEIN

Eight-Week Lymph Node Toxicity Study in the Rat

Body weight (g)

Group mean values

TYPE OF DIET	/ BIOPROTEIN IN DIET	DAY -7				DAY 1				DAY 8				DAY 15			
		MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
Altromin 1321	0(group 1)	80.8	5.0	5		121.0	8.1	5		154.0	10.9	5		192.2	10.4	5	
	+(group 2)	83.0	4.4	5		121.2	10.4	5		153.8	15.7	5		194.6	18.3	5	
Semi-synthetic	0(group 3)	80.4	4.6	5		121.2	5.1	5		150.2	5.8	5		191.6	9.2	5	
	+(group 4)	83.2	3.9	5		121.8	7.2	5		158.4	9.8	5		204.4	10.8	5	
Synthetic	0(group 5)	78.8	4.0	5		121.6	4.1	5		155.2	3.6	5		196.2	6.3	5	
	+(group 6)	80.8	3.6	5		121.8	6.3	5		156.2	9.7	5		198.8	12.9	5	

TYPE OF DIET	/ BIOPROTEIN IN DIET	DAY 22				DAY 29				DAY 36				DAY 43			
		MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
Altromin 1321	0(group 1)	223.2	14.6	5		248.8	18.7	5		268.8	25.2	5		280.0	23.5	5	
	+(group 2)	224.0	20.6	5		252.4	21.3	5		270.4	20.9	5		285.8	18.8	5	
Semi-synthetic	0(group 3)	221.6	11.6	5		249.4	14.2	5		269.4	17.1	5		287.8	19.5	5	
	+(group 4)	238.4	8.0	5		265.0	7.5	5		284.2	10.0	5		301.6	10.8	5	
Synthetic	0(group 5)	228.2	9.1	5		255.0	10.6	5		278.6	11.5	5		295.8	15.7	5	
	+(group 6)	233.0	14.8	5		263.2	19.2	5		283.2	20.5	5		299.0	22.7	5	

+ means for Altromin 1321 and synthetic diet = 15 % and for semi-synthetic diet = 22 %

Means with different letters are significantly different ($p<0.05$)

S.D. = standard deviation N = number of animals

BIOPROTEIN

Eight-Week Lymph Node Toxicity Study in the Rat

Body weight (g)

Group mean values

TYPE OF DIET	/ BIOPROTEIN IN DIET	DAY 50				DAY 57				BODY WT GAIN 1-57			
		MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
Altromin 1321	0(group 1)	297.2	28.0	5		311.4	29.8	5		190.4	22.2	5	
	+(group 2)	301.0	20.6	5		319.0	22.2	5		197.8	12.6	5	
Semi-synthetic	0(group 3)	303.6	22.4	5		321.8	25.8	5		200.6	26.6	5	
	+(group 4)	314.0	10.3	5		331.2	14.2	5		209.4	19.1	5	
Synthetic	0(group 5)	310.4	19.7	5		326.4	21.4	5		204.8	23.2	5	
	+(group 6)	315.6	23.6	5		332.6	22.3	5		210.8	18.3	5	

+ means for Altromin 1321 and synthetic diet = 15 % and for semi-synthetic diet = 22 %

p>0.05

S.D. = standard deviation N = number of animals

BIOPROTEIN

Eight-Week Lymph Node Toxicity Study in the Rat

Food consumption (g)

Group mean values

TYPE OF DIET	/ BIOPROTEIN IN DIET	DAY 8				DAY 15				DAY 22			
		MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
Altromin 1321	0(group 1)	152.0	14.7	5	b	161.2	5.9	5	b	189.0	25.2	5	c
	+(group 2)	139.0	10.1	5	ab	150.8	8.2	5	ab	164.2	9.5	5	ab
Semi-synthetic	0(group 3)	128.2	14.9	5	a	139.4	15.4	5	a	151.0	17.4	5	a
	+(group 4)	144.0	9.7	5	b	156.2	7.7	5	b	176.0	6.9	5	bc
Synthetic	0(group 5)	148.0	7.2	5	b	159.4	5.5	5	b	175.6	6.8	5	bc
	+(group 6)	140.8	11.2	5	ab	148.4	12.9	5	ab	169.4	12.8	5	ab

TYPE OF DIET	/ BIOPROTEIN IN DIET	DAY 29				DAY 36				DAY 43			
		MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
Altromin 1321	0(group 1)	180.6	22.4	5		180.8	21.6	5	b	178.4	19.8	5	b
	+(group 2)	167.2	11.3	5		166.8	10.1	5	ab	166.4	6.8	5	ab
Semi-synthetic	0(group 3)	150.8	19.0	5		150.2	14.6	5	a	155.4	13.6	5	a
	+(group 4)	167.8	8.0	5		170.0	9.1	5	b	169.8	10.7	5	ab
Synthetic	0(group 5)	171.0	7.6	5		174.0	5.7	5	b	177.4	7.2	5	b
	+(group 6)	168.2	16.6	5		165.2	16.5	5	ab	170.2	28.6	5	ab

+ means for Altromin 1321 and synthetic diet = 15 % and for semi-synthetic diet = 22 %

Means with different letters are significantly different ($p<0.05$)

S.D. = standard deviation N = number of animals

BIOPROTEIN

Eight-Week Lymph Node Toxicity Study in the Rat

Food consumption (g)

Group mean values

TYPE OF DIET	/ BIOPROTEIN IN DIET	DAY 50				DAY 57				TOTAL 1-57			
		MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
Altromin 1321	0(group 1)	176.6	31.3	5		180.0	34.5	5		1398.6	163.1	5	b
	+(group 2)	162.5	11.1	4		164.2	11.8	5		1273.3	79.6	4	ab
Semi-synthetic	0(group 3)	152.8	15.0	5		152.2	16.8	5		1180.0	124.0	5	a
	+(group 4)	164.8	8.4	5		162.0	7.1	5		1310.6	57.7	5	ab
Synthetic	0(group 5)	171.2	12.7	5		168.2	8.9	5		1344.8	37.6	5	b
	+(group 6)	160.6	20.3	5		164.2	12.9	5		1287.0	120.0	5	ab

+ means for Altromin 1321 and synthetic diet = 15 % and for semi-synthetic diet = 22 %

Means with different letters are significantly different ($p<0.05$)

S.D. = standard deviation N = number of animals

BIOPROTEIN

Eight-Week Lymph Node Toxicity Study in the Rat

Haematology

Group mean values

TYPE OF DIET	/ BIOPROTEIN IN DIET	% NEUTRO				% LYMPHO				% EOS			
		MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
Altromin 1321	0(group 1)	11.4	5.0	5	a	86.4	5.9	5	b	0.4	0.5	5	
	+(group 2)	16.0	6.4	5	b	80.4	6.3	5	a	0.8	0.8	5	
Semi-synthetic	0(group 3)	9.8	4.3	5	a	87.6	4.2	5	b	1.6	1.9	5	
	+(group 4)	22.0	7.1	5	b	74.8	6.8	5	a	0.8	1.1	5	
Synthetic	0(group 5)	10.6	4.4	5	a	86.4	3.2	5	b	1.4	0.5	5	
	+(group 6)	22.8	7.9	5	b	73.0	8.0	5	a	1.8	1.6	5	

TYPE OF DIET	/ BIOPROTEIN IN DIET	% BASO				% MONO			
		MEAN	S.D.	N	p	MEAN	S.D.	N	p
Altromin 1321	0(group 1)	0.0	0.0	5		1.8	1.3	5	a
	+(group 2)	0.0	0.0	5		2.8	1.6	5	b
Semi-synthetic	0(group 3)	0.0	0.0	5		1.0	1.0	5	a
	+(group 4)	0.0	0.0	5		2.4	1.1	5	b
Synthetic	0(group 5)	0.0	0.0	5		1.6	1.1	5	a
	+(group 6)	0.0	0.0	5		2.4	0.9	5	b

+ means for Altromin 1321 and Synthetic diet = 15 % and for Semi-synthetic diet = 22 %

Means with different letters are significantly different ($p<0.05$)

S.D. = standard deviation N = number of animals

BIOPROTEIN

Eight-Week Lymph Node Toxicity Study in the Rat

Absolute (mg) and relative (% of body weight) organ weights

Group mean values

TYPE OF DIET	/ BIOPROTEIN IN DIET	BODY WT, g DAY 57				ILIAC LYMPH NODE (left) ABSOLUTE				ILIAC LYMPH NODE (left) RELATIVE			
		MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
Altromin 1321	0(group 1)	311.4	29.8	5		15.0	5.1	5		0.00487	0.00180	5	
	+(group 2)	319.0	22.2	5		18.4	3.4	5		0.00574	0.00075	5	
Semi-synthetic	0(group 3)	321.8	25.8	5		22.0	7.1	5		0.00697	0.00266	5	
	+(group 4)	331.2	14.2	5		18.6	10.8	5		0.00555	0.00319	5	
Synthetic	0(group 5)	326.4	21.4	5		20.2	3.6	5		0.00624	0.00141	5	
	+(group 6)	332.6	22.3	5		24.4	9.0	5		0.00725	0.00239	5	

+ means for Altromin 1321 and Synthetic diet = 15 % and for Semi-synthetic diet = 22 %

p>0.05

S.D. = standard deviation N = number of animals

BIOPROTEIN

Eight-Week Lymph Node Toxicity Study in the Rat

Absolute (mg) and relative (% of body weight) organ weights

Group mean values

TYPE OF DIET	/ BIOPROTEIN IN DIET	LIVER				LIVER				MESENTERIC LYMPH NODE			
		ABSOLUTE				RELATIVE				ABSOLUTE			
		MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
Altromin 1321	0(group 1)	10340	920.8	5		3.33	0.10	5	a	243.4	54.6	5	a
	+(group 2)	10757	926.2	5		3.37	0.12	5	a	320.0	66.1	5	b
Semi-synthetic	0(group 3)	10726	1294.4	5		3.33	0.22	5	a	302.3	79.1	4	a
	+(group 4)	11206	924.2	5		3.38	0.18	5	a	342.6	72.1	5	b
Synthetic	0(group 5)	10180	1091.8	5		3.11	0.15	5	b	242.2	45.4	5	a
	+(group 6)	10226	1234.1	5		3.07	0.20	5	b	336.6	54.7	5	b

+ means for Altromin 1321 and Synthetic diet = 15 % and for Semi-synthetic diet = 22 %

Means with different letters are significantly different ($p<0.05$)

S.D. = standard deviation N = number of animals

BIOPROTEIN

Eight-Week Lymph Node Toxicity Study in the Rat

Absolute (mg) and relative (% of body weight) organ weights

Group mean values

TYPE OF DIET	/ BIOPROTEIN IN DIET	MESENTERIC LYMPH NODE RELATIVE				SPLEEN ABSOLUTE				SPLEEN RELATIVE			
		MEAN	S.D.	N	p	MEAN	S.D.	N	p	MEAN	S.D.	N	p
Altromin 1321	0(group 1)	0.0784	0.0174	5	a	615.8	104.9	5	a	0.197	0.017	5	a
	+(group 2)	0.1010	0.0241	5	b	717.2	111.1	5	b	0.226	0.041	5	b
Semi-synthetic	0(group 3)	0.0940	0.0254	4	a	691.4	88.1	5	a	0.214	0.014	5	a
	+(group 4)	0.1032	0.0203	5	b	766.4	145.2	5	b	0.230	0.034	5	b
Synthetic	0(group 5)	0.0741	0.0121	5	a	643.0	63.4	5	a	0.197	0.009	5	a
	+(group 6)	0.1011	0.0137	5	b	788.6	124.1	5	b	0.236	0.023	5	b

+ means for Altromin 1321 and Synthetic diet = 15 % and for Semi-synthetic diet = 22 %

Means with different letters are significantly different ($p<0.05$)

S.D. = standard deviation N = number of animals

In case you would like to look into the appendices of this report (the pages 27 to 73), please do not hesitate to contact us on fax no. +45 6593 2312.

Supplement

DOSSIER

BioProtein

Registration under directive 82/471/EEC

Norferm AS

Submission October 2004

Additional information on the safety of BioProtein from studies on piglets, pigs for fattening, broiler chicken, Atlantic Salmon and rats

1 SUMMARY

This dossier provides additional information in support of the pending application for the extended use of BioProtein as an animal feed ingredient within the European Union. As well as new data generated in farmed salmon, pigs and broiler chickens, it provides the results of further research in the laboratory rat initiated to investigate earlier findings observed in rats fed a non-commercial, experimental sample with a reduced nucleic acid content, produced for research purposes.

1.1 Target Animal Studies

Five new studies in target animals are presented:

- Weaned piglets receiving feed containing 9% BioProtein.
- Fattening pig trial where the pigs received 6% BioProtein.
- A field study of around 1000 fattening pigs on commercial farms receiving 6% BioProtein.
- A broiler chicken trial with feed containing 6% BioProtein.
- A trial in farmed Atlantic Salmon (*Salmo salar*) where levels of 4, 9, 18 and 36% BioProtein were tested.

The results of all these studies confirmed that BioProtein is well tolerated by farm animals with no adverse health effects. Growth and performance were at least as good as the control animals and often better results were obtained as a result of feeding BioProtein.

In the first of the above trials, the piglets receiving the BioProtein diet had a significantly better weight gain over the experimental period than the controls and in the fattening pig trial BioProtein gave weight gains that were not significantly different from the control animals. In both trials, feed intake and feed conversion ratio were not significantly affected.

The field study involving 15 commercial farms and around 1000 fattening pigs relied on the assessments of the farmers recorded through questionnaires. The records included their assessment of feed quality, feed intake, growth performance and health status of the pigs, as well as information regarding the environmental conditions on the farms. The results indicated that 6% BioProtein gave comparable or even better weight gains than the normal feed. Feed intake was unaffected and BioProtein did not adversely affect the health status of the pigs.

The broiler chicken trial lasted from day-old to 36 days of age and the level of BioProtein tested was 6% of the feed. The BioProtein group had a significantly higher weight gain to 36 days ($p<0.05$) and also during the period to 14 days of age ($p<0.02$). Although the higher feed intake of the BioProtein feed was not significantly different from the control feed, the feed conversion of the BioProtein group was significantly improved ($p<0.03$). In addition, there were no differences in mortality, carcass dressing percentage or litter quality as a result of including BioProtein in the feed.

The nine week salmon trial, involving 270 salmon, showed a progressive increase in specific growth rate as the inclusion of BioProtein increased. The difference with the

controls became significant at 18 and 36% BioProtein ($p<0.05$). This pattern was also evident in the final weights of the fish, though not quite reaching the conventional level of significance ($p=0.051$). BioProtein also improved feed efficiency and differences became significant at the highest inclusion level of BioProtein. The higher levels of BioProtein also significantly improved both energy and nitrogen retention. Higher plasma and liver levels of urea may have been indicative of catabolism of nucleic acids, but the growth and other results demonstrate that this is well within the capacity of the salmon to handle without adverse effects.

At termination of the two controlled pig trials and the broiler chicken trial, animals were subjected to necropsy and selected organs were examined for histological changes. There were no adverse effects attributable to BioProtein in any of these studies.

1.2 Rat Studies

Two new studies have been completed in rats:

- A single generation study
- A study in juvenile rats from weaning to 32 weeks of age.

Antibody studies were undertaken on blood samples taken from rats in these two studies, including samples from immunised rats from the Single Generation Study,

In the single generation study BioProtein was tested at dietary inclusion levels of 6% and 12%. Diets containing the same levels of Brewer's Yeast were also included in the study in order to have a conventional source of protein of a similar nature to form an additional basis of comparison with BioProtein.

The study in juvenile rats used the 12% BioProtein diet, but it was fed for different periods of time in the various treatment groups. The BioProtein diet was introduced from weaning or after 6 weeks of age and both groups remained on the diet until 12 weeks of age. An additional group of rats fed BioProtein from 6 weeks continued on the diet until 32 weeks of age, while another group was returned to the Casein control diet from12 weeks to 32 weeks of age

BioProtein showed no effects on reproductive performance and did not affect growth and development of the offspring to 12 weeks of age. Similarly, growth and general well being were unaffected in the Juveniles Study in rats fed BioProtein from weaning or 6weeks of age to either 12 or 32 weeks of age.

The main findings of interest were that parent animals in both the BioProtein and Brewer's Yeast groups showed a degree of enlargement of mesenteric lymph nodes, which was not unexpected in the light of results from previous studies. In the BioProtein groups this was accompanied by minimal to moderate accumulation of macrophages. In the offspring maintained to 12 weeks of age, only males had a significant increase in mesenteric lymph node weights, without any dose-response relationship. Minimal to slight macrophage accumulation was seen in the lymph nodes of only 10% of the BioProtein-fed rats.

Juvenile rats fed the 12% BioProtein diet from weaning or from 6 weeks of age to 12 weeks of age showed some lymph node enlargement, mostly in females. This was accompanied by minimal to slight macrophage accumulation in only 3 females in each of these groups, with none in males. Increased mesenteric lymph node weights remained evident in a group that continued to receive BioProtein to 32 weeks of age. The degree of macrophage accumulation was minimal to moderate. The group returned to the Control diet from 12 to 32 weeks of age showed some weight increase in males only, but without any accumulation of macrophages. This indicates that the small effect on mesenteric lymph nodes is reversible when animals are returned to the control diet.

Analysis of blood samples from rats that had received Control or BioProtein diets from the above studies was undertaken for IgG, IgG1, IgG2a and IgGA antibodies against BioProtein. Whereas parent animals receiving BioProtein had higher levels of antibodies than Control animals, offspring in the BioProtein groups showed no increase relative to the Controls.

The levels of antibodies to BioProtein in serum from immunised rats fed BioProtein were low, compared to the levels in the immunised Control rats. The levels of BioProtein antibodies were not increased over the feeding period from 21 to 45 days of age, indicating no increase in response with time.

The significance of the antibody levels compared to the levels in rats fed the casein Control diet was dependent on the age of the rats. Control rats have a low level of antibodies reacting with BioProtein, which may be explained by the existence of non-specific, cross-reacting antibodies. The level was shown to increase with age, which may be a consequence of a continuously and more diverse production of antibodies towards ingested antigens with age.

Early presentation of BioProtein was also shown to reduce the immunological response. Rats receiving BioProtein from 3 weeks of age responded with a significantly lower BioProtein antibody titre than animals receiving BioProtein from 6 weeks of age.

In BioProtein fed offspring of parents fed BioProtein no significant level of antibodies was found. Also, following immunisation of offspring, rats fed BioProtein had only slightly increased BioProtein antibody titres. On the other hand, a very high response was found in BioProtein immunised Control rats.

The antibody response to Brewer's Yeast in the diet seemed to follow the same trend as that observed for BioProtein. However, with Brewer's Yeast a more pronounced antibody response was observed. .

The antibody studies and the increase in mesenteric lymph node weights, together with the low-grade macrophage accumulation seen in some rats, are indicative of an immune response to a dietary antigen. Furthermore, the response is diminished by parental exposure or early introduction into the diet and the effects are also reversible, indicating the absence of any toxic effect.

The animals studied *post mortem* in the pig and broiler chicken trials showed no evidence of immune responses as seen in the rat, including absence of changes to lymph tissue associated with the intestines.

The effects on the immune system seen in these studies with commercially produced BioProtein were far less than in the previous study at the Danish Veterinary and Food Administration using a specially prepared sample of experimental material with a reduced nucleic acid content. It is also interesting that in earlier 8 weeks rat study, Brewer's Yeast produced effects comparable to the experimental sample, whereas the effects of Brewer's Yeast seen in the recent Single Generation Study were much less and only evident in the parents. Also, the sexes have not exhibited entirely consistent effects between the various past and present studies.

1.3 Conclusions

BioProtein is further confirmed to give very good performance in target animals, i.e. weaned piglets, fattening pigs, broiler chickens and farmed Atlantic salmon. There were no adverse health effects in these species as confirmed by pathological assessment.

The recent rat studies demonstrated that BioProtein supports normal reproductive performance and normal growth and development in offspring or juvenile rats where BioProtein was introduced into the diet at or soon after weaning. Increased weight of mesenteric lymph nodes, accompanied by low-grade macrophage accumulation in some animals, is believed to be a result of an immune response to an unfamiliar ingredient. The effect was shown to be reversible after withdrawal of the BioProtein diet

The laboratory rat appears to be particularly sensitive in its immune responses and may not be an appropriate model when assessing the suitability of BioProtein for farm animals. No such responses were seen in the target farm animals.

1.4 Proposal for inclusion.

In this Dossier, Norferm AS presents further documentation supporting the safety of the BioProtein product pending application according to Council Directive 82/47/EEC concerning certain products used in animal nutrition. The pending application for extended authorization has the following proposal for inclusion;

PROPOSAL FOR INCLUSION INTO THE ANNEXES OF DIRECTIVE 82/471

1 Name of product group	2 Name of product	3 Designation of nutritive principle or identity of micro-organism	4 Culture substrat (specifications if any)	5 Composition characteristics of product	6 Animal species	7 Special provisions
1.1.2 Bacteria cultivated on natural gas	1.1.2.1 Protein pro-duct of fermentation from natural gas ob-tained by culture of: Methy-lococcus capsulatus (Bath), DB3 (Alcaligenes acido-vorans), et DB5 (Bacillus firmus) and DB4	Methylococcus capsulatus (Bath) NCIMB strain 11132 Alcaligenes acido-vorans NCIMB strain 12387 (13287 = Ralstonia sp.) Bacillus firmus NCIMB strain 13280 (= Brevibacillus agri 13289) (Bacillus brevis NCIMB strain 13288 = Aneurini bacillus sp.)	Natural gas: (approx. 91% methane, 5% ethane, 2% propane, 0.5% isobutane, 0.5% n-butane, 1% other compo-nents), ammonia, mineral salts	Crude protein: min. 65%	- Pigs from piglets to slaugther - Calves from 80 kg on - Salmon - Broilers - Cats - Dogs	Declarations to be made on the label or the packaging of the product: the name: **BioProtein®** - crude protein - crude ash - crude fat - moisture content - instructions for use - maximum incorporation rate in the feed: - 15% cats - 12% dogs - 6% poultry - 8% pigs for fattening - 8% calves - 19% salmon (freshwater) - 33% salmon (seawater) - declaration of the words: “avoid inhalation” Declarations to be made on the label or packaging of the compound feedingstuffs: - The name: “Protein product obtained by bacterial fermentation of natural gas” - amount of the product contained in the feedingstuffs

2 INTRODUCTION

BioProtein is an animal feed ingredient produced from methane by continuous fermentation with methanotropic bacteria. The commercial product consists of the heat-inactivated, dried, fermentation biomass containing its native level of nucleic acids.

BioProtein is has been approved since 1995 for use in the feed of the following animal types:

- Up to 8% in the feed of pigs from 25kg to 100kg live weight;
- Up to 8% in feed for veal calves from 80kg live weight;
- Up to 19% in feed for salmon raised in fresh water;
- Up to 33% in feed for salmon raised in seawater.

Following a request to extend the uses of BioProtein in animal feed, the scientific Committee on Animal Nutrition (SCAN) issued a favourable opinion for additional uses of:

- Up to 8% in the feed of piglets and fattening pigs to slaughter weight;
- Up to 6% for broiler chickens.

Later on, an application for extended approval of BioProtein was submitted to the Commision also to include pets at inclusion levels of 15% for cats and 12% for dogs.

These recommendations were not implemented due to the result of a study in rats using a sample of non-commercial, nucleic acid- reduced material produced on a single occasion for research purposes. This has been the subject of further investigation since that time.

BioProtein is being used very successfully in the Scandinavian salmon farming industry, with very good results in terms of growth and product quality. The restricted weight range for which the inclusion of BioProtein in the feed is permitted has limited use for pigs, and current economics of veal calf production do not favour the use of BioProtein.

In the light of developments over recent years, notably concerns over the use of bovine, ovine and caprine protein ingredients, issues associated with genetically modified crops and a shortage of fishmeal, BioProtein offers an alternative and much-needed source of protein. Furthermore, the BioProtein technology seems rather attractive for gas producing developing countries with limited domestic production of feed proteins.

The purpose of this submission is to provide the Commission with new information to support the pending application for the extended safe use of BioProtein in the target species, i.e. farm animals, pets and farmed salmon. Also, new data are provided from studies in the laboratory rat aimed at understanding the effects first seen in the nucleic acid-reduced material.

3 BACKGROUND

Following the approval in 1995 for BioProtein to be used in the feed of the specified farm animals, 7000 tonnes of BioProtein have been consumed in feeding stuffs in two countries. A new large-scale plant, capable of producing 10,000te/yr, has been built at a cost of 50 million Euros and came on stream in 2001.

From studies leading to the commercial approval in 1995, from subsequent studies and from market feedback, there is nothing to suggest that BioProtein has any adverse effect on the health, well being and performance of farm animals and farmed salmon that consume it.

The sample provided to the Danish Veterinary and Food Administration (DVFA) for research purposes was, unlike commercial BioProtein, a sample of fermentation biomass that had been further processed to reduce its nucleic acid (RNA and DNA) content. The process for reducing the nucleic acid content was undertaken on a laboratory scale and included the following processing steps:

- The cells were subjected to heat shock at 90 degrees C in order to activate endogenous RNases/DNases as well as for inactivation of proteases.
- Iron was added at a concentration of 10x the content of commercial BioProtein to stimulate RNase/DNase activity.
- Cells were incubated at 60 degrees C to allow degradation of nucleic acids
- Degraded nucleic acids and other solubles were removed by centrifugation.

The material used for the study at the DVFA was based on a mixture of material that had already been spray dried plus a proportion of fresh biomass from the pilot fermenter.

Chemical analysis in comparison with commercially produced BioProtein showed no unexpected changes in proximate analysis. There were, however, significant increases in the levels of iron (10x) and copper (2.5x). (Detailed results are provided in Annex 1.)

In the 90 days study conducted at the DVFA the animals grew well and showed no adverse clinical signs. There were, however, increases in the size (weight) of mesenteric lymph nodes, changes in white blood cells and histological changes in the lymph nodes and some other organs *(*Mølk *et al,* 2002. A copy is provided as Annex 2).

A separate study in mice (Christensen *et al*, 2003.A copy is provided as Annex 3) investigated immune responses to the nucleic acid reduced-material and to BioProtein. Both samples gave similar antibody responses, but the authors suggested that particle size could influence the immune response.

Comparative analysis of nucleic acid reduced material and commercial BioProtein, following *in vitro* digestion by stomach proteases, have shown that the particle size of commercial BioProtein is larger than the experimental material.(Annex 1). In the same study it was found that Brewers Yeast had a particle size similar to the nucleic acid reduced material.

The results obtained in the RNA-reduced sample prompted investigations in the rat to identify whether similar immunological responses were to be seen with BioProtein itself.

These studies included: 4 week and 8 week rat studies; an 8 week rat study on Brewer's Yeast; a single generation study in the rat; a 4 month study in blue foxes: a 4 month study in pigs (25 to 110kg); an 8 week tolerance study in cats. These studies were reported to the Commission in earlier submissions.

Brewer's Yeast was included as a reference point for protein sources common in farm animal nutrition, but which normally the laboratory rat would not consume.

In all these studies, BioProtein supported normal growth and development with no adverse effects on reproduction in the rat.

The pig study included pathology assessment and no adverse effects were seen.

In the rat studies the weights of mesenteric lymph nodes were often increased, but to a lesser degree than by the nucleic acid-reduced experimental sample. The exception to this was the female offspring retained to 12 weeks of age in the single generation study, where no increase occurred. Cats and blue foxes showed slight increases in lymph node weights at the highest inclusion levels (12% and 20%).

An independent review (previously submitted) of the pathology from all these studies concluded that histological changes, such as macrophage accumulation in the lymph nodes, were at a low level compared to the changes seen in the DVFA study with nucleic acid-reduced material. However, Brewer's Yeast produced lymph node enlargement and histology comparable to that of the nucleic acid-reduced material.

In order to further support the safe commercial use of BioProtein, new investigations have been undertaken in pigs, broiler chickens and farmed salmon. These have included blood analysis and examination of tissues involved in the immune system. The results of these are given in Section 4.1 and are presented to support the extension of the use of BioProtein in farm animals as proposed previously.

At the same time, two new studies have been completed with the aim of gaining further understanding of the immunological response in the rat. The results are given in Section 4.2.

4 NEW STUDIES

4.1 Studies in farm animals

4.1.1 Introduction

Additional studies documenting the efficacy and safety of BioProtein for the target species: weaned piglets, pigs for fattening, broiler chickens and Atlantic salmon have been completed.

- Study 1. BioProtein in diets for piglets
- Study 2. Effect of BioProtein on growth performance of fattening pigs
- Study 3. Field trial with in Norway in co-operation with Felleskjopet Fôrutvikling. BioProtein as a feed ingredient for fattening pigs.
- Study 4. Broiler chicken trial with BioProtein
- Study 5. BioProtein as a protein source in diets for Atlantic salmon (Salmo salar)

4.1.2 Efficacy trials with BioProtein on target species

The target animal categories are weaned piglets, pigs for fattening, broiler chickens and Atlantic salmon. The recommended inclusion rate is 8% for weaned piglets, 8% for pigs for fattening, 6% for broiler chickens and 33% for Atlantic salmon in seawater.

Individual efficacy studies are presented in section 4.1. Table 4.1.1 summaries experimental studies concerning the efficacy of BioProtein in target species.

Table 4.1.1. Overall results from efficacy studies with BioProtein on target species: weaned piglet, pigs for fattening, broiler chickens, and Atlantic salmon

Commented [A1]: Should other efficacy results be included too?

Study	Species	Number of individuals	Level, g/kg diet	% change vs. control		
				Feed intake	Weight gain	Feed efficiency
Study 1	Piglets	100	90	+5.6	+11.4[a]	-5.1
Study 2	Pigs for fattening	64	60	+2.1	-1.2	+3.4
Study 2	Field trial with Pigs for fattening	ca. 1000	60	No change	Increased	-
Study 3	Broiler chickens	120	60	+2.4	+4.0[a]	-1.3[a]
Study 4	Atlantic salmon	270	40	+2.1	-0.7	+2.2
			90	+2.1	+8.0	-4.3
			180	+6.3	+15.2[a]	-5.8[a]
			360	+1.0	+13.0[a]	-10.8[a]

[a]Significant improvement compared with control ($P < 0.05$)

The studies with BioProtein in diets for target species on growth performance can be summarized as follows:

- Study 1 Demonstrates an improvement in weight gain of weaned piglets of BioProtein at the inclusion rate of 90 g per kg diet ($P<0.05$).
- Study 2 Demonstrates no significant changes in feed intake, growth rate or feed efficiency of fattening pigs fed diets with 60 g per kg BioProtein.
- Study 3 Showed improvement in weight gain, and no changes in feed intake of fattening pigs fed diets with 60 g per kg BioProtein as judges subjectively by the pig producer.
- Study 4 Demonstrates that 60 g per kg diet of BioProtein significantly improved weight gain and feed efficiency of broiler chickens ($P<0.05$).
- Study 5 Demonstrated an improvement in weight gain and feed efficiency of Atlantic salmon fed BioProtein at 180 and 360 g per kg of diet ($P<0.05$).

Table 4.1.2. Overall results on health observations from efficacy studies with BioProtein on target species: weaned piglet, pigs for fattening, broiler chickens, and Atlantic salmon

Commented [A2]: Should other efficacy results be included too?

Study	Species	Main parameters	Health observations
Study 1	Weaned piglets	Growth Clinical health Autopsy Pathology	Improved weight gain, No changes in feed intake, No negative effect on clinical health. No effects seen.
Study 2	Pigs for fattening	Growth Clinical health Autopsy Pathology	No changes in weight gain, No changes in feed intake, No negative effect on clinical health No effects seen.
Study 3	Field trial, pigs for fattening	Growth Clinical health Subjective observation by producer	Improved weight gain, No changes in feed intake, Tendency to increased diarrhoea during early stages of fattening period in 2 out of 9 farms
Study 4	Broiler chickens	Growth Litter quality Clinical health Autopsy Pathology	Improved weight gain, Improved feed intake, No adverse effect on litter quality No negative effect on clinical health No effects seen.
Study 5	Atlantic salmon	Growth Clinical health	Improved weight gain, Improved feed intake, No negative effect on clinical health

Adding BioProtein to diets for target species demonstrated no adverse effect on weight gain or feed intake or clinical health of the animals. In the field trial, the slight increase in the incidence of diarrhoea was concluded not to be related to BioProtein. No adverse effect on litter quality of broiler chickens was observed.

Based on clinical observation and absence of adverse pathology, it is concluded that the use of BioProtein as a protein source for the target species, weaned piglets, pigs for fattening, broiler chickens and Atlantic salmon at levels ranging from 60 to 90 g per kg

in diets for pigs, 60 g per kg in diets for broiler chickens, and 40 to 360 g per kg of diet for farmed fish, is safe for the target animals.

4.1.3 BioProtein in diets for piglets (Annex 4)

Materials and Methods

A trial was conducted at Tomb Jordbruksskole in Norway to investigate the effect of BioProtein on growth performance of piglets. From A total of 100 piglets (12.2 kg initial weight) 50 pigs were assigned to each of two treatments on the basis of initial weight and sex. The dietary treatments consisted of a conventional fishmeal based control diet and one test diet containing 90 g/kg BioProtein. Pigs had free access to feed and water. The piglets were observed daily for any clinical sign of toxicity, ill health or other abnormalities. Feed consumption and weight gain was measured throughout the trial. At the termination of the trial a gross and histopathological examination was performed at the pathology laboratory at the Norwegian School of Veterinary Science, Oslo, Norway on six piglets per treatment. Tissue samples were taken from liver, kidney, spleen, thymus, and ileum.

Results and discussion

Health
All animals were in normal health prior to the study. One pig died on the control diet and one pig on the BioProtein diet was omitted from the trial due to a very poor growth rate. No incidence of diarrhoea was observed. Overall, the piglets were kept under highly hygienic conditions and no clinical health problems related to any specific dietary treatment have been encountered during the experimental period. Offering diets *ad libitum* to piglets throughout this experiment reflects on their relatively fast daily growth rates of 527.5 g on average at their feed conversion ratio (FCR) of 1.53 on average. These growth performance characteristics should be regarded as very satisfactory. Also, this implies that the use of BioProtein as a protein source for weaned piglets had no detrimental effect on their clinical health.

Growth performance
The effects of BioProtein on body weight changes, growth rates, feed intake, and FCR are presented in Table 4.1.3

During week 1, the weight gain was similar between the control and the BioProtein diet. During week 2 and 3, the weight gain was numerically higher for the pigs receiving BioProtein. Overall, the weight gain of piglets receiving the BioProtein diet increased by 53 g per day compared with the control pigs. This resulted in a significant increase in total weight gain by 11.4% (1.19 kg) compared with the control during the overall period. There were no significant differences in feed intake between piglets receiving the control and the BioProtein diet. Feed intake of piglets receiving the BioProtein diet was, however, 5.5% higher than the control piglets. The FCR during week 1, week 2, and week 3 was lower for the dietary treatment containing BioProtein. Overall, the improvement in FCR with BioProtein was 5.1%.

Table 4.1.3. Effect of BioProtein on growth performance of pigs

Diets	Control	BioProtein
Number of pigs	49	49
Initial weight, kg	12.2	12.1
Final weight, kg	22.8	23.8
Average daily gain, g/day, d 41-47	333	308
Average daily gain, g/day, d 48-54	491	582
Average daily gain, g/day, d 55-62	679	773
Average daily gain, g/day, d 41-62	501	554
Total weight gain, kg	10.43 [b]	11.62 [a]
Average daily feed intake, g/day, 41-54 d	657	675
Average daily feed intake, g/day, 55-62 d	1031	1128
Average daily feed intake, g/day, 41-62 d	782	826
Total feed intake, kg	16.4	17.3
Feed conversion ratio, 41-54 d	1.60	1.52
Feed conversion ratio, 55-62 d	1.52	1.46
Feed conversion ratio, 41-62 d	1.57	1.49

These results suggest that adding up to 90 g/kg BioProtein to diets for weaned piglets had a positive effect on growth performance. The increase in weight gain was a result of both increased feed intake as well as an improved feed efficiency of diets containing the BioProtein. These results demonstrate that BioProtein is a suitable protein source in diets for weaned piglets.

Pathology
The results from gross and histopathological examination showed that there were no significant differences between the control and the BioProtein group for any of the organs examined. (Annex 5)

Conclusions

The results showed that replacing fishmeal with 90 g/kg BioProtein significantly improved weight gain, and numerically improved feed efficiency and feed intake of weaned piglets.

Addition of 90 g/kg BioProtein in the diet gave at least as good growth performance as the control diet. This together with the clinical health observations and pathology findings indicate there is no adverse effect on the health of weaned piglets administered BioProtein at 90 g/kg in the diet.

4.1.4 Effect of BioProtein on growth performance of fattening pigs (Annex 6)

Materials and methods

A growth performance study was carried out to investigate the effects of BioProtein on growth performance of fattening pigs. The study was carried out at the Norwegian

cooperative experimental station, Rogaland, Norway in the period from October 2003 until December 2003. The objectives were to evaluate 1) the effect of adding 60 g/kg BioProtein as a protein feedstuff to fattening pig diets on growth performance and carcass traits and 2) fatty acid composition of fat and meat of fattening pigs.

The study was carried out with 64 fattening pigs (38.7 kg and 112.9 kg initial and final weight) from 10 litters. Pigs were obtained from a commercial farm. The experimental period lasted during the growing period and the fattening period until pigs received commercial slaughter weights. Pigs were allotted on the basis of initial weight, sex and litter to each of two dietary treatments with four replicate pens and 32 pigs per treatment. The treatments consisted of a soybean and canola meal based control diet and one-test diets containing 60 g/kg BioProtein. Feed and water was provided ad libitum.

Weight gain and feed consumption was measured and health status of the animals was continually assessed. All pigs were slaughtered at the same day at a commercial slaughterhouse. Samples from subcutaneous fat of the *longissmus dorsi* muscle were taken from each carcass after one day after slaughter for determination of fatty acid composition and iodine value. At the termination of the trial a gross and histopathological examination was performed at the pathology laboratory at the Norwegian School of Veterinary Science, Oslo, Norway on 15 pigs per treatment. Tissue samples were taken from liver, kidney, spleen, thymus, ileum, and lymph nodes in the distal jejunum. Blood samples were taken to determine the effect of BioProtein on liver and kidney function at the beginning of the trial, in the middle, and the day before the autopsy.

Results and discussion

Health
All animals were in normal health prior to the study. One pig died on the BioProtein diet. Acute heart failure, moderate constipation in the colon and mycoplasma pneumonia was revealed at necropsy. No incidence of diarrhoea was observed. No clinical health problems related to any specific dietary treatment have been encountered during the experimental period. The mean body weight at the end of the trial of 112.9 kg and mean body weight gain of 1045 g per day in average for fattening pigs in both treatment groups can be seen to increase at an acceptable level for the duration of the study. There is no discernible effect of treatment on body weight data. This indicates that the use of BioProtein as a protein source for fattening pigs had no detrimental effect on their clinical health.

Growth performance and carcass traits
There were no significant differences in daily weight gain, feed intake or FCR between the pigs fed the control diets and those fed the diet containing 60 g/kg BioProtein (Table 4.1.4). The lack of response of BioProtein on growth performance was probably a result of the similar content of essential amino acids between the two diets.

Table 4.1.4. Effect of BioProtein on growth performance

Diets	Control	BioProtein	P-values
Number of pigs	32	32	
Initial weight, kg	39.2	38.2	
Final weight, kg	113.8	111.9	
Days to market	71	71	
Daily weight gain, g	1051	1038	NS
Feed conversion ratio, kg/kg	2.91	3.03	NS
Feed intake, kg	194	198	
Slaughter weight	80.6	78.1	
Dressing percentage	70.8	69.8	0.08
Carcass lean percentage	56.7	56.2	NS

There were no significant differences between treatments for carcass lean percentage, but the control pigs had slightly higher carcass lean percentage than the BioProtein pigs. There was a tendency ($P < 0.08$) towards a lower dressing percentage for the pigs receiving the BioProtein diet.

Fatty acid composition of fat

The results from the fatty acid analyses of carcass fat showed that the iodine value was numerically lower in the carcass fat of the pigs fed the diet containing BioProtein compared with the control pigs (77.8 vs. 76.8) (Table 4.1.5). A low iodine value indicates a lower content of unsaturated fatty acid. Also, the content of unsaturated fatty acids in the carcass fat was numerically lower for the control pigs compared with those fed BioProtein (30.5 vs. 31.1%). The reduction in iodine value and decrease in content of unsaturated fatty acids in the carcass fat with BioProtein was probably a result of the increase in saturated fatty acid in the diet by exchanging soybean meal by BioProtein. Reduced content of unsaturated fatty acids and iodine value of carcass fat indicate that BioProtein has a positive effect on product quality.

Table 4.1.5. Effect of BioProtein on fatty acid composition of carcass fat

Diets	Control	BioProtein	P-value
Iodine value in fat	77.8	76.8	NS
Saturated fatty acids	30.5	31.1	-
Monounsaturated fatty acids	51.8	51.7	-
Polyunsaturated fatty acids	17.6	17.1	-
Sum C22:5/C22:6	0.25	0.26	-

Pathology

The results from gross and histopathological examination showed that there were no significant differences between the control and the BioProtein group for any of the organs examined. (Annex 7). Analysis of blood samples showed that there were no significant differences between the control pigs and those receiving BioProtein for aspartate aminotransferase, gammaglutamyl transferase or alkaline phosphatase, blood urea or creatinine (Table 4.1.6). The level of glutamate dehydrogenase was significantly lower for pigs receiving BioProtein in the terminal samples. The differences were small

and are not believed to be of clinical relevance. The results suggest that there was no adverse effect of BioProtein on liver and kidney function.

Table 4.1.6. Effect of BioProtein on blood enzymes of weaned pigs

Diets	Control	(±SD)	BioProtein	(±SD)	P-value
Aspartate					
Aminotransferase					
Initial	66.65	(33.76)	56.05	(14.85)	NS
Middle	45,06	(11.52)	39.55	(9.34)	NS
Terminal	43.7	(16.48)	36.45	(6.62)	NS
Average	52.03	(25.0)	44.01	(13.73)	NS
γ-glutamyltransferase					
Initial	76.8	(32.71)	84.95	(28.81)	NS
Middle	92.5	(47.96)	92.3	(51.09)	NS
Terminal	66.65	(20.53)	65.25	(19.05)	NS
Average	78.17	(35.99)	80.83	(36.84)	NS
Glutamic dehydrogenase					
Initial, 13.10.2003	2.45	1.73	2	0.46	NS
Middle,17.11.2003	1.5	0.51	1.35	0.59	NS
Terminal, 15.12.2003	2.2	0.41	1.75	0.44	0.003
Average	2.07	1.14	1.7	0.56	NS
Alkaline Phosphatase					
Initial	481.6	(92.0)	464.9	(98.1)	NS
Middle	396.9	(87.5)	389.5	(71.8)	NS
Terminal	317.8	(59.9)	319.7	(48.6)	NS
Average	398.8	(104.9)	391.3	(95.4)	NS
Creatinine					
Initial	107.3	(12.6)	105.8	(9.2)	NS
Middle	122.1	(13.4)	120.8	(10.4)	NS
Terminal	129.9	(13.8)	130	(12.0)	NS
Average	119.7	(16.2)	118.9	(14.5)	NS
Urea					
Initial	3.95	(0.959	3.81	(0.58)	NS
Middle	4.72	(0.97)	4.62	(1.41)	NS
Terminal	5.59	(1.03)	5.52	(1.01)	NS
Average	4.75	(1.19)	4.65	(1.26)	NS

Conclusions

The results from this trial shows that the use of 60 g/kg BioProtein in diets for fattening pigs have no adverse effect on growth performance or carcass quality.

Adding BioProtein to diets for fattening pigs appeared to decrease the iodine value and level of unsaturated fatty acids in carcass fat.

There was no adverse effect of BioProtein on liver and kidney function.

The weight gain, feed intake, animal observations, and necropsy findings all indicate there is no negative effect on the health of fattening pigs administered BioProtein at 60 g/kg in the diet.

4.1.5 Field trial within Norway in co-operation with Felleskjøpet Fôrutvikling. BioProtein as a feed ingredient for fatting pigs (Annex 8)

Materials and methods

A field trial was carried out to study the effect of BioProtein under practical farm conditions. A total of 15 commercial farms and approximately 1000 fattening pigs were included in the trial. The test period lasted from November 2003 until February 2004. The test diet contained 60 g/kg BioProtein and was identical to the BioProtein test diet used in study 2A. A total of 740 metric tones of the test diet were used for the field trial. All farms were asked to fill out a questionnaire regarding growth performance, health status and pellet quality at the end of the test period. Most farms had pigs with good health status, but one farm reported good to medium health status. Most farms reported good environmental conditions in the pig house, but three farms reported medium environmental conditions.

Results and discussion

Slaughtering
All pigs were slaughtered at a commercial slaughterhouse. Comments from the veterinary control agency indicate that all pigs were in normal health. The results from the questionnaire are presented in Table 4.1.7

Table 4.1.7. Results from the questionnaire, number of answers for each of the three options from the different questions

	Changes in a positive direction	No changes	Changes in a negative direction
Feed intake	0	9	0
Weight gain	4	5	0
Sickness/mortality	2	7	0
Diarrhoea	0	7	2[1]
Pellet quality	1	8	0

1 Both producers whom reported increased incidence of diarrhoea remarked that this only occurred in the early stages of the fattening period, and that this was temporary.

Out of the nine producers that had filled out the questionnaire, none have reported changes in feed intake of the pigs. Four producers reported increase in weight gain of the pigs, and two producers reported a reduction in mortality rates and incidence of sickness. Two producers reported an increase in the incidence of diarrhoea during the early stages of the growing-finishing period. One of these cases, this may have been associated with the lower health status and environmental conditions reported at the farm. The increased incidence of diarrhoea did not, however, adversely affect growth performance of these pigs during the testing period. With respect to pellet quality, one producer reported improvement in pellet quality, while eight producers reported no

changes in pellet quality. Two producers reported a slight reduction in carcass lean meat percentage during the test period, while the remaining farms reported no changes in lean meat percentage.

Conclusion

The results from the field trial suggested that the addition of 60 g/kg BioProtein in diets for fattening pigs did not have any adverse effect on growth performance of the pigs. Rather, it appeared that the dietary inclusion of BioProtein had a positive effect on growth performance. Two out of nine producers reported a slight decrease in carcass lean meal percentage after they started to use the BioProtein diet.

The only adverse clinical health observation reported during the three-month testing period was a tendency for an increased incidence of diarrhoea at two farms during the early phase of the fattening period. This is not believed to be caused by BioProtein and it did not adversely affect growth performance of these pigs.

4.1.6 Broiler chicken trail with BioProtein (Annex 9).

Materials and methods

A growth trial was carried out with broiler chickens at the Experimental farm at the Agricultural University of Norway, Ås, Norway, during the period from February until April 2004. The main objective of the study was to determine the effect of BioProtein in diets for broiler chicks on: 1) weight gain, feed intake, feed efficiency and dressing percentage and 2) clinical health and target species safety. The trial was carried out with 120 day-old Ross 208 broiler chicks. On receipt, the birds were subjected to a health inspection and randomly allocated into two dietary treatment groups. There were two experimental diets: a control diet and a diet with reduced soybean meal supplied with 60 g/kg BioProtein. Feed and water were provided ad libitum.

Weight gain and feed consumption was registered per pen throughout the trial. Twelve birds per treatment were used for determination of slaughter weight and dressing percentage. Litter condition per pen was judged at 21 days and at 35 days of age using a score from 1 to 5, with 1 for dry and porous litter and 5 for wet and caking litter.

The birds were observed at least twice daily for any clinical sign of toxicity, ill health or other abnormalities. Any deviation from normal was recorded. Dead birds were autopsied at the pathology laboratory at the Norwegian School of Veterinary Science, Oslo, Norway.

At the termination of the trial a gross and histopathological examination was performed at the pathology laboratory at the Norwegian School of Veterinary Science, Oslo, Norway on 12 birds per treatment.
Tissue samples were taken from liver, kidney, heart, proximal small intestine, spleen, and bursa fabricius.

Results and discussion

Health
All birds were in normal health prior to the study. Three birds died on the control diet and three birds died on the BioProtein diet (Necropsy report, Annex 10). The mortality rates were 5%, which are considered to be typical. No clinical health problems related to any specific dietary treatment have been encountered during the experimental period. The birds had normal growth rates and feed intakes throughout the study period.

Growth performance
The effects of BioProtein on body weight changes, growth rates, feed intake, and FCR are presented in Table 4.1.8.

Table 4.1.8 Effect of BioProtein on growth performance, dressing percentage and litter quality of broiler chickens[1].

	Level of BioProtein (g/kg)		SEM [2]	P-value
	0	60		
No. of chickens	60	60		
No. of deaths	3	3	-	-
Individual initial weight, g	42.6	42.2	-	-
Individual final weight, g	2136	2219	-	-
Mortality rate, %	5	5	-	-
Weight gain, d 0-14	354[a]	389[b]	7.6	0.02
Feed intake, d 0-14	492	517	9.3	0.11
Feed to gain, d 0-14	1.39	1.33	0.04	NS
Weight gain, d 14-36	1739	1788	17.2	0.10
Feed intake, d 14-36	2817	2869	42	NS
Feed conversion ratio, d 14-36	1.62	1.60	0.01	NS
Weight gain, 0-36 d, g	2094[a]	2177[b]	23.7	0.05
Feed intake 0-36 d, g	3308	3386	41.3	NS
Feed conversion ratio, d 0-36	1.58[a]	1.56[b]	0.006	0.03
Dressing percentage, n= 12, %	63.1	62.4	0.06	NS
Litter quality score, d 0 -36[3]	1.75[b]	2.38[ab]	-	NS
Litter quality score, d 0 – 21[3]	1.00	1.50	-	NS

1 Growth performance data are expressed as pen average.

2 SEM = standard error of the mean.

3 Litter quality; score 1 –5, where 1 is dry and porous litter and 5 is wet and caking litter.

At day 36, individual body weights of chicken fed the BioProtein diet were 83 g higher than for the control birds. Average weight gain per pen from 0 to 14 days was significantly ($P < 0.02$) higher for the chicken fed the BioProtein diet compared to the

control chickens. Although not significant, feed intake of the chickens fed the BioProtein diet was also higher (P = 0.11) compared to the control chickens. There were, however, no significant differences in FCR between treatments during this period.

During the period from day 14 to day 36, weight gain tended (P < 0.10) to be higher for the chickens fed the BioProtein diet, but there were no significant differences between treatments for feed intake or FCR during this period. During the period from day 0 until day 36, chickens fed the BioProtein diet had a significantly (P < 0.05) higher weight gain compared with the control chickens. There were no significant differences in feed intake between the chickens receiving the control and the BioProtein diet during this period, but the birds receiving the 60 g/kg BioProtein diet had better (P < 0.03) FCR compared with the control birds.

The weight gain and feed intake indicate there is no negative effect on the health of the chickens administered BioProtein up to 60 g/kg in the diet.

There were no differences in dressing percentage among treatments. Furthermore, there were no significant differences in litter quality of birds receiving the control or the 60 g/kg BioProtein diet at d 0 – 36 or at d 0-21.

Pathology
The results from gross and histopathological examination showed that there were no significant differences between the control and the BioProtein group for any of the organs examined (Annex 11).

Conclusion

Replacing soybean meal with 60 g/kg BioProtein improved growth rate and feed conversion compared to a conventional soybean meal-based diet. BioProtein also had a positive effect on feed intake of the broiler chickens, especially during the first 14 days of the experiment. Thus, BioProtein could be used as a major protein source in diets for broiler chicks.

The weight gain, feed intake, and clinical health observations and necropsy findings indicate there is no negative effect on the health of broiler chicks given diets containing 60 g/kg BioProtein.

4.1.7 Bacterial protein as a protein source in diets for Atlantic salmon (Salmo Salar) (Annex 12).

Materials and Methods

A growth trial with Atlantic salmon was carried out at Akvaforsk, Institute of Aquaculture research, Sunndalsøra, Norway during 2003 until 2004. The aim of this trial was to investigate BioProtein as a protein source for Atlantic salmon, with the main focus on growth, feed utilization, energy budget and nitrogen metabolism. There were a total of five dietary treatments. The dietary treatments consisted of a fishmeal based control diet and four test diets containing 45, 90, 180, and 360 g BioProtein g/kg. A total of 270 Atlantic salmon (*Salmo salar*) with 170 g initial weight were used in the trial. Each of the five diets was fed to three replicate groups of 18 fish per group. The trial

lasted nine weeks. Weight gain and feed intake was registered throughout the trial. Urea levels in plasma and liver were analysed in fish from the 0, 180, and 360 g/kg BioProtein group, heat increment was measured during the last week.

Results and discussion

Health
There was no mortality in either treatment group. No clinical health problems related to the dietary treatment have been encountered during the experimental period. Some incidences of cataracts were observed, but there were no differences in occurrence of this condition between the treatments groups. The growth rate and feed intake is considered to be good for fish this size at the present facilities.

Growth performance
There tended (P=0.051) to be a difference in final weight among the treatments (Table 4.1.9). Final weights were highest for the fish receiving diets containing 180 and 350 g/kg BioProtein. The specific growth rates were significantly higher for the 180 and 360 g/kg BioProtein group compared to the control and the 45 g/kg BioProtein group. The addition of 90 g/kg BioProtein gave an intermediate response in specific growth rates.

There were no significant differences in feed intake of the fish, but the feed efficiency ratio was significantly higher for the 360 g/kg BioProtein group than the 0 and 45 g/kg group. The feed efficiency of fish receiving intermediate levels of BioProtein of 90 and 180 g/kg was intermediate. The weight gain and feed intake indicate there is no negative effect on the health of the fish administered BioProtein up to 360 g/kg in the diet.

Table 4.1.9. Effect of BioProtein on growth performance of Atlantic Salmon

	Level of BioProtein, g/kg				
Treatment	0	45	90	180	360
Final weight, g	330	327	345	364	360
Specific growth rate, SGR	1.38^{b}	1.37^{b}	1.49^{ab}	1.59^{a}	1.56^{a}
Intake, % BW day	0.96	0.98	0.98	1.02	0.97
Gain: feed	1.39^{bc}	1.36^{c}	1.45^{abc}	1.47^{ab}	1.54^{a}

Nitrogen and energy retention
N-retention was significantly higher in the 90, 180, and 360 g/kg BioProtein group compared to the control and the 45 g/kg BioProtein group (Table 4.1.10). The group receiving the highest level of 360 g/kg BioProtein had the highest N-retention. This shows that the inclusion of BioProtein in the diets has a N-sparing effect on the fish. There were also significant differences among diets for energy retention. The fish receiving 180 and 360 g/kg BioProtein had significantly higher energy retention than the fish receiving the control and the 45 g/kg BioProtein diet.

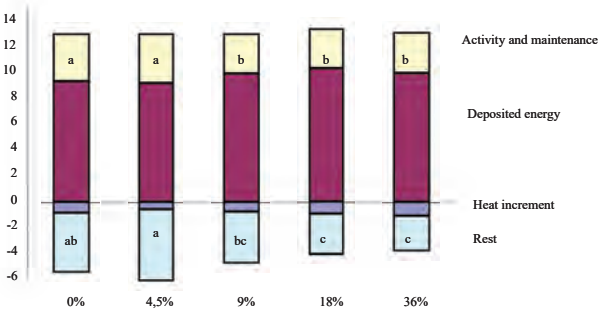
Table 4.1.10. Effect of BioProtein on retention of nitrogen and energy of Atlantic Salmon, whole body retention

	Level of BioProtein, g/kg				
Treatment	0	45	90	180	360
Nitrogen retention	46.2[b]	45.5[b]	52.7[a]	52.7[a]	55.1[a]
Energy retention	50.5[bc]	48.3[c]	55.8[ab]	58.8[a]	58.6[a]

Energy utilization

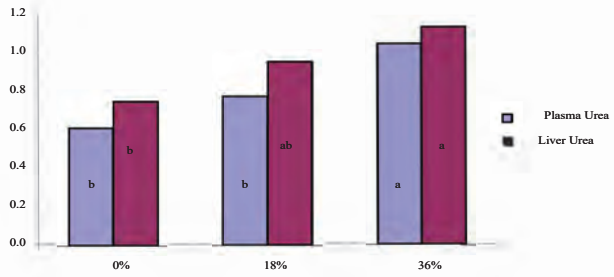
The results on energy utilization are shown in Figure 4.1.11. There were no significant differences in deposited energy per kg gain or in heat increment among treatments. The rest fraction, which is energy in urea, ammonia, faeces and the cumulative error, is significantly lower in the groups receiving the highest levels of 180 and 360 g/kg BioProtein. Percentage energy retention was highest for the 180 and 360 g/kg BioProtein groups. The groups receiving 0 and 45 g/kg BioProtein used more energy for activity and maintenance than the other groups. The results suggest that these fish utilized the energy more efficiently; less is used for maintenance and activity, thus their growth rates and feed efficiency ratio are improved.

Figure 4.1.11. Effect of BioProtein on energy utilization of Atlantic salmon, MJ per kg gain



The level of urea in plasma and liver increased with increasing levels of BioProtein in the diet (Figure 4.1.12). Plasma urea was significantly higher in the fish receiving the 360 g/kg BioProtein diet than those receiving the 0 and 180 g/kg BioProtein diet while the liver urea level was significantly higher in the fish receiving the 360 g/kg BioProtein diet than those receiving the 0 kg BioProtein diet. Since most of the N from amino acids metabolism in fish is excreted as ammonia, the increase in urea levels is probably due to the breakdown of the nucleic acids in the BioProtein. The higher feed utilization and improved growth rates in the groups receiving the highest levels of BioProtein indicate that the fish is able to handle the increased levels of the nucleic acids in the diets.

Figure 4.1.12. Effect of BioProtein on urea concentration in plasma (mmol/L) and liver (mmol/kg) of Atlantic salmon



Conclusion

Replacing fishmeal and wheat with increasing levels of lipoprotein in diets for Atlantic salmon weighing 170 g live weight resulted in:

Dose dependent improvement in specific growth rate and feed utilization.
Significant improvement in specific growth rate and feed utilization for fish receiving the 180 and 360 g/kg BioProtein diet.
Significant improvement in nitrogen and energy retention for fish receiving the 90, 180 and 360 g/kg BioProtein diet.
Significant increases in plasma and liver urea concentration with increasing levels of BioProtein which are believed to be related to the break down of nucleic acids.
No adverse effect on clinical health of Atlantic salmon given diets containing up to 360 g/kg BioProtein.

4.1.8 Discussion; studies on target animals

These new trials on BioProtein have confirmed its suitability for growing pigs (weaned piglets and fattening pigs), broiler chickens and Atlantic Salmon. It gave good growth and overall performance in all species and it was particularly impressive that increasing inclusion rates of BioProtein up to 36% (360g/kg) in salmon feed produced a linear dose/response in growth rate and feed utilisation.

Based on clinical observations in all trials and additional pathology investigations in pigs and chickens, BioProtein had no adverse effects on health.

These results add weight to previous successful feeding trials conducted with BioProtein in pigs at up to 150g/kg of feed (Øverland 2001, Annex 13), in broiler chickens at up to 60g/kg of feed (Skrede *et al* 2003, Annex 14) and up to 12g/kg in the feed of blue foxes (Skrede and Ahlstrøm, 2002, Annex 15).

4.2 Studies in rats

4.2.1 Introduction

Two new studies have been completed in rats:

A single generation study
A study in juvenile rats from weaning to 32 weeks of age.

In addition, antibody studies were undertaken on blood samples taken from rats in these two studies, including samples from immunised rats from the Single Generation Study.

In the single generation study BioProtein was tested at dietary inclusion levels of 6% and 12%. Diets containing the same levels of Brewer's Yeast were also included in the study in order to have a conventional source of protein of a similar nature to form an additional basis of comparison with BioProtein.

The study in juvenile rats used the 12% BioProtein diet, but it was fed for different periods of time in the various treatment groups.

BioProtein showed no effects on reproductive performance and did not affect growth and development of the offspring to 12 weeks of age. Similarly, growth and general well-being were unaffected in the Juveniles Study in rats fed BioProtein from weaning or 6weeks of age to either 12 or 32 weeks of age.

The main findings of interest were that parent animals in both the BioProtein and Brewer's Yeast groups showed a degree of enlargement of mesenteric lymph nodes, which was not unexpected in the light of results from previous studies. In the BioProtein groups this was accompanied by minimal to moderate accumulation of macrophages. In the offspring maintained to 12 weeks of age, only males had a significant increase in mesenteric lymph node weights, without any dose-response relationship. Minimal to slight macrophage accumulation was seen in the lymph nodes of only 10% of the BioProtein-fed rats.

Juvenile rats fed the 12% BioProtein diet from weaning or from 6 weeks of age to 12 weeks of age showed some lymph node enlargement, mostly in females. This was accompanied by minimal to slight macrophage accumulation in only 3 females in each of these groups, with none in males. Increased mesenteric lymph node weights remained evident in a group that continued to receive BioProtein to 32 weeks of age. The degree of macrophage accumulation was minimal to moderate. The group returned to the Control diet from 12 to 32 weeks of age showed some weight increase in males only, but without any accumulation of macrophages. This indicates that the small effect on mesenteric lymph nodes is reversible when animals are returned to the control diet.

Analysis of blood samples from rats fed that had received Control, BioProtein or Brewers Yeast (single generation) diets from the above studies was undertaken for IgG, IgG1, IgG2a and IgGA antibodies against BioProtein. Whereas parent animals receiving BioProtein had higher levels of antibodies than Control animals, offspring in the BioProtein groups showed no increase relative to the Controls. A similar pattern was

seen for rats fed Brewers Yeast although with a more pronounced response than BioProtein.

In the Juveniles Study, antibody levels were generally somewhat higher in the BioProtein groups than in the Controls, but introduction of BioProtein at weaning or returning to the control diet after 12 weeks of age, reduced the antibody response to BioProtein.

These data suggest that the increase in mesenteric lymph node weights and low-grade macrophage accumulation seen in some rats are indicative of an immune response to an unfamiliar ingredient. Furthermore, the response is diminished by parental exposure or early introduction into the diet and the effects are also reversible, indicating the absence of any toxic effect.

4.2.2 Single Generation Study

This study was conducted at Scantox, Hestehavevej 36A, Ejby, DK-4623 Lille Skensved, Denmark, in accordance with the OECD Guideline for testing of chemicals No 415 'One Generation Reproduction Toxicity Study' (adopted on 26 May 1983) and in compliance with the Principles of Good Laboratory Practice.

A copy of the study report is provided in Annex 16. (Scantox study number 5261: 'BioProtein and Brewer's Yeast- One generation reproduction toxicity study in rat')

The objective of the study was to obtain information concerning the effects of BioProtein on male and female reproductive performance, including gonadal function, oestrous cycle, mating behaviour, conception, pregnancy, parturition, lactation, weaning and on the growth and development of the selected offspring up to the age of 12 weeks. Brewer's Yeast was included as an additional comparator, representing a conventional ingredient of a similar nature to BioProtein.

4.2.2.1 Study design

One hundred and twenty male and 120 female Wistar rats of the strain BrlHan:WIST@Mol(GALAS), aged 5-6 weeks (males) or 8-9 weeks (females) at start of treatment were allocated to five groups of 24 animals/sex/group. The treatments consisted of five individually formulated and manufactured diets as follows:-

1. Casein Control
2. 6% BioProtein
3. 12% BioProtein
4. 6% Brewer's Yeast
5. 12% Brewer's Yeast

The diets were based on cereals, with Casein as the supplementary protein source in the control diet. BioProtein and Brewer's Yeast were introduced in place of appropriate proportions of Casein. Using information on the nutritional analysis of the ingredients, all diets were formulated to be equal in:

- Protein
- Methionine + cystine
- Fat
- Metabolisable energy
- Calcium
- Phosphorus
- Magnesium

Also, each diet was formulated to satisfy minimum requirements for essential fatty acids and other potentially limiting amino acids. The diets were fed *ad libitum.*

Males were treated for 10 weeks before mating, during the mating period and until necropsy. Females were treated for 2 weeks before mating and until weaning the offspring when they reached 21 days of age. On completion of mating the males were killed and subjected to gross pathological examination. At weaning, female parents and offspring not selected for extended treatment were killed and subjected to a gross pathological examination

From weaning, 20 male and 20 female offspring selected randomly from each group were maintained to 12 weeks of age on their respective diets.

For each group, time to mating, fertility, length of the gestation period and the gestation rate were calculated. The following parameters recorded between from birth to weaning were included:

1. Number and sex of pups, number of stillbirths and live births as soon as possible after delivery (Day 0-1).
2. Litter size including dead pups on the morning after birth (Day 0-1).
3. Number of male and female pups (Day 1).
4. Number of survivors on Day 1, 4, 7, 14 and 21.
5. Number of pups with grossly visible abnormalities
6. Number of pups with physical or gross behavioural abnormalities after parturition and during the lactation period.

Clinical signs were recorded daily for all animals including pups throughout the study. Body weight for each female was recorded on arrival, on the first day of treatment and weekly thereafter during pre mating period. The dams were weighed on Days 0, 7, 14 and 20 of gestation, and on Days 0-1, 4, 7, 14 and 21 of lactation. Pups were weighed collectively (i.e. the litters, males and females separately), on Days 0-1, 4, 7 and 14 and individually on postnatal Day 21. Selected pups for extended treatment were weighed weekly. Males were weighed on arrival and weekly in the pre-mating period, during mating period.

Food consumption for all animals recorded weekly throughout the study, except during the mating period and during lactation. In the latter case it was recorded on the same days as the weighing of dams and pups.

Prior to necropsy, blood samples from parent animals (10 males and 10 females per group) and selected F_1 offspring (10 males and 10 females per group) were taken for haematology and coagulation test.

After completion of extended treatment of the selected offspring, 3 offspring of each sex from control and 12 % BioProtein group were selected for immunological investigation. Blood samples as above were also taken for antibody analysis (see Section 4.2.4).
At necropsy all parents, pups not selected for extended treatment and selected offspring were examined macroscopically. Implantation sites of the female parents were counted. Abnormalities, mesenteric lymph nodes, spleen, liver, cervix, coagulation gland, epididymidis, ovaries, prostate, seminal vesicles, testes, uterus, vagina where appropriate from all parents were collected, weighed and fixed and histological examination was performed on these tissues/organs. Any abnormalities, mesenteric lymph nodes, spleen and liver from selected offspring were collected, weighed and fixed. Histological examination was performed on these tissues/organs of the control and high dose group of Brewer's Yeast and all animals of all groups treated with BioProtein.

4.2.2.2 Results

No treatment related clinical signs were observed in parents or in selected F_1 offspring.

No treatment related changes were seen in the body weight of parents during the pre-mating period and during gestation and lactation periods in females. Body weights of selected offspring for the extended treatment also remained unaffected due to the treatment with BioProtein and Brewer's Yeast.

Occasionally, food consumption was significantly reduced during the early weeks of the study in both sexes of parent animals. Occasional treatment related increases and decreases in food consumption of offspring of 6% BioProtein, 12% BioProtein and 6% Brewer's Yeast group were found during the period of extended treatment. There were no significant differences in total food consumption over the period of treatment.

No statistically significant difference was observed in reproduction parameters and on litter data of all animals treated with BioProtein and Brewer's Yeast.

Prothrombin time in male parents and selected male offspring from Groups 2 and 3 treated with 6 % and 12 % BioProtein, respectively was statistically significantly higher than for the controls. However, the values were within the historical range of data fro the laboratory and the differences were minimal in absolute terms. They were therefore considered to be of no biological significance. Female parents and offspring were comparable to the control group.

There were no other significant differences in haematological parameters in male parents and male offspring. In female parents, 6% BP had a significantly higher white blood cell count than the control, though 12% BP did not. Both the yeast groups were raised in a dose related manner.
Increased neutrophils and lymphocytes were associated with raised white blood cell counts

In female offspring there was a reduction in white cell count in the 6% BioProtein rats ($p<0.05$), though not in those receiving 12%, but there were no changes in any of the sub-groups of whites cells. The 12% Yeast group exhibited an increase in neutrophils ($p<0.05$).

Rats receiving 6% or 12% Brewers Yeast exhibited higher prostate weights.

Statistically significant increases were found in both absolute and relative weights of mesenteric lymph nodes in both sexes of parent animals. These increases were significant in both the BioProtein groups and in the 12% Yeast group. They tended, though not consistently so, to be dose related. In offspring taken to 12 weeks of age, weights were only increased significantly in males receiving BioProtein.

Table 4.2.1. Weights of mesenteric lymph in parents

	Control	6% BP	12% BP	6% BY	12% BY
Male- Abs.	192	247**	276**	204	241*
- Rel	0.0428	0.0548**	0.0605**	0.0456	0.0542**
Fem - Abs	276	354**	341**	316*	358**
- Rel	0.1037	0.1228**	0.1230**	.01142	0.1274**

Table 4.2.2 Weights of mesenteric lymph nodes in offspring at 12 wks of age

	Control	6% BP	12% BP	6% BY	12% BY
Male- Abs	198	273**	263**	218	220
- Rel	0.0549	0.0760**	0.0724**	0.0528	0.0592
Fem - Abs	200	236	231	198	229
- Rel	0.0893	0.1064	0.1010	0.0881	0.0968

Relative liver weights were increased in male and female parents of the 12% Yeast group. In the offspring the relative weights were significantly increased in males of the 12% Yeast group, but were reduced in the BioProtein groups.

Relative weights of spleen in female parents fed both levels of Yeast were significantly increased and although 6% BioProtein also produced a significant increase, 12% BioProtein did not. In the offspring of both BioProtein groups weights were significantly and dose dependently higher when compared with controls.

Microscopically, dose related increased macrophage accumulation in the mesenteric lymph nodes of the rats treated with BioProtein was found, ranging from minimal to moderate in degree. The effect was more pronounced in the male rats than in the female rats. However, in offspring treated up to 12 weeks of age the incidence of macrophage accumulation in the mesenteric lymph node was graded from minimal to slight and only affected about 10% of the rats fed with BioProtein.

No other treatment-related histological changes were observed.

Conclusions: Daily dietary administration of 6%, 12% BioProtein and 6%, 12% Brewer's Yeast to BrlHan: WIST@Mol (GALAS) rats during the pre-mating, gestation and lactation did not show any adverse effect on reproduction. Occasional significant decrease in the food consumption in both sexes during pre-mating period of parent animals was considered of no relevance for the reprotoxicological outcome of the study. Daily dietary administration of 6%, 12% BioProtein and 6%, 12% Brewer's Yeast to offspring up to the age of 12 weeks did not show any effect on growth and development.

Minor changes seen in various parameters, particularly mesenteric lymph nodes and spleen are considered to be related to an immune- mediated response to feeding of BioProtein.

4.2.3 Study in Weanling Rats

This study was conducted at Scantox, Hestehavevej 36A, Ejby, DK-4623 Lille Skensved, Denmark. The objective of this study was to obtain information on the effect of BioProtein on male and female juvenile rats.

A copy of the study report is provided in Appendix 17 (Scantox study number 52692: 'BioProtein – Study in Juvenile Rats')

The objective of the study was investigate the effects of introducing BioProtein into the diet of young rats at different ages and to further investigate the effects of returning animals to the control diet for an extended period of treatment.

4.2.3.1 Study design

Twenty pregnant female SPF Wistar rats of the strain BrlHan:WIST@Mol (GALAS) from Taconic M&B A/S, DK-4623 Lille Skensved, Denmark were used as mothers to give offspring for allocation to the various treatment groups at weaning (lactation day 21). Ten male and 10 female offspring were allocated to each of six treatment groups. The treatments consisted of various dietary regimes as follows:-

1. Control (casein) weaning to 12 weeks
2. 12% BioProtein weaning to 12 weeks
3. Control weaning to 6 weeks, 12% BioProtein to 12 weeks
4. Control to 32 weeks
5. Control to 6 weeks, 12% BioProtein to 32 weeks
6. Control to 6 weeks, 12% BioProtein to 12 weeks, Control to 32 weeks

Clinical signs were recorded daily for all selected animals. Body weights were recorded on the day of selection (i.e. lactation Day 21) and weekly thereafter. Food consumption was recorded weekly throughout the study.

Prior to necropsy, blood samples were collected for the haematology and coagulation tests. Blood samples were also collected for antibody analysis (see Section 4.2.4).

After completion of the treatment periods at 12 or 32 weeks of age, all animals were subjected to necropsy. Liver, mesenteric lymph node and spleen, plus any abnormalities, was collected, weighed, fixed and histological examination was performed on these tissues/organs.

4.2.3.2 Results

No treatment related clinical signs were observed during entire period of the study.

There were no statistically significant differences in final body weight or body weight gain at 12 or 32 weeks of age. Occasional statistically significant differences in the body

weight of males found during the early weeks of the study were attributed to lower body weights at initiation of the treatment when compared with controls.

No statistically significant group mean difference was observed in the body weight of the females at any time point when compared with control.

Food consumption remained unaffected due to treatment with 12% BioProtein during the entire period of the study.

A statistically significant increase in the white blood cells (WBC) and lymphocytes in the females of Group 5 was found when compared with controls. At 12 weeks of age, prothrombin time in both sexes of Groups 2 and 3 was statistically significantly higher than the controls. At Week 32 of age also a statistically significantly increase in prothrombin time and decrease in fibrinogen was observed in Group 5. However, the difference in prothrombin time on both occasions was minimal in absolute terms and therefore is considered to be of no biological significance.

No significant group mean differences were noticed in the other haematology parameters tested when compared with controls.

At week 12 of age, a statistically significant increase in weights of mesenteric lymph nodes (both absolute and relative) was seen in the females of Groups 2 and 3. A similar effect in males was observed, however, the difference was statistically significant only for the absolute weights of males from Group 3.

At 32 weeks of age, a statistically significant increase in weights of mesenteric lymph nodes (both absolute and relative) was seen in males of Groups 5 and 6. A similar effect was observed in females. However, the group mean difference became statistically significant only in Group 5. This increase in weights of mesenteric lymph nodes in both sexes suggested a treatment related effect. Weights for mesenteric lymph nodes in Group 6, which had been returned to the Casein Control diet from 12 weeks of age, were significantly lower than those of animals in Group 5, indicating reversibility of the effect.

Table 4.2.3 Weights of mesenteric lymph nodes

	1-Control	2	3	4- Control	5	6
M- Abs	202	248	282*	159	260**	241*
- Rel	0.0537	0.0659	0.0732	0.0320	0.0541**	0.0480**
F – Abs	188	236**	233*	161	265**	163
- Rel	0.0828	0.1038**	0.1006*	0.0630	0.0968**	0.0620

Absolute and relative spleen weights were higher in both sexes of Group 5. However, microscopic evaluation revealed no treatment related changes in the organ.

No significant group differences were seen in the liver weights of treated animals when compared with controls at 12 or 32 weeks.

Microscopically, incidence of minimally to slightly increased macrophage accumulation was recorded in the mesenteric lymph nodes of 3 female rats of each of Groups 2 and 3.

However, no increased accumulation of macrophages was recorded in the mesenteric lymph nodes of the males from Groups 2 and 3.

In 7 male and 10 female rats of Group 5, minimally to moderately increased macrophage accumulation was recorded in the mesenteric lymph nodes. No increased accumulation of macrophages was recorded in the mesenteric lymph nodes of the males from Group 6 or in the mesenteric lymph nodes of the control group.

Conclusions: Daily dietary administration of 12 % BioProtein to the juvenile male and female rats from the age of 3 weeks to 12 weeks (Group 2 and 6) and from the age of 7 weeks to 32 weeks (Group 5) did not show any effect on growth or development. Minor changes seen in white blood cells, mesenteric lymph nodes and spleen are concluded to be related to immune- mediated responses to feeding of BioProtein. The study also demonstrated that these changes are reversible.

4.2.4 Antibody Studies (Annex 18)

4.2.4.1 Procedures

In the Single Generation Study, blood samples were taken prior to necropsy from all male and female parents and from all offspring retained to 12 weeks of age. In addition, 3 male and 3 female offspring from the Control and 12% BioProtein groups were immunised with BioProtein by intraperitoneal injection on days 1 and 14 of this phase of the study. Blood samples were taken on day 1 and at termination of these animals on day 24.

In the Juveniles Study, blood samples were taken from all animals prior to necropsy. On these and the above samples, serum analysis was undertaken for IgG, IgG1, IgG2a and IgGA antibodies to BioProtein.

4.2.4.2 Results

Whereas parent animals receiving BioProtein had higher levels of antibodies than the Controls, offspring in the BioProtein groups showed no increase relative to the Controls.

The levels of antibodies to BioProtein in serum from immunised rats fed BioProtein were low, compared to the levels in the immunised Control rats. The levels of BioProtein antibodies were not increased over the feeding period from 21 to 45 days of age, indicating no increase in response with time.

The significance of the antibody levels compared to the levels in rats fed the casein Control diet was dependent on the age of the rats. Control rats have a low level of antibodies reacting with BioProtein, which may be explained by the existence of non-specific, cross-reacting antibodies. The level was shown to increase with age, which may be a consequence of a continuously and more diverse production of antibodies towards ingested antigens by age.

Early presentation of BioProtein was also shown to reduce the immunological response. Rats receiving BioProtein from 3 weeks of age responded with a significantly lower BioProtein antibody titre than animals receiving BioProtein from 6 weeks of age.

In BioProtein fed offspring of parents fed BioProtein no significant level of antibodies was found. Also, following immunisation of offspring, rats fed BioProtein had only slightly increased BioProtein antibody titres. On the other hand, a very high response was found in BioProtein immunised Control rats.

The antibody response to Brewer's Yeast in the diet seemed to follow the same trend as that observed for BioProtein. However, with Brewer's Yeast a more pronounced antibody response was observed.

Conclusions: The antibody study indicates that there are immune responses in rats to BioProtein and Brewers Yeast in the diet. Early presentation resulted in very low levels of BioProtein-reacting antibodies and to tolerance development. This is supported by the result from both the Single Generation and Juveniles studies. The significance of the antibody level in BioProtein fed rats was less pronounced for the rats at 32 weeks of age than at 12 weeks of age and it was significantly lower than observed for immunised Control rats.

5 DISCUSSION

The new studies in farm animals, including farmed salmon, demonstrate that BioProtein is well tolerated by the target species and produces good performance under controlled and commercial conditions.

The results further confirm that BioProtein is a feed ingredient of high nutritional value that supports good weight gain, food utilisation and clinical health. In the salmon trial there was an improved performance as the inclusion of BioProtein increased to the maximum dietary level of 36%. There were no adverse health effects attributable to BioProtein and pathology undertaken on pigs and broiler chickens gave no indication of any adverse effects. This absence of effects included examination of examination of tissues involved in the immune system.

The results of new studies in rats throw further light on the responses originally seen in study on nucleic acid reduced experimental material and seen to a much lesser degree in commercially produced BioProtein.

BioProtein supported good reproductive performance, growth and development of animals in these studies, with absence of any adverse clinical signs.

The principal finding was an enlargement (increased weight) of mesenteric lymph nodes accompanied by a degree of macrophage accumulation in the lymph nodes. There were no other histological changes of significance in the organs examined. Parent rats in the Single Generation Study exhibited enlarged lymph nodes in the BioProtein and Brewer's Yeast groups, whereas in offspring retained to 12 weeks of age significant increases occurred in BioProtein-fed males only. Minimal to slight macrophage accumulation was seen in only 10% of the rats. It is interesting that the responses to Brewer's Yeast seen in the recent study were of the same order, or slightly less than, BioProtein, whereas in the earlier 8 week study the response to Brewer's Yeast was much more severe and comparable to the nucleic acid-reduced material.

In the Juveniles Study lymph node effects were reduced by introduction of BioProtein at weaning, as opposed to 3 weeks later, and were reversible when rats were returned to the control diet after 12 weeks of age.

These results, coupled with the antibody data from rats in both studies, lead to an improved understanding of the responses to BioProtein seen in the rat.

No changes were observed in target species (pigs, broiler chickens and Atlantic salmon) exposed to BioProtein. In some investigations an enlargement of mesenteric lymph nodes has been recorded that was accompanied in some instances by a minimal to moderate accumulation within lymph nodes of macrophages. Some degree of mesenteric lymph node enlargement was observed with a control protein source, Brewer's Yeast, which is considered safe. It is not clear that the changes observed in lymph nodes are anything other than a reflection of the fact that exposure of mature animals to large amounts of a foreign protein source for the first time is associated with immune activation in draining lymph nodes. The data obtained with Brewer's Yeast are consistent with this interpretation. Moreover, it is important to emphasise that no such changes were observed in the relevant target species.

As indicated above, exposure of immunologically mature animals to a new dietary protein source in large amounts might be expected to result in the elicitation of a specific immune response.

One of the factors governing immune recognition of, and responses to, food proteins is immunological tolerance. This is best described as an immunological hyporesponsiveness to dietary proteins that aids in preventing unnecessarily vigorous or inappropriate immune reactions to food components. This immunological safety measure is most effective in limiting responses to food proteins experienced neonatally. However, given that oral tolerance is rarely complete, it comes as no surprise that food protein-specific IgG antibodies are found in normal subjects with no history of food allergy or adverse reactions to food or food components. Indeed, it is likely the case that the elaboration of an IgG antibody response is the normal consequence of dietary exposure to food proteins, particularly when a food protein is experienced for the first time after infancy.

Against this background, experiments were conducted to examine the induction in rats of immune responses to dietary BioProtein. In a study of juvenile animals IgG and IgA antibody responses were measured at various periods following exposure to BioProtein. Rats that received BioProtein (12%) in their diets continuously from 3 to 12 weeks of age displayed only relatively low-grade serum antibody concentrations at 12 weeks. Somewhat higher antibody responses were seen in rats that received the same dietary concentrations of BioProtein from 6 weeks to 12 weeks of age. Thus, in this latter group, despite a lower total cumulative dietary exposure by 12 weeks of age, levels of antibody were found to be higher. These data are therefore consistent with the view that first exposure to a novel dietary protein later in life is likely to provoke a more vigorous antibody response. The corollary is that, in this instance, exposure earlier in development resulted in only modest antibody responses despite a higher total cumulative exposure over a 12 weeks period.

Further support for this derived from serological studies performed in the context of a single generation rat study. In the F0 generations there were modest IgG and IgA antibody responses to 12% BioProtein. However, in the F1 generation (born to dams exposed to BioProtein) specific antibody responses were reduced or absent.

Finally, a small series of experiments were conducted in which the impact of dietary exposure to BioProtein, or to a control protein (casein), on the subsequent stimulation of specific immune responses following immunisation with BioProtein were examined. The data clearly revealed that animals that had received dietary BioProtein from 3 weeks of age were significantly less able than control (casein-fed) rats to respond immunologically to the same protein. The interpretation is that exposure of young rats to dietary BioProtein caused immunological hyporesponsiveness (immunological tolerance) resulting in a significant inhibition, or complete absence, of specific immune responses to the same protein following challenge later in life.

Collectively, these data indicate dietary exposure to BioProtein of adult rats (that have not previously experienced this protein) results in an immune response. This is a normal response that would be expected following first exposure of adult animals to a novel protein that had not previously been experienced in the diet, or during development *in*

utero or during lactation. This view is supported by the results obtained from feeding Brewer's Yeast, which also would have been 'novel' to the rats.
It has been suggested that the failure of rats exposed early in life to BioProtein to respond to a subsequent immunisation with the same protein later in life with a normal immune response might be suggestive of immunotoxicity or non-selective immunosuppression. There are two important points to make: (1) the reduced immunological responsiveness to BioProtein as described above is wholly consistent with specific oral tolerance to a dietary protein experienced neonatally or early in life, and (2) that evidence from all studies, in all species tested, with BioProtein indicates very clearly that there are no alerts whatsoever for immunotoxicity or immunosuppression. Thus, for instance, there are no haematological parameters suggestive of immunosuppression (and importantly, no changes in leukocyte numbers) and no changes to the major lymphoid organs. The conclusion drawn is that BioProtein is without adverse effects on the immune system and fails to induce immunotoxicity or immunosuppression.

6 CONCLUSIONS

BioProtein is further confirmed to give very good performance in target animals, i.e. weaned piglets, fattening pigs, broiler chickens and farmed Atlantic salmon. There were no adverse health effects in these species as confirmed by pathological assessment.

The recent rat studies demonstrated that BioProtein supports normal reproductive performance and normal growth and development in offspring or juvenile rats where BioProtein was introduced into the diet at or soon after weaning. Increased weight of mesenteric lymph nodes, accompanied by low-grade macrophage accumulation in some animals, is believed to be a result of an immune response to an unfamiliar ingredient. The effect was shown to be reversible after withdrawal of the BioProtein diet

The laboratory rat appears to be particularly sensitive in its immune responses and may not be an appropriate model when assessing the suitability of BioProtein for farm animals. No such responses were seen in the target farm animals.

It is Norferm`s opinion that the new studies presented in this Dossier along with those conducted previously , further confirms that BioProtein is safe for use in animal feed.

LIST OF ANNEXES.

ANNEX 1: Chemical and Physical Analysis; comparison of commercial BioProtein and nucleic acid reduced experimental sample.

ANNEX 2; Publication by Mølck. et.al; " Immunotoxicity of nucleic acid reduced BioProtein, a bacterial derived single cell protein in Wistar rats". *Toxicology* (2002) **174**,183-200.

ANNEX 3. Publication by Christensen et.al; "The oral immunogenicity of BioProtein, a bacterial single cell protein, is affected by its particulate nature". *British Journal of Nutrition* (2003) **90**, 169 - 178

ANNEX 4. "BioProtein in diets for piglets". Kari Helga Kløvstad, Norgesfòr, Oslo, Norway.

ANNEX 5. "BioProtein in diets for piglets: Gross and histopathological examination". Thor Landsverk, Norwegian School of Veterinary Science.

ANNEX 6. "Effect of BioProtein on growth performance of fattening pigs". By Hallgeir Sterten, Felleskjøpet Fòrutvikling, Trondheim, Norway.

ANNEX 7. "Histopathological examination of organs collected from a feeding trial with BioProtein". By Thor Landsverk, Norwegian School of Veterinary Science.

ANNEX 8. "Field trial with BioProtein in co-operation with Felleskjøpet Fòrutvikling". By Inger Johanne Karlengen, Dept. of Anim. Sci., Agricultural Univ. of Norway.

ANNEX 9. "Broiler chicken trial with BioProtein". By H.F. Schøyen, H. Hetland and Anders Skrede, Ag. Univ of Norway and T. Landsverk Norwegian School of Veterinary Science.

ANNEX 10. "Necropsy report: Trials with bacterial protein". By Thor Landsverk, Norwegian School of Veterinary Science.

ANNEX 11. "BioProtein in diets for chicken: Gross and histopathological examination". By Thor Landsverk, Norwegian School of Veterinary Science.

ANNEX 12. "Bacterial protein as a protein source in diets for Atlantic salmon (Salmo salar)". By T.B Aas, B. Grisdale-Helland, B.F. Terjesen and S.J. Helland. Akvaforsk, Inst. of Aquaculture Research, Sunndalsøra, Norway.

ANNEX 13. Publication by Øverland et.al. Agri. Univ. of Norway. " Bacterial Protein Grown on Natural Gas as Feed for Pigs". *Acta . Agric. Scan. Sect. A, Animal Sci*. (2001) **51**, 97 – 106

ANNEX 14. Publication by Skrede et.al. " The effect of bacterial protein grown on natural gas on growth performance and sensory quality of broiler chickens". *Canadian Journal of Animal Science.(2003) 229-237.*

ANNEX 15. Publication by Skrede at.al." Bacterial Protein Produced on Natural Gas: A New Potential Feed Ingredient for Dogs Evaluated Using the Blue Fox as a Model". *J. Nutr*. **132,** 2002, 1668S – 1669S

ANNEX 16. "One Generation Reproduction Study in Rats". Scantox Study No 52691.

ANNEX 17. "Study in Juvenile Rats". Scantox Study No 52692.

ANNEX 18. "BioProtein Antibody Responses in Feeding Studies". Report by Helle N. Thestrup, Norferm Denmark AS

SECOND AMENDMENT TO GRAS NOTICE FOR DRIED *METHYLOCOCCUS CAPSULATUS* PRODUCT

Submitted by: Keller and Heckman LLP
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On behalf of our client

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April 11, 2021

I. Introduction

The purpose of this amendment is to address questions raised by the U.S. Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM) during the March 11, 2021 teleconference, and corresponding meeting minutes (dated March 12, 2021), regarding the February 28, 2020 submission of the Generally Recognized As Safe (GRAS) Notice for Calysta, Inc.'s Dried *Methylococcus capsulatus* Product (hereinafter "FeedKind®"). A first amendment was submitted on July 17, 2020 and the GRASN was filed on August 13, 2020 and designated as GRAS Notice No. AGRN 40. This second amendment specifically addresses CVM's questions regarding the identity, method of manufacture, and specifications for FeedKind®, as well as the identity, method of manufacture, and specifications for the raw materials used in the continuous fermentation process.

For clarity, we have repeated CVM's questions in **bold** below, followed by our responses.

a. DRIED MCP QUESTIONS AND COMMENTS
IDENTITY AND COMPOSITION OF DRIED MCP

1. **The notifier should quantify the contents of constituents that account for nearly 100% of the composition. For example, the percent composition determined as the sum of quantified constituents (crude protein, crude fat, crude fiber, moisture and minerals) is 90.5%. The percent composition is 94.3%, when ash is used in place of minerals content. Examples of constituents that may significantly contribute to the composition are inorganic compounds (anions/cations such as sulfate, nitrate), carbohydrates and organic acids. In addition, crude protein corresponds to approximately 73.46% of the biomass, but the sum of amino acids ranges from 56.48 to 60.7%, averaging 59.26%. Thus, there is approximately 14.2% non-protein nitrogen present. The presence of nucleic acid, nucleotides, biogenic amines, and similar nitrogenous entities that are commonly present in fermentation biomass products is not addressed in the notice and should be discussed.**

   In addition, the notice does not contain the contents of certain mineral anions and cations that could contribute to the composition. For example, based on the batch analysis summarized in Table 6 in the notice, the average mineral content, which is 2.9 g/100g, is derived mainly from the contents of sodium, calcium and phosphorus. However, because the source of calcium is calcium chloride, the contents chloride could be significant. Furthermore, the molybdate content may be significant as one of the sources of sodium is sodium molybdate dihydrate. And, phosphorus mainly exists as phosphate, which contains four oxygen atoms. In addition, several mineral nutrients added to the fermenter provide a source of sulfate, which is not accounted for.

The reported composition for the three submitted lots ranges from 93.9-94.6%. The unaccounted for 5-6% is primarily composed of soluble carbohydrates. Calysta has developed

laboratory scale data indicating that typical carbohydrate content is approximately 8%. A summary of the lab scale test is provided here:

Calysta used levels of levulinate after hydrolysis to estimate total carbohydrate levels in the form of glycogen, glucose, or other sugars. Calysta prepared multiple samples of biomass, all of 0.35g of biomass. Glucose was spiked into samples at different concentrations to estimate carbohydrate losses during the conversion to levulinate. 0, 5, 25, 50, and 100 ug of glucose were added.

All samples were subjected to acid hydrolysis and assayed for levulinate (the degradation product of glucose/glycogen). Glucose (6C) is decomposed to Levulinate (5C) according to the molar ratio:

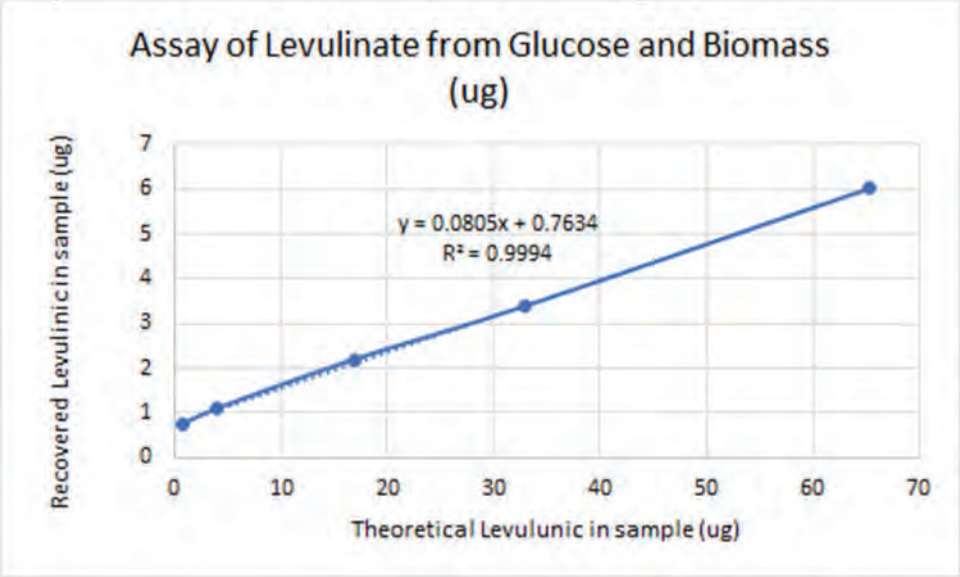
levulinate/Glucose $C_5H_8O_3/C_6H_{12}O_6$ = 116.1/180 = 116.1/180 = 0.645

Ten replicates of biomass sample (no spiked glucose) showed an average concentration of 0.638% levulinate. Values from spiked samples showed an 8.05% recovery rate. Average levulinic acid in Wild type biomass (n=10) is 6.38 g/kg, with recovery of 8.05% it brings levulinic before analysis to 79.25 g/kg. This is the sum of decomposed glucose, glycogen and any other glucose containing sugars in the biomass.

Table 1: Summary of Lab Scale Test

1 OD pellet (ug)	Glucose Spike (ug/ sample)	Theoretical Levulinate from Glucose (ug)	Levulinate from pellet (ug)	Theoretical Levulinate from Glucose and Biomass (ug)	Assay of Levulinate from Glucose and Biomass (ug)
350	0	0.0	0.76	0.76	0.76
350	5	3.2	0.76	3.99	1.105
350	25	16.1	0.76	16.89	2.2
350	50	32.3	0.76	33.01	3.395
350	100	64.5	0.76	65.27	6.01

Figure 1: Assay Levulinate from Glucose and Biomass (ug)



While Calysta does not directly test for carbohydrates (beyond fiber), the remaining product not accounted for by the current specifications is expected to be carbohydrates, and this is supported by the laboratory scale data above. Because of the nature of the product, the expected inaccuracy of available methods, and because of FeedKind®'s intended function (sources of protein), we believe it to be unnecessary to go through method development and validation for such an analysis. For nitrogen, CVM has indicated that the firm should discuss the approximately 14% of nitrogen content not accounted for by the protein content. The DUMAS method employed by Calysta to determine "crude protein" will indeed report results for all nitrogen containing compounds such as nucleic acids and biogenic amines. From the previously submitted stability results, we know that biogenic amine content ranges from 3,000-5,000 ppm (0.3-0.5%). The remaining nitrogen content is nucleic acids. While Calysta does not assay for nucleic acid content this level is consistent with a microbial biomass products in general and FeedKind® specifically as illustrated in Skrede *et al.* 1998 (cited in AGRN 40) which indicates a nucleic acid content of ~9.5%. Further, this would not present a safety concern to animals and would be broken down in the animal's digestive tract and not passed into the human food supply.

CVM has asked for clarity regarding the mineral content. Phosphorus is reported as elemental phosphorus. When accounting for the fact that phosphorus is typically present as phosphate (PO_4), it accounts for approximately 4-5g/100g of product. Chloride is reported as salt content. Molybdenum is expected to be present in FeedKind® at approximately 8-12mg/kg based on microbial media usage rates, and therefore we do not believe analysis for molybdate content is required.

MANUFACTURE

2. **The amendment dated July 17, 2020 states that periodic testing conducted during the manufacture includes analyses of potential heavy metal contaminant concentration in the continuous fermentation run, including testing for lead (Pb),**

cadmium (Cd), and arsenic (As). The firm should explain why mercury is not included in periodic testing.

Mercury is not included in periodic testing because as noted in the first amendment (dated July 17, 2020), Calysta has set a per lot specification for mercury of ≤0.01 ppm. As every lot is tested for mercury, the firm did not see a need to test periodically. We are also providing mercury testing data for the three lots provided as batch analyses in AGRN 40 in Table 2 below.

Table 2: Mercury Content of AGRN 40 Batches

	TEES 09/63	**TEES 09/84**	**TEES 09/102**
Mercury (mg/kg)	(b) (4)		

3. **The notice states that natural gas and solutions of minerals are passed through appropriate filters when fed into the fermenter. However, it is not clear what contaminants and impurities are removed by these filters. This should be explained.**

The filter used is a 0.2um filter intended to remove microbial contaminants from the components used in the fermentation media prior to addition to the fermenter.

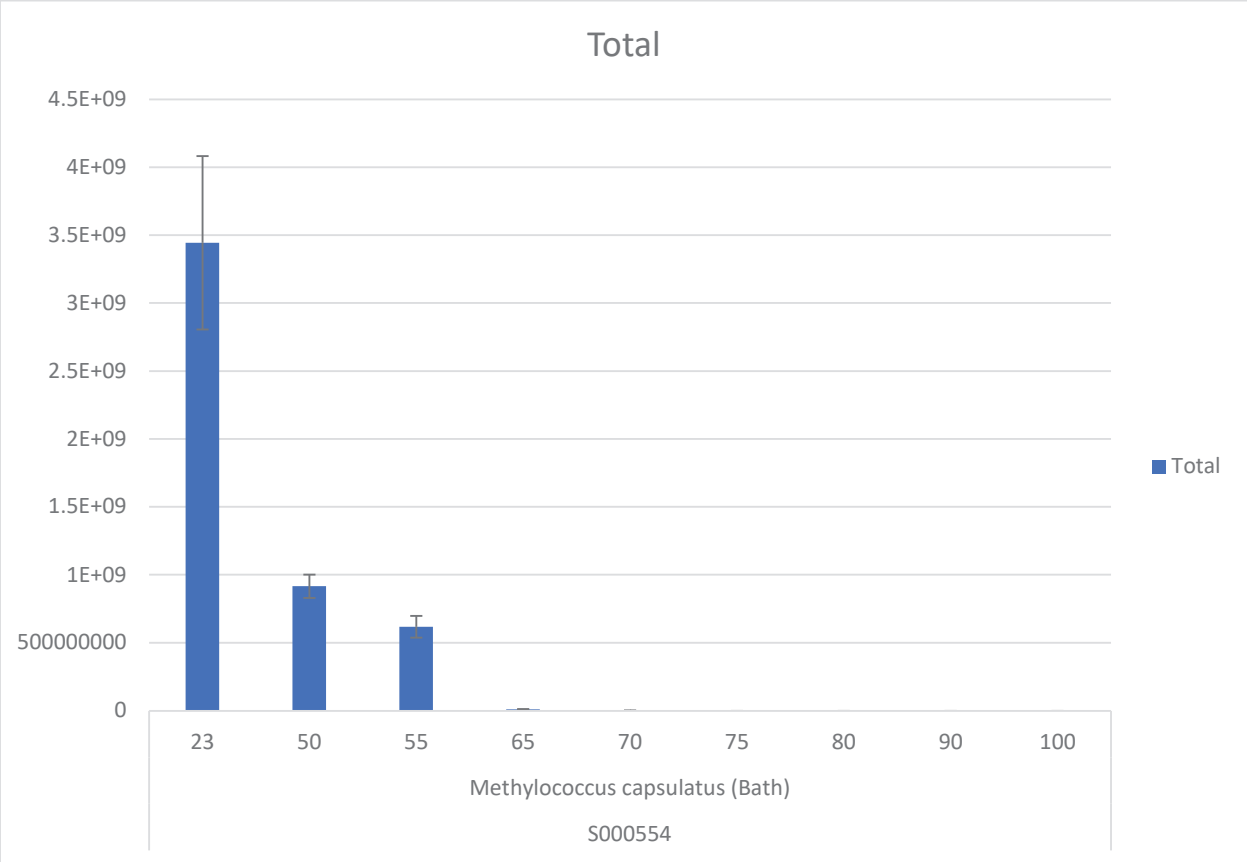
BATCH ANALYSIS AND SPECIFICATIONS

4. **Based on information summarized in Table 2 of the notice, it is not clear that the viability of the production organism and the three heterotrophic microorganisms are reduced by 2 orders of magnitude (2 log units). For example, Table 2 does not show the identity of the microorganisms for which the test results are obtained; and, the notice does not contain a description of the conditions used to grow the microorganisms, which should be optimal for the specific microorganism. The notifier should provide data and information demonstrating that the production organism and the three heterotrophic microorganisms are not viable in the notified substance using validated methods. This information should detection. We note that pH and temperature during the continuous fermentation are maintained at 6.2 ± 0.5 and 45°C ± 5°C, respectively.**

The total plate count method utilized for testing of each batch allows for enumeration of both remaining heterotrophic organism from the production run as well as any potential contaminating microorganisms. This method is based on EN ISO 4833:2013 and is validated for use in animal feed. Internally, Calysta uses plate count agar (PCA) to culture the 3 heterotrophic organisms. Typical time and temperature is 45°C for 24 hours. Sciantec Analytical, the United Kingdom-based laboratory that tested Calysta's sample lots, utilizes an aerobic plate count method based on EN ISO 4833:2013 which also utilizes PCA as a growth medium. Typical time and temperature is 37°C for 72 hours. While this growth temperature is below that utilized by Calysta internally, any remaining viable heterotrophic production organisms will be enumerated by this method.

M. capsulatus is not captured by this test method as it requires a carbon source containing only a single carbon (*i.e.* methane or methanol) for growth. *M. capsulatus* is difficult to culture in a way which would easily permit enumeration on a per lot basis. To address this, Calysta has developed internal data which indicates that *M. capsulatus* is entirely inactivated by the heat treatment process employed during production. Figure 2 indicates that *M. capsulatus* is inactivated in as little as 30 seconds at 75°C. The included heat kill data combined with the total plate count specification clearly indicates that the conditions of manufacture for FeedKind® reduce the number of viable production organisms by more than 2 logs (99%).

Figure 2: *M. capsulatus* Heat Kill Curve



SPECIFICATIONS

5. **The notifier should provide a specification acceptance criterion for maximum mesophilic aerobic plate count, mold count, yeast count and ash content that are more closely aligned with the batch analysis results. The specification acceptance criterion for mesophilic aerobic plate count (500,000 colony forming units per gram (CFU/g)) is 500 times larger than that observed in the batch analysis (1,000 cfu/g); And, it does not include a maximum limit. Furthermore, the notifier should clarify if the manufacturing process includes an "Ultra High Temperature (UHT)" heating step. When the notified substance undergoes a UHT step, average total aerobic plate**

count is less than 1000 CFU/g, but if the UHT treatment is not done, the total aerobic plate count can reach 170,000 CFU/g. The specification acceptance criteria for mold count (5,000 CFU/g) is more than 2 logs greater than the batch analysis results. The specification acceptance criteria for yeast count (5,000 CFU/g) is more than 4 times greater than the batch analysis results. The specification acceptance criteria for ash content, ≤12%, is significantly higher than ash content determined in the batch analysis, which is in the range of 6.0% to 7.4%.

First, it is unclear what CVM would consider an "ultra-high temperature" heating step. Table 1 in AGRN 40 indicates the time and temperatures during the production process and includes a "heat treatment" step which is 121°C for 1-2 minutes and further "evaporator" and "spray dryer" steps which occur at 80°C for a total of 5-11 minutes. The tests on batches which did not undergo the "heat treatment" step were conducted and included in order to better understand the effects of the "heat treatment" step and to give CVM additional comfort that this step results in at least a 2 log reduction in viable production organisms. Calysta agrees that the specification acceptance criteria for maximum mesophilic plate counts and mold and yeast counts should be lowered and clarified. Calysta agrees to set the specification for mesophilic plate count to ≤ 250,000 cfu/g, ≤ 1,000 cfu/g for mold and ≤ 2,000 cfu/g for yeast. Calysta believes that as there is no safety concern with this level (or indeed at higher levels) and this specification is suitable to ensure safety for a microbial biomass product while still giving the firm the flexibility necessary given the inherent variability of microbial testing. Calysta further notes that while the counts for these specifications were very low for the submitted batches, these numbers represent the low end typically seen. For ash, Calysta would note that ash content represents residual mineral content and due to the nature of the product (a microbial fermentation product) is variable by nature. For this reason, Calysta requires flexibility on the specification and proposes to leave the specification limit at ≤ 12%.

6. **The notifier should provide the citation for the validated method used to determine the nickel content or a copy of the validated method including a validation summary.**

As detailed in Appendix 1, Sciantec has provided additional information regarding validation of various analytical methods. Sciantec has developed an in-house validated method for the detection of nickel with an LOD of 0.1 mg/kg in animal feed. The method and validation summary are attached in Appendices 2 and 3.

7. **The notifier should provide the citation for the validated method used to determine the mercury content or a copy of the validated method including a validation summary.**

Per our conversation with CVM on March 29, 2021, we attach a method summary for the detection of mercury. The method is validated and accredited by UKAS. As with Question 6, the method summary is attached in Appendix 1 for detection of mercury at an LOD of 0.01 mg/kg.

STABILITY

8. **The notifier should provide citations to the validated methods used in the stability study or a copy of the validated methods including validation summaries. The tests performed are for the determination of crude protein, crude fat, ash, moisture and crude fiber, amino acid profile, fatty acid profile, microbiology and biogenic amines. Microbial testing performed are anaerobic plate count, aerobic plate count, yeasts, and molds.**

Please see Appendix 1 for relevant method descriptions for UKAS validated and accredited methods, as well as validation summaries for those methods not UKAS accredited. Yeast and mold methods are not accredited, and summaries are included as Appendix 4.

9. **The notifier should explain how it can demonstrate the stability of aerobic count of the notified substance for 52 weeks given that the microbial testing results for aerobic plate count for one of the three batches in the stability study (TEES005/28) significantly deviates starting after week 26. The aerobic count is 300 CFU/g at week 26 and 700,000 CFU/g at week 52. If additional batch data is available, it should be provided.**

The firm includes additional stability data below in Table 3 to specifically address CVM's question regarding microbial stability for Batch TEES005/28. We have included the aerobic plate count data previously provided in AGRN 40 (0-52 weeks) and have added new data for weeks 72 and 104. This additional data clearly indicates that the test at 52 weeks (700,000 cfu/g) was an outlier. Tests at 72 and 104 weeks show results in line with the other time points. Full additional results for other analyses are included in Table 4 as requested.

Table 3: Week 72 and 104 Aerobic Plate Count Results

Batch TEES005/28 25ºC/60%RH	
Time	Aerobic (cfu/g)
0 Weeks	(b) (4)
4 Weeks	
8 Weeks	
12 Weeks	
26 Weeks	
52 Weeks	
78 Weeks	
104 Weeks	

Table 4: Batch Analyses for TEES004/29, TEES004/29a, TEES004/11, and TEES05/28

Batch TEES004/29 25°C/60%RH (no UHT; real time)					
Nutritional Analysis					
Duration (Weeks)	Moisture (Max 10%)	Crude Fat (Min 5%)	Crude Protein (Min 68%)	Crude Fiber (Max 1%)	Ash (Max 12%)
72	(b) (4)				
104					
156					
Microbiological Analysis					
Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Molds cfu/g	
72	(b) (4)				
104					
156					
Batch TEES004/29a 25°C/60%RH (real time)					
Nutritional Analysis					
Duration (Weeks)	Moisture (Max 10%)	Crude Fat (Min 5%)	Crude Protein (Min 68%)	Crude Fiber (Max 1%)	Ash (Max 12%)
72	(b) (4)				
104					
156					
Microbiological Analysis					
Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g	
78	(b) (4)				
104					
156					
BatchTEES004/11 25ºC/60%RH					
Nutritional Analysis					
Duration (Weeks)	Moisture (Max 10%)	Crude Fat (Min 5%)	Crude Protein (Min 68%)	Crude Fiber (Max 1%)	Ash (Max 12%)
78	(b) (4)				
104					
156					
Microbiological Analysis					
Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g	
78	(b) (4)				
104					
156					
Batch TEES005/28 25ºC/60%RH					
Nutritional Analysis					

Duration (Weeks)	Moisture (Max 10%)	Crude Fat (Min 5%)	Crude Protein (Min 68%)	Crude Fiber (Max 1%)	Ash (Max 12%)
78	(b) (4)				
104					

Microbiological Analysis				
Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
78	(b) (4)			
104				

ANALYTICAL METHODS

10. Proximate analysis: Because method AOAC 994.12 is not applicable to the determination of the amino acids tyrosine and tryptophan, the notifier should describe the method modifications that allow these determinations.

Please see Appendix 1 for methods and validation information for tryptophan. As discussed in our March 29, 2021 call, a true determination of tyrosine would require a second, separate analysis which would incur significant expense. Tyrosine numbers reported are those derived from the method listed above, even though it is not strictly appropriate for this determination. We believe this is of little consequence as tyrosine content is not directly safety related. See Appendix 5 for tyrosine performance testing data.

11. Proximate analysis: The notice does not contain a citation for the method used in the provide the citation for the validated ICP OES method used to determine mineral contents or a copy of the method including a validation summary.

Please see Appendix 1 for LOD and method summaries for minerals. Sciantec uses an in-house method based on BS EN 15510:2017, which is validated for detection of minerals in animal feed.

12. Specifications: It is not clear that method AOAC 2011.03, 2003.09 is applicable as for the determination of microorganisms that grow aerobically at mesophilic temperatures (25 to 40°C). Methods AOAC 2011.03 and 2003.09 are applicable for the determination of *Salmonella* in a variety of foods and in specific foods, respectively.

AOAC 2011.03 and 2003.09 were listed in error. Aerobic and anaerobic methods used are EN ISO 4833:2013, which is validated for detection of microbes in animal feed at a LOD 10 cfu/g. Please see Appendix 1 for additional method summary.

B. STARTING RAW MATERIALS QUESTIONS AND COMMENTS

The starting materials methane/natural gas, nickel chloride hexahydrate and nitric acid have no regulatory status for use in the manufacture of animal food (or human food).

Pipeline natural gas as a source of methane

Identity and specifications

It is not clear from information contained in the notice that the composition of the pipeline natural gas used in the manufacture of the notified substance has a consistent profile as natural gas derived from different producing regions have different constituent profiles, and natural gas derived from different producing regions are mixed before becoming pipeline natural gas used by consumers. The study by Chao (1993) (which is contained in the notice) determined the content ranges of numerous trace constituents in pipeline natural gas derived from different producing regions, including benzene in the range of <0.2 to 471 ppm by volume, toluene in the range of <0.1 to 100 ppm by volume, and hexanes in the range of <0.2 to 1156 ppm by volume. In addition, some of the results in the study by Chao (1993) may be affected by the quality of the analytical methods used in this dated study. In addition, it is not clear based on the data and information contained in the notice that downstream processing steps in the manufacture of the notified substance remove all the undesirable trace constituents that may be found in pipeline natural gas, and that may accumulate in the notified substance. We note that removal of certain volatile constituents during processing may be difficult due to strong nonbonding interactions as occurs between aromatic hydrocarbons and the aromatic side chains of amino acids in proteins. Because undesirable trace constituents may be present in the pipeline natural gas used to manufacture the notified substance and the process controls used to manage the accumulation of these constituents are not validated, the notice does not establish a qualitative and quantitative relationship between the notified substance and test articles used in safety studies. The notifier should provide the identities and contents of potential unwanted constituents in the natural gas and more comprehensively describe how these unwanted constituents are controlled to ensure that they do not become contaminants that adversely affect the safety of the notified substance.[1]

Chao and Attari (1995) reported the results of a 3-year program performed to survey the detailed compositions of pipeline gas from major producing areas in the US, imported gas from natural gas from Canada, and natural gas used to generate electricity at 4 power plants in the U.S. The natural gas stream samples were collected from 19 separate sampling points (including 4 power plants) in 10 states across the continental U.S. from October 1990 to 1993.[2] The origins of the natural gas sampled ranged from the on-shore and off-shore Gulf coast to Northern California and Canada. The samples were analyzed to measure the concentrations of a comprehensive list of major, minor, and trace constituents utilizing a complete field sampling and analysis system that had been developed and validated, including proportional sampling, cryogenic sampling, sorbent sampling, and on-line measurement techniques. The method detection limits (MDLs) included 0.1 ppmv for toluene and 0.2 ppmv for benzene, hexanes, and cyclohexane.[3] Table 5 presents the summary statistics for these natural gas constituents.

[1] On March 16, 2021, Calysta requested clarification from CVM regarding reference to a body of evidence regarding the interaction between cyclic/aromatic hydrocarbon constituents and aromatic side chains of amino acids, that was mentioned during the March 11, 2021 teleconference. On March 16, 2021, CVM responded with citations to the specific references and further context for the question. See Appendix 7.

[2] Chao and Attari (1995), Figure 1, page 12.

[3] Chao and Attari (1995), Table 1, page 8.

Table 5: Summary Data for Selected Natural Gas Constituents Reported by Chao and Attari (1995)[4]

Constituent(s)	# of Samples	Range (ppmv)	Median (ppmv)	Sample ID
Hexanes	19	<0.2 to 1156	170	IGT-041
Cyclohexane	17[5]	<0.2 to 146	24	IGT-082
Benzene	17	<0.2 to 471	7	IGT-022
Toluene	17	<0.1 to 100	6	IGT-022

The samples with the highest concentrations of these analytes were IGT-041 for hexanes, IGT-082 for cyclohexane, and IGT-022 for benzene and toluene.

Black and Veatch reported the compositions of 3 examples of pipeline quality natural gas from US-based interstate facilities in the year 2000 and later, which illustrate the range of natural gas compositions that meet minimum pipeline specifications for consumer use.[6] The concentrations of constituents presented for these examples are consistent with those reported by Chao and Attari (1995). Specifically, the concentration ranges reported were:

- Benzene: 44 to 470 ppmv
- Toluene: 18 to 100 ppmv

In the screening-level safety assessment presented below, we assumed that the concentrations of each natural gas constituent were the maximum values reported in Chao and Attari (1995), which equaled or exceeded the corresponding maximum concentrations reported by Black & Veatch. The concentrations considered for the screening assessment are presented in Table 6.

Table 6: Maximum Reported Concentrations of Selected Natural Gas Constituents

Constituent(s)	Concentration (ppmv)	Concentration (ppmw)[7]	Sample ID
Hexanes	1156	5009	IGT-041
Cyclohexane	146	695	IGT-082
Benzene	471	1924	IGT-022
Toluene	100	477	IGT-022

Like methane, all of the constituents listed in Table 6 are susceptible to substantial metabolic degradation by *M. capsulatus* (Bath) and will be reduced substantially during the

[4] Chao and Attari (1995), Table 7, page 50;
[5] Cyclohexane was not measured in 2 of the 19 samples, identified as IGT-011 and IGT-012, which were the 2 samples analyzed the earliest in the survey. BTEX analytes were measured in these 2 samples but, like cyclohexane, BTEX is not included in the summary statistics. The reason for this is that a relatively low resolution GC column was used for these earliest analyses, so that cyclohexane, C8 hydrocarbons, and C9 hydrocarbons were not adequately separated from benzene and toluene, respectively. The issue was remedied to measure these analytes in the subsequent 17 samples.
[6] Black & Veatch (2021). Natural Gas technical Paper. Prepared for Calysta, 7 pp.
[7] Conversion from ppmv to ppmw was accomplished by multiplying the concentration of each constituent (ppmv) in a natural gas sample by its molecular weight and adding the products of the multiplications together, then dividing the product of each constituent by the sum of the products and multiplying the result by 10^6.

manufacturing of FeedKind®. For example, Colby *et al.* (1977) demonstrated the very broad substrate specificity that the methane mono-oxygenase of *M. capsulatus* possesses, which catalyzes a variety of different oxygen-incorporation reactions.[8] Colby *et al.* (1977) showed that this mono-oxygenase effectively catalyzed the aerobic oxidation of C1, C2, C3, C4, C5, C6, C7 and C8 n-alkanes with a specific activity of 85, 63, 68, 68, 69, 39, 27, and 9 milli-units/mg protein, respectively, to produce the corresponding alcohols. In addition, they showed that this enzyme catalyzed cyclohexane, benzene, and toluene under the same conditions with a specific activity of 62, 62, and 52 milli-units/mg protein, respectively, to yield cyclohexanol, phenol, and benzyl alcohol, respectively. Thus, the safety assessment calculations presented below considers the ability of *M. capsulatus* to metabolically detoxify n-hexanes, other n-alkanes, cyclohexane, and other aromatics.

In addition, approximately 5% of the natural gas that enters the fermenter is off-gassed from the fermenter and is vented into the combustion chamber that operates at approximately 800°C, where no less than 99.5% of the volatile organic carbon (VOC), including any benzene and toluene that may be present in the off-gas, is decomposed to yield carbon dioxide (CO_2). Thus, essentially all of the n-alkanes, including n-hexane, in the off-gas will be destroyed in the combustion chamber. Unlike the cyclic and aromatic VOCs including cyclohexane, benzene and toluene, methane and n-alkanes are not subject to potential induced-dipole to induced-dipole interactions with the aromatic amino acids of proteins. However, the loss of n-alkanes through off-gassing was not accounted for in the calculations, which contributes to the conservatism of calculations.

In sum, we assumed, conservatively, that:

- 100% of n-hexane, cyclohexane, benzene and toluene that enter the fermenter with natural gas during fermentation are present in the FeedKind® biomass after centrifugation.
- Cyclohexane, benzene and toluene may accumulate in the biomass because of potential induced-dipole to induced-dipole interactions with the aromatic amino acids of the proteins of the biomass
- 100% of the “hexanes” that enter the fermenter is present as n-hexane and can accumulate in the biomass, although n-hexane is:
    - Not susceptible to induced-dipole to induced-dipole interactions.
    - A well-known neurotoxicant at sufficiently high inhalation concentrations but has not been demonstrated to be neurotoxic to humans by ingestion or dermal exposure.

Furthermore, there are at least 2 steps of the FeedKind® manufacturing process that substantially reduce the potential levels of any residual hexane, cyclohexane, and benzene that may remain in the finished product, namely the evaporation step and the spray drying step. The

[8] Colby J, Stirling DI, Dalton H (1977). The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath): Its ability to oxygenate n-alkanes, n-alkenes, ethers, and alicyclic, aromatic and heterocyclic compounds. Biochem. J. 165: 395-402; For review see Jiang H, Chen Y, Murrell JC, Jiang P, Zhang C, Xing X-H, Smith TJ (2010). Methanotrophs: Multifunctional bacteria with promising applications in environmental bioengineering. Biochem. Engineer. J. 49:277-288.

product is harvested continuously from the fermenter by conveying the culture to a centrifuge, which separates the biomass from the bulk of the culture medium and recycles approximately 52,000 kg water/hour back to the fermenter. The water that remains with the harvested biomass and is not recycled from the centrifuge to the fermenter is continuously replaced with fresh makeup water in the fermenter. During the post-centrifugation steps, on the other hand, substantial levels of water yet remaining with the harvested biomass after centrifugation are removed from the biomass at temperatures approximately equal to or greater than the boiling points of benzene (80.1°C), hexane (68°C), and cyclohexane, respectively. Specifically, we calculated that only 1.05% of the water removed from the fermenter/hour remains in FeedKind® after the evaporation and spray drying steps of the process at 80°C.

These values were calculated as follows:

- 1145 kg FeedKind® produced/fermenter/hour.[9]
- 5600 kg water removed from FeedKind® through evaporation after centrifugation.
- 3000 kg water removed from FeedKind® through spray drying after evaporation.
- 91.5 kg water retained in FeedKind®/fermenter/hour.[10]
- 1.05% of water present after centrifugation remains in FeedKind® after evaporation and spray drying.[11]

Thus, 98.95% of the water associated with the FeedKind® leaving the centrifuge is lost through the evaporation and spray drying steps to produced finished FeedKind®.

The enthalpies of evaporation and the boiling points of hexane, cyclohexane, and benzene are lower than the corresponding values for water, and the enthalpy of evaporation for toluene is lower than that of water, as shown in Table 7.[12]

Table 7: Boiling Points and Enthalpy of Vaporization of Selected Natural Gas Constituents

Constituent	Boiling Point (°C)	Molar Enthalpy of Vaporization (kJ/mol)	Sample ID
n-Hexane	68	31.5	B&V Example 1
Cyclohexane	80.7	33.5	IGT-082
Benzene	80.1	30.7	IGT-022
Toluene	110.6	38.1	IGT-022
Water	**100**	**40.7**	--

[9] (10,000 tonnes FeedKind® produced/fermenter/year x 1000 kg/tonne) ÷ [(24 hours/day x 7 days/week x 52 weeks/year] = 1145 kg produced/fermenter/hour.

[10] 8% water in finished FeedKind® x 1145 kg FeedKind® produced/fermenter/hour = 91.5 kg water in finished FeedKind®.

[11] (91.5 kg water retained in FeedKind®/fermenter/hour ÷ (5600 kg water removed from FeedKind® through evaporation after centrifugation + 3000 kg water removed from FeedKind® through spray drying after evaporation + 91.5 kg water retained in FeedKind®/fermenter/hour) x 100 = 1.05%

[12] Kotz JC, Treichel P (1999). Chemistry and Chemical Reactivity. 4th Edition, Saunders College Division.

It is reasonable to expect that n-hexane, cyclohexane and benzene will evaporate more readily than water when subjected to the same conditions because the boiling points and vaporization enthalpies of these substances are lower than the corresponding values for water. Thus, we assumed conservatively that, like water, 98.95% of each of these constituents associated with the FeedKind® leaving the centrifuge is lost through the subsequent evaporation and spray drying steps and 1.05% of each constituent remains in finished FeedKind®. We assumed, conservatively, that there is no loss of toluene because the boiling point of toluene exceeds that of water.

Table 8 presents the maximum concentration of selected natural constituents in finished FeedKind® and in salmonid food assumed in the screening level safety assessment calculations below.

Table 8: Maximum Concentrations of Natural Gas Constituents in FeedKind® and Salmonid Food

Constituent(s)	Natural Gas Sample ID	Maximum Concentration in Natural Gas (ppmw)	Percent Retained in FeedKind® (%)	Maximum Concentration in FeedKind® (ppm)[13]	Maximum Concentration in Finished Salmonid Food (ppm)[14]
Hexanes	IGT-041	(b) (4)	1.05%	(b) (4)	(b) (4)
Cyclohexane	IGT-082		1.05%		
Benzene	IGT-022		1.05%		
Toluene	IGT-022		100%		

Experiments with salmon liver microsomes have demonstrated that salmonids have the capacity to metabolize cyclohexane, benzene and chemically related compounds and, therefore eliminate these compounds effectively and rapidly. For example, Kennish *et al.* (1988) demonstrated that hepatic enzymes of adult Chinook salmon metabolized toluene to yield benzyl alcohol with very similar kinetics as Kennish *et al.* (1985) described earlier for the metabolism of cyclohexane by Coho salmon liver enzymes to yield cyclohexanol under the same optimal conditions.[15] Kennish *et al.* (1988) noted that optimal conditions of temperature (15°C to 25°C), pH and ionic strength for the catalysis of cyclohexane and toluene were identical across salmon species tested in their studies. Kennish *et al.* (1985) noted that the optimal temperature (i.e.

[13] Each 12-week production begins with injecting 20 kg/hr natural gas into the inoculated culture medium in the fermenter, followed by gradually ramping up the injection rate over the next 5 to 10 days to achieve a steady-state rate of approximately 2400 kg/hour natural gas. The time weighted average natural gas flow rate is approximately 2289 kg/hour over a 12-week cycle, assuming that the flow rate is ramped up from over the first 7 days of the cycle. Therefore, the proportion of natural gas consumed in the process to FeedKind® produced is approximately 2 on a per weight basis (i.e. 2289 kg/hour natural gas consumed ÷ 1145 kg/hour FeedKind® produced = 2 kg natural gas/kg FeedKind®). Thus, for example, 1924 ppmw benzene in natural gas x 1.05% benzene assumed to be retained in FeedKind® x 2 kg natural gas/kg FeedKind® = 40.4 ppm benzene assumed to be retained in FeedKind®.

[14] For example, 40 ppm benzene in FeedKind® x 18% maximum FeedKind® use level in salmonid food = 7.2 ppm benzene in salmonid feed.

[15] Kennish JM, Gillis D, Hotaling K (1988). Metabolic conversion of toluene and ethylbenzene by Pacific salmon microsomal preparations. Mar. Environ. Res. 24: 69-71; Kennish JM, Montoya C, Whitsett J, French JS. (1985). Metabolic conversion of cyclohexane by Pacific salmon microsomal preparations. Mar. Environ. Res. 17: 129-132.

20°C) yielding the maximum rate in salmon is substantially lower than the optimal temperature reported for mammalian systems, which is attributable to genetic, developmental and environmental factors.[16]

Roubal *et al.* (1977) reported that benzene was rapidly metabolized and eliminated from the bodies of young Coho salmon following intra-peritoneal (*i.p.*) injection of uniformly labelled ^{14}C-benzene (198 µCi/mg).[17] We estimate that the total dose of benzene administered to each fish in this study was approximately 12 mg/kg bw.[18] Injections *i.p.* bypass first-pass metabolism in the gut but not in the liver. Roubal *et al.* (1977) found only 0.066%, 0%, 0.02%, 0.01%, and 6.22% of the radioactivity administered to the fish in the flesh, brain, liver, gall bladder, and carcass, respectively, 6 hours post exposure (i.e. total ~6.3% of the administered dose remaining in the animals). Only 0.006%, 0%, 0%, and 0.22% of the radioactivity remained in the flesh, brain, liver, gall bladder, and carcass, respectively, 24-hours post-exposure. The results clearly demonstrated that benzene was readily metabolized in the liver and benzene and its metabolites were rapidly eliminated from the bodies of the fish after exposure.

Furthermore, sound U.S. and global aquacultural practices require fasting and feed withdrawal periods prior to slaughter. Benefits include complete gut evacuation, a clean digestive tract, good water quality by minimizing ammonia and fecal excretion during transport, reduced metabolism, and the elimination of xenobiotics, among other reasons.[19] Accordingly, for example, the quality regulations of Norwegian food laws indicate that the fish should be starved to empty the gut before harvesting to ensure proper hygiene for further processing. Among the benefits of this practice includes the reduction of physical activity, fighting among the fish related to the instinct to maintain dominance hierarchies, and stresses related to acute crowding and other factors during transportation.[20] The common current practice is to starve the fish for 3 to 4 days before harvest and, under low temperature conditions, the fish should be starved for at least 5 to 7 days pre-harvest. Thus, if there were any residual n-hexane, cyclohexane, benzene or toluene from natural gas in salmonid food containing FeedKind®, it is reasonable to expect that none of these substances would remain in the bodies of the fish after 3 or more days of starvation prior to transport and slaughter.

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[16] Kennish et al. (1985) cites Forlin L, Anderson T, Koivusaari U and Hansson T (1984). Influence of biological and environmental factors on hepatic steroid and xenobiotic metabolism in fish: Interaction with PCB and β-naphthoflavone. Mar. Environ. Res. 14: 47-58.

[17] Roubal WT, Collier K, Malins DC (1977). Accumulation and metabolism of carbon-14 labeled benzene, naphthalene, and anthracene by young coho salmon (Oncorhychus Kisutch). Arch. Environ. Contam. Toxicol. 5: 513-529.

[18] 2.5 µCi ^{14}C-benzene ÷ (198 µCi/mg benzene x 1 g bw) x 1000 g/kg = 12.6 mg benzene/kg bw; the body weight of fingerling Coho salmon was assumed to be similar to that reported by Luzzanna U, Hardy RW, Halver JR (1998). Dietary arginine requirement of fingerling coho salmon (Oncorhychus kisutch). Aquaculture 163: 137-150 (i.e. mean 0.9 ± 0.02 g S.E.M.).

[19] Waagbo R, JHorgensen SM, Timmerhaus G, Breck O, Olsvik PA (2017). Short-term starvation at low temperature prior to harvest does not impact the health and acute stress response of adult Atlantic salmon. Peer J 5:e3273; DOI 10.7717/peerj.3273: https://peerj.com/articles/3273.pdf.

[20] VKM (2008). Opinion of the Panel on Animals Health and Welfare of the Norwegian Scientific Committee for Food Safety: Transportation of fish within a closed system. VKM Report 2008: 23, 07/806-Final. 14 May 2008. 63 pp. (https://vkm no/download/18.d44969415d027c43cf154e6/1500390477876/577c2a6603.pdf).

Based on these published reports, we assumed, conservatively for our screening-level safety assessment calculations, that 0.066% (i.e. the percent of the administered radiolabel remaining in flesh after only 6 hours reported by Roubal *et al.* 1977) of the daily oral intake of benzene in salmonid food remains in the edible tissue of the fish when it is harvested and consumed. In addition, we assumed that other natural gas constituents are metabolized in the fish and/or by *M. capsulatus* to the same overall extent, based on the published reports of Kennish *et al.* (1985, 1988), Colby *et al.* (1977) and studies cited therein, which clearly demonstrated the capacity of fish liver enzymes and *M. Capsulatus* to metabolize these substances rapidly.

High-end exposures were estimated for human consumption of salmon and trout raised on diets containing 18% FeedKind® based on the highest calculated concentrations of natural gas constituents in the salmonid feed (Table 8). Additional assumptions included:

- Cumulative feed consumed by the target animal per weight of edible tissue (i.e. 1.77 and 2.14 kg feed/kg edible body weight for Atlantic salmon and trout, respectively)[21]
- 100% of the intake of each constituent from the feed accumulates in the edible fish tissue
- High chronic daily consumption of salmon or trout by humans is equal to the 90th percentile daily ingestion level of all finfish (i.e., 0.17 kg/day)[22]
- Body weight 70 kg[23]

Maximum estimated daily intakes (EDIs) of the constituents from the consumption of salmon and trout, based on these highly exaggerative assumptions, are presented in Table 9.[24]

Table 9: Maximum Estimated Daily Intake (EDI) of Natural Gas Constituents from Fish Consumption

Constituent(s)	Maximum Concentration in Edible Salmon Tissue (ppm)	Maximum Concentration in Edible Trout Tissue (ppm)	Maximum EDI from Salmon Consumption (mg/kg bw/day)	Maximum EDI from Trout Consumption (mg/kg bw/day)
Hexanes	(b) (4)			
Cyclohexane				
Benzene				
Toluene				

[21] *See* Table 2 in Fry JP, Mailloux NA, Love DC, Milli MC, Cao L (2018). Feed conversion efficiency in aquaculture: do we measure it correctly? Environ. Res. Lett. 13: 024017: https://iopscience.iop.org/article/10.1088/1748-9326/aaa273/pdf.

[22] *See* Table 2.055 in Smiciklas-Wright H, Mitchell DC, Mickle SJ, Cook AJ, Goldman JD (2002). USDA 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII 1994-1996).

[23] NRC (2005) specifies an MRL of 0.0003 mg Hg/kg bw/day for a 70-kg person

[24] For example, [7.27 ppm benzene in salmonid feed x 1.77 kg feed/kg edible salmon tissue x 0.066% benzene oral intake remaining in edible tissue x 0.17 kg salmon/day]/70 kg bw = 2.06 x 10^{-5} mg benzene/kg bw/day.

Toxicity reference values for risk assessment have been developed by US EPA Integrated Risk Information System (IRIS) Program for all of the substances assumed to remain in the edible tissue of salmonids fed FeedKind® at the highest use level in fish food (i.e. 18%), including hexane, cyclohexane, benzene and toluene. These toxicity levels include a cancer slope factor (CSF)[25] for benzene, non-cancer reference doses (RfDs)[26] for chronic oral exposures to benzene and toluene, and a reference concentrations (RfCs) for chronic inhalation exposure of n-hexane and cyclohexane. As noted above, oral exposures to n-hexane and cyclohexane, unlike inhalation exposures to these substances, have not been shown to be associated with oral toxicity or developmental toxicity, respectively. However, we derived a chronic oral RfDs from the inhalation RfCs for n-hexane and cyclohexane in an abundance of caution in this screening level safety assessment.[27] The toxicity values used in this assessment are presented in Table 10.

Table 10: Toxicity Values for Selected Natural Gas Constituents

Constituent(s)	Chronic Oral RfD (mg/kg bw/day)	Cancer Slope Factor $(mg/kg\ bw/day)^{-1}$	Critical Effect(s)	Reference
n-Hexane	0.2[28]	ND[29]	Peripheral neuropathy	n-Hexane | IRIS | US EPA
Cyclohexane	1.7[30]	ND	Reduced pup weights in 2-generation rat developmental toxicity test	Cyclohexane (CASRN 110-82-7) | IRIS | US EPA
Benzene	4×10^{-3}	0.015	Decreased lymphocyte count; leukemia	Benzene (CASRN 71-43-2) | IRIS | US EPA
Toluene	0.08	ND	Increased kidney weight in rats	Toluene (CASRN 108-88-3) | IRIS | US EPA

[25] Oral Slope Factor: "An upper bound, approximating a 95% confidence limit, on the increased cancer risk from a lifetime oral exposure to an agent. This estimate, usually expressed in units of proportion (of a population) affected per mg/kg-day, is generally reserved for use in the low-dose region of the dose-response relationship, that is, for exposures corresponding to risks less than 1 in 100." IRIS Glossary | Integrated Risk Information System | US EPA."

[26] Reference dose: An "estimate, with uncertainty spanning perhaps an order of magnitude, of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime"; IRIS Glossary | Integrated Risk Information System | US EPA.

[27] For approach to converting RfCs to RfD see, for example, https://rais.ornl.gov/tutorials/toxvals html#2.4%20Derivation%20of%20Inhalation%20RfDs%20and%20Slope%20Factors.

[28] n-Hexane RfD = 0.7 mg/m^3 RfC x 20 m^3/day ÷ 70 kg bw = 0.2 mg/kg bw/day.

[29] ND = not determined; there are no data indicating an association between cancer and human exposure to these substances.

[30] Cyclohexane RfD = 6 mg/m^3 RfC x 20 m^3/day ÷ 70 kg bw = 1.7 mg/kg bw/day.

The toxicity reference values presented in Table 10 were used to calculate the upper bound cancer risk estimate for benzene and hazard quotients (HQs)[31] for potential non-cancer effects presented in Table 11.

Table 11: Worst-Case Risk Estimates for EDI of Potential Gas Constituents through Fish Consumption

	Salmon consumption		Trout Consumption	
Constituent(s)	**HQ for Potential Non-Cancer Effects (unitless)**	**Upper-Bound Cancer Risk Estimate (unitless)**	**HQ for Potential Non-Cancer Effects (unitless)**	**Upper-Bound Cancer Risk Estimate (unitless)**
Hexanes	2.68×10^{-4}	NA	3.25×10^{-4}	NA
Cyclohexane	24.34×10^{-6}	NA	5.25×10^{-6}	NA
Benzene	5.16×10^{-3}	3.09×10^{-7}	6.230×10^{-3}	3.74×10^{-7}
Toluene	6.08×10^{-3}	NA	5.88×10^{-3}	NA

Table 12 presents the corresponding central tendency risk estimates calculated by substituting the highest concentration by the median concentration of each constituent of natural gas reported in Chao and Attari (1995).

Table 12: Central Tendency Risk Estimates for EDI of Gas Constituents through Fish Consumption

	Salmon consumption		Trout Consumption	
Constituent(s)	**HQ for Potential Non-Cancer Effects (unitless)**	**Upper-Bound Cancer Risk Estimate (unitless)**	**HQ for Potential Non-Cancer Effects (unitless)**	**Upper-Bound Cancer Risk Estimate (unitless)**
Hexanes	3.96×10^{-5}	NA	4.79×10^{-5}	NA
Cyclohexane	7.14×10^{-7}	NA	8.64×10^{-7}	NA
Benzene	7.68×10^{-5}	4.61×10^{-9}	9.29×10^{-5}	5.57×10^{-9}
Toluene	3.66×10^{-4}	NA	3.54×10^{-4}	NA

The results presented in Table 11 and Table 12 clearly show the upper bound cancer risk estimate for benzene is less than 10^{-6} (i.e. *de minimis*), and all HQs for all natural gas constituents would be orders of magnitude less than 1 even at the maximum concentrations of constituents reported in natural gas and exaggerative worst case exposure assumptions. Thus, there is no reasonable expectation of harm associated with the consumption of salmonids fed FeedKind® up to the highest use level in salmonid food (i.e. 18%).

The results of the safety assessment presented above also support the safety of the target animals, namely salmonid. This is because, in addition to the exaggerative exposure assumptions, the toxicity values used to estimate the non-cancer and cancer risks are at least 300-fold less than the no observed effect levels (NOAELs) or Benchmark Dose Low (BMDL = lower

[31] Hazard quotient: the ratio of the potential exposure to a substance (i.e. the EDI) and the level at which no adverse effects are expected (i.e. the RfD).

confidence limit of the BMD) for n-hexane, cyclohexane, and benzene and 3000-fold less than the BMDL for toluene from animal studies, which indicates that the margin of safety for the target animals is orders of magnitude greater than the margin of safety for the protection of human health.

As noted above, the natural gas available to users in the U.S. may contain a wide range of benzene, toluene, cyclohexane and hexanes concentrations, based on the survey of Chao and Attari (1995) and benzene and toluene concentrations based on the 3 examples representing the spectrum of natural gas products in the U.S. reported by Black and Veatch (2021). These values are presented in Table 13 below.

Table 13: Range and Median of Constituent Concentrations Reported in Natural Gas in the US

	Chao and Attari (1993)		Black and Veatch (2021)	
Constituent(s)	**Range (ppmv)**	**Median(ppmv)**	**Range (ppmv)**	**Median (ppmv)**
Hexanes	<0.2 to 1156	170	NR[32]	NR
Cyclohexane	<0.2 to 146	24	NR	NR
Benzene	<0.2 to 471	7	44 to 470	230
Toluene	<0.1 to 100	6	5 to 100	18

In coordination with its suppliers, Calysta will monitor the natural gas received to its facility with gas chromatography to ensure that the benzene concentration does not exceed 40 ppmv. Further, in the site selection process for production facilities, Calysta will preferentially choose gas supplies and regional locations with reliably low contaminant levels. Calysta will not use natural gas that contains ≥40 ppmv benzene to produce FeedKind®. This approach will also ensure that the natural gas used to produce FeedKind® will also contain toluene and other constituents at the lower end of the respective ranges reported for these compounds in natural gas in the U.S., and substantially lower than the 40 ppmv threshold for benzene because:

- The levels of compounds like toluene are characteristically lower than the corresponding benzene concentration in the natural gas.
- The predominant method in North America for the removal of aromatics and natural gas liquids (NGLs) from natural gas is cryogenic expansion. The efficiency of this removal process is largely a function of the boiling point of the respective gases. As benzene has the lowest boiling point of the targeted aromatic contaminants in natural gas, a maximum limit on benzene will in effect limit toluene and cyclohexane as well.

Ensuring that the concentration of benzene does not exceed 40 ppmv ensures that the natural gas used to produce FeedKind® contains no more than approximately 8.5% of the maximum concentration of benzene reported in the natural gas surveys, and that FeedKind®

[32] Black and Veatch (2021) presented data for "n-Hexane+", which covers all alkanes ≥C6 in size, and did not provide values specifically for hexanes or cyclohexane.

cannot possibly contain more than approximately 3.4 ppm benzene.[33] No benzene or related compounds are expected to be present in FeedKind®. However, it is clear that the worst-case risk estimates presented in Table 11 for benzene and the other natural gas constituents are overestimated by at least an additional factor of 10.

In an abundance of caution, we calculated risk estimates assuming that the concentrations of the constituents in the natural gas used to produce FeedKind® is 8.5% of the respective maximum concentrations reported in the natural gas, using the same approach as above for non-cancer endpoints except that we assumed that none of the constituents is metabolized in the fermenter or detoxified by the fish and that all of the constituents consumed by the fish with FeedKind®. The results are presented in Table 14.

Table 14: Worst-Case Risk Estimates for Potential Gas Constituents Assuming No Metabolism in the Fermenter or Detoxification in Fish

	Salmon	Trout
Constituent(s)	**HQ for Potential Non-Cancer Effects (unitless)**	**HQ for Potential Non-Cancer Effects (unitless)**
Hexanes	0.0346	0.0418
Cyclohexane	0.000559	0.000676
Benzene	0.664	0.803
Toluene	0.783	0.758

All of the HQs for these constituents are less than 1, indicating that there is no reasonable expectation of harm from the high-end consumption of salmon or trout fed food containing up to 18% FeedKind® manufactured using natural gas containing no more than 40 ppmv benzene.

In addition, we calculated risk estimates for the cancer endpoint for benzene based on the same assumptions, except that we assumed that 1%, rather than 99.934% (i.e. 100%-0.066%), of the dose was not effectively detoxified in the bodies of the fish. The resultant cancer risk estimates were 3.98×10^{-7} and 4.82×10^{-7} for salmon and trout consumption, respectively. Again, these risk estimates are *de minimis*.

Overall, the results of these calculations, based on the exaggerative exposure assumptions and the safety factors used to calculate potential lifetime human health risks, show that there is no reasonable expectation of harm to the target animals or to consumers from the intended use of FeedKind® in fish food.

We do not have data to characterize the constituents of the natural gas used to manufacture the BioProtein® that was tested in the animal studies. However, it is clear from the analysis presented above that maintaining a threshold of 40 ppmv benzene in the natural gas used

[33] 40 ppmv benzene threshold ÷ 471 ppmv benzene maximum reported x 100 = 8.49%; 40.4 ppm benzene in FeedKind® from 471 ppmv maximum reported benzene natural gas x 8.49% = 3.43 ppm maximum benzene concentration in FeedKind®.

to manufacture FeedKind® used as intended presents no safety concern to salmon or to consumers.

NITRIC ACID COMMENTS AND QUESTIONS

SPECIFICATIONS

1. **The specifications for nitric acid in the notice do not include a test for heavy metals. The notifier should provide nitric acid specifications appropriate for use in the manufacture of animal food, including specifications for iron, mercury, arsenic, cadmium and lead.**

Information from Calysta's nitric acid supplier regarding the heavy metals analysis of the nitric acid ingredient is provided in Appendix 6. While this does not constitute a "specification" *per se*, it does indicate that for all of metals listed above (except iron) the concentration is below detection limits (<0.1 ppm for arsenic, cadmium and lead, 0.005 ppb for mercury). Iron is present at very low levels (0.23 ppm) however this is a negligible amount of iron in comparison to the iron added to the fermentation media (as iron sulfate) which is a required nutrient for the growth of the microbes. Nitric acid is used at a rate of approximately 0.07 mg per kg of finished feed, with iron being present in the nitric acid at 0.23 ppm. Iron sulfate is added to ensure an iron concentration of 300-350 ppm, and therefore any potential contribution to the overall amount of iron present from nitric acid (0.23 ppm in the nitric acid) is negligible

STABILITY

2. **The notifier should explain why byproducts of photochemical reactions that may take place during storage of nitric acid do not pose a safety concern when the nitric acid is used in the fermentation process.**

Nitric acid is stored in opaque (stainless steel) containers and is not stored in direct sunlight. Therefore, the possible photochemical reactions will not take place and there is no related safety concern.

Appendices

Appendix 1 – Sciantec Analytical Methods – Summary

Appendix 2 – Determination of Heavy Metals by ICP-MS

Appendix 3 – Nickel by ICP-MS Validation Summary

Appendix 4 – Direct Enumeration of Yeasts and Moulds by the Colony Count Method using OGYE Agar

Appendix 5 – Tyrosine PT Data 2019 – Present

Appendix 6 – Nitric Acid Metals Content

Appendix 7 – 03.16.21 CVM Email Response to Calysta Follow-up Questions

SECOND REVISED Part 7 - References

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Cerrito, Chelsea

From: Drozen, Melvin S. <Drozen@khlaw.com>
Sent: Wednesday, June 16, 2021 10:29 AM
To: Carlacci, Louis
Cc: Animalfood-premarket; Skasko, Mark; M. S. Tomas Belloso Ph. D. (tbelloso@calysta.com)
Subject: [EXTERNAL] Third Amendment to AGRN 40/Responses to CVM Questions on Second Amendment
Attachments: 06.16.21_Third Amendment to Calysta FeedKind GRASN.zip; M-000091-Z-0007-OT-AA_MTG_dsign.pdf

Dear Dr. Lou,

On behalf of Calysta, Inc., attached please find the Third Amendment to GRAS Notice for Dried *Methylococcus Capsulatus* product (hereinafter "FeedKind®"), which was originally submitted to CVM on February 28, 2020. The Amendment addresses questions raised during the June 3, 2021 teleconference and corresponding meeting minutes sent to us on June 4(attached) regarding the (1) the heat kill curve illustrated in Figure 2 of the second amendment, (2) the specification for Mesophilic plate count, and (3) the justification for the ash content acceptance criteria. This third amendment also provides an updated specification table for FeedKind®.

The attached zip file contains (1) the Third Amendment to the GRASN and (2) associated appendices. No additional references were cited in the Third Amendment, therefore we did not provide a revised Part 7 reference list.

Please let us know if you have any questions or if you have any difficulty accessing the materials. In the meantime, we look forward to receiving a "no questions" letter in the foreseeable future.

Best,
Mel.

From: Drozen, Melvin S.
Sent: Friday, June 4, 2021 11:52 AM
To: Carlacci, Louis <Louis.Carlacci@fda.hhs.gov>
Cc: Skasko, Mark <Mark.Skasko@fda.hhs.gov>; Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>; M. S. Tomas Belloso Ph. D. (tbelloso@calysta.com) <tbelloso@calysta.com>
Subject: FW: [EXTERNAL] RE: Question on Second Amendment to GRAS Notice No. AGRN 40

Hi Lou,

Thanks. We will let you know if we have any questions. Best. Mel.

Melvin S. Drozen
Partner
tel: +1 202.434.4222 | fax: +1 202.434.4646 | drozen@khlaw.com
1001 G Street NW, Suite 500 West | Washington, DC 20001

From: Carlacci, Louis <Louis.Carlacci@fda.hhs.gov>
Sent: Friday, June 4, 2021 11:15 AM
To: Drozen, Melvin S. <Drozen@khlaw.com>
Cc: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>; Skasko, Mark <Mark.Skasko@fda.hhs.gov>; M. S. Tomas Belloso Ph. D. (tbelloso@calysta.com) <tbelloso@calysta.com>
Subject: RE: [EXTERNAL] RE: Question on Second Amendment to GRAS Notice No. AGRN 40

Hi.
Please find attached our letter and meeting minutes for the June 3, 2021 teleconference.
Thanks.
Lou

Louis Carlacci, Ph.D.
Chemist

Center for Veterinary Medicine
Office of Surveillance and Compliance
Division of Animal Feeds
U.S. Food and Drug Administration
Tel: 240-402-2921
louis.carlacci@fda.hhs.gov

From: Drozen, Melvin S. <Drozen@khlaw.com>
Sent: Thursday, June 03, 2021 3:24 PM
To: Carlacci, Louis <Louis.Carlacci@fda.hhs.gov>
Cc: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>; Skasko, Mark <Mark.Skasko@fda.hhs.gov>; M. S. Tomas Belloso Ph. D. (tbelloso@calysta.com) <tbelloso@calysta.com>
Subject: [EXTERNAL] RE: Question on Second Amendment to GRAS Notice No. AGRN 40

Hi Lou,

Thanks to you and Mark for your time today. We plan to move forward in preparing responses to your questions and submit them in an amendment. In the meantime, if you can send us the minutes of the meeting via email, that will help us in putting together the responses and amendment. This will also confirm that providing the minutes via email is fine.

Regards,

Mel.

From: Carlacci, Louis <Louis.Carlacci@fda.hhs.gov>
Sent: Wednesday, June 2, 2021 3:26 PM
To: Drozen, Melvin S. <Drozen@khlaw.com>
Cc: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>; Skasko, Mark <Mark.Skasko@fda.hhs.gov>
Subject: Question on Second Amendment to GRAS Notice No. AGRN 40

Hi Mel.

Please provide a time that I can use to arrange a conference call to ask a few questions on the CMC information in the last amendment. These questions should be clearly addressed in a short amendment. Mark Skasko (Team leader on the CMC team) and I will be the only ones on the call on the CVM side.

Thanks.

Lou

Louis Carlacci, Ph.D.
Chemist

Center for Veterinary Medicine
Office of Surveillance and Compliance
Division of Animal Feeds
U.S. Food and Drug Administration
Tel: 240-402-2921
louis.carlacci@fda.hhs.gov

THIRD AMENDMENT TO GRAS NOTICE FOR DRIED *METHYLOCOCCUS CAPSULATUS* PRODUCT

Submitted by: Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington, DC 20001

On behalf of our client

Calysta, Inc.
1140 O'Brien Drive
Menlo Park, CA 94025
United States

June 16, 2021

I. Introduction

The purpose of this amendment is to address questions raised by the U.S. Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM) during the June 3, 2021 teleconference, and corresponding meeting minutes (dated June 4, 2021), regarding the February 28, 2020 submission of the Generally Recognized As Safe (GRAS) Notice for Calysta, Inc.'s Dried *Methylococcus capsulatus* Product (hereinafter "FeedKind®"). A first amendment was submitted on July 17, 2020 and the GRASN was filed on August 13, 2020 and designated as GRAS Notice No. AGRN 40. A second amendment was submitted on April 11, 2021. This third amendment specifically addresses CVM's questions regarding (1) the heat kill curve illustrated in Figure 2 of the second amendment, (2) the specification for Mesophilic plate count, and (3) the justification for the ash content acceptance criteria. This third amendment also provides an updated specification table for FeedKind®.

For clarity, we have repeated CVM's questions in **bold** below, followed by our responses.

HEAT KILL CURVE

1. **Specifically, CVM has questions on the heat kill curve illustrated in Figure 2 and questions on the specification acceptance criteria.**

   CVM asked that Figure 2 in the amendment date April 11, 2021 be redone as logarithm of cell count versus temperature to illustrate the heat kill curve of the production organism. CVM asked that the data points used for the plot be provided.

As requested, Calysta has revised Figure 2. "*M. capsulatus* Heat Kill Curve" from the second amendment, dated April 11, 2021, to be presented with a logarithmic scale on the Y axis and temperature (°C) on the X axis. The updated figure and associated data points are provided in Appendix 1.

MESOPHILIC PLATE COUNT

2. **CVM noted that the specification acceptance criteria for Mesophilic plate count contained in the amendment dated April 11, 2021 are not aligned with the results of the batch analysis, and that the amendment did not contain adequate justification for this. The firm indicated that additional analysis results that show the absence of several pathogenic microorganisms could be provided to demonstrate safety of potential higher microbial counts. The firm indicated that the analysis of other batches demonstrates the need for the proposed specification acceptance criteria for Mesophilic plate count.**

Calysta has further reviewed the historical mesophilic plate counts available to the firm and has determined that a specification of ≤ 200,000 cfu/g is most appropriate. Calysta has previously tested 276 separate lots of FeedKind® produced during research and development phases to optimize the production process. Of these lots, 8 results were extremely high (> 500,000 cfu/g) and were excluded from further analyses. The average of the remaining lots is ~31,000 cfu/g with a standard deviation of ~82,000 (note for the purposes of these calculations

lots which were below the LOD of < 10 cfu/g were treated as if the analysis result was 10 cfu/g). The average plus 2 SD is 195,000 and supports a final specification of ≤ 200,000 cfu/g. This specification would cover 90% of historical lots. Individual lot data is contained in Appendix 2.

To further address the safety of this specification we note that the total mesophilic plate count is not a safety concern. As described in the submitted notice, the elevated fermentation temperatures and defined fermentation media which contains a limited carbon source make contamination of the fermentation process with pathogenic microbes exceptionally unlikely. Further processing steps, including a heat treatment step, further reduce the likelihood of such contamination. Additionally, a review of the most similar previous animal GRAS notices (*i.e.* microbial biomass ingredients) indicates that Calysta's specification is not out of line with previous notices for which CVM has provided "no questions" letters. KnipBio has submitted two separate GRAS Notices (AGRN 26 and 33) for "Dried *Methylobacterium extorquens* biomass," neither of which contain a specification related to total bacterial counts. DSM's Notice (AGRN 20) for "Inactivated modified *Saccharomyces cerevisiae*" set a specification for total bacterial count of ≤ 10^6 cfu/mL, which is far higher than the specification set by Calysta.

ASH CONTENT ACCEPTANCE CRITERIA

3. **CVM also noted that the ash content acceptance criteria is not aligned with the batch analysis results and that the amendment did not contain adequate justification for this. CVM noted that based on ash content batch results provided, four standard deviations above the mean ash content (which is 10% ash) contains 99.95% of the population following a normal distribution. The firm indicated that the proposed acceptance criteria for ash content is needed and that more analysis results to justify the acceptance criteria for ash content could be provided. CVM requested that the firm provide an updated specification table containing a summary of the tests, acceptance criteria, and analytical method citations to capture revisions incurred through the amendment process.**

Appendix 3 contains analyses of 289 lots. This data shows that a true average value for ash is 8.2% with a standard deviation of 2%. The average plus 2 SD is 12.2% and supports a specification of 12%. Ash fluctuates predictably due to fermentation stage and productivity. Startup and low productivity levels in the fermenter deliver higher ash level while high productivity or steady state operations have lower ash levels. The representative samples had low ash because they happened to be taken during periods of high productivity. Given that ash is primarily salts and minerals present in the media, and higher ash is not seen as a health risk because Calysta concurrently monitors for heavy metals and contaminants directly, we believe that leaving the ash specification at 12% is appropriate. Ash is not used as a proxy for any other measurements.

SPECIFICATION TABLE

Below we provide an updated specifications table for FeedKind® which reflects all of the changes made subsequent to the initial GRAS Notice submission on February 28, 2020. The updates include the following:

- A specification for Mercury
- An updated method for total mesophilic plate count
- Lowered specification for mesophilic plate count of ≤ 200,000 cfu/g
- Lowered specification for mold of ≤ 1,000 cfu/g
- Lowered specification for yeast of ≤ 2,000 cfu/g

It is Calysta's belief that the updated specification table encompasses all agreed upon changes from the previous amendments.

UPDATED FeedKind® Specifications

Specifications				
Chemical Composition	**Minimum**	**Maximum**	**Units**	**Test Method**
Crude Protein		(b) (4)	% dry weight	DUMAS method
Crude Fat			% dry weight	modified Weibull Acid Hydrolysis Method
Crude Fiber			% dry weight	AOCS Ba 6a-05, Ba 6-84 AOAC 962.09, S 1022 using Gravimetry
Ash			% dry weight	AOAC 942.05, S 1024 using Gravimetry
Moisture			w/w	AOAC 934.01, 930.15, S 1024 using Gravimetry

Nickel	(b) (4)	*mg/kg*	ICP-MS Internal Method
Mercury		*mg/kg*	ICP-MS Internal Method
Microbiological Limits	**Limits**		**Test Method**
Mesophilic Aerobic Plate Count	(b) (4)		EN ISO 4833:2013
Mold			AOAC 997.02; FDA/BAM Chapter 18
Yeast			AOAC 997.02; FDA/BAM Chapter 18

Average of Calculated CFU/ml OD:

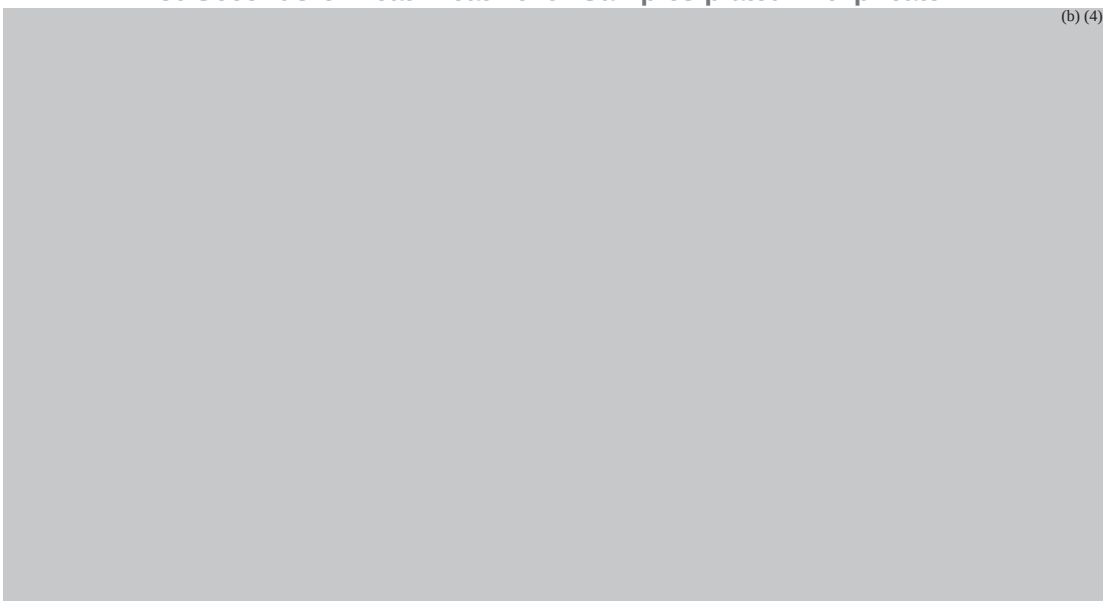
MC Bath Heat Treatment Experiment 02
30 Seconds of Heat Treatment. Samples plated in triplicate.

(b) (4)

Average of Calculated CFU/ml OD:

MC Bath Heat Treatment Experiment 02
30 Seconds of Heat Treatment. Samples plated in triplicate.

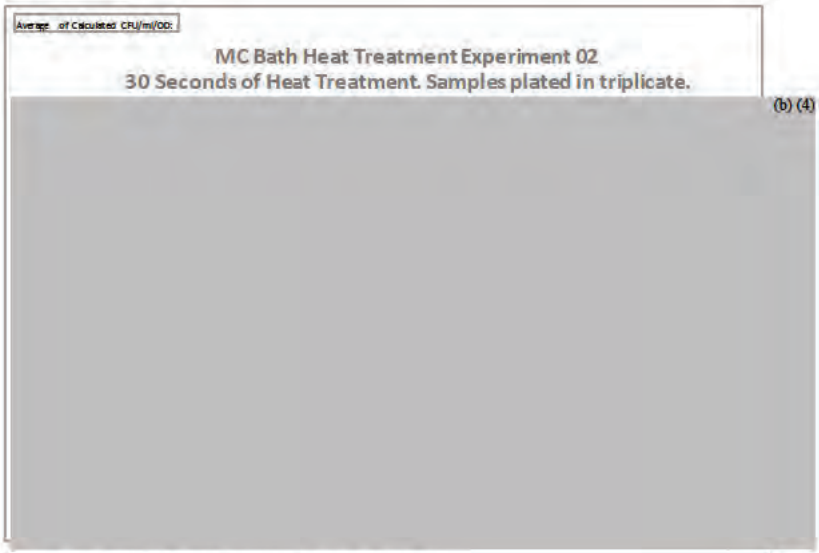
(b) (4)



Row Labels	StdDev of Calculated CFU/ml/OD:
Methylococcus capsulatus (Bath)	1485056324
Grand Total	**1485056324**

Row Labels	Average of Calculated CFU/ml/OD:
5000554	554293425.9
Methylococcus capsulatus (Bath)	554293425.9
23	3444444444
50	916666666.7
55	618055555.6
65	9375000
70	99166.66667
75	0
80	0
90	0
100	0
Grand Total	554293425.9

Row Labels	StdDev of Calculated CFU/ml/OD:
5000554	1106015678
Methylococcus capsulatus (Bath)	1106015678
23	637831250.2
50	85264091.37
55	79558605.82
65	3608439.182
70	10112.21758
75	0
80	0
90	0
100	0
Grand Total	1106015678



(b) (4)

*Samples were heated at the tested temperature for exactly 30 seconds.

Actual Number of Samples:	Replicate Number:	Sample Description:	Strain#	Targeted OD Normalized Sample:	Actual Final OD of Sample:	Volume of Heat Treated Sample (ml)	Heat Treatment Temperature (C):	Incubation Duration:	Plating Media:	Plating Dilution	Volume Plated (ml):	Plating Method:	Plating Incubation Temperature (C):	Plating Incubation Time (Hrs):	CFU Count:	Calculated CFU/ml:	Calculated CFU/ml/OD:
1	1	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	23	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			(b) (4)
2	2	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	23	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
3	3	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	23	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
4	1	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	50	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
5	2	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	50	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
6	3	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	50	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
7	1	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	55	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
8	2	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	55	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
9	3	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	55	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
10	1	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	60	30 seconds	MMS2.0	100000	0.1	Sterile Glass Beads	42	120			
11	2	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	60	30 seconds	MMS2.0	100000	0.1	Sterile Glass Beads	42	120			
12	3	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	60	30 seconds	MMS2.0	100000	0.1	Sterile Glass Beads	42	120			
13	1	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	65	30 seconds	MMS2.0	100000	0.1	Sterile Glass Beads	42	120			
14	2	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	65	30 seconds	MMS2.0	100000	0.1	Sterile Glass Beads	42	120			
15	3	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	65	30 seconds	MMS2.0	100000	0.1	Sterile Glass Beads	42	120			
16	1	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	70	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
17	2	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	70	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
18	3	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	70	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
19	1	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	75	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
20	2	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	75	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
21	3	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	75	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
22	1	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	80	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
23	2	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	80	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
24	3	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	80	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
25	1	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	90	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
26	2	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	90	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
27	3	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	90	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
28	1	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	100	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
29	2	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	100	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
30	3	Methylococcus capsulatus (Bath)	S000554	1	0.96	0.08	100	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			

TPC		Average	30972.86
10		Median	980
10		Standard Deviation	82314.01
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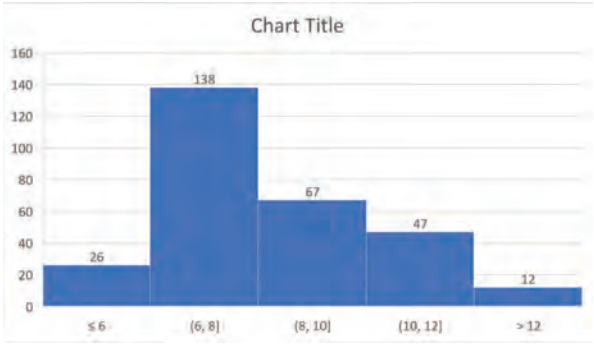
BATCH NUMBER	Ash (g/100 g)	Percentile
TEES-005/24	4.6	0.00%
TPP-009/04	5.1	0.30%
TPP-009/06	5.3	0.60%
TEES-004/18	5.4	1.00%
TPP-004/01	5.4	1.00%
TPP-009/05	5.4	1.00%
TPP-013/06	5.4	1.00%
TEES-004/12	5.6	2.40%
TEES-004/15	5.6	2.40%
TEES-004/17	5.6	2.40%
TPP-007/08	5.7	3.40%
TPP-013/02	5.7	3.40%
TEES-004/2	5.8	4.10%
TEES-004/59	5.8	4.10%
TPP-004/08	5.8	4.10%
TPP-004/09	5.8	4.10%
TPP-013/04	5.8	4.10%
TPP-013/05	5.8	4.10%
TEES-009/79	5.9	6.20%
TPP-004/02	5.9	6.20%
TPP-004/12	5.9	6.20%
TPP-004/13	5.9	6.20%
TEES-004/3	6	7.60%
TEES-009/102	6	7.60%
TPP-004/14	6	7.60%
TPP-013/07	6	7.60%
TEES-004/6	6.1	8.90%
TEES-009/74	6.1	8.90%
TPP-004/04	6.1	8.90%
TPP-009/01	6.1	8.90%
TPP-009/09	6.1	8.90%
TEES-004/19	6.2	10.70%
TEES-006/RESEARCH2	6.2	10.70%
TEES-009/75	6.2	10.70%
TPP-004/03	6.2	10.70%
TEES-004/23	6.3	12.10%
TEES-004/4	6.3	12.10%
TEES-009/73	6.3	12.10%
TPP-004/01	6.3	12.10%
TPP-007/09	6.3	12.10%
TPP-013/03	6.3	12.10%
TEES-004/11	6.4	14.10%
TEES-007/02b	6.4	14.10%
TEES-009/36	6.4	14.10%
TEES-009/37	6.4	14.10%
TEES-009/93	6.4	14.10%
TEES-009/101	6.5	15.90%
TEES-005/01	6.5	15.90%
TEES-009/34	6.5	15.90%
TEES-009/87	6.5	15.90%
TPP-007/01	6.5	15.90%
TPP-007/05	6.5	15.90%
TPP-007/07	6.5	15.90%
TPP-009/08	6.5	15.90%
TPP-013/08	6.5	15.90%
TEES-004/10	6.6	19.00%
TEES-004/16	6.6	19.00%
TEES-004/35	6.6	19.00%
TEES-004/39	6.6	19.00%
TEES-004/40	6.6	19.00%
TEES-004/49	6.6	19.00%
TEES-009/1	6.6	19.00%
TEES-009/33	6.6	19.00%
TEES-009/42	6.6	19.00%
TEES-009/76	6.6	19.00%
TPP-004/11	6.6	19.00%
TPP-007/06	6.6	19.00%
TPP-007/10	6.6	19.00%
TEES-004/13	6.7	23.50%
TEES-004/45	6.7	23.50%
TEES-004/5	6.7	23.50%
TEES-004/56	6.7	23.50%
TEES-006/RESEARCH1	6.7	23.50%

	Overall	In-spec samples only
Mean	8.2	7.97
Median	7.6	7.45
Std. Deviatio ı	2.0	1.7

Total	290	
6	26	9%
6 to 8	138	57%
8 to 10	67	80%
10 to 12	47	96%
>12	13	100%



TEES-009/38	6.7	23.50%
TEES-009/40	6.7	23.50%
TEES-009/68	6.7	23.50%
TEES-009/84	6.7	23.50%
TPP-007/03	6.7	23.50%
TEES-004/47	6.8	26.90%
TEES-004/48	6.8	26.90%
TEES-004/53	6.8	26.90%
TEES-004/55	6.8	26.90%
TEES-004/58	6.8	26.90%
TEES-007/01b	6.8	26.90%
TEES-009/28	6.8	26.90%
TEES-009/39	6.8	26.90%
TEES-009/49	6.8	26.90%
TEES-009/78	6.8	26.90%
TEES-009/83	6.8	26.90%
TPP-008/01	6.8	26.90%
TEES-004/51	6.84	31.10%
TEES-004/46	6.85	31.40%
TEES-004/28	6.9	31.80%
TEES-004/30	6.9	31.80%
TEES-004/37	6.9	31.80%
TEES-004/42	6.9	31.80%
TEES-004/44	6.9	31.80%
TEES-004/51	6.9	31.80%
TEES-004/52	6.9	31.80%
TEES-005/29	6.9	31.80%
TEES-009/14	6.9	31.80%
TEES-009/81	6.9	31.80%
TEES-009/82	6.9	31.80%
TEES-009/88	6.9	31.80%
TPP-004/06	6.9	31.80%
TEES-004/36	6.92	36.30%
TEES-004/37	6.95	36.60%
TEES-004/49	6.97	37.00%
TEES-004/26	7	37.30%
TEES-004/41	7	37.30%
TEES-009/32	7	37.30%
TEES-009/41	7	37.30%
TEES-009/70	7	37.30%
TEES-009/77	7	37.30%
TEES-004/35	7.08	39.40%
TEES-005/26	7.1	39.70%
TEES-009/12	7.1	39.70%
TEES-009/71	7.1	39.70%
TEES-004/30	7.18	40.80%
TEES-004/38	7.18	40.80%
TEES-004/47	7.18	40.80%
TEES-004/32	7.2	41.80%
TEES-004/33	7.2	41.80%
TEES-009/30	7.2	41.80%
TEES-009/72	7.2	41.80%
TEES-009/85	7.2	41.80%
TPP-004/07	7.2	41.80%
TPP-004/10	7.2	41.80%
TEES-004/28	7.23	44.20%
TEES-004/40	7.23	44.20%
TEES-004/46	7.3	44.90%
TPP-009/03	7.3	44.90%
TEES-004/55	7.34	45.60%
TEES-004/39	7.38	46.00%
TEES-005/27	7.4	46.30%
TEES-005/28	7.4	46.30%
TEES-009/29	7.4	46.30%
TEES-009/63	7.4	46.30%
TEES-009/80	7.4	46.30%
TEES-009/86	7.5	48.00%
TPP-007/02	7.5	48.00%
TPP-007/04	7.5	48.00%
TEES-004/31	7.51	49.10%
TEES-005/25	7.6	49.40%
TEES-009/15	7.6	49.40%
TEES-009/21	7.6	49.40%
TEES-009/31	7.6	49.40%
TEES-009/19	7.7	50.80%
TEES-009/20	7.7	50.80%

TEES-009/26	7.7	50.80%
TEES-004/52	7.71	51.90%
TEES-004/42	7.74	52.20%
TEES-005/23	7.8	52.50%
TEES-004/24	7.9	52.90%
TEES-004/34	7.9	52.90%
TEES-004/54	7.9	52.90%
TEES-004/9	7.9	52.90%
TEES-004/53	7.94	54.30%
TEES-004/48	7.98	54.60%
TEES-004/27	8	55.00%
TEES-004/38	8	55.00%
TEES-009/52	8	55.00%
TEES-009/58	8	55.00%
TEES-009/59	8	55.00%
TEES-004/43	8.02	56.70%
TEES-004/54	8.03	57.00%
TEES-004/31	8.1	57.40%
TEES-009/61	8.1	57.40%
TEES-009/62	8.1	57.40%
TPP-004/05	8.1	57.40%
TEES-005/03	8.2	58.80%
TEES-009/64	8.2	58.80%
TEES-009/67	8.2	58.80%
TEES-004/25	8.3	59.80%
TEES-009/16	8.3	59.80%
TEES-009/25	8.3	59.80%
TEES-009/51	8.3	59.80%
TPP-011/01	8.3	59.80%
TEES-005/41	8.4	61.50%
TEES-005/42	8.4	61.50%
TEES-009/17	8.4	61.50%
TEES-009/24	8.4	61.50%
TEES-005/38	8.5	62.90%
TEES-005/40	8.5	62.90%
TEES-005/43	8.5	62.90%
TEES-009/18	8.5	62.90%
TEES-009/23	8.5	62.90%
TPP-008/02	8.5	62.90%
TEES-004/44	8.55	65.00%
TEES-005/31	8.6	65.30%
TEES-005/35	8.6	65.30%
TEES-005/39	8.6	65.30%
TEES-005/47	8.6	65.30%
TEES-009/22	8.6	65.30%
TEES-009/53	8.6	65.30%
TPP-013/01	8.6	65.30%
TEES-005/36	8.7	67.80%
TEES-009/35	8.7	67.80%
TEES-009/54	8.7	67.80%
TEES-009/55	8.7	67.80%
TEES-005/45	8.8	69.20%
TEES-009/60	8.8	69.20%
TEES-005/44	8.9	69.80%
TEES-005/48	8.9	69.80%
TEES-009/69	8.9	69.80%
TEES-004/45	9.03	70.90%
TEES-004/26	9.07	71.20%
TEES-005/46	9.1	71.60%
TEES-009/27	9.1	71.60%
TEES-004/36	9.2	72.30%
TEES-009/56	9.2	72.30%
TEES-004/57	9.25	73.00%
TEES-005/13	9.3	73.30%
TEES-005/17	9.3	73.30%
TEES-009/57	9.3	73.30%
TEES-009/39	9.4	74.30%
TEES-005/30	9.5	74.70%
TEES-005/37	9.5	74.70%
TEES-004/50	9.6	75.40%
TEES-004/22	9.62	75.70%
TEES-005/18	9.7	76.10%
TEES-004/14	9.8	76.40%
TEES-004/43	9.8	76.40%
TEES-005/05	9.8	76.40%
TEES-005/10	9.8	76.40%

TEES-005/51	9.8	76.40%
TEES-009/90	9.8	76.40%
TEES-005/33	9.9	78.50%
TEES-009/50	9.9	78.50%
TPP-008/03	9.9	78.50%
TEES-009/66	10	79.50%
TEES-005/12	10.1	79.90%
TEES-005/50	10.1	79.90%
TEES-004/8	10.12	80.60%
TEES-005/08	10 2	80.90%
TEES-005/54	10 2	80.90%
TEES-005/49	10 3	81.60%
TEES-009/44	10 3	81.60%
TEES-009/89	10 3	81.60%
TEES-009/91	10 3	81.60%
TEES-005/02	10.4	83.00%
TEES-005/52	10.4	83.00%
TEES-009/45	10.4	83.00%
TEES-004/7	10 5	84.00%
TEES-005/06	10 5	84.00%
TEES-005/11	10 5	84.00%
TEES-005/21	10 5	84.00%
TEES-009/10	10 6	85.40%
TEES-009/48	10 6	85.40%
TEES-009/9	10 6	85.40%
TEES-005/20	10.7	86.50%
TEES-009/65	10,7	86.50%
TEES-009/2	10 8	87.10%
TEES-009/46	10 8	87.10%
TEES-005/15	10 9	87.80%
TEES-009/97	10 9	87.80%
TEES-005/19	11	88.50%
TEES-005/53	11	88.50%
TEES-005/22	11.1	89.20%
TEES-009/43	11.1	89.20%
TEES-009/94	11.1	89.20%
TEES-004/56	11.16	90.30%
TEES-004/50	11.24	90.60%
TPP-009/02	11 3	91.00%
TEES-004/41	11.36	91.30%
TEES-004/29	11.38	91.60%
TEES-009/11	11.4	92.00%
TEES-009/47	11.4	92.00%
TEES-009/95	11 5	92.70%
TEES-009/96	11 5	92.70%
TEES-005/16	11 6	93.40%
TEES-009/7	11 6	93.40%
TEES-009/98	11 6	93.40%
TEES-009/12	11.7	94.40%
TEES-004/59	11.72	94.80%
TEES-009/92	11 8	95.10%
TEES-009/101	11 9	95.50%
TEES-005/32	12	95.80%
TEES-005/34	12 2	96.10%
TEES-009/99	12 3	96.50%
TEES-009/44	12 4	96.80%
TEES-009/45	12 4	96.80%
TEES-009/43	12 5	97.50%
TEES-009/100	12 6	97.90%
TEES-009/3	13.1	98.20%
TEES-009/4	14	98.60%
TEES-009/6	14	98.60%
TEES-009/5	14 5	99.30%
TEES-009/8	14 8	99.60%
TEES-009/13	15.1	100.00%

Sciantec

GRAS Notice M000091Z0007
Dried *Methylococcus capsulatus* Product
for use as a source of protein in salmonid species
Calysta, Inc.[1]

Memorandum of June 3, 2021 Teleconference

FDA-CVM Participants	**Firm Participants, Keller and Heckman LLP [2]**
Dr. Mark Skasko, HFV-224	Mr. Melvin S. Drozen
Dr. Louis Carlacci, HFV-224	Dr. Preston A. Fulmer
	Ms. Jill M. Mahoney
	Dr. Ivan J. Boyer
	Firm Participants, Calysta, Inc.
	Dr. Tomas Belloso
	Ms. Lori Giver
	Mr. Allan LeBlanc

Background

Calysta, Inc. (the notifier) submitted a generally recognized as safe (GRAS) notice dated February 28, 2020 (M-000091-A-0000) through its representative Mr. Melvin S. Drozen, Keller and Heckman LLP. This notice informs the Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM) of the notifier's conclusion that Dried *Methylococcus capsulatus* Product (MCP) is GRAS through scientific procedures as a source of protein in food for salmonid species at levels up to 18% of the diet. Following an April 23, 2020 meeting with the notifier (M-000091-Z-0001) and after receiving an amendment dated July 17, 2020 (M-000091-T-0002), the notice was filed on August 13, 2020 (M-000091-N-0003) and designated as GRAS Notice No. AGRN 40.

Meeting Notes

The purpose of this meeting is to request information to clarify questions identified in the amendment dated April 11, 2021 and discuss how these could be addressed. Specifically, CVM has questions on the heat kill curve illustrated in Figure 2 and questions on the specification acceptance criteria.

CVM asked that Figure 2 in the amendment date April 11, 2021 be redone as logarithm of cell count versus temperature to illustrate the heat kill curve of the production organism. CVM asked that the data points used for the plot be provided.

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[1] 1140 O'Brien Drive, Menlo Park, California 94025; T: 314-368-7114.

[2] Regulatory Counsel for the Notifier: 1001 G Street, N.W., Suite 500 West, Washington, D.C. 20001; T: 202-434-4100; F: 202-434-4646; Contact: Mr. Melvin S. Drozen, Partner; 202-434-4222; e-mail: drozen@khlaw.com.

CVM noted that the specification acceptance criteria for Mesophilic plate count contained in the amendment dated April 11, 2021 are not aligned with the results of the batch analysis, and that the amendment did not contain adequate justification for this. The firm indicated that additional analysis results that show the absence of several pathogenic microorganisms could be provided to demonstrate safety of potential higher microbial counts. The firm indicated that the analysis of other batches demonstrates the need for the proposed specification acceptance criteria for Mesophilic plate count. CVM also noted that the ash content acceptance criteria is not aligned with the batch analysis results and that the amendment did not contain adequate justification for this. CVM noted that based on ash content batch results provided, four standard deviations above the mean ash content (which is 10% ash) contains 99.95% of the population following a normal distribution. The firm indicated that the proposed acceptance criteria for ash content is needed and that more analysis results to justify the acceptance criteria for ash content could be provided. CVM requested that the firm provide an updated specification table containing a summary of the tests, acceptance criteria, and analytical method citations to capture revisions incurred through the amendment process.

Process Moving Forward and Timeline

CVM explained that the notifier may provide an amendment to address the questions and comments raised by CVM during the June 3, 2021 teleconference. CVM stated that minutes of this teleconference will be sent to the notifier via e-mail by June 7, 2021. The amendment can be sent to animalfood-premarket@fda.hhs.gov within 2 weeks. If no amendment is received, CVM will proceed with evaluation of the notice.

{see appended electronic signature page}
Louis Carlacci, Ph.D.
Chemist
Chemistry, Manufacturing, and Controls Team, HFV-224
Division of Animal Feeds

Electronic Signature Addendum for Submission ID

M-000091-Z-0007-OT-AA

Signing Authority (Role)	Letter Date
Louis Carlacci: Reviewer	6/4/2021

This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.