KnipBio

Lawrence Feinberg, CEO
110 Canal street
Lowell, MA 01852

June 17th 2019 Revised August 9, 2019

To the attention of

David Edwards, Ph.D.
Division of Animal Feeds, HFV-220,
7519 Standish Place,
Rockville, MD 20855

Re: Gras Notification submission for "**Dried** *Methylobacterium extorquens* biomass for use as a replacement for soybean or fish meal at levels up to 6% of diets for crustacean species in aquaculture"

Dear Dr. Edwards,

Please find associated with this letter, a notification of GRAS status for Dried *Methylobacterium extorquens* biomass for use as an ingredient in crustaceans feed. This submission has been revised based on a conversation between Dr. Smedley and you, on August 6, 2019 at the AAFCO meeting.

We are incorporating by reference the KnipBio Animal GRAS Notice submission (AGRN 26) and all amendments covering the use of dried *M. extorquens* biomass in aquaculture feeds. This submission received a letter of no questions on February 11, 2019. The current GRAS notice is specific to the use of dried *M. extorquens* biomass in crustacean feed, and we refer to the specific sections and page numbers of AGRN 26 that are being relied on for support of the notice.

Methylobacterium extorquens is not a pathogen, does not produces toxins, is used at relatively low inclusion rates (6%) and did not show any harm in all our trials. KnipBio does not anticipate any safety issue for human consumption of crustaceans fed KBM.

Dr. Kristi Smedley is KnipBio's point of contact and, please do not hesitate to contact her should you need additional information or to ask follow-up questions.

We thank you in advance for your consideration of this notification.

Sincerely,

Lawrence Feinberg, CEO

RECEIVED
AUG 16 2019
By____________

KnipBio

Dr. Kristi Smedley's contact information

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
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GRAS Notice

Dried *Methylobacterium extorquens* biomass

for use as a replacement for soybean or fish meal at levels up to 6% of diets for crustacean species in aquaculture

Submitted by:
KnipBio, Inc.
110 Canal Street
Lowell, MA 01854
Phone: (978) 636-5647

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Part 1. Introductory Information

(1) The undersigned is hereby submitting a GRAS Notice in accordance with 21 CFR Subpart E, Section 570.

(2) The name and address of the organization is:

KnipBio, Inc.
110 Canal Street
Lowell, MA 01854
Phone: (978) 636-5647
info@knipbio.com

(3) Name of the notified substance:

Dried *Methylobacterium extorquens* biomass

(4) Intended conditions of use of the notified substance:

The substance will be used as a as a source of protein for crustacean species at a level up to 6% of the diet, in crustacean.

(5) KnipBio, Inc. has concluded, through scientific procedures in accordance with §570.30(a) and (b), that the substance has GRAS status for the intended use.

(6) KnipBio, Inc. has concluded that the notified substance is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on the aforestated conclusion that the notified substance is GRAS under the conditions of its intended use.

(7) If the Center for Veterinary Medicine (CVM) asks to see the data and information that are the basis for this conclusion of GRAS status, either during or after its evaluation of this notice, KnipBio Inc. will:

(i) agree to make the data and information available to CVM; and
(ii) agree to both of the following procedures for making the data and information available to CVM:
(A) Upon CVM's request, KnipBio, Inc. will allow CVM to review and copy the data and information during customary business hours at the above-stated address, where these data and information will be available to CVM; and
(B) Upon CVM's request, KnipBio, Inc. will provide CVM with a complete copy of the data and information either in an electronic format that is accessible for its evaluation or on paper.

(8) Certain of the data and information in Parts 2 through 7 of this GRAS Notice are exempt from disclosure under the provisions of §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 in black-bordered boxes.

(9) The undersigned hereby certifies that, to the best of KnipBio's knowledge, this GRAS Notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to KnipBio and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

(10) The name and title of the person who has signed this GRAS Notice is:

By: ______________________

Name: Larry Feinberg

Title: Chief Executive Officer

Address: KnipBio, Inc.
110 Canal Street
Lowell, MA 01854

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

Cf. Part 2 of AGRN26

(a) Scientific data and information that identifies the notified substance. Same as Part 2 (a) AGRN26 (pages 7-8), Appendix 2-1

(b) A description of the method of manufacture of the notified substance in sufficient detail to evaluate the safety of the notified substance as manufactured;

Same as Part 2 (b) AGRN26 (page 8).

(b)(1) Construction and Characterization of the Production Microorganism (Pre-fermentation)

Same as Part 2 (b) (1)AGRN26 (pages 8-9), Appendices 2-2 and 2-3. August 8, 2018 Supplemental information (pages 2-6 and pages 20-21).

(b)(2) Fermentation of the Production Microorganism to Manufacture the Notified Substance

Same as Part 2 (b) (2)AGRN26 (pages 9-10), August 8, 2018 Supplemental information (pages 7-9, and 21).

(c) Specifications for material that is of appropriate grade for use in animal food.

Same as Part 2 (c) AGRN26 (page 10-16) August 8, 2018 supplemental information (pages 9-11), September 27, 2018 supplemental information revised Table 3 (page 3)(and supportive information, pages 2-5).

(d) The information specific to stability is also referenced as found in Part 2 (c) AGRN26 (pages 16-20).*When necessary to demonstrate safety, relevant data and information bearing on the physical or other technical effect the notified substance is intended to produce, including the quantity of the notified substance required to produce such effect.*

Dried *Methylobacterium extorquens* biomass is a valuable source of protein for use in crustacean feeds when used at levels up to 6% of the feed. The analytical data demonstrate that the product will be at least 50% protein, with a balanced complement of amino acids. Part 6-5 of this Notice provides a summary information on the composition of the product. A full description of the product was the object of **AGRN26** and its amendments, as well as in the Supplementals from August 9 and September 27, 2019. There is a number of similar biomass products that are used as protein sources in animal (including aquaculture) feed listed as AAFCO defined ingredients 36.15 and 36.16 and assorted fermentation products (AAFCO 36.2-36.13). Consideration of microbial proteins as valuable for feeds have been covered in a number of recent publications (Nasseri *et al.*, 2011; Matassa *et al.* , 2016; Anupama and Ravindra, 2000; Ritala *et al.*, 2017; Qiu *et al.*, 2018; Martínez-Córdova *et al.*, 2017). Moreover, there is significant new interest in feeding bacterial biomass in aquaculture feed (cf for review Sharifuzzaman and Austin, 2017; Banerjee and Ray, 2017; Das *et al.*, 2017; Chauhan and Singh, 2019; Wang *et al.*, 2019). KnipBio has studied the availability of the notified substance for use in aquaculture with a number of studies with various aquaculture species. Many of these studies have been published or presented at international congress (Pujol-Baxley, 2018a, b). All are considered supportive of the analytical data demonstrating the nutritive value of the protein source.

The notified substance has utility in the use as a protein supplement for crustacean aquaculture. The pivotal study that supports the utility, showing that dried *Methylobacterium extorquens* biomass provides available protein needed for the growth of crustacean in aquaculture, is attached in **Appendix 2-1** (Tlusty *et al.*, 2017). Tlusty *et al.* describes a feeding study with Pacific white shrimp (*Litopenaeus vannamei*), looking at animal's growth and consumer taste preference, a study for which FDA has reviewed the protocol. In the described study, animals performed equivalently when fed diets containing dried *Methylobacterium extorquens* biomass as when fed a standard aquaculture diet. In **AGRN26**, KnipBio presented data supporting the use of the substance as a valuable source of protein at a 10% inclusion for finfish species. In this notice, KnipBio present data supporting the use of the substance as a valuable source of protein at a 6% inclusion for crustaceans.

Tlusty *et al.* (2017) includes a comprehensive summary of the methodology used in these studies. The results reported in the paper demonstrate the potential broad applicability of dried *Methylobacterium extorquens* biomass, made from *Methylobacterium extorquens*, as a viable protein source for use in aquafeeds. When fed to crustacean up to 6.3% inclusion it resulted in equivalent performance in weight gain and specific growth rate (SGR). The feed conversion ratio of shrimp was best when there was 50% substitution with the notified substance, yet growth (weight gain and SGR) was greatest in the control diets. One possible reason for this discrepancy is that the consumption of feed between the different diets was not equivalent. Shrimp are bottom feeders and it is very likely that the shrimp that were fed the high inclusion biomass received fewer available pellets in the water column, which may account for the reduced weight gain and SGR. As stated in the manuscript," there were air bubbles in the shrimp diet SHR-KH (100% KBM replacement) that did not sink as well as the other two diets that did not have air bubbles (SHR-LK and SHR-C). While all pellets were consumed by the shrimps, those fed SHR-HK would have needed to swim in the water column to retrieve some of the pellets, while those fed SHR-LK and SHR-C mostly fed off the tank floor, and such increased activity may account for the reduced weight gain and SGR. Another possibility is that the SHR-KH diet may have been less palatable, as the shrimps had a lower feed efficiency of this diet than the other diets. Whether this is an absence of an attractant not replaced in SHR-KH or the absence of a critical nutritional component like methionine in the diet ([redacted] (b) (4) [redacted] (b) (4)), the exact cause is unknown at this time".

In other completed studies (in the attached **Appendix 2-2**) the notified substance KBM was fed at 0, 1, 2, 4, 6, 12, 13.3 and 26.6 % of the feed. The studies included a growth trial (using the four identified diets) as well as a digestibility study and demonstrated no significative difference in growth up to the 6% inclusion and the control group when expressed as biomass, mean weight, weight gain or feed conversion ratio.

Table 2-1 in the submitted GRAS Notice and the companion **Appendix 2-2**, as well as the published Tlusty *et al.* study (2017), clearly demonstrate that the notified substance is an available and safe protein source when fed at levels up to 6% of the feed for crustacean.

Table 2-1. Summary of Shrimp Feeding Studies with the Notified Substance.

Date and location	Goal	# of animals	% Notified Substance	Trial Length	Start size gm	Survival %	Conclusion
[redacted] (b) (4) (Tlusty *et al.* 2017)	FM replacement-Growth and survival	720	50-100	60-105 days			No effect on survival. Slight growth defect w/ 100% replacement. No difference in human taste trial.
[redacted] (b) (4) [redacted] (b) (4)(A. Davis, Appendix 2-2)	Soybean meal replacement. Growth and survival	96	6 - 12	6 weeks	~ 1.51	> 97	No statistical difference in weight gain compared to control diet.
[redacted] (b) (4) [redacted] (b) (4)(A. Davis, Appendix 2-2)	Replacement of SBM. Retention efficiency of AA.	240	1 - 12	6 weeks	~ 0.98	92.5 - 100	No statistical difference up to 6% inclusion.
[redacted] (b) (4) [redacted] (b) (4) (A. Davis, Appendix 2-2)	Replacement of SBM. Digestibility	240	6-26.6	6 weeks	~ 0.15	97.5 – 100	No statistical difference up to 12% inclusion.

The data shown in Tlusty *et al.* (2017), as well as procedures and data for additional studies conducted by KnipBio to date, are shown in **Appendix 2-2. Table 2-1** above summarizes the results of the studies described in Tlusty *et al.*, as well as other studies that KnipBio has commissioned, in which shrimp were fed the notified substance. In this Table, the studies published in the Tlusty paper are highlighted in blue. The other studies have not been published, and in some cases, were not designed to produce publishable data, but they all show that the notified substance caused no harmful effects on the crustacean. Full study records for these experiments, particularly including the studies published in Tlusty *et al.* manuscript can be made available by KnipBio at FDA's request.

The data presented in this Section and its Appendices clearly show that the notified substance has utility in providing a source of protein for the target crustacean species: shrimp (Pacific white shrimps, *Litopenaeus vannamei*). Furthermore, for reasons explained in detail in the "**Narrative**" section below, KnipBio contends that the data from investigations support the broader use of the notified substance up to 6% for all crustacean feed, with the shrimp species named above fulfilling all of the required criteria of covering a broad diversity of crustacean species that are well-studied, sensitive to testing, and commercially relevant.

Part 3. Target animal and human exposures.

In this Part 3 of the Notice, KnipBio provides data and information about exposure to the target animal and to humans consuming human food derived from food-producing animals.

(a) Exposure to the target animal.

(a)(1) The amount of the notified substance that different target animal species are likely to consume in the animal food (including drinking water) as part of the animal's total diet, including the intended use and all other sources in the total diet

The notified substance is dried *Methylobacterium extorquens* biomass This substance is characterized by a protein content of 50-53% and is suitable for use as a replacement for soybean or fish meal protein to constitute up to 6% of the total diet in crustacean feed (Pacific white shrimp, *Litopenaeus vannamei*).

(a)(2) When applicable, the amount of any other substance that is expected to be formed in or on food because of the use of the notified substance (e.g., hydrolytic products or reaction products)

KnipBio believes that there will be no other substances formed in or on food because of the use of the notified substance, including any possible hydrolytic products, reaction products or other such substances.

(a)(3) When applicable, the amount of any other substance that is present with the notified substance either naturally or due to its manufacture (e.g., contaminants or by-products)

The microbial strain on which the notified substance is based, *Methylobacterium extorquens*, is a natural producer of polyhydroxybutyrates (PHBs). The notified substance is therefore expected to contain certain amounts of PHBs, which might range from 15-25% depending on the fermentation conditions. As summarized below from **AGRN26** (pages 22-29) and amendment from August 9, 2018 (pages 14-16), KnipBio believes that the expected levels of PHBs in the notified substance will be well below the levels that might be expected to be harmful to the target species.

Other substances that might be present in the notified substance due to its manufacture are methanol (used as a feedstock in the fermentation) and formaldehyde. As discussed in section Part3 (a)(3) of **AGRN26** (pages 22-29) and amendments from August 9, 2018 (pages 11, 16-20) and September 27, 2018 (pages 3-5), KnipBio believes that the expected levels of both compounds in the notified substance will be well below the levels that might be expected to be harmful to the target species.

M. extorquens is a natural producer of the C30 class of carotenoids, but these compounds, when ingested by aquatic animals, do not impart color to the flesh (Takaichi, 2009; Konovalova *et al.*, 2007).

The data and information that is relied on to establish the amounts of these substances in the notified substance were presented in the following section of **AGRN26.**

(a)(4) The data and information you rely on to establish the amount of the notified substance and the amounts of any other substance in accordance with paragraphs (a)(1) through (a)(3) of this section that different target animal species are likely to consume in the animal food (including drinking water) as part of the animal's total diet

Information showing the composition of the notified substance, including its main components, protein, moisture, fat and ash, any other substances that might be present in the notified substance as well as the analytical methods used to determine the composition has been presented in **AGRN26** Section Part 3 (a)(4) pages 23- 28 and in the revised supplemented data provided on August 8, 2018, pages 9-11, 14-18 and September 27, 2018, pages 1-5.

Concentration of PHBs in the notified substance.

The concentration of PHBs in the notified substance is described in **AGRN26** pages 23-24 in **Appendix 3-2** as well as in the Supplemental data from August 9, 2018 (pages 14-16).

Concentration of carotenoids in the notified substance

The concentration of carotenoids in the notified substance is detailed in **AGRN 26** pages 24-26 and the analytical methods provided in **Appendix 3-3** as well as in the Supplemental data from August 9, 2018 (pages 1-7).

Concentration of methanol and formaldehyde in the notified substance.

The concentration of methanol and formaldehyde in the notified substance has been described in **AGRN26** pages 26-28 and in **Appendix-4.** More details and methods verification were provided in the Supplemental information from August 9, 2018 pages 11, 16-18, 18-20 and September 27, 2018, pages 3-9.

The concentrations of methanol and formaldehyde, which might arise in the notified substance due to its method of manufacture, are expected to be no more than 0.03% (300 ppm) and 0.0025% (25 ppm) respectively. Both substances are therefore expected to be present at levels below the maximum allowed under applicable regulations: for example, 21 CFR 573.460(b)(1) for formaldehyde and methanol, as discussed in Section 3(a)(3) of **AGRN26** and as specified in the Supplemental information dated September 27, 2018 (pages 7-9).

(b) When the intended use is in food for food-producing animals, you must provide:

> *(b)(1) The potential quantities of any residues that humans may be exposed to in edible animal tissues, including:*
> *(i) Residues of the notified substance;*
> *(ii) Residues of any other substance that is expected to be formed in or on the animal food because of the use of the notified substance; and*
> *(iii) Residues from any other substance that is present with the notified substance whether naturally, due to its manufacture (e.g., contaminants or by-products), or produced as a metabolite in edible animal tissues when the notified substance is consumed by a food producing animal*

As with all ingested protein sources, the content of the notified substance that is being provided to the target species will be digested into amino acids or other available compounds and metabolized by the animal to be used in the growth of crustacean. Therefore there would not be any safety concerns regarding human consumption of the product, nor is there the need for any specific exposure assessment for humans.

With regard to the potential presence of PHBs in the notified substance, as discussed above it is expected that any such concentrations in the substance will not exceed 25%, and that such concentrations will be diluted at least 16-fold because the notified substance will be incorporated into crustacean diets at no greater than 6% of the total diet. It is further expected that the PHBs will be metabolized or digested in the GI tract of the shrimp to some extent (Defoirdt *et al.*, 2009; Liu *et al.*, 2010; Gao *et al.*, 2019; Gowda and Shivakumar, 2019; Defoirdt *et al.*, 2018) and further that, as a high molecular weight substance, PHBs are unlikely to be deposited in fish flesh to any appreciable degree. It is thus expected that PHB would also unlikely be deposited in crustacean and therefore human exposure to any potential PHB presence will be extremely low.

> *(b)(2) The data and information you rely on to establish, in accordance with paragraph (b)(1) of this section, the potential quantities of any residues that humans may be exposed to in edible animal tissues.*

As noted above, at the level of inclusion in crustacean aquaculture feed that KnipBio intends (up to 6% w/w in the total diet) the maximum level of PHB to which the target species would be exposed would be approximately 1-1.5%. The literature summarized in **Part 6** of the Notification (as well as **Part 6** of **AGRN26**) provides evidence that PHBs would be degraded to fatty acids in crustacean intestinal tract, such that human consumers of crustacean that have been fed the notified substance would not be expected to be exposed to significant levels of PHBs, and so there would be no adverse effects on human consumers of such crustacean.

Part 4. Self-limiting levels of use.

In circumstances where the amount of the notified substance that can be added to animal food is limited because animal food containing levels of the notified substance above a particular level would become unpalatable or technologically impractical, in Part 4 of your GRAS notice you must include data and information on such self-limiting levels of use.

There are no self-limiting levels of use for the notified substance.

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

If the statutory basis for your conclusion of GRAS status is through experience based on common use in animal food, in Part 5 of your GRAS notice you must include evidence of a substantial history of consumption of the notified substance for food use by a significant number of animals of the species to which the substance is intended to be fed prior to January 1, 1958; and evidence of a substantial history of consumption by humans consuming human foods derived from food-producing animals prior to January 1, 1958.

This GRAS Notice is not based on common use in food prior to 1958.

Part 6. Narrative.

Summary

The data and literature described throughout this dossier unequivocally support KnipBio's conclusion that use of dried *Methylobacterium extorquens* biomass, when incorporated at 6% or less of aquaculture feed for crustacean is safe. This conclusion is supported by a number of studies, described above, in which shrimp were fed this biomass preparation, with no adverse effects seen, and no effect on one of the most sensitive parameters: growth of the animal. This conclusion is also supported by ample evidence from the literature and other experimental data derived from KnipBio and others.

The following sections summarize the basis for this determination.

(a)(1) You must explain why the data and information in your notice provide a basis for your view that the notified substance is safe under the conditions of its intended use for both the target animal and for humans consuming human food derived from food producing animals.

The data and information included in this Notice provide a basis for KnipBio's view that the notified substance is safe under the conditions of its intended use for both the target animal and for humans consuming human food derived from food producing animals. The following is a summary of this information (also described in AGRN26). Except as explicitly noted, all the data and information described below is available to the public.

1) Target Species; Suitability of the Notified Substance for Aquaculture Feed.

According to the United Nations Food and Agriculture Organization (UN-FAO), there are as many as 580 aquatic species currently farmed all over the world, representing the protein sector with the greatest genetic diversity (Ababouch *et al.*, 2016). Aquaculture species having economic value encompasses finfishes, mollusks, crustaceans, amphibians, invertebrates and aquatic plants. In all, the world market for aquaculture products for the decade between 2005-2014 in aquaculture grew by ~6%.
(http://www.nmfs.noaa.gov/aquaculture/aquaculture_in_us.html).
Moreover the global aquaculture market is experiencing robust growth, and is expected to accelerate through the year 2022, according to a report from the market research firm Technavio. (https://www.seafoodsource.com/features/technavio-report-global-aquaculture-markets-growth-accelerating-through-2022.) An important part of the aquaculture market is the crustacean market that comprises marine shrimp, fresh water shrimp (prawn), lobsters, crayfish, crabs and brine shrimps.
According to the analysis done by Persistence Market Research, [redacted] (b) (4)

https://www.persistencemarketresearch.com/market-research/crustacean-market.asp

Marine shrimp continued to dominate crustacean aquaculture, with shrimp production in 2000 reaching >1 MMT (66.0% of global crustacean aquaculture production) and valued at >US $B7(73.4% of total value). Aquaculture currently provides just over a quarter (26.1%) of total global shrimp landings. The main cultivated species are the giant tiger prawn (*Penaeus monodon*), the fleshy prawn (*P. chinensis*) and the whiteleg shrimp (*L. vannamei*), these three species accounting for over 86% of total shrimp aquaculture production in 2000. As of 2015, *L. vannamei* was representing more than 43% of the World Production of Shrimp (Capture Fisheries and aquaculture combined) and~70-75% of the shrimp species in aquaculture, as presented in **Fig. 6-1**.

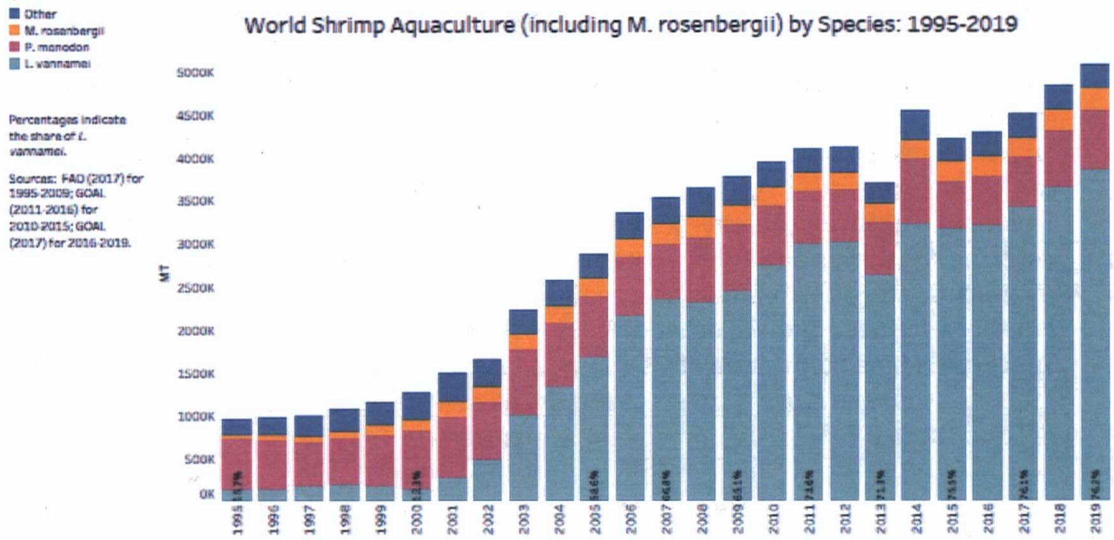

Fig.6-1: World shrimp aquaculture by species: 1995-2019 (Anderson, Valderrama & Jori, GOAL 2017)

Here, KnipBio proposes that the data from investigations of the species reported in this GRAS Notice, (*Lito*)*Penaeus vannamei* (Pacific white shrimp), is sufficient to support the use of the notified substance for crustacean feed, with this species fulfilling all of the required criteria of covering a broad diversity of crustacean that are well-studied, sensitive to testing, and commercially relevant.

A Google Scholar search of "*(Lito)Penaeus vannamei*" returns approximately 51,700 results, encompassing, but not limited to, physiology, microbiology, aquaculture, disease. Shrimp are particularly sensitive to diseases, including bacterial and viral infections due to the low auto-immune memory generally afflicting crustaceans (Thitamadee *et al.,* 2016; Flegel, 2009). While there a few variety of species raised for economical gain in crustacean aquaculture, Pacific white shrimp (*L. vannamei*) represent approximately 75% of the total commercial value for the shrimp industry (Tacon, 2002; Anderson, J., GOAL 2017).

- Pacific white shrimp are an important model for crustaceans. Production is conducted responsibly, in highly monitored laboratory settings, in compliance with the Institutional Animal Care and Use Committee (IACUC).
- Broodstock of *P. vannamei* can be collected naturally, harvested from ponds, or purchased from tank-reared SPF/SPR broodstock from the United States of America representing a diverse genetic pool (http://www.fao.org/tempref/FI/CDrom/aquaculture/I1129m/file/en/en_whitelegshrimp.htm).
- The life cycle and rearing of Pacific white shrimp is well understood (http://www.fao.org/fishery/culturedspecies/Penaeus_vannamei/en).
- Digestion and transit time in the animal gut have been studied (Beseres *et al.*, 2006).
- *P. vannamei* is very efficient at utilizing Biofloc in ponds or consuming formulated feeds representing a diverse, omnivorous diet. Under intensive culture conditions, lower protein content feeds are used representing a greater range of diet formulations. For example, lower cost, less-processed terrestrial proteins like soy can be incorporated (Yun *et al.*, 2016; Kim *et al.*, 2014).
- The major disease problems suffered by *P. vannamei* include viruses and bacteria entering the ponds and other systems. *P. vannamei* have little to no immune-memory so the effect of nutrition against invasive microorganisms or viruses is more pronounced than in other animal systems (Selvin, 2010; Flegel, 2009).
- Super-intensive cultivation strategies (*i.e.* indoor raceway systems enclosed in greenhouses) are biosecure, eco-friendly, have a small ecological footprint and can produce cost-efficient, quality shrimp (Suantika *et al.,* 2018).

For these reasons, KnipBio believes that the data and information presented in this dossier, while primarily focused on Pacific white shrimp is sufficient to support the finding that the notified substance is Generally Recognized as Safe for use in any crustacean aquaculture feed, when incorporated at 6% or less of the feed.

2) *Methylobacterium extorquens.*

a) Natural history, biology

As described in **AGRN26** (pages 34-35), the notified substance is dried *Methylobacterium extorquens* biomass. The α-proteobacterium *M. extorquens* is a facultative aerobic Gram-negative naturally occurring bacterium found in nature as a leaf symbiont (Balachandar *et al.*, 2008; Kutschera, 2007) which has the ability to use C1 compounds, such as methanol, as a carbon source (Chistoserdova and Kalyuzhnaya., 2018; Šmejkalová *et al.*, 2010; Andersen, 2014).

The complete genome sequences are now available for 7 strains of *M. extorquens* as well as about 20 other species in the genus (NCBI) Reference sequences can be found on the following websites:

[redacted] (b) (4)

http://hamap.expasy.org/proteomes/METEP.html
http://www.ebi.ac.uk/Tools/dbfetch/expasyfetch?CP000908

[redacted] (b) (4)

https://biocyc.org/

The ability of *M. extorquens* to use renewable carbon sources such as methanol makes it a promising candidate for new biotechnology development (Dourado *et al.*, 2015; Strong *et al.*, 2016).

A great body of work has been published about improvement of bioprocess for increasing production of valuable molecules using C1 compounds as a principal carbon source (Delaney *et al.*, 2013; Peyraud *et al.*, 2012; Lamarche *et al.*, 2018; Zhang *et al.*, 2018). Scale-up in large fermenters has been published for production either of PHA/PHB or biomass for agriculture applications (Bogosian, 2016; Miguez *et al.*, 2006; Groleau *et al.*, 1994).

b) Evidence of lack of pathogenicity or toxicity.

KnipBio believes that the production organism for the notified substance, *Methylobacterium extorquens*, is a safe organism that will not pose any health risks to the target species that will be fed the notified substance, or to human consumers who eat such aquatic animals. We base this belief on extensive literature searches which show that there is no evidence that this species is pathogenic, toxic or allergenic, as previously described in **AGRN26** (pages 35-39).

Pathogenicity

An updated Google Scholar search "*Methylobacterium extorquens* pathogenicity" on April 24, 2019 yielded 364 new hits compared to the search done on October 2017 (**AGRN26, Appendix 6-1**), most of which related to plant pathogenicity. When the word plant was removed from the search, 59 new hits are generated (**Appendix 3-1**). Nothing significant from the earlier literature search discussed in **AGRN26** (cf. **Appendix 6-1**) and relevant to *M. extorquens* pathogenicity was identified.

Toxicity

A Google Scholar search "*Methylobacterium extorquens* toxicity" on April 24, 2019 yielded 521more hits than the search performed for **AGRN26** (**Appendix 6-2**) . When the word plant is removed from the search, 142 new hits were generated, **(Appendix 3-2)**. Again, nothing significant from the earlier literature search discussed in **AGRN26** (cf. **Appendix 6-2**) and relevant to *M. extorquens* toxicity was found. These hits are provided in **Appendix 3-2** of this Notice.

A search conducted on March 28, 2019 in the ARDB-Antibiotic Resistance Genes Database (Center for Bioinformatics and Computational Biology- University of Maryland- College Park, MD 20742) yielded the same result as described in AGRN26 and KnipBio has confirmed that its strain is resistant to bacitracin.

Symbiotic Relationships

As noted above, *M. extorquens* occurs in nature as a leaf symbiont (Knief *et al.*, 2010). It is not believed that this species maintains any other symbiotic relationships other than with the plant species described in these references.

Summary

KnipBio believes that, based on the literature identified and discussed above, the production organism for the notified substance, *Methylobacterium extorquens*, is a safe organism that will not pose any health risks to the target species that will be fed the notified substance, or to human consumers who eat such crustacean.

3) Production strain

a) Genetic manipulation to create production strain

The notified substance is dried *Methylobacterium extorquens* biomass Strain KB203. Description of the genetic make-up of strain KB203 was presented in **Part 2** of **AGRN26** and its **Appendices 2-1, 2-2, and 2-3** and were discussed in **AGRN 26 Part 6** (page 40).

b) Effects of genetic manipulation.

These effects were presented in **Part 2 of AGRN26** and its **Appendices (2-1, 2-2, and 2-3)** and discussed in **section 6** (page 40).

c) Safety of KB203 and impact of genetic manipulation on safety.

M. extorquens has been classified as Biosafety Level 1 (ATCC, 2017). As described in **Appendix 2-3** and **Part 2** of **AGRN26**, the genetic manipulations made to the starting strain consist only of gene deletions.

The most direct evidence for the safety of the production strain KB203 are the various feeding studies that were described in detail in **Part 2** of **AGRN26** and its Appendices, as well as in this GRAS Notification for the target animal. A summary is found in **section 6** of **AGRN 26** (page 40).

A discussion of the lack of genetic spillover was provided in **August 9, 2018** supplemental information (pages 20-21).

4) Fermentation and product formulation.

a) Fermentation process.

As described in detail in **Part 2** of **AGRN26**, the notified substance is produced from biomass of *Methylobacterium extorquens*, which has been grown to sufficient volume via fermentation using conditions well-established for this microorganism. At the conclusion of each production run, the biomass is separated from the spent media by centrifugation and is then spray-dried for preparation of the dried *Methylobacterium extorquens* biomass material, as described in **Appendix 2-4 of AGRN26.**

KnipBio currently expects that commercial production of the notified substances may involve one or more toll manufacturers, operating under contract with KnipBio. All such manufacturing activities will be conducted in compliance with the Food Safety Modernization Act and will utilize Standard Operating Procedures in accordance with Good Manufacturing Practice.

b) Food-grade materials used in production: safety implications.

Throughout the manufacturing process, food-grade materials of suitable purity are used, as discussed in **AGRN 26 Part 6** (page 41) and described in the Tables in **Appendix 2-4** of **AGRN26.**

c) Safety implications of fermentation and the production process.

Production of the notified substance will take place using the most appropriate procedures in accordance with Good Manufacturing Practice, using only materials that are of known purity and are suitable for use in animal food. Fermentation workers will be appropriately trained, and all safety precautions consistent with Good Industrial Large Scale Practice for microorganisms designated as Biosafety Level 1 will be utilized. Furthermore, it is necessary for the efficiency and productivity of the fermentation for strict controls to be in place to prevent contamination of the production organism with other microorganisms, and so the resulting biomass from each production run should be free of any such contamination.

5) Dried Methylobacterium extorquens biomass product (the "notified substance")

a) Background

The notified substance is a dried *Methylobacterium extorquens* biomass, as described in **AGRN26**, from cultures of *Methylobacterium extorquens* fermented using methanol as its carbon source.

b) Composition

The notified substance is dried *Methylobacterium extorquens* biomass that provides an excellent source of protein for the target crustacean species, and in particular includes concentrations of specific amino acids comparable to other sources of protein. Detailed information on the composition of the notified substance is provided in **AGRN26, Part 2** and in **Appendix 2-8.** Also presented in **Part 2** of **AGRN26** were comparisons of the composition of the notified substance with that of other sources of protein provided by fishmeal or soybean meal, showing that the notified substance is a comparable source of protein as compared to soybean meal and some fishmeal (i.e. tuna or local mixed species), although it is not as good a source of fat (<1% dry weight) or fiber (<0.5% dry weight) as those other meals.

Table 6-1 Specifications of the Notified substance as described in AGRN26 and Supplemental data from August 9, 2018.

	Method	Value
Moisture %	AOAC 930.15	<7
Protein (crude) %	AOAC 990.03	>50
PHB %	Adapted from Karr *et al.* (1983)	< 25
Methanol (mg/g)	Adapted from Anthon *et al.* (2004)	<0. 3
Lead ppm	AOAC 990.08	<0.05
Total coliform (cfu/g)	MFHPB-34	<5
Appearance (color)		Light pink to reddish color
Appearance (form)		Fine powder

As summarized in **AGRN26, Appendix 2-8**, the notified substance is also expected to contain certain low concentrations of metals. **Table 6-2** below shows the expected concentrations of these metals in the notified substance, derived from the analyses reported in **AGRN26, Appendix 2-8** , and discussed further in the Supplementals information from August 9, 2018 (Pages 9-11) and September 27, 2018 (Pages1-2) . Cd and Pb are categorized as toxic elements. Al, Cu, Fe, Mn, Zn, Mo and Se are essentials elements. Because the notified substance will make up no greater than 6% of the overall diet of the targeted species, the maximum concentrations of these metals to which the target species will be exposed would be at least 16-fold lower than the figures shown in the first column of the **Table 6-2.** This **Table** also shows reported literature values for the daily requirements for shrimp, for certain of these metals (where such data are available in the literature). It can be seen that the expected exposure to these metals in feed containing the notified substance would fall within the species' dietary requirements for that metal, making it highly unlikely that the metals present in the notified substance would have any deleterious effect on the target species.

Table 6-2 Metal Concentrations in the Notified Substance and Dietary Requirements for the Target Species.

	KBM203	Requirement (mg/kg dry diet)	6% inclusion
Mineral	mg/kg	Shrimp (L. v.)	mg/kg
Calcium	260-440	5,000-20,000	15.6-26.4
Phosphorus	9,400-10,500	300-7,000	564-1630
Sodium	3,000-4,000		180-240
Chloride	240-390		14.4-23.4
Magnesium	820-900	2,600-3,500	49.2-54
Manganese	65.4-76.80		3.9-4.6
Iron	167-263		10.0-15.8
Zinc	40.7-65.8	15	2.4-3.9

Copper	11.9-19.7	16-32	0.7-1.2
Potassium	4,960-6,200		297.6-372
Cobalt	5.7-6.2		0.3-0.4
Molybdenum	12.00-14.8		0.1-0.9
Sulfur	3,430-4,050		205.8-243
Selenium	0.05< -0.11	0.2-0.4	<0.001

Sources: National Research Council (2005, 2011a, 2011b); Wu and Chen, (2005). http://www.fao.org/fishery/affris/other-species/en/

As discussed in **AGRN26, Part 2** (pages 14,15) and in the Supplemental information from August 9, 2018 (page 21), the notified substance, although produced from live microorganisms, is not expected to contain any appreciable levels of live cells

c) Potential contaminants

i) Polyhydroxybutyrates (PHBs)

As described in AGRN26, the microbial strain on which the notified substance is based, *Methylobacterium extorquens*, is a natural producer of polyhydroxybutyrates (PHBs) (Korotkova and Lidstrom, 2001). The notified substance is therefore expected to contain certain amounts of PHBs, which might range from 15-25% depending on the fermentation conditions. KnipBio believes that the expected levels of PHBs in the notified substance will be well below the levels that might be expected to be harmful to the target species, and furthermore there is evidence in the literature that PHBs may have beneficial effects in animal diets.

Polyhydroxybutyrates are ubiquitous compounds, naturally produced by a broad range of microorganisms (Raza *et al.,* 2019) and used by such bacteria as an intracellular carbon and energy storage compound. PHBs have been found by several investigators to be produced in *M. extorquens*, at concentrations ranging from 25-33%, depending on growth conditions (Bourque *et al.*, 1992; Höfer *et al.*, 2011).

There is a considerable body of literature attesting to the testing of PHBs in crustacean diets, as summarized in **Table 6-3** below. No deleterious effects were seen from the presence of these compounds in animal feed, and in some cases positive effects were noted. For example, reports in the literature mention that PHA/PHB polymers can be degraded into β-hydroxy-short chain fatty acids (SFCA) (Laranja and Bossier, 2019). Numerous other publications in the literature report seeing positive effects of PHBs in animal (Ludevese-Pascual *et al.*, 2017, 2019; Situmorang *et al.*, 2016; Laranja *et al.*, 2018; Sivagnanavelmurugan *et al.*, 2018), with several noting antimicrobial effects of PHBs as well as SCFA, the product of PHB degradation.

Table 6-3 below summarizes numerous studies in the literature, including those cited above, in which crustacean animals were fed diets that included PHBs as one component, and sums up the findings and outcomes of these studies. No significant adverse effects were seen in any of those studies, and some showed beneficial effects on parameters such as growth rate.

Table 6-3. Literature Reports testing Polyhydroxybutyrates in crustacean Diets.

Species	Age/ size	% PHB	PHB origin	Trial length	Challenge	Outcome	Reference
Shrimp Penaeus monodon	PL1-PL30	3.4-4.1	Bacillus spp.	30 days	*Vibrio campbellii*	No effect on body weight or body length	Laranja *et al.* (2014)
Shrimp Penaeus monodon	PL5	55% in Bacillus sp.	Bacillus spp.	16 days	*Vibrio campbellii*	Improvement of immune response	Laranja *et al.* (2017)
Macrobrachium rosenbergii		0.01-0.08%	Artemia + PHB	20 days		No effect on growth. Increased survival	Thai *et al.* (2014
		0.01-0.08%	Artemia + PHB	9 days	*Vibrio harveyii*	Increase survival	Thai *et al.* (2014)
Brine shrimp Artemia franciscana	nauplii	29-55% Bacillus DCW	Bacillus spp.	48h	*Vibrio campbellii*	Bacillus sp. Containing PHB more effective than amorphous PHB	Laranja *et al.* (2018
Brine shrimp Artemia franciscana	nauplii	10% (vol)	Chemical	48h	*Vibrio campbellii*	3-HB released form PHB induces immune response of shrimp	Defoirdt *et al* (2018)
Shrimp Litopenaeus vannamei	PL6	69.9% Halomonas DCW	Artemia + Halomonas-PHB	15 days	*Vibrio anguillarum*	Improved growth, survival and robustness	Gao *et al.* 2019
Tiger shrimp Penaeus monodon	PL30	PHB substratum	Chemical	61 days	*Vibrio campbellii*	Enhancement robustness and resistance against pathogens and environmental conditions	Ludevese-Pascual *et al.* (2019)
Chinese mitten crab Eriocheir sinensis	larval stage 1	0.01?	rotifers		*Vibrio anguillarum*	Increase in survival rate	Sui *et al.* (2012)
Giant freshwater prawn Macrobrachium rosenbergii	larval stage 1	0.5	Artemia nauplii + PHB		none	increase in survival rate of larvae	Nhan *et al.* (2010)
Giant river prawn Macrobrachium rosenbergii		0.5					Liu *et al.* (2010)
Brine shrimp Artemia franciscana	nauplii			48h	*Vibrio campbellii*		Liu *et al.* (2010)
Brine shrimp Artemia franciscana	nauplii	0.01- 0.1		48h	*Vibrio campbellii*	increased survival rate	Baruah *et al.* (2015)
Brine shrimp Artemia franciscana	nauplii	1-2 ?		48h	*Vibrio harveyi*	PHB decreases colonization by Vibrio	Van Cam *et al.* (2009)

Brine shrimp Artemia franciscana		0.01- 0.1		48h	*Vibrio campbellii*	Increase survival	Defoirdt *et al.* (2007)
Pacific white shrimp Litopenaeus vannamei	~4 gm	2		6 weeks		Slightly higher survival, lower total bacterial count in the intestine.	da Silva *et al.* (2016) J.
Brine shrimps Artemia franciscana	nauplii	0.001-0.02	*B. casei* MSI04	48h	*Vibrio*		Kiran *et al.* (2016)
Pacific white shrimp Litopenaeus vannamei	~ 5 gm	1-5		35 days		No effect on growth, FCR, weight gain	Duan *et al.* (2017
Brine shrimp Artemia franciscana	nauplii	32% VSS		48h	*Vibrio campbellii*	Improved survival	Halet *et al.* (2007)
Giant Tiger prawn Penaeus monodon	post-larvae	artemia +PHB		15 days	*Vibrio campbellii*	Improved survival	Ludevese-Pascual (2017)
Pacific white shrimp Litopenaeus vannamei	post-larvae		Granules added to Biofloc	40 days		PHB can be used as an additional carbohydrate for biofloc nurseries in brackish water	Luo *et al.* (2019)

DCW: Bacterial Dry Cell Weight

Shrimp intestine is continuously exposed to foreign substances found in the environment as well as its food, including microbes, pathogens, and other substances. Common to all animals, shrimp digestive tract harbor a diverse microbial community which is linked to its health and plays many functions related to immunity and pathogen resistance (Duan *et al.*, 2018).

During its catabolism process, intestine microbial population can produce short-chain fatty acids (SCFAs) which can subsequently reduce the intestine pH, promote the growth of beneficial bacteria, and inhibit the growth of pathogenic bacteria (Koh *et al.*, 2016).

There is significant reason to expect that PHB-producing bacteria are naturally found in shrimp guts, largely because production of PHBs by bacteria is so ubiquitous across many genera and species. A wide variety of bacteria and fungi have the ability to degrade extracellular PHB as they are able to secrete extracellular PHB depolymerase enzymes (Egusa *et al.*, 2018; Jendrossek and Handrick, 2002; Martínez-Tobón *et al.*, 2018; Mohanrasu *et al.*, 2018). Defoirdt *et al.* (2009) indicate that PHB particles are partially degraded in the gut of brine shrimps nauplii. Liu *et al.* (2010) isolated PHB-degrading bacteria from the gastrointestinal environment of a few aquatic animals (sturgeon, European sea bass, prawns). Any PHBs found in the feed would be expected to be degraded by such bacteria, therefore making it very unlikely that human consumers would be exposed to any significant levels of PHBs. Studies by De Schryver *et al.*, (2010), suggest that

PHB (used at 5% w/w in a diet) is degraded during the gastrointestinal passage of juvenile sea bass. Defoirdt *et al.* (2007) provided evidence to show that PHB particles were at least partially degraded in the intestines of nauplii of the brine shrimp *Artemia fransiscana*. In 2018, Defoirdt *et al.* reported that the degradation product of PHB, 3-hydroxybutyrate (3-HB), can be found in the intestinal tract of PHB fed brine shrimp. The 3-HB lead to a decrease in the production of virulence factor by the pathogen, and a protection of the gnotobiotic brine shrimp.

Moreover, it was demonstrated that the digestion and transit time in the gut of penaeid shrimp gut is pretty short (~60 min or so) and one would expect that non digested PHB should be released in the water (Beseres *et al.*, 2006).

In addition, as noted above, at the level of inclusion in aquaculture feed that KnipBio intends (up to 6% w/w in the total diet) the maximum level of PHB would be approximately 1-1.5%. The literature cited and discussed above indicates that such levels would not be expected to have an effect of the health of the crustacean, and since any PHBs in the notified substance would be expected to be degraded in the crustacean gut, there would be no adverse effects on the health of humans consuming crustacean which have fed on the notified substance.

ii) Other Potential Contaminants

As described in AGRN26, no other contaminants are expected to be found in the notified substance.
The agency previously raised concerns about fermentation products from Gram negative bacteria and brought to KnipBio's attention two publication from the European Food Safety Authority (*EFSA FEEDAP Panel, 2017a,b).*

After reviewing the publication, KnipBio completed a safety assessment for the use of *M. extorquens*, a Gram-negative organism, and noted that it does not share any of the potentially problematic traits of certain other gram-negative microorganisms. As described in the Supplemental from September 27, 2018 (Pages 5-7), there is no evidence that *M. extorquens* produces harmful endotoxins, lipopolysaccharides (LPS), or any other substance identical or similar to such substances that are produced by some strains of *E. coli* or other Gram-negative microorganisms that are known to be pathogens. KnipBio has confirmed through whole-genome sequencing of the production organism that there is no antibiotic resistance gene present in the production organism, and that no unexpected genetic rearrangement arose after the genetic manipulations performed.
As presented in **AGRN26, Appendix 2-2**, *M. extorquens* genome has no homology to any known toxins. In addition, KnipBio has not introduced any heterologous open reading frames into the production organism, so there would not be expected to be any impact of the genetic engineering that might have introduced sequences coding allergens or toxins. KnipBio maintains that they have assessed the safety of *M. extorquens*, a Gram-negative microorganism, as suggested by the FEEDAP document.

d) Target Animal studies

The notified substance has utility in the use as a protein supplement for crustacean aquaculture. KnipBio has carried out a number of studies in which the notified substance was fed to crustaceans. These studies have shown the utility of the notified substance as a source for protein in the crustacean diet and have also demonstrated that no adverse effects arose from the inclusion of the notified substance in the diet.

KnipBio's safety and utility argument is based on the comparison of the composition of dried *Methylobacterium extorquens* biomass with conventional feed ingredients and feeds and is also based on the compositional analysis. This is a typical approach for major ingredients in the diet. We are supporting this assessment with live animal studies. Some of this supporting data is found in the published paper (Tlusty *et al.*, 2017). In **Appendix 2-2** and **Table 2-1** of this GRAS Notification, KnipBio provides a number of additional studies that can be used to support the safety and utility of the biomass as a protein source.

The first report published in Tlusty *et al.* (2017) describes feeding studies on Pacific white shrimp (*Litopenaeus vannamei*) growth and consumer taste preference, a study for which FDA has reviewed the protocol. The shrimp diets in the growth studies sought to replace 50% and 100% of fishmeal (FM) of a diet considered to be commercially relevant and are described as % FM. These percentages correlate to an inclusion rate of 6.3% and 12.6% respectively. The diet formulation description is found in Table 1 of the Tlusty paper, which describes the inclusion rate as (g kg^{-1}) (**Appendix 2-1**).

In this study it was reported that the shrimp on the high KBM diet (12.6% KBM) had a decreased growth rate; but an increase in feed conversion ratio, indicating that there was not an issue with availability of the nutrition of the dried *Methylobacterium extorquens* biomass diet observed. The paper reported that there was some feed manufacturing error and air bubbles were seen in the pellets prepared for the shrimp diet SHR-KH (100% KBM replacement, which corresponds to a 12.6% inclusion for the total diet) and therefore the pellets likely did not properly sink in the water column while the other two diets (control and 6.3% inclusion) did. Shrimp are bottom feeders and it is very likely that the shrimp that were fed the high inclusion biomass received fewer available pellets in the water column, which may account for the reduced weight gain and SGR (Specific Growth Rate).

The safety of the notified substance for use in shrimp feed is also corroborated by studies done in at the [redacted] (b) (4) (2016- **Appendix 2-2**). Three trials were conducted to evaluate the biological response of shrimp to dried *Methylobacterium extorquens* biomass in soy-based (SBM) diets in terms of growth. <u>In trial 1</u>, three experimental diets were formulated to contain increasing levels (0, 6, and 12%) of dried *Methylobacterium extorquens* biomass as a replacement of SBM. <u>In trial 2</u>, six experimental diets were formulated to supplement with increasing levels (0, 1, 2, 4, 6, and 12%) of KBM as a replacement of SBM.

In the first trial, a significant increase in survival was observed with shrimp that were fed the notified substance as part of their diet. At an inclusion of 6%, no significant difference was found in the final mean weight or weight gain compared to the control diet.

In trial 2, significant improvements in final mean weight and Weight Gain (WG) but dramatically reduced FCR were determined in shrimp fed with the diet containing low levels of dried *Methylobacterium extorquens* biomass (1%) compared to those fed with diet supplemented with >6% dried *Methylobacterium extorquens* biomass. No significant difference was found in survival (92.5%–100%) across all the treatments.

In trial 3, five experimental diets were formulated to include 0, 6, 12, 13.3. and 26.6% of dried *Methylobacterium extorquens* biomass, with the last two diets being formulated on a protein digestibility basis. Shrimp fed with diets containing 13.3 and 26.6 % dried *Methylobacterium extorquens* biomass exhibited significantly improved protein and reduced lipid contents. Again, with a 6% inclusion, no significant difference could be observed in the final biomass, final mean weight, weight gain as well as the proximate composition of the shrimp.

Overall, the results indicated that no significant differences were observed in terms of growth performance and FCR when the diets were supplemented with dried *Methylobacterium extorquens* biomass up to 6% of the diet.
Based on the totality of the data, the composition of the biomass, the published study, and the corroborative studies there is ample evidence to demonstrate the potential broad applicability of dried *Methylobacterium extorquens* biomass as a viable protein source for use in crustacean aquafeeds and that use of the notified substance at levels up to 6% in shrimp diets is safe.

Table 6-3 and **Appendix 2-2** of this Notice summarize the results of the studies conducted with shrimp that were fed the notified substance (in some cases, early formulations of the notified substance). These studies all show that the notified substance caused no harmful effects on the shrimp at a 6% inclusion. Full study records for these studies, particularly including the studies published in Tlusty *et al.*, are available at the KnipBio offices should FDA be interested in reviewing them.

6) Summary of Safety Argument; Assertion of GRAS Status

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

a) Safety to target animals

The notified substance is based on a naturally-occurring microorganism, *Methylobacterium extorquens*, classified as Biosafety Level 1, that has never been reported to have pathogenic, toxic, or other hazardous properties (as confirmed in the company's literature searches), although strains of this species and other *Methylobacterium* species have been isolated from healthcare-associated infections in immunocompromised hosts. The starting wild type strain has been subjected to genetic manipulation only to remove two biosynthetic pathways, and since no heterologous, foreign or synthetic coding DNA has been introduced into the strain, the manipulation has not introduced any new biochemical functions into the strain.

The notified substance will be manufactured commercially using a well-understood, well-characterized growth medium, using standard fermentation procedures. AAFCO approved and/or food-grade materials of suitable purity will be used for all components of the growth media for all fermentations, and Good Manufacturing Practice and suitable Standard Operating Procedures will be used at all stages of manufacture.

The notified substance will have a well-characterized composition that will provide an excellent source of protein for the target species. The product will contain no impurities that might cause harm to the target species. The only impurity that is expected to be present in the notified substance would be certain levels of polyhydroxybutyrates, but since the notified substance will be added to crustacean diets at a maximum of 6% by weight, the levels of PHBs to which the target species would be exposed would likely be no greater than 1-1.5% by weight in the total diet. Data from the literature summarized in this Notice indicate that the presence of PHBs in crustacean diets do not cause any adverse or negative effects and in fact may offer some benefits to the fish, such as enhanced growth rates.

Finally, the feeding studies conducted by KnipBio, including the published studies (Tlusty *et al.*, 2017), indicate that the dried *Methylobacterium extorquens* biomass can be safely ingested by the target species with no adverse effects.

b) Safety to humans

KnipBio also believes that there will not be any adverse effects on the health of humans who consume crustacean that have been fed the notified substance, because there will be no exposure of humans to any deleterious substance. As described above, the notified substance itself is safe for ingestion by the target species and is unlikely to have any harmful component. The notified substance will largely consist of protein and amino acids, which when ingested by the target species will be metabolized and incorporated into proteins and other molecules within the crustacean gut. All components of the notified substance will be digested in the gut of the target species like normal feed ingredients. The entire product will therefore be metabolized by the aquatic animal like normal ingredients, and we do not anticipate any safety issue for human consumption. As discussed above, although the notified substance is expected to contain levels of PHBs no greater than 25%, such levels will be diluted at least sixteen-fold in the crustacean diet. Furthermore there is strong evidence, as discussed above, that microorganisms capable of degrading PHBs into short chain fatty acids can be found in the gut of many crustacean species, and so it is expected that there will be no residual concentrations of PHBs in the tissue of fish to which the notified substance has been fed, thus posing no health risk to humans who consume such fish.

Finally, KnipBio has conducted taste-testing studies in which human subjects ingested small amounts of crustacean biomass that had been fed preparations consisting of KnipBio's dried *Methylobacterium extorquens* biomass, and although those studies were not designed to test safety per se, there were no adverse effects noted in any individuals taking part in these studies.

For these reasons, KnipBio maintains that the notified substance is Generally Recognized as Safe for use in crustacean feed for the target species, when used as an additive of up to 6% by weight in fish feed.

7) Discussion of (any) data inconsistent with GRAS determination

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

8) Identification, justification for claims of confidentiality

All the data in this dossier are nonconfidential and are available to the public, except for specific information which constitutes trade secrets of KnipBio, Inc., and which the company claims as confidential in accordance with 21 CFR Part 171.1(h)(1). The two categories of information that are claimed as confidential trade secrets are (a) information regarding the manufacturing process for the notified substance as provided in **AGRN26** and its Appendices as well as the Supplemental data from August 9 and September 27, 2018; and (b) specific Standard Operating Procedures developed and maintained by KnipBio, which are used in laboratory and fermentation processes in the manufacture of the notified substance, or for quality control in manufacture of the notified substance. Public disclosure of information in each of these categories would result in substantial harm to KnipBio and its business, by providing the company's competitors a significant advantage in allowing them to recreate the company's proprietary processes.

Although some of the studies described in **Appendix 2-2** have not been published in the scientific literature, KnipBio has relied on these studies in its substantiation of GRAS status and it has been cited herein as corroborative evidence.

KnipBio, Inc. maintains internal procedures and practices to maintain the confidentiality of its trade secrets and other information. Documents containing confidential or trade secret information are marked as Confidential. Disclosure of such information within the Company is on a need-to-know basis and all Company employees have signed employment contracts that include strict confidentiality provisions, including a prohibition on unauthorized disclosure of information such as the confidential or trade secret information. Any individual or company outside of the Company who needs to know the confidential or trade secret information in the course of their business with the Company must, before receiving any such information, sign a written nondisclosure agreement to hold the information confidential and proprietary to the Company. KnipBio can provide further information to FDA CVM regarding its procedures and policies for maintaining the confidentiality of its trade secret information.

Part 7: List of supporting data and information in this GRAS notice.

This GRAS Notice relies on the following literature references in support of the finding of GRAS status for the notified substances. All these references are available in the public domain. All the references that are not highlighted can be found in AGRN26. New references highlighted in blue are provided as PDF documents in the CD.

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From:	Kristi Smedley
To:	Carlacci, Louis; Wong, Geoffrey K
Cc:	Catherine Pujol-Baxley, Ph. D.
Subject:	RE: Amendment for AGRN 33--Knipbio
Date:	Tuesday, May 05, 2020 10:14:03 PM
Attachments:	FDA AGRN33 Amendment KB-050520v2.pdf

Lou:

I apologize for not catching that change.

I hope the attached letter is satisfactory.

Received Date
May 12, 2020

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell [redacted] (b) (6)
Fax 703-580-8637

From: Carlacci, Louis [mailto:Louis.Carlacci@fda.hhs.gov]
Sent: Tuesday, May 05, 2020 5:11 PM
To: Kristi Smedley; Wong, Geoffrey K
Subject: RE: Amendment for AGRN 33--Knipbio

Kristy:
Sorry for any confusion, but the acceptance criterion for formaldehyde content in the specification table and the label statement are not consistent.
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: Kristi Smedley <smedley@cfr-services.com>
Sent: Tuesday, May 05, 2020 4:58 PM
To: Carlacci, Louis <Louis.Carlacci@fda.hhs.gov>; Wong, Geoffrey K <Geoffrey.Wong@fda.hhs.gov>
Subject: Amendment for AGRN 33--Knipbio

Dr. Carlacci and Mr. Wong:

Thank you for your call earlier today.

We have modified the specifications in accord with your requests, to include all the information (including formaldehyde) in the specification table.

Should you have any other questions, please let me know.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell [redacted] (b) (6)
Fax 703-580-8637

Version 2 of Amendment – attached to Dr. Kristi Smedley's May 5, 2020 e-mail (10:14 pm)

KnipBio

May 5th, 2020

Louis Carlacci, Ph.D.
Chemist, Ingredient Safety Team
Division of Animal Feeds
Center for Veterinary Medicine
U.S. Food and Drug Administration
7519 Standish Place
Rockville, MD 20855

RE: GRAS Notice No. AGRN 33

Dear Dr. Carlacci:

KnipBio, Inc., would like to thank you and your colleagues at CVM for reviewing the GRAS Notice AGRN 33 and your request for updating the Specification of the notified substance, as per your phone conversation with Dr Kristi Smedley.

Updated Table 6.1 of AGRN33: Specification of the notified substance

	Method	Value
Moisture %	AOAC 930.15	<7
Protein (crude) %	AOAC 990.03	>50
PHB %	Adapted from Karr *et al.* (1983)	< 25
Methanol (mg/g)	Adapted from Anthon *et al.* (2004)	<0.3
Formaldehyde (mg/g)	Adapted from Anthon *et al.* (2004)	<0.002
Lead ppm	AOAC 990.08	<0.05
Total coliform (cfu/g)	MFHPB-34	<5
Appearance (color)		Light pink to reddish color
Appearance (form)		Fine powder

In the finished commercial product for crustaceans, KnipBio will specify on the label of the notified substance that the levels of formaldehyde are guaranteed to be below 0.002 mg/g (or 0.2% w/w).

Thank you very much for the opportunity to address these questions. Please contact the Dr Kristi Smedley if there are any additional questions we can address.

Sincerely,

Larry Feinberg

CEO

From: Kristi Smedley
To: Carlacci, Louis; Wong, Geoffrey K
Subject: Amendment for AGRN 33--Knipbio
Date: Tuesday, May 05, 2020 4:58:36 PM
Attachments: FDA AGRN33 Amendment KB050520.pdf

Dr. Carlacci and Mr. Wong:

Thank you for your call earlier today.

We have modified the specifications in accord with your requests, to include all the information (including formaldehyde) in the specification table.

Should you have any other questions, please let me know.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell [redacted] (b) (4)
Fax 703-580-8637

Version 1 of Amendment – attached to Dr. Kristi Smedley's May 5, 2020 e-mail (4:58 pm)

KnipBio

May 5th, 2020

Louis Carlacci, Ph.D.
Chemist, Ingredient Safety Team
Division of Animal Feeds
Center for Veterinary Medicine
U.S. Food and Drug Administration
7519 Standish Place
Rockville, MD 20855

RE: GRAS Notice No. AGRN 33

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Appearance (form)		Fine powder

In the finished commercial product for crustaceans, KnipBio will specify on the label of the notified substance that the levels of formaldehyde are guaranteed to be below 0.003 mg/g (or 0.3% w/w).

Thank you very much for the opportunity to address these questions. Please contact the Dr Kristi Smedley if there are any additional questions we can address.

Sincerely,

Larry Feinberg

CEO

T-1

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Road * Woodbridge, VA 22192-5755
Telephone 703 590 7337 * Fax 703 580 8637
Smedley@cfr-services.com

September 13, 2019

Louis Carlacci, Ph.D.
Chemist, Ingredient Safety Team
Division of Animal Feeds
Center for Veterinary Medicine
U.S. Food and Drug Administration
7519 Standish Place
Rockville, MD 20855

RE: GRAS Notice Dried *Methylobacterium extorquens* Biomass for Crustaceans --KnipBio

Dear Dr. Carlacci:

Based on your phone call this morning, we are amending the Animal GRAS Notice as file on August 9, 2019 specific to Dried *Methylobacterium extorquens* biomass for Crustaceans' diets by KnipBio.

Specific to Page 27, Safety please add the following:

The safety of Dried *Methylobacterium extorquens* biomass is based on the composition of the substance (as reported by laboratory assessment and in published papers) and the live animal research as published in Tlusty , 2017 and other live animal studies provided.

Should you have any other issues specific to this GRAS notice, please contact me.

Sincerely,

Kristi O. Smedley

Kristi O. Smedley, Ph.D.
Consultant to KnipBio

cc: KnipBio

RECEIVED
SEP 16 2019
By__________

Appendix 2-1. Tlusty *et al.*(2017)

A transdisciplinary approach to the initial validation of a single cell protein as an alternative protein source for use in aquafeeds

Michael Tlusty[1,2], Andrew Rhyne[1,2,3,4], Joseph T. Szczebak[4], Bradford Bourque[3], Jennifer L. Bowen[5], Gary Burr[6], Christopher J. Marx[7] and Lawrence Feinberg[7]

[1] Anderson Cabot Center for Ocean Life at the New England Aquarium, New England Aquarium, Boston, MA, United States

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[3] Department of Arts and Sciences, Roger Williams University, Bristol, RI, United States

[4] Department of Biology and Marine Biology, Roger Williams University, Bristol, Rhode Island, United States

[5] Northeastern University, Nahant, MA, United States

[6] National Cold Water Marine Aquaculture Center, USDA ARS, Franklin, ME, United States

[7] KnipBio Inc., Lowell, MA, United States

ABSTRACT

The human population is growing and, globally, we must meet the challenge of increased protein needs required to feed this population. Single cell proteins (SCP), when coupled to aquaculture production, offer a means to ensure future protein needs can be met without direct competition with food for people. To demonstrate a given type of SCP has potential as a protein source for use in aquaculture feed, a number of steps need to be validated including demonstrating that the SCP is accepted by the species in question, leads to equivalent survival and growth, does not result in illness or other maladies, is palatable to the consumer, is cost effective to produce and can easily be incorporated into diets using existing technology. Here we examine white shrimp (*Litopenaeus vannamei*) growth and consumer taste preference, smallmouth grunt (*Haemulon chrysargyreum*) growth, survival, health and gut microbiota, and Atlantic salmon (*Salmo salar*) digestibility when fed diets that substitute the bacterium *Methylobacterium extorquens* at a level of 30% (grunts), 100% (shrimp), or 55% (salmon) of the fishmeal in a compound feed. In each of these tests, animals performed equivalently when fed diets containing *M. extorquens* as when fed a standard aquaculture diet. This transdisciplinary approach is a first validation of this bacterium as a potential SCP protein substitute in aquafeeds. Given the ease to produce this SCP through an aerobic fermentation process, the broad applicability for use in aquaculture indicates the promise of *M. extorquens* in leading toward greater food security in the future.

Subjects Agricultural Science, Aquaculture, Fisheries and Fish Science, Biotechnology, Food Science and Technology, Microbiology

Keywords Biotechnology, Aquaculture, Single cell protein, Shrimp, Salmon, Methylotrophs, Alternate protein, Food security, Microbiome, Smallmouth grunt

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Appendix 2-2. Shrimp Feeding Studies

Appendix 2-2. Summary of KnipBio Shrimp Trials

This appendix summarizes the results of studies reported in KnipBio's published paper Tlusty *et al.* (2017) as well as other unpublished studies that KnipBio has conducted or sponsored in which aquatic animals were fed the notified substance (in some cases, early formulations of the notified substance). These studies all show that the notified substance caused no harmful effects on the crustacean. Full study records for these studies, particularly including the studies published in Tlusty *et al.*, are available at the KnipBio offices should FDA be interested in reviewing them.

This report includes Pacific White Shrimp (L. vannamei) studies performed at

1. [redacted] (b) (4) (published in Tlusty *et al.* (2017))
2. [redacted] (b) (4).

1.<u>Study 1</u> [redacted] (b) (4) (*included in Tlusty et al. 2017*)

<u>*a. Experimental design*</u>

Experimental diets used for all animal trials were produced using commercial manufacturing methods. Diets were stored in polypropylene plastic bags at room temperature until fed. All diets were fed within 4 months of manufacture.

Hatchery-raised Pacific white shrimp (*L. vannamei*) were acquired from [redacted] (b) (4) [redacted] (b) (4) USA) and stocked at 60 shrimp/tank into twelve 110L glass aquaria. Animal care and procedures used in this trial were approved by [redacted] (b) (4) [redacted] (IACUC protocol R-13-12-20).

To determine the effect of KBM on shrimp growth and survival, three diets of varying KBM inclusion were formulated (Table). Each of the 12 experimental tanks was randomly assigned one of the three diets, totaling four replicates per treatment. Each tank was fed to apparent satiation four times/day.

Ingredient	Composition (g kg^{-1} as fed)		
	SHR-C	SHR-KL	SHR-KH
Menhaden fish meal	1200.0	600.0	0.0
KnipBio meal	0.0	630.0	1260.0
Soybean meal	3800.0	3800.0	3800.0
Menhaden fish oil	307.0	371.0	435.0
Corn starch	348.0	174.0	0.0
Whole wheat	3400.0	3400.0	3400.0
Trace mineral premix	50.0	50.0	50.0
Vitamin premix	180.0	180.0	180.0
Choline clorine	20.0	20.0	20.0
Vitamin C	10.0	10.0	10.0
CaP-diebasic	200.0	280.0	360.0
Lecethin	100.0	100.0	100.0
Cholesterol	5.0	5.0	5.0
Empareal 75 CGM	380.0	380.0	380.0

Composition of three experimental feeds used to test the efficacy of KnipBio single cell protein (KnipBio meal; KBM) as a fishmeal substitute using Pacific white shrimp (*L. vannamei*), where SHR-C = **SHR**imp **C**ontrol feed (711500245) and SHR-KL and SHR-KH are control feed with fishmeal replaced with KBM; KL = **K**nipBio meal **L**ow (50% replacement) and KH = **K**nipBio meal **H**igh (100% replacement).

The gross wet weight (g) of all shrimp per tank was measured at day 0 (N=60), day 60 (N=45-55, depending on tank), and day 105 (N=19-20, depending on tank). At day 60, 20 shrimps from each tank (N=80 per treatment) were randomly selected and returned to their original tank and maintained according to the above experimental design for an additional 90 days. The remaining shrimps not used for the second 90-day trial (N=25-35, depending on tank) were euthanized, placed on ice, and wet weight (g), and carapace length (mm) were measured for each individual. On day 150, the remaining shrimps in each tank were enumerated and wet weight (g) and carapace length (mm) were measured for each individual.

b. Results

Diet had no effect on shrimp survival (one-way ANOVA, $F_{2,9} = 2.4$, $p > 0.1$, combined average = 84.7±5.6%); however, diet did influence shrimp growth (one-way ANOVA, % weight gain, $F_{2,9} = 5.4$, $p < 0.5$; SGR, $F_{2,9} = 8.6$, $p < 0.01$). Shrimp fed diet with 100% FM replacement (SHR-KH) grew less than those fed the control diet (SHR-C), and shrimp fed diet with 50% FM replacement (SHR-KL) showed growth intermediate to, and not statistically different from either SHR-C or SHR-KH. Diet influenced shrimp feed efficiency (one-way ANOVA, $F_{2,9} = 5.27$, $p < 0.5$). The food conversion ratio (FCR) of shrimp fed diets containing KBM were not statistically different than those fed the control diet.

c. Conclusion.

The results of this study show that, in Pacific White shrimp during a 15-week growth period, the notified substance even when constituting 50% or 100% of the fish diet had no effect on shrimp survival with only minor differences in growth rate.

2.Study 2 ([redacted] (b) (4)

a.*Experimental design*

In this Study, three trials were conducted to evaluate the biological response of shrimp to KBM (Bacterial Biomass) in soy-based diets in terms of growth. In the trial 1 and 2, test diets were formulated to be isonitrogenous and isolipidic (35% protein and 8% lipid). All experimental diets were produced at the [redacted] (b) (4) (b) (4) sing the standard procedures for the shrimp feeds described by (Qiu and Davis, 2016)). Dry pellets were crumbled, packed in sealed bags, and stored in a freezer until use.

The growth trials were conducted at the [redacted] (b) (4) (b) (4) USA). Pacific white shrimp post larvae (PL) were obtained from [redacted] (b) (4) and nursed in an indoor recirculating system. PLs were fed a commercial feed [redacted] (b) (4) using an automatic feeder for ~1 week, and then switched to crumbled commercial shrimp feed ([redacted] (b) (4)) for ~1- 2 weeks.

<u>In trial 1</u>, three experimental diets (T_1D_1 - T_1D_3) were formulated to contain increasing levels (0, 6, and 12%) of KBM as a replacement of SBM.

Composition (% as is) of test diets utilized in trial 1.

Ingredient	Diet code		
	T_1D_1	T_1D_2	T_1D_3
Soybean meal[1]	54.10	47.40	40.50
Corn protein concentrate[2]	8.00	8.00	8.00
Whole wheat[3]	25.00	25.00	25.00
KBM	0.00	6.00	12.00
Fish oil[2]	6.05	6.14	6.24
Trace mineral premix[5]	0.50	0.50	0.50
Vitamin premix[6]	1.80	1.80	1.80
Choline chloride[3]	0.20	0.20	0.20
Stay C[7]	0.10	0.10	0.10
Mono-dicalcium phosphate[8]	2.50	2.50	2.50
Lecithin[9]	1.00	1.00	1.00
Cholesterol[3]	0.05	0.05	0.05
Corn starch[3]	0.70	1.31	2.11

[1] De-hulled solvent extract soybean meal, [redacted] (b) (4).

[redacted] (b) (4).

[3] [redacted] (b) (4)

[5] [redacted] (b) (4) [redacted]

[6] [redacted] (b) (4) [redacted].

[7] [redacted] (b) (4) [redacted]

[8] [redacted] (b) (4)

[9] [redacted] (b) (4).

Proximate composition (% as is) and amino acid profile (% as is) of the test diets used in trial 1.

Composition[1]	T_1D_1	T_1D_2	T_1D_3
Crude Protein	37.67	36.37	36.77
Moisture	5.41	8.34	6.66
Crude Fat	9.54	8.71	8.49

Crude Fiber	4.05	3.48	3.05
Ash	6.06	5.65	5.51

[1] Diets were analyzed at [redacted] (b) (4)

The recirculating system consisted of 12 aquaria (160 L) with four replicate groups of shrimps (1.51 g initial mean weight; 8 shrimps/ tank) that were offered diets using a standard feeding protocol over 6 weeks. Based on historic results, feed inputs were pre-programmed and daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality.

Results for trial 1

Performance of juvenile shrimp *L. vannamei* (Initial weight 1.51g) offered diets with different bacterial biomass levels (0, 6, and 12 %) for six weeks in trial 1.

Diet	KBM levels (%)	Final biomass (g)	Final mean weight (g)	WG^3 (%)	FCR^2	Survival (%)
T_1D_1	0	[redacted] (b) (4)	8.26^{a}	[redacted] (b) (4)		
T_1D_2	6		6.96^{ab}			
T_1D_3	12		5.72^{b}			
PSE^1			0.1646			
P-value			0.0014			

[1] PSE: Pooled standard error.

[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).

[3] WG: Weight gain = (Final weight - Initial weight) / Initial weight × 100%.

Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

b. In trial 2, to confirm the results in trial 1 and investigate the effects of low inclusion levels of KBM, six experimental diets (T_2D_1 - T_2D_6) were formulated to supplement with increasing levels (0, 1, 2, 4, 6, and 12%) of KBM as a replacement of SBM.

Composition (% as is) of test diets utilized in trial 2.

Ingredient	Diet code					
	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6
Fish meal[1]	6.00	6.00	6.00	6.00	6.00	6.00
Soybean meal[2]	53.00	51.90	50.80	48.60	46.50	40.10
Corn protein concentrate[3]	8.00	8.00	8.00	8.00	8.00	8.00
KBM	0.00	1.00	2.00	4.00	6.00	12.00
Fish oil[2]	5.92	5.93	5.94	5.95	5.97	6.01
Trace mineral premix[6]	0.50	0.50	0.50	0.50	0.50	0.50

Vitamin premix[7]	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride[5]	0.20	0.20	0.20	0.20	0.20	0.20
Stay C[8]	0.10	0.10	0.10	0.10	0.10	0.10
Mono-dicalcium phosphate[9]	2.50	2.50	2.60	2.60	2.80	2.90
Lecithin[10]	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol[5]	0.08	0.08	0.08	0.08	0.08	0.08
Methionine[11]	0.05	0.05	0.04	0.04	0.04	0.02
Lysine[11]	0.00	0.01	0.01	0.03	0.04	0.07
Corn starch[5]	20.85	20.93	20.93	21.10	20.97	21.22

1 [redacted].

11 [redacted].

Proximate composition (% as is) of the test diets used in trial 2.

Composition[1]	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6
Crude protein	36.33	35.52	36.42	34.29	34.48	36.10
Moisture	7.15	8.57	7.34	9.47	9.42	8.08
Crude fat	9.39	9.44	8.94	9.36	9.83	8.15
Crude fiber	3.21	3.28	3.01	2.99	2.73	2.69
Ash	6.86	6.75	6.70	6.62	6.60	6.55

1 Diets were analyzed at [redacted]

The recirculating system consisted of 24 aquaria (135 L) with four replicate groups of shrimps (10 shrimp / tank). Shrimps were offered diets using standard feeding protocol over 6 weeks. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality.

Results for trial 2

<u>Growth Performance</u> of juvenile shrimp *L. vannamei* (Initial weight 0.98g) offered diets with different bacteria biomass levels (0, 1, 2, 4, 6, and 12 %) for six weeks in trial 2.

Diet	KBM levels (%)	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_2D_1	0	[redacted]	8.4^{ab}	[redacted]		
T_2D_2	1		9.2^{a}			
T_2D_3	2		8.6^{ab}			
T_2D_4	4		8.5^{ab}			
T_2D_5	6		7.7^{b}			
T_2D_6	12		5.8^{c}			
PSE[1]			0.1031			

P-value	(b) (4)	<0.0001	(b) (4)		

[1] PSE: Pooled standard error.

[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).

Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Proximate composition (moisture: % as is; protein and lipid: % dry weight) and amino acid profile[2] (% dry weight) of whole shrimp body.

Diet	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6	PSE[1]	*P*-value	Adjust *P*-value
KBM levels (%)	0	1	2	4	6	12			
Moisture	75.65^{ab}	75.48^{ab}	75.79^{ab}	75.17^{b}	76.93^{ab}	77.23^{a}	0.2091	0.0117	0.0351
Protein	75.08^{b}	74.97^{b}	74.39^{b}	74.80^{b}	75.28^{b}	77.77^{a}	0.1807	<0.0001	0.0003
Lipid	6.37^{b}	6.54^{ab}	7.92^{a}	7.26^{ab}	5.76^{b}	3.62^{c}	0.1706	<0.0001	<0.0001
Alanine	4.27	4.33	4.44	4.28	4.40	4.31	0.0365	0.5092	0.5555
Arginine	5.45^{bc}	5.23^{c}	5.43^{c}	5.47^{bc}	5.72^{b}	6.17^{a}	0.0325	<0.0001	<0.0001
Aspartic Acid	6.76	6.94	6.79	6.71	6.84	6.86	0.0322	0.2140	0.3425
Cysteine	0.60	0.61	0.61	0.60	0.62	0.63	0.0039	0.0673	0.1614
Glutamic Acid	10.18	10.41	10.16	10.06	10.22	10.31	0.0559	0.3439	0.4855
Glycine	5.01^{cd}	4.83^{d}	5.01^{cd}	5.19^{bc}	5.49^{b}	6.08^{a}	0.0350	<0.0001	<0.0001
Histidine	1.49	1.52	1.49	1.48	1.50	1.52	0.0115	0.6861	0.7159
Hydroxylysine	0.14	0.15	0.17	0.18	0.17	0.17	0.0070	0.5032	0.5555
Hydroxyproline	0.20	0.22	0.23	0.21	0.21	0.20	0.0051	0.4981	0.5555
Isoleucine	2.95	3.00	2.95	2.93	2.97	2.95	0.0111	0.3829	0.5105
Leucine	4.95	5.03	4.94	4.92	4.97	5.01	0.0162	0.1896	0.3250
Lysine	4.92	5.04	4.98	4.93	5.04	5.11	0.0239	0.0874	0.1907
Methionine	1.46^{c}	1.49^{c}	1.50^{bc}	1.51^{abc}	1.55^{ab}	1.57^{a}	0.0065	0.0002	0.0010
Phenylalanine	3.16	3.23	3.20	3.18	3.20	3.27	0.0169	0.3398	0.4855
Proline	4.09^{a}	4.15^{a}	4.18^{a}	4.00^{ab}	3.88^{ab}	3.67^{b}	0.0415	0.0036	0.0143
Serine	2.35	2.38	2.35	2.34	2.36	2.36	0.0195	0.9934	0.9934
Threonine	2.62^{b}	2.76^{a}	2.62^{b}	2.60^{b}	2.64^{b}	2.61^{b}	0.0135	0.0049	0.0169
Tryptophan	0.87	0.85	0.85	0.85	0.85	0.88	0.0048	0.1132	0.2263
Tyrosine	2.51	2.33	2.49	2.52	2.45	2.57	0.0405	0.4536	0.5555
Valine	4.10	4.19	4.12	4.16	4.17	4.00	0.0257	0.1632	0.3014
Total	68.05	68.66	68.49	68.08	69.23	70.23	0.2581	0.0629	0.1614

[1] PSE: Pool standard error.

[2] Proximate composition and amino acid profile of whole body samples were analyzed at (b) (4)

(b) (4)

Values within a row with different superscripts are significantly different based on Tukey's multiple range test.

iii.In trial 3, five experimental diets (T_3D_1 - T_3D_5) were formulated (Table 3). Additionally, a reference diet was utilized to determine digestibility coefficients in conjunction with 1% chromic oxide as an inert marker and 70:30 replacement strategy.

Composition (% as is) of test diets utilized in trial 3.

Ingredients	Diet code				
	T_3D_1	T_3D_2	T_3D_3	T_3D_4	T_3D_5
Fish meal[1]	6.00	6.00	6.00	6.00	6.00
Soybean meal[2]	53.00	46.50	46.50	40.10	40.10
Corn protein concentrate[3]	8.00	8.00	8.00	8.00	8.00
KBM	0.00	6.00	13.30	12.00	26.60
Fish oil[2]	5.92	5.97	5.81	6.01	5.70
Trace mineral premix[6]	0.50	0.50	0.50	0.50	0.50
Vitamin premix[7]	1.80	1.80	1.80	1.80	1.80
Choline chloride[5]	0.20	0.20	0.20	0.20	0.20
Stay C[8]	0.10	0.10	0.10	0.10	0.10
Mono-dicalcium phosphate[9]	2.50	2.80	2.80	2.90	2.90
Lecithin[10]	1.00	1.00	1.00	1.00	1.00
Cholesterol[5]	0.08	0.08	0.08	0.08	0.08
Methionine[11]	0.05	0.04	0.03	0.02	0.02
Lysine[11]	0.00	0.04	0.05	0.07	0.09
Corn starch[5]	20.85	20.97	13.83	21.22	6.91

Proximate composition (% as is) of the test diets used in trial 3.

Composition[1]	T_3D_1	T_3D_2	T_3D_3	T_3D_4	T_3D_5
Crude protein	35.7	33.7	38.4	34.7	41.1
Moisture	8.7	11.71	8.39	9.7	10.46
Crude fat	6.71	7.57	8.2	7.64	7.26
Crude fiber	3.1	2.47	2.64[2]	2.62	2.39[2]
Ash	7.08	6.67	7.08	6.61	6.56

[1] Diets were analyzed at (b) (4)

[2]Diets were analyzed at (b) (4)

The recirculating system consisted of 24 aquaria (135L) with four replicate groups of shrimps (10 shrimps/ tank) that were fed as described for trial 2 for 6 week.*Results for trial 3*

Performance of juvenile shrimp *L. vannamei* (Initial weight 0.15g) offered diets formulated to partially replace soybean meal on a digestible protein basis for six weeks in trial 3.

Diet	KBM levels (%)	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_3D_1	0	42.68^{a}	4.74^{a}	3160.39^{a}	1.72^{c}	90.0
T_3D_2	6	43.15^{ab}	4.30^{ab}	2813.38^{ab}	1.90^{bc}	100.0
T_3D_3	13.3	45.38^{ab}	4.54^{a}	2732.16^{abc}	1.73^{c}	100.0
T_3D_4	12	38.48^{ab}	3.84^{bc}	2438.14^{bc}	2.11^{ab}	100.0
T_3D_5	26.6	35.05^{b}	3.60^{c}	2304.94^{c}	2.26^{a}	97.5
PSE[1]		1.1420	0.0710	57.1783	0.0338	1.9084
P-value		0.0406	0.0002	0.0008	0.0001	0.3194

[1] PSE: Pooled standard error.

[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).

[3] WG: Weight gain = (Final weight - Initial weight) / Initial weight × 100%.

Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Proximate composition of whole shrimp body and protein retention efficiency (PRE) offered diets formulated to utilize bacterial biomass (KBM) partially replace soybean meal on a digestible protein basis for six weeks in trial 3.

Diet	KBM levels (%)	Protein[2] (%)	Moisture (%)	Lipid[2] (%)	Fiber[2] (%)	Ash[2] (%)	PRE[3] (%)
T_3D_1	0	70.83^{b}	76.1	8.40^{a}	5.25	11.50^{c}	30.50^{a}
T_3D_2	6	70.68^{b}	76.7	7.89^{a}	5.26	11.80^{c}	29.37^{a}
T_3D_3	13.3	72.52^{ab}	76.6	5.07^{b}	5.30	12.56^{bc}	27.97^{a}
T_3D_4	12	72.59^{ab}	77.0	6.00^{ab}	5.48	13.35^{ab}	25.43^{ab}
T_3D_5	26.6	73.55^{a}	77.4	4.07^{b}	5.68	14.01^{a}	20.11^{b}
P-value		0.0107	0.2379	0.0003	0.3623	<0.0001	0.0002
PSE[1]		0.2819	0.1932	0.2803	0.0849	0.1399	0.6234

[1] PSE: Pool standard error.

[2] Dry weight basis.

[3] Protein retention (%) = (Final weight × Final protein content) - (Initial weight × Initial protein content) × 100 / Protein offered.

Conclusion.

The results of this study show that, during a 12-week growth period, the notified substance can be included up to 6% by replacing soybean meal in shrimps without significantly affecting growth performance, FCR, and protein as well as amino acids retention efficiency. Moreover, an increase in survivability was observed in the first trial, suggesting a beneficial effect of the notified substance in the conditions tested.

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Appendix 3-1. Updated Literature Google Scholar search results: *M. extorquens* Pathogenicity

Appendix 3-2. Updated Literature Google Scholar search results: *M. extorquens* Toxicity