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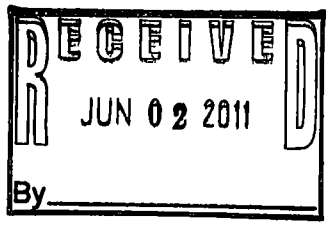
May 27, 2011

Mr. Geoffrey K. Wong
Division of Animal Feeds (HFV-224)
Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Pl.
Rockville, MD 20855
240-453-6879
Geoffrey.Wong@fda.hhs.gov

RE: *Bacillus cereus* var. *toyoi* (*B. toyoi*) Animal Feed Ingredient GRAS Notification

Dear Mr. Wong:

In accordance with the Notice of Pilot Program published in the Federal Register 75 FR 31800-31803 (a notice of a claim for exemption based on a GRAS determination), I am submitting in triplicate, as the agent of the notifier, Rubinum S.A. Animal Health, a GRAS notification for the use of *Bacillus cereus* variant *toyoi* (*B. toyoi*) for use in swine, fattening cattle and calves, chicken broilers and turkeys, fattening rabbits and breeding does to help maintain the animal's gut microflora. A GRAS expert panel dossier setting forth the basis for the GRAS determination, as well as *curriculum vitae* of the members of the GRAS panel and all cited references, are enclosed.



Best regards,

Ray A. Matulka

Raymond A. Matulka, Ph.D.
Director of Toxicology

1. GRAS Exemption Claim

A. Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR 170.36(c)(1)

Rubinum S. A. Animal Health, on the advice of qualified experts, has concluded *B. toyoi,* a non-toxigenic strain of *Bacillus cereus*, to be generally recognized as safe (GRAS) as an animal feed ingredient and therefore, exempt from the requirement of premarket approval, under the conditions of its intended use as described below. The common name for this ingredient is *Bacillus cereus* variant *toyoi* (*B. toyoi*) for consumption by swine (fattening pigs, sows and gilts, and piglets), fattening cattle and calves, chicken broilers and turkeys, fattening rabbits and breeding does to help maintain the animal's gut microflora. The basis for this finding is described in the following sections.

Signed,

Ram A. Matulka 27MAY2011

Raymond A. Matulka, Ph.D. **Date**
801 N. Orange Avenue Suite 710
Orlando, FL 32801

(i) Name and Address of the Notifier

Rubinum S.A. Animal Health
P.O. Box 283
Av. de la Llana No. 123,
E-08191, Rubí (Barcelona), Spain

Agent of the Notifier:

Raymond A. Matulka, Ph.D.
Director of Toxicology
Burdock Group
801 N. Orange Ave. Suite 710
Orlando, FL 32801
Telephone: 407-802-1400
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(ii) Common Name of the Notified Substance

The common name of the ingredient *Bacillus cereus* variant *toyoi* (*B. toyoi*), for the purposes of this GRAS Notification has been defined as:

Bacillus cereus variant *toyoi* (*B. toyoi*)

The common name will be abbreviated to *B. toyoi* throughout the remainder of this notification and the use of the term "Toyocerin®" within this notification denotes the use of the commercial product that contains *B. toyoi*.

(iii) Conditions of Use

B. toyoi is manufactured as a finished product under the commercial name Toyocerin® to be used at levels up to 2.0 x 10^9 CFU[1] *B. toyoi*/kg feed, for consumption by swine (fattening pigs, sows and gilts, and piglets), fattening cattle and calves, chicken broilers and turkeys, fattening rabbits and breeding does (Table 1).

[1] In the context of this GRAS, CFU equates to a single colony forming unit of *B. toyoi,* typically in spore form.

Table 1. Typical use of *B. toyoi* by swine, bovine, poultry and rabbits

Animal Species	Toyocerin® level in feed (g/kg feed)	*B. toyoi* intake (CFU/kg feed)
Swine		
Fattening pigs	0.1	1.0×10^9
Sows and gilts	0.2	2.0×10^9
Piglets	0.1	1.0×10^9
Bovine		
Fattening cattle	0.02	2.0×10^8
Calves	0.1	1.0×10^9
Poultry		
Chicken broilers	0.1	1.0×10^9
Turkeys	0.1	1.0×10^9
Rabbits		
Fattening rabbits	0.1	1.0×10^9
Breeding does	0.1	1.0×10^9

CFU = colony-forming units

Utility: *B. toyoi* is to be provided to help maintain/support the animal's gut microflora. The ability of *B. toyoi* to help maintain or support the animal's gut microflora has been reported in several published manuscripts. *B. toyoi* has been shown to germinate and form vegetative cells in both broiler chickens and suckling piglets (Jadamus *et al.*, 2001), which has been stated by the Federal and Drug Administration (FDA) as necessary for use of microbial products in animal feeds.[2] Thelen *et al.* (2004) evaluated the use of *B. toyoi* ($1x10^9$ CFU/kg of feed) in early weaned piglets, and reported that the use of *B. toyoi* had a flora stabilizing effect, although it did not have an effect on the average daily live weight gain or the overall feed intake. *B. toyoi* use did not have a significant effect on the total numbers of aerobic or anaerobic bacteria growing in the duodenum or caecum of the piglets. Feeding of *B. toyoi* did not significantly change lactobacilli or enterococci in the duodenum, caecum or rectum. *B. toyoi* fed to piglets before and after weaning had a stabilizing effect on the total anaerobic bacterial growth throughout the intestine, as well as lactic acid bacteria and enterobacterial growth capacities in mucosal tissue samples in the duodenum, jejunum, ileum, caecum and colon (Jadamus *et al.*, 2000).

Jadamus *et al.* (2002) evaluated the effect of *B. toyoi* on enterobacterial growth in digesta samples from piglets before and shortly after weaning. At the start of the study, pregnant sows (n = 3/group) were fed basal diet or a diet supplemented with Toyocerin® ($1x10^9$ CFU/kg feed), starting 14 days before farrowing. The sows farrowed and, starting at 14 days of age, the suckling piglets (n > 10 piglets/sow) had *ad libitum* access to prestarter feed without Toyocerin®, respectively. Three piglets from each dose group were killed at the age of 13, 21, 28 (weaning) and 32 days, and the contents of the stomach, duodenum, jejunum ileum, caecum and colon were collected and evaluated for enterobacteria. Access to the prestarter diet at the time of weaning generally did not significantly change the development of enterobacterial growth capacities, although a significant increase in growth was noted in the ileum. Four days after weaning, Toyocerin® consumption helped to maintain enterobacteria growth throughout the digestive system, while the control pigs had decreased enterobacteria concentrations in the ileum and colon, compared to levels reported in the jejunum and caecum (Jadamus *et al.*, 2002).

A report by the Asahi Chemical Industry Company (ZEN-NOH, 1978) showed that the addition of *B. toyoi* (100 ppm[3], or $1x10^9$ *B. toyoi* CFU/kg milk replacer) to dairy calves (n = 6) helped maintain total bacterial counts and *Lactobacillus* counts in the duodenum and caecum

[2] http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm074707.htm; CPG Sec. 689.100 Direct –Fed Microbial Products; site last visited May 3, 2011.

[3] ppm = parts *per* million

over a 21 day period. *Escherichia coli* counts in the caecum were not different from control values, although a decrease was noted in the duodenum.

Dietary supplementation of *B. toyoi* to rabbits did not change the total cellulolytic bacteria counts, dry matter or crude protein digestibility coefficients, although the reduction in the fecal excretion of nitrogen was significantly affected ($P < 0.05$) by *B. toyoi* administration (Pascual *et al.*, 2008). There were no significant effects on the microbiological parameters evaluated.

Overall, the data cited above indicates that the consumption of *B. toyoi* helps to maintain the animal's gut microflora without adverse effects. This work has been conducted in a variety of the target animal species at the indicated levels of use.

(iv) Basis of GRAS Determination

Pursuant to 21 CFR § 570.35 and the Pilot Program described in the Federal Register (Volume 75, Number 107, June 4, 2010, pages 31800-31803), the use of *B. toyoi* as an animal feed ingredient in the animal species and intended typical feed intake shown in Table 1, has been determined GRAS by scientific procedures for its intended conditions of use. The safety of *B. toyoi* for this use is supported by the information contained in the attached Dossier in Support of the Generally Recognized As Safe (GRAS) Status of *Bacillus cereus* var. *toyoi* as an Animal Feed Ingredient and the Amendment to the Dossier in Support of the Generally Recognized As Safe (GRAS) Status of *Bacillus cereus* var. *toyoi* as an Animal Feed Ingredient. The dossier and amendment discuss the studies utilized to determine the safety-in-use of *B. toyoi*, which includes feeding trials in which rabbits, pigs, chickens, turkeys and cattle (the species of interest) have been fed diets containing *B. toyoi* at or above the recommended intake of *B. toyoi per* day.

(v) Availability of Information

The data and information that serve as a basis for this GRAS determination are available for FDA review and copying at reasonable times at:

Burdock Group
801 N. Orange Ave. Suite 710
Orlando, FL 32801
Telephone: 407-802-1400
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Alternatively, data and information that serve as a basis for this GRAS determination may be sent to FDA upon request.

2. Detailed Information about the Identity of the Notified Substance

A. Identity

B. toyoi is a naturally-occurring, non-toxigenic strain of *Bacillus cereus* that is manufactured to the final product, Toyocerin®, a dry, odorless, and white to grayish-brown powder that includes calcium carbonate and corn flour carriers. *B. toyoi* was originally isolated from the soil and has not been genetically altered in any fashion and therefore not considered to provide an adverse effect on the environment. Bacilli are capable of anaerobic growth and fermentation of carbohydrates, and *B. toyoi* has been reported to non-significantly increase short-

chain fatty acid production in piglets (Jadamus *et al.*, 2002). The general descriptive parameters of Toyocerin® are presented in Table 2. After 5 - 10 hours incubation on nutrient agar (pH 7.2, 37 °C aerobic conditions), *B. toyoi* colonies are circular, white, diffuse colonies that have slightly undulated edges. At 20 – 30 hours, each colony has a round convex elevation formed on top of the colony. The vegetative cells are Gram-positive rods, 1.0-1.2 µm x 3.0-5.0 µm, motile, stain red with malachite green/safranin stain while with Gram stain *B. toyoi* stains blue, and grows in short to long tangled chains. *B. toyoi* spores are ellipsoidal and occupy a central or paracentral position in the bacterial cell, and the spores stain green with malachite green/safranin stain. *B. toyoi* has been deposited in four internationally recognized culture collection systems (Table 3).

Table 2. General description of Toyocerin® containing *B. toyoi*

Bacterial ingredient (synonym)	*Bacillus cereus* var. *toyoi* (*B. toyoi*)
Color	White to grayish-brown
Molecular formula	N/A
Molecular weight	N/A
Odor	Odorless
Physical state	Dry powder
Taste	N/A
Storage	Closed package in a cool, dry place between 15 °C and 25 °C

N/A = Not available

Table 3. International depository of *B. toyoi*.

Depository	Accession No.	Date of Deposit
Collection Nationale de Cultures de Micro-organisms (CNCM). Institute Pasteur. 25-28 Rue du Docteur Roux-75015. Paris. France	CNCM No. 1-1012	November 5, 1990
The National Collection of Industrial and Marine Bacteria, Ltd. (NCIMB) Fersuson Building. Caribstone Estate, Bucksburn. Aberdeen. AB21 9YA. Scotland.	NCIMB No. 40112	February 9, 1989
Colección Espanola de Cultivos Tipo (CECT). Universidad de Valencia. Edificio de Investigación. Campus Burjassot. 46100 Burjassot. Valencia. Spain	CECT No. 876	January 11, 1989
Fermentation Research Institute, Agency of Industrial Science and Technology, Japan	FERM-P. No. 1214	December 8, 1971

Identification of *B. toyoi* and differentiation of *B. toyoi* from wild-type *Bacillus cereus* isolates may be performed by an antibiotic susceptibility test and Fourier transform infrared spectroscopy (FT-IR) (Mietke *et al.*, 2010). The antibiotic susceptibility test is conducted by means of the agar diffusion test on Mueller-Hinton agar using filter paper discs loaded with cefamandole, penicillin G and tetracycline. Inhibition zones greater than 15 mm indicated antibiotic sensitivity. *B. toyoi* will be susceptible to the β-lactam antibiotics (*i.e.*, penicillin and cefamandole) and resistant to tetracycline. Most wild-type strains will be resistant to β-lactam antibiotics and susceptible to tetracycline. The FT-IR analysis will provide spectra that are distinctly different for each strain analyzed (Mietke *et al.*, 2010). Cluster analysis of the *B. cereus* strains analyzed showed that *B. toyoi* is clearly distinguishable and far removed from all other strains included in the study (Mietke *et al.*, 2010). In addition, differences in biochemical characteristics (API System testing), sensitivity to phi-7 phage (specific to *B. toyoi*) (Kozasa and

et al, 1975), serological cross-reactivity, pyrolysis mass spectrometry (Shute and Linton, 1987), DNA-homology, rRNA restriction patter, Multiplex-PCR and pulsed field gel electrophoresis (PFGE) may be further utilized for the classification and unique identification of *B. toyoi* as a variant (*i.e.*, subspecies) strain of *Bacillus cereus* (Table 4) (Hattori, 1986, Leuschner *et al.*, 2002, Pecoraro and Bucher, 2002, Atanassova, 2009). *B. toyoi* may also be identified according to Bergey's manual of systematic bacteriology and differentiated from other *B. cereus* bacteria by the fact that *B. cereus* gives a strong response in the utilization of citrate, while *B. toyoi* provides a negative or weak reaction (Sakamoto *et al.*, 1989).

Table 4. Characteristics of *B. toyoi*

Characteristics	*B. cereus*	*B. toyoi*
API system testing (biochemical characteristics)		
Utilization of citrate (API test)	+	- or w
Acid from *a*-Methyl-*D*-glucoside (API Test)	-	+
Acid from *D*-turanose (API Test)	-	+
Sensitivity to phi-7 phage	-	+
Serology	No cross-reactivity with *B toyoi*	No cross-reactivity with *B. cereus*
Pyrolysis mass spectrometry	Typical for *B. cereus*	Atypical for *B. cereus*
DNA-Homology	Not homologous with *B toyoi*	Not homologous with *B. cereus*
rRNA restriction patterns (Ribotyping)	Distinguishable with *B. toyoi*	Distinguishable with *B. cereus*
Multiplex-PCR & RAPD	Distinguishable with *B. toyoi*	Distinguishable with *B. cereus*
Fourier transform infrared spectroscopy (FT-IR)	Distinguishable with *B. toyoi*	Distinguishable with *B. cereus*
Pulsed Field Gel Electrophoresis (PFGE)	Distinguishable with *B. toyoi*	Distinguishable with *B. cereus*

+ = positive; - = negative; w = weak positive; API = Analytical Profile Index; *B. cereus* = *Bacillus cereus*; *B toyoi* = *Bacillus toyoi*; PCR = Polymerase Chain Reaction; RAPD = Random Amplification of Polymorphic DNA

A variety of studies have been conducted on *B. toyoi* that confirm that *B. toyoi* does not produce antibiotics, does not transfer resistance to the antibiotics tetracycline, chloramphenicol and sulfamethoxazole to other strains of *Bacillus* and *Escherichia coli*, does not receive plasmids from other bacterial strains, and that plasmids from *B. toyoi* do not confer antibiotic resistance to other bacterial strains, as described in unpublished studies and stated in a publicly available safety assessment of *B. toyoi* by the European Commission (Ogimoto and Inamoto, 1993, Kataoka, 1994, EC, 2001). The European Commission (EC, 2001) further cites *in vitro* studies show that *B. toyoi* does not contain the complete genetic complement necessary to produce functional enterotoxins or an emetic toxin, as well as unpublished study reports (Bauerfeind and Wieler, 1993). This information provides further corroborative data confirming the safety of *B. toyoi.*

B. toyoi was also analyzed to determine antibiotic resistance by evaluating the minimum inhibitory concentration (MIC) of various antimicrobials, which included antibiotics relevant in human or veterinary medicine (Table 5). The lower the MIC number, the more sensitive *B. toyoi* is to the antimicrobial agent, and the data in Table 5 shows that *B. toyoi* is sensitive to a variety of antibiotics. Overall, *B. toyoi* is sensitive to antibiotics used in human and veterinary medicine except for tetracyclines, chloramphenicol and sulfonamides.

Table 5. Minimum inhibitory concentrations (MIC) of antimicrobial agents and coccidiostats against *B. toyoi* (Rubinum, 2010)

Antimicrobial agent	MIC (µg/ml)	Antimicrobial agent	MIC (µg/ml)
Ampicillin*	< 0.20	Bacitracin	100
Vancomycin*	1.56	Cefalexin	12.5
Gentamicin*	< 4	Oxacillin	< 0.20
Kanamycin*	6.25	Penicillin G	< 0.20
Streptomycin*	3.13	Dicloxacillin	0.20
Erythromycin*	3.13	Oleandomycin	0.20
Clindamycin*	< 4	Virginiamycin	6.25
Quinupristin + Dalfopristin*	0.5	Chlortetracycline	50
Tetracycline*	> 100	Flavophospholipol	6.25
Chloramphenicol*	> 100	Spiramycin	3.13
Sulfonamides	> 100	Carbadox	50
Polymyxin B	100	Furazolidone	3.13
Oxytetracycline	> 100	Apramycin	0.05
Colistin	> 100	Tylosin	1.56
Nalidixic acid	> 100	Kitasamycin (Leucomycin)	0.20
Fradiomycin (Neomycin)	3.13	Tiamulin	0.20
Avilamycin	3.13		
Monensin sodium	0.78	Maduramycin	1.25
Lasalocid sodium	0.78	Nicarbazin	> 50
Salinomycin sodium	0.39	Naracin	1.25
		Nicarbazin + Narazin	1.56

*Relevant antimicrobials in human or veterinary medicine whose MICs are required to be tested for any micro-organism intended for use as feed additive in accordance with the EFSA's Technical Guidance "*Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance*"; EFSA = European Food Safety Authority

Methods for the analysis of *B. toyoi* in the product (*i.e.*, Toyocerin®), and the subsequent feeds have been submitted and evaluated by the European Union (EU) Community Reference Laboratory for Feed Additives (CRL-FA) for authorizations for use of Toyocerin® in animal feeds in the EU. Three reference samples of Toyocerin® have been sent to CRL-FA and have been stored under the CRL sample number 0001-2009-223-0001. The general methodology to quantitate the *B. toyoi* contained in Toyocerin® and in feeds has been evaluated by the EU, published in the Journal of AOAC International,[4] and found to be appropriate for the enumeration of *Bacillus* species when used as feed ingredients (Leuschner and Bew, 2002, 2003). The method may be applied to enumerate bacilli in an additive, premixture or feedingstuff assuming that the probiotic bacilli is present in far higher numbers than any other bacilli. Additional use of the Fourier transform infrared spectroscopy (FT-IR) would separate *B. toyoi* from wild-type *Bacillus cereus/Bacillus thuringiensis/Bacillus mycoides/Bacillus weihenstephanensis* strains by means of hierarchical cluster analysis (Mietke *et al.*, 2010).

B. Composition

The composition of Toyocerin® is summarized in Table 6. Toyocerin® is a mixture of *B. toyoi* spore concentrate, corn flour and calcium carbonate.

[4] AOAC = Association of Analytical Communities; http://www.aoac.org/about/aoac.htm; site last visited May 3, 2011

Table 6. Composition of Toyocerin®

Analysis	Average
B. toyoi concentrate (*Percent* of net weight of the product) (CNCM I-1012/NCIMB 40112)	6%
B. toyoi viable spores (CFU)	1×10^{10} CFU/g product (Range of $0.75 \times 10^{10} - 1.25 \times 10^{10}$ CFU/g product)
Calcium carbonate (*Percent* of net weight of the product)	90%
Corn flour (*Percent* of net weight of the product)	4%

CFU = colony forming units

C. Method of Manufacture of Toyocerin®

(b) (4)

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[5] 21CFR§184.1553 Peptones.
[6] 21CFR§184.1277 Dextrin.
[7] AAFCO OP 48.24 Condensed Fermented Corn Extractives.
[8] AAFCO OP (2011) 96.11 Yeast Extract.
[9] 21CFR173.340 Defoaming agents.
[10] http://www.fami-qs.org/index.htm; site last visited May 19, 2011.
[11] http://www.dnvba.com/Global/food-beverage/food-safety/Pages/animal-feed-and-ingredients.aspx; site last visited May 19, 2011.

(b) (4)

Figure 1. Schematic of the Toyocerin® manufacturing process

D. Specifications for *B. toyoi* as a Feed Ingredient

The Physical and chemical specifications provided in Table 7 for *B. toyoi* include inert carriers, heavy metal analysis, and microbial evaluations.

Table 7. Specifications for Toyocerin®

Parameter	Specification
Microorganism contained in Toyocerin®	1 x 10^{10} viable CFU of *B. toyoi**
Carriers	
Calcium carbonate	~ 90% of net weight of product
Corn flour	~ 4% of net weight of product
Particle size (to pass through 18 mesh sieve)	< 850 microns
Heavy metals	
Arsenic	< 2 ppm
Cadmium	< 1 ppm
Lead	< 10 ppm
Mercury	< 0.3 ppm
Microbiological assays	
Aflatoxin B-1	< 0.05 ppm
Escherichia coli	Less than L.O.D. (3 MPN/g product)
Coliforms	Less than L.O.D. (3 MPN/g product)
Salmonella	Absent in 25 g product
Shelf life	Maximum of 18 months from date of packaging when stored in a cool, dry place

CFU = colony forming units; L.O.D. = Limit of detection; MPN = most probable number; ppm = parts *per* million; *Approximately 6% of net weight of Toyocerin®

3. Self-Limiting Levels of Use

The quantity of *B. toyoi* used in animal feed is as self-limiting as any other similar direct-fed microbial might be.

4. Stability

The stability of the viable spores in *B. toyoi* in Toyocerin® was examined during the pelleting process of feeds for pigs, rabbits, laying hens and turkeys (Table 8). The results indicate that Toyocerin® remains stable during the pelleting process for each feed. The stability of Toyocerin® (*i.e.*, analysis for the loss of viability of *B. toyoi* spores) during the storage of the processed (*i.e.*, pelleted) feed has been analyzed (Table 9), with the results demonstrating that Toyocerin® is stable in pelleted pig feed. Toyocerin® remains around 70% stable during a three-month storage period under high temperature (30-44°C) and high humidity (60-80% relative humidity). The stability of Toyocerin® has also been analyzed after the addition to chicken feed and rabbit feed and stored at room temperature, or elevated (30°C) temperature for up to three months. The theoretical final concentration of *B. toyoi* in the feed was $1x10^6$ CFU/g feed (Table 9).

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Table 8. Stability of Toyocerin® during pelleting of feeds (Rubinum S. A., 2010)

Pigs					
Place of Assay	**Feed Sample and Type**	***B. toyoi* CFU/g sample**			
		Test 1 (Pig starter feed)		**Test 2 (Pig grower feed)**	
		Sample results	**Mean**	**Sample results**	**Mean**
Quality Control Laboratory of Rubinum, S. A.	Mash Feed	1) 1.13×10^6 2) 1.10×10^6 3) 1.05×10^6	1.09×10^6 (100)[b]	1) 0.98×10^6 2) 1.12×10^6 3) 1.01×10^6	1.04×10^6 (100)
	Pelleted Feed	1) 1.02×10^6 2) 1.03×10^6 3) 1.11×10^6	1.06×10^6 (96)	1) 1.00×10^6 2) 0.89×10^6 3) 1.08×10^6	0.99×10^6 (95)
CEINAL[a]	Mash Feed	1.02×10^6 (100)		1.14×10^6 (100)	
	Pelleted Feed	1.06×10^6 (104)		1.03×10^6 (90)	
Rabbits					
Place of Assay	**Feed Sample and Type**	***viable B. toyoi* spores CFU/g sample**			
		Test 1 (Rabbit feed)		**Test 2 (Rabbit feed)**	
		Sample results	**Mean**	**Sample results**	**Mean**
Quality Control Laboratory of Rubinum, S. A.	Mash Feed (before pelleting)	1) 1.12×10^6 2) 1.08×10^6 3) 0.94×10^6	1.05×10^6 (100)[b]	1) 1.05×10^6 2) 1.21×10^6 3) 1.28×10^6	1.18×10^6 (100)
	Pelleted Feed (after pelleting)	1) 0.92×10^6 2) 0.99×10^6 3) 0.87×10^6	0.93×10^6 (89)	1) 0.98×10^6 2) 1.05×10^6 3) 1.15×10^6	1.06×10^6 (90)
CEINAL[a]	Mash Feed	0.86×10^6 (100)		1.02×10^6 (100)	
	Pelleted Feed	0.81×10^6 (94)		0.98×10^6 (96)	
Laying Hens					
Place of Assay	**Feed Type**	***viable B. toyoi* spores CFU/g sample**			
		Sample results		**Mean**	
Quality Control Laboratory of Rubinum, S. A.	Mash Feed (before pelleting)	1) 0.98×10^6 2) 0.95×10^6 3) 1.19×10^6		1.04×10^6 (100)[b]	
	Pelleted Feed (after pelleting)	1) 0.99×10^6 2) 1.14×10^6 3) 0.91×10^6		1.02×10^6 (98)	
CEINAL[a]	Mash Feed	1.13×10^6 (100)			
	Pelleted Feed	1.06×10^6 (94)			
Turkeys*					
Place of Assay	**Feed Type**	***viable B. toyoi* spores CFU/g sample**			
		Feed Group	Results (x 10^6 ***B. toyoi*** **CFU/g)**		**Difference results Pellet/mash (%)**
Quality Control Laboratory of Rubinum, S. A.	Mash feed (before pelleting)	T1-Started feed	0.808		-
		T2-Grower feed	0.807		-
		T3-Finisher feed	0.857		-
		Mean of three samples:	0.827[a] *(83)*		-
	Pelleted Feed (after pelleting)	T1-Started feed	0.812		100%
		T2-Grower feed	0.826		101%
		T3-Finisher feed	0.751		88%
		Mean of three samples:	0.796[a] *(80)*		96%

CEINAL-SILLIKER (Independent Laboratory)	Mash feed (before pelleting)	T1-Started feed	0.990	-
		T2-Grower feed	1.039	-
		T3-Finisher feed	0.830	-
		Mean of three samples:	0.953[a] *(95)*	-
	Pelleted Feed (after pelleting)	T1-Started feed	0.827	84%
		T2-Grower feed	0.885	85%
		T3-Finisher feed	0.810	98%
		Mean of three samples:	0.841[a] *(84)*	88%

a,b: Mean values with different superscript in the same column are statistical significant ($P < 0.05$)
Figures in parenthesis express the *percentage* of the expected final concentration
*Stability **after** pelleting of feeds for turkeys; CFU = colony forming units

Table 9. Stability of Toyocerin (viable *B. toyoi* spores) in pelleted feed stored at room temperature and elevated temperatures and relative humidity for 3 months (Rubinum S. A., 2010)

	***B. toyoi* CFU count[a] *per* gram of feed sample**			
Storage conditions	**Month of storage**			
	0	**1**	**2**	**3**
	Piglet pelleted starter feed			
Room temperature	1.13×10^6 (100)[b]	1.05×10^6 (93)	1.01×10^6 (89)	0.98×10^6 (87)
30°C	1.13×10^6 (100)	1.01×10^6 (90)	0.96×10^6 (85)	0.94×10^6 (82)
	Pig pelleted feed			
Room temperature	0.97×10^6 (100)	1.02×10^6 (105)	0.95×10^6 (98)	0.93×10^6 (96)
30°C 60% RH	0.97×10^6 (100)	0.82×10^6 (85)	0.73×10^6 (75)	0.71×10^6 (93)
44°C 80% RH	0.97×10^6 (100)	0.85×10^6 (88)	0.80×10^6 (82)	0.67×10^6 (69)
	Broiler feed			
Room temperature	1.11×10^6 (100)[b]	1.06×10^6 (95)	0.98×10^6 (88)	0.94×10^6 (85)
30°C	1.11×10^6 (100)	0.93×10^6 (84)	0.89×10^6 (80)	0.84×10^6 (76)
	Rabbit feed			
Room temperature	1.05×10^6 (100)[b]	1.00×10^6 (93)	0.93×10^6 (85)	0.91×10^6 (79)
30°C	1.05×10^6 (100)	0.98×10^6 (93)	0.89×10^6 (85)	0.83×10^6 (79)
	Turkey Feed			
Room temperature	0.76×10^6 (100)	N/A N/A	N/A N/A	0.790×10^6 (99)

[a] Mean of 3 Toyocerin Lot No's -5 feed samples assayed/Lot No/Time point
[b] Figures in parentheses are *percentage* survival rates in comparison with initial values.
N/A = Not analyzed

5. Basis of GRAS Determination

The determination that *B. toyoi* is GRAS is on the basis of scientific procedures, as described in the attached "Dossier in Support of the Generally Recognized as Safe (GRAS) Status of *Bacillus cereus* var. *toyoi* (*B. toyoi*) as an Animal Feed Ingredient" and the attached "Amendment to the Dossier in Support of the Generally Recognized As Safe (GRAS) Status of *Bacillus cereus* var. *toyoi* as an Animal Feed Ingredient".

No adverse effects were noted in acute studies in laboratory rats and mice when *B. toyoi* was administered orally *via* gavage at up to $2x10^{10}$ (rats) and $1x10^{11}$ CFU/kg bw[12] (mice), or when administered *via i.p.*[13] at up to $5x10^{9}$ CFU/kg bw in mice, as discussed in the attached GRAS dossier. Other *B. cereus* species have been found to be nonpathogenic and has been utilized in the production of protease enzyme for the production of milk rennet for human food (Burdock, 1997). Subchronic feeding studies in laboratory animals administered *B. toyoi* at up to $3x10^{11}$ CFU/kg bw/day did not report effects on body weight gain(s), compared to control animals. Nor was *B. toyoi* shown to be toxic in a 12-month rat study when *B. toyoi* was administered at up to $2x10^{9}$ CFU/kg bw/day (the NOAEL for this study, the highest concentration administered), and did not affect reproductive endpoints in pigs, as discussed in the attached GRAS dossier. Rats were also administered *B. toyoi* and evaluated for the distribution of *B. toyoi* from the gut to selected tissues (liver, kidney, spleen, lung and blood) (Kobayashi *et al.*, 1973). *B. toyoi* was isolated from the digestive tract for up to 72 hours after the acute oral dose of $1x10^{11}$ CFU/animal, but was not isolated at any time point from any of the tissues analyzed (assay limit was < $1x10^{3}$ *B. toyoi* CFU/g). *B. toyoi* spores injected directly into the intraperitoneal cavity at up to $1x10^{8}$ CFU/animal produced no evidence of bacteremia or inhibition of mouse (four-week-old male ddY) growth during the nine-day period following the intraperitoneal injection, compared to controls (Hoshino and Obara, 1975). *B. toyoi* does not exhibit toxicity in the rabbit intestinal tract, as shown by the intestinal loop method in rabbits described in the attached GRAS dossier. These data and information show that *B. toyoi* does not exit the intestinal tract into the tissues or exhibit localized toxicity when in the intestinal tract; therefore, humans will not be directly consuming *B. toyoi* from commercial meat-producing animals, exposure to *B. toyoi* by humans is remote and would not constitute a concern because of the lack of toxicity noted in a variety of studies and further corroborates the safety of *B. toyoi* as discussed in the attached GRAS determination.

The GRAS determination discusses the studies critical in determining the safety of *B. toyoi* in the selected target species. Additional studies have been published which corroborate the determination of safety. Lodemann *et al.* (2008) assessed the effect of feed supplementation with *B. toyoi* on the transport and barrier properties of the pig jejunum. Sows were fed *B. toyoi*-supplemented diet from Day 24 after mating and the subsequent piglets received *B. toyoi* from Day 15 after birth through Day 56. At Days 14, 28, 35 and 56 after birth, piglets (n = 5/group) were killed and tissue samples from the mid jejunum were assessed for absorptive and secretory properties. The authors reported that the addition of *B. toyoi* did not cause significant effects on the absorptive capacities, but may have only caused a stabilizing effect on glucose transport in the jejunum. Sows (n = 18/group) fed either a basal diet or a diet containing *B. toyoi* ($2.5x10^{5}$ CFU/g dry weight food) beginning 24 days after mating (*i.e.*, 90 days before birth) until birth. The authors reported that "none of the sows included in this study showed any clinical signs of disease during the course of the study" and that "[D]iagnostic screening for intestinal bacterial

[12] bw = Body weight
[13] i.p. = intraperitoneal

pathogens on days 90, 30 and 10 *ante partum*[14] showed only sporadic isolations of putative pathogens." The authors concluded that "[B]ased on the clinical health status of the sows and the low isolation frequencies of putative pathogens, we conclude there were no apparent differences in the prevalence of pathogenic bacteria during pregnancy or difference between the control and the probiotic group." The number of live-born piglets and dead-born piglets were not significantly different between control and *B. toyoi*-treated groups, but the birth weight of the live-born piglets per sow tended to be lower, after taking into account the differences in the number of piglets *per* sow. Pascual *et al.* (2008) reported that the administration of *B. toyoi* to rabbits (n = 50/group) for 30 days did not affect rabbit growth rate, feed intake or feed conversion rate during the experimental period, but did significantly reduce mortality and a sanitary risk index[15] ($P < 0.05$). The authors concluded that addition of *B. toyoi* could have provided positive changes in hindgut microflora, although no significant differences for any of the microbiological parameters studied were found. Trocino *et al.* (2005) found that administration of *B. toyoi* ($2x10^5$ or $1x10^6$ CFU/g diet) to rabbits (n = 72/group in Trial 1 and n = 60/group in Trial 2) for 35 days did not affect mortality or sanitary risk, while an increase in weight gain was reported for the low-dose *B. toyoi* feed group that did not occur in the high-dose group. The authors concluded that:

> The supplementation of *B. cereus* var. *toyoi* at the dose of $2x10^5$ spores/g diet by means of Toyocerin® improved moderately the growth performance, and reduced only the morbidity, but not the mortality or the health risk of rabbits kept in commercial farms both in the absence and presence of severe health problems. Increasing *B. cereus* supplementation until $1x10^6$ spores/g diet did not produce any improvement of performance or health status (Trocino *et al.*, 2005).

Taras *et al.* (2005) also published a study in sows that corroborates the safety of *B. toyoi* when fed to sows (Day 90 ante partum until Day 28 post-partum) and piglets (Day 15 – 56). The authors reported that "[C]ompared to the control group, supplementation of piglet diets with *B. cereus* var. *toyoi* probiotic led to no apparent difference over the total trial period from day 0 to 56 and resulted in comparable bw on day 56 (18.0 *vs.* 17.8 kg, P = 0.790)." Feed intake of lactating sows *per* sum of nursing day was not significantly different between control and *B. toyoi*-treated animals. No increased mortality was noted in the *B. toyoi*-treated sows or piglets during the study (Taras *et al.*, 2005). *B. toyoi* has also been fed to pregnant sows (n = 40/group) from 14 days prior to farrowing until 28 days post-partum (*i.e.*, piglet weaning), with no adverse effects on number of piglets born/litter, number of piglets weaned/litter, piglet body weight at birth or weaning, or in sow health (Stamati *et al.*, 2006).

B. toyoi spores have also been fed to turkeys (n = 36 BUT-9 male turkeys/dose group) for twelve weeks, with an additional four-week withdrawal period (Biedrzycka *et al.*, 2003). The performance parameters of live body weight, feed conversion rate and mortality did not change with *B. toyoi* consumption. *B. toyoi* did not affect the fecal counts of Bifidobacterium and Lactobacillus, or spore-saccharolytic bacteria during the *B. toyoi*-feeding phase. Although increased fecal *E. coli* counts were noted during *B. toyoi* administration (an increase of 0.69 log cycle), the health of the turkeys was not adversely affected (Biedrzycka *et al.*, 2003). An additional study which corroborates the safety-in-use of *B. toyoi* in chickens was conducted by Vila *et al.* (2009) who administered *B. toyoi* (at up to $1x10^9$ CFU/kg feed) to Ross 308 broiler and Single Comb White Leghorn Hy-Line W98 male one-day-old chickens in two different experimental protocols, and the birds were inoculated at days 3, 7 or 14 with *Salmonella enterica*

[14] ante partum; of or occurring in the period before childbirth
[15] The authors defined the sanitary risk index as the sum of mortality and morbidity.

var. Enteritidis. The birds were fed *B. toyoi* for 42 days and evaluated at 10, 17, 28 and 42 days for average daily gain, average daily feed intake, and the feed conversion ratio. Although this study did not contain an un-inoculated group that was fed *B. toyoi*, the authors did state that "[N]o significant interactions were detected between the main factors of the experiment (inoculation and feeding treatment) for any of the performance variables studied, except for global feed conversion rate. The inclusion of Toyocerin® in feed significantly ($P < 0.05$) improved the feed conversion rate at the end of the 42 day trial only when birds were inoculated at three days of age, but no significant differences were detected when birds were inoculated at 7 or 14 days of age (data not shown)." The safety of *B. toyoi* (as included in the product Toyocerin®) has also been corroborated with the administration of Toyocerin® to four-week-old Japanese quail (*Coturnix japonica*) for 12 weeks (Homma and Shinohara, 2004). Administration of $1x10^9$ CFU/kg feed had no effect on bodyweight, weight gain, feed intake or feed efficiency. Administration of *B. toyoi* when fed a standard diet that contained 23.5% crude protein and 11.7 MJ/kg metabolizable energy increased rectal body temperature, while birds fed a high-energy diet and *B. toyoi* did not have an increase in body weight during weeks 5-8 of the study. *B. toyoi* administration increased body temperature in both standard and high-energy diets during weeks 9-12 of the study. No adverse effects or mortalities due to *B. toyoi* administration were stated by the authors (Homma and Shinohara, 2004). An unpublished study in which Toyocerin® contained in milk replacer was fed to dairy calves from 6 until 62 days of age corroborates the published safety-in-use of *B. toyoi* (up to $1x10^9$ CFU/kg feed) in cattle, as no adverse effects were noted in the following endpoints: diarrhea, pneumonia, body weight, hematology (*i.e.*, erythrocyte, leukocyte, lymphocyte, monocyte, eosinophil, hemoglobin and basophil blood counts), plasma electrolytes, plasma enzyme levels (alanine-aminotransferase, aspartate-aminotransferase, *gamma*-glutamyl transferase, alkaline phosphatase and acetylcholinesterase), total cholesterol, triglycerides, urea, glucose, albumin and total protein (Simon and Manner, 2009).

The GRAS Expert Panel determined that *B. toyoi* is GRAS under the intended conditions of use for use as an animal feed ingredient in the diets of swine, cattle, poultry and rabbits. This determination is based on the views of experts who are qualified by scientific training and experience to evaluate the safety of substances used as ingredients in food. In addition, the Scientific Committee on Animal Nutrition of the European Commission (SCAN), which has since been modified to form the European Food Safety Authority (EFSA) has commented in publicly available documents on the addition of *B. toyoi* to feedingstuffs and has agreed that *B. toyoi* is safe at the proposed levels of target species consumption (EC, 2001, EFSA, 2007, EFSA, 2008), as was described in the amendment to the GRAS determination (please see attached GRAS dossier amendment). A review discussing the use of bacterial spore-forming bacteria in human food and animal feed stated that "[I]n the case of Toyocerin® this contains a strain of *B. cereus* var *toyoi* that has been deemed safe for animal use because of its failure to produce enterotoxins and its failure to transfer antibiotic resistance" (Hong *et al.*, 2005).

On the basis of the data and information described in the attached dossier, the data described in this notification, and other publicly available information, there is consensus among experts qualified by scientific training and experience to evaluate the safety of substances added to food, that *B. toyoi* is GRAS under the intended conditions of use.

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