EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
C2 - Management of scientific committees; scientific co-operation and networks

REPORT OF THE SCIENTIFIC COMMITTEE ON ANIMAL NUTRITION ON PRODUCT TOYOCERIN® FOR USE AS FEED ADDITIVE

(Adopted on 5 December 2001)

1. BACKGROUND

The additive Toyocerin® is a product whose active ingredient consists of viable spores of a microorganism: *Bacillus cereus* var. *toyoi* (CNCM I 1012 / NCIB-40112)

It has been authorised provisionally as a feed additive for use with pigs (until 17 July 1999).

The producer, ASAHI Vet SA, Spain, request:

– a permanent Community authorisation of the product for use as a feed additive in pigs of all physiological stages including piglets, growing-finishing animals and reproductive sows at the recommended levels shown in Table 1

– the prolongation of the provisional Community authorisations for use with calves including use in milk replacers and grower feeds, beef cattle, rabbit does and growing rabbits and poultry, including layers and broiler chickens without a withdrawal period as summarised in Table 1.

It has therefore submitted dossiers including the relevant data to the Commission through Spain, acting as national Member State rapporteur.

Following the SCAN Opinions of 17 February 2000 on the safety of use of *Bacillus* species in animal nutrition and of 3 July 2001 on antibiotic resistance, additional data on the ability of the strain to produce toxins and on the genetic basis of its antibiotic resistance were requested by the Commission and provided by the company.

2. TERMS OF REFERENCE

The Scientific Committee on Animal Nutrition is requested to assess the safety of product Toyocerin® for a possible use in animal nutrition as prescribed in Table 1 hereafter. The particular safety aspect of toxin production should be carefully considered.

In addition, as permanent Community authorisation of a feed additive requires that efficacy be demonstrated, the Scientific Committee is requested to assess the efficacy of the product for use with pigs at the levels recommended by the company.

Table 1

Additive	Chemical formula, description	Species or category of animal	Maximum age	Minimum content	Maximum content
				CFU/kg of complete feedingstuff	
Toyocerin ® *Bacillus cereus* CNCM I 1012 / NCIMB - 40112	Mixture of *Bacillus cereus* Containing a minimum of 1 x 10^9 CFU/g of the additive	Piglet	From weaning to 2 months	10^9	10^9
		Pigs for fattening	From 2 to 4 months (25-60 kg) From 60 kg until slaughter	$0.5x10^9$ $0.2x10^9$	10^9 10^9
		Sows	From service to weaning	$0.5x10^9$	$2x10^9$
		Chicken for fattening Laying hens	-	$0.2x10^9$	10^9
		Cattle for fattening	From 6 months to marketing	$0.2x10^9$	$0.2x10^9$
		Calves	6 months	$0.5x10^9$	10^9
		Rabbits for fattening Breeding does	-	$0.1x10^9$	$5x10^9$

3. GENERAL DESCRIPTION

The Toyocerin® product was first considered by SCAN in an Opinion published 13th January, 1995 in which various questions relating to the safety of the product were addressed. In preparing the present Opinion, SCAN was cognisant of the conclusions reached in this earlier Opinion and, to avoid unnecessary duplication, gave greater emphasis to those issues which arisen since 1995 or where the SCAN had previously identified areas where it was considered desirable to see more information[1].

3.1. Specifications of the active substance

The active constituent of the additive is a preparation of viable spores of a strain of *Bacillus cereus* var. *toyoi* originally isolated from soil. The strain has been deposited at the Fermentation Research Institute Agency of Industrial Science and Technology under the accession number FERM-P.N°.1214, at the Colection Espanola de Cultivos Tipo Departamento de Microbiologia Facultad de Ciencias Biologicas Universidad de Valencia with the accession number CECT N° 876, at the UK National Collection of Industrial and Marine Bacteria, Ltd. with the accession number NCIMB N° 40112 and at the Collection Nationale de Cultures de Microorganismes Institut Pasteur with the accession number N° 1-1012. It has been identified according to criteria given in Bergey's Manual of Systematic Bacteriology and is not genetically modified.

The product strain has been extensively characterised biochemically and by molecular methods and shown to be genetically stable and to contain seven plasmids.

3.2. Characterisation and use of the additive

The bacterial strain is incorporated into the product as spores and the final product contains at least 1 x 10^9 spores of *B. cereus* var. *toyoi*/g, the remainder consisting of maize flour (4% by weight) and calcium carbonate (90 % by weight). Routine monitoring for heavy metals (<30 ppm), contaminating microorganisms and mycotoxins is undertaken.

The strain demonstrates the resistance typical of bacterial endospores to temperature, desiccation and other environmental factors likely to kill or reduce the viability of the vegetative form and, consequently, is highly stable. Viability was retained for at least one year under recommended conditions of storage and after pelleting of pig and rabbit feeds.

The product is intended for use with sows, and pigs of all ages for fattening, laying hens, chickens for fattening, rabbit does and growing rabbits up to

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[1] First report of the Scientific Committee for Animal Nutrition on question 58 by the Commission on the use of spores of *Bacillus toyoi* in feedingstuffs for calves (milk replacers), cattle for fattening, chickens for fattening, laying hens, piglets, pigs rabbits and sows for breeding – Opinion expressed on 13 January 1995

slaughter, calves up to six months for fattening and beef cattle at the application rates specified in Table 1. Permanent authorisation is sought for use with pigs.

4. STUDIES CONCERNING THE SAFETY OF USE OF THE ADDITIVE

4.1. Tolerance tests

Tolerance tests have been done in laboratory animals including mice (x 1000), rats (x100) and in all target species at 10 to 100 times the recommended maximum dose of the product in the feed. The duration of tests varied: 15 days for layers, breeding does and rabbits, two months in the case of broilers, up to six months for calves and pigs, up to one year for cattle, and a reproductive cycle of sows. No adverse effects on general health, growth rate and productivity were observed in any case.

4.2. Effect on the gastro-intestinal microflora

The effects of Toyocerin® on the microbial flora of each target species was examined when given at the recommended dose or at higher doses. In all cases, *B. cereus* var. *toyoi* was detected in the gastrointestinal tract of the treated target species (piglets, rabbits, broilers, calves). Although no multiplication of the product strain was observed (or would be expected), the strain showed some persistence in the gut of treated animals after withdrawal of the additive. An examination of faeces after a 5-day administration of 10^9 cfu per kg feed to chickens and pigs, showed that it took two weeks in chickens and three weeks in pigs before *B. cereus* var. toyoi was no longer detectable.

The addition of *B. cereus* var. *toyoi* had no adverse effect on the gastrointestinal microflora of any target species. No significant changes to the microflora were observed although on occasions a decrease in numbers of *E. coli* was observed. This was sometimes accompanied by small increase in counts of lactobacilli, as in the case of calves after 21 days application. Similarly, in chickens submitted to intermittent heat stress (2 experiments), Toyocerin® used at 10^{10} cfu / kg feed maintained a slightly higher number (x 10) of lactobacilli and a reduced number of coliforms in the upper part of the small intestine. *B. cereus* var. *toyoi* also limited proliferation of *E. coli* in the alimentary tract of rabbits.

The effect of *B. cereus* var. *toyoi* ($1x10^6$ spores /g feed) on consistency and bacteriology of faeces in the piglet after oral challenge with *E. coli* was investigated. The treatment reduced the severity of diarrhoea and decreased the morbidity in piglets challenged with *E. coli* 0 149 K 88 and K 99. Culture of the faecal samples showed a reduction in the number of total coliforms in the treated animals at day 7 after the inoculation. An increase in the number of enterococci in animals of the treated group was also observed.

4.3. Resistance to antibiotics

The strain is sensitive to relevant antibiotics[2] except tetracyclines, chloramphenicol and sulfonamides. The question of tetracycline and chloramphenicol resistance was extensively dealt with in a separate dossier received in September 2001. The work was done taking account of the SCAN Opinion on the criteria for assessing the safety of micro-organisms resistant to antibiotics of human clinical and veterinary importance[2].

Transformation tests indicated that resistance to tetracycline and chloramphenicol was chromosomal and not located on any of the seven plasmids known to be present.

Three studies considered the conjugative transfer of resistance. These demonstrated firstly, that the Toyocerin® strain was very unlikely to acquire R-plasmids from other closely related strains, and that, secondly, in filter mating experiments transfer frequencies were very low ($<10^{-8}$ with related bacilli and $<10^{-10}$ with *E.coli* as recipients). These results should be seen in the context of bacilli as transient, non-colonising species in the digestive tract.

Primers for all known and sequenced genes conferring resistance to tetracyclines (33) were used in PCR studies made in four laboratories. Of the 33 genes, 20 encoded membrane-bound proteins which increased the efflux of the antibiotic, nine encoded cytoplasmic proteins protective of ribosomal function, one encoded an enzyme able to degrade tetracyclines (*tetX*) and three genes (*tetU, ortc, tet32*) produced products with as yet unknown mechanisms of action.

With the exception of *tetT* encoding a ribosomal protection protein, no PCR products indicative of the presence of known tetracycline resistance genes were found. In the case of *tetT*, one study found a product of unexpected size. In a second study, a BLAST search showed no similarity of the amplified products produced with primers targeting the *tetT* with any *tetT* sequence and the absence of *tetT* was concluded.

No known genes conferring resistance to tetracyclines were found present in *Bacillus cereus* var. toyoi.

The absence of *cat* encoding chloramphenicol resistance was also demonstrated by PCR.

Transposon mutagenesis (Tn917) produced a mutant strain with increased sensitivity to both tetracycline and chloramphenicol implying a disabling insertion of the transposon at the site in chromosomal DNA conferring resistance. Cloning and sequencing of the DNA adjacent to the insertion site showed that the transposon had inserted in the flanking region of two co-regulated genes (ORF 1 and 2) located between the known chromosomal

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[2] Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of micro-organisms resistant to antibiotics of human clinical and veterinary importance, adopted on 3 July 2001. Available at: http://europa.eu.int/comm/food/fs/sc/scan/outcome_en.html

genes *ger*IC and *nuc*B. ORF 1 and 2 show no homology to any known tetracycline (or chloramphenicol) resistance gene and when cloned into a sensitive strain of *E.coli* did not confer any antibiotic resistance.

The data provided clearly indicates that the resistance to chloramphenicol and tetracycline are closely interrelated and may represent a multiple drug resistance mechanism possibly unique to the Toyocerin® strain. The fact that the two genes in which the resistance is located are located between recognised housekeeping genes indicates that they are particular to the organisms and not externally acquired. Therefore the probability of transfer to other organisms is not a concern.

Consequently, according to the SCAN opinion on the criteria for assessing the safety of micro-organisms resistant to antibiotics of human clinical and veterinary importance, the results of the tests carried out demonstrated that tetracycline resistance in *Bacillus cereus* var *toyoi* is not transferable, not associated with known resistance genes and is chromosomally located.

4.4. Toxin production

Initial evidence of absence of toxicity, considered by SCAN in 1995[1], was based on ileal loop tests with rabbits (negative but no positive control) and vero cell cytoxicity test (dye based detection). Both tests proved negative. The absence of the emetic toxin was assumed because of the amylolytic properties of the strain, an inverse relationship appearing to exist between cerulide (the emetic toxin) and amylase production. The only positive effects seen were in an erythrocyte lysis assay when the production strain was described as mildly haemolytic.

Following the adoption by SCAN of the "Opinion on the Safety of the use of *Bacillus* species in animal nutrition ", the Commission services informed the manufacturers of products derived from or consisting of species of this genus that a more exhaustive testing for toxin production was now required. A recommended (but not mandatory) scheme for testing was included in the annex to the Opinion.

The company responded to this request generally following the testing scheme recommended by SCAN. Any deviations from the methods recommended were described and valid reasons for the modifications provided.

- PCR test were made using primers for each of the three components of the Hbl and Nhe toxins. Fragment only of the HblA (HblC and HblD not detected) and Nhe A genes were present (Nhe B and Nhe C absent);

- SDS PAGE was used to detect the presence of one 105kDa component of the Nhe toxin from x100 fold concentrated supernatant. (No sequence data is available). A 105kDa band was detected in the positive control but was absent in both the probiotic strain and the negative control;

- Antibodies raised against each of the three components of the Hbl toxin were used for Western blots. All three were detected in the positive control but none were present in the probiotic strain (x100 culture filtrate) or the

negative control. The gene fragment detected by PCR, either must not be expressed or the expressed, part of the protein fails to carry the binding epitope for the antibody.

– Two cytotoxicity tests in Vero cells used x5 concentrated supernatant leading to higher concentration than that recommended by the SCAN. Detection by the MTT assay and concentration ultrafiltration using a 10kDa cut-off membrane proved negative for the probiotic strain;

– A Hep-2 cell vacuolation test was performed to detect the emetic toxin. No response has been observed for the probiotic strain compared with a positive control;

– A preliminary clinical evaluation of safety performed during one month in 1979 on five male adults also attests to the safety of the product for humans.

On the basis of the above information, it can be concluded that the strain used in Toyocerin® is a non toxigenic strain. Primers for EntK enterotoxin could not be defined because of absence of sequence data. SCAN strongly recommends that this PCR test should be made as soon as an adequate sequence for the gene encoding this toxin has been published.

4.5. Worker safety

Particle size analysis obtained by dry sieving showed that approximately 20 % of the product passed a 150 μm screen, and 10% a 9.8 μm screen. Since the diameter of a bacterial spore is approximately 1μm it is likely that this potentially respirable fraction contains microbial material. Consequently, and in the absence of evidence to the contrary, the product should be treated as a respiratory sensitiser (R42) and the use of appropriate protection stipulated in the Safety Data Sheet.

An irritation test on the ocular mucosa of rabbits (Draize test) has been performed and demonstrated that the product had no or minimal irritant effect: and it can be considered as a non irritant for the human eye.

4.6. Consumers safety

In its Opinion of 1995, SCAN concluded that the organism did not colonise the digestive tract of target species and that there was no evidence of absorption/invasion. Consequently there were no issues relating to metabolic fate.

Carcass contamination represents a potential route for the live organisms to transfer to human consumers and to contribute to the transient flora of the digestive tract. However, evidence showing that the strain lacks the capacity to produce toxins and will not transfer antibiotic resistance to other organisms in the digestive tract, indicates that there is no cause for concern for consumer safety.

5. EFFICACY

In its Opinion (expressed on 18th February 2000) on how the efficacy of microbial products seeking permanent authorisation should be established, SCAN distinguished two post-weaning growth periods for pigs for fattening: from weaning to approximately two months of age or around 25kg live weight, and from the end of this period until slaughter weight, (usually around six months of age).

The company reported in detail three series of experiments grouped by age of animal:

(1) Post weaning piglets (9 experiments);
(2) Growing-fattening pigs (3 experiments);
(3) Sow reproduction (6 experiments).

(1) The nine experiments with weaned piglets (5.5 to 10.4 kg live weight, aged between 21 and 44 days at the beginning of the trials) were made in five different European countries (see table 2 in annex). In each experiment, animals were generally divided in two groups *i.e.* a control and a treated group receiving the recommended dose of $1x10^9$ cfu *B. cereus* var. toyoi/kg of complete feedingstuff, the studies varying in size between 12-365 animals. The average weight at the end of the experiment varied between 11.1 and 28.7 kg. Overall, the results demonstrated an improvement in the average daily gain (ADG) of 9% and an improvement of 6.3% in the feed: gain ratio (FGR). Statistical treatment of the data within experiments showed that in three trials ADG was significantly improved ($P<0.05$), in three trials the feed:gain ratio was improved ($P <0.05$) and in one experiment scour frequency was reduced ($P<0.05$). A trend towards an improvement in final weight, ADG and FGR was observed in all trials where statistical significance was not reached.

Data was also collected from 3,800 piglets over nine additional field trials on production farms. While results generally supported those observed in the more carefully controlled trials (an overall improvement in ADG of 6.8% and feed : gain ratio of 3.8% which reached significance in 6/9 locations), some experiments were confounded by the absence of negative control groups and others by the inclusion of various antibiotic growth promoters in combination with the additive *B. cereus* var. *toyoi*.

(2) In growing and fattening pigs, three experiments were run in two different locations, one in Spain and the other two in Germany, with 72, 48 and 108 pigs, respectively. Three dose levels (0.2 ; 0.5 and $1.0x10^9$ *B. cereus* var. *toyoi* cfu /kg of complete feed) were compared, arranged in different combinations during the growing and the finishing periods (see table 3 in annex). Where a company recommends a dose range rather than a single value, the assessment of safety is made with the upper value and efficacy with the lower value. In this case the lower values claimed are $0.5x10^9$ cfu/g for the growing period and $0.2x10^9$ cfu/g for the fattening period. The data demonstrated a significant improvement in ADG and FGR compared to control animals during the growing period in two trials. A fourth trial did not use the minimum dose claimed during the growing period and so was

excluded from consideration. All three trials used the minimum dose claimed for the finishing period, but no significant change in final weight, ADG or feed: gain ratio was observed. The product was evidently at its most effective in younger animals and not during the latter stages of growth. However, the data as presented did not allow the growing and finishing phases to be combined and the overall results tested for significance.

(3) In the reproductive sow, the experiment concerning the efficacy of the additive compared different levels of supply: $0.5x10^9$; $1.0x10^9$ cfu / kg complete feedingstuff and $3.0x10^9$ *B. cereus* / head / day, from one week prior to farrowing until weaning. Experiments were conducted at six different sites from 1992 to 1998 with a minimum of 6 and a maximum of 89 sows / treatment (see table 4 in annex). In two experiments, animals were monitored during two successive cycles. The data demonstrated a significant ($P<0.05$) improvement in the survival rate of piglets whatever the minimum recommended dose in three experiments and an improvement in the number of piglets weaned in two experiments. Additional significant effects were found on litter weight at weaning, digestibility of dietary nutrients and in the diarrhoea score of piglets during the suckling. A series of 23 field trials each comparing a control group of sows to a group treated with the additive, involved a total of 3225 animals. In 13 of the trials, an improvement of less than 4 % in the survival rate was observed; in seven trials, the improvement was 4 to 8 %, and in 3 trials 10 to 20 %. An overall analysis showed a significant increase in numbers of piglets weaned and in viability at weaning ($P<0.05$), corroborating the experiments run on reproductive sows kept in experimental conditions.

Overall, sufficient data has been provided by the company to show that the inclusion of Toyocerin® in the diet of pigs can have a significant beneficial effect on growth and feed/gain ratio of piglets up to two months of age, *i.e.* 25 kg of liveweight at the level of 1.0×10^9 cfu /kg of complete feedingstuff. Data on the growing-finishing pigs showed a significant improvement only in 2/3 studies made with growing pigs and no significant improvement in the three studies with finishing animals. SCAN is unable to draw conclusions from this limited data on the efficacy of the product when used with growing pigs to market weight and at the minimum recommended dose. The product does have a positive effect on sow performance expressed as the survival rate and the litter weight at weaning and on the number of animals weaned in sows fed a diet containing the minimum recommended dose of Toyocerin®. Consequently, the incorporation of the product in the diet could be recommended for piglets up to 2 months of age / 25 kg live weight and for sows from one week before farrowing to weaning.

6. CONCLUSION

SCAN concludes, on the basis of the information supplied and evidence provided by the company, that the product Toyocerin®:

- does not produce, nor has the capacity to produce, the toxins found in other strains of *B. cereus*;
- will not transfer the resistance to antibiotics to other bacteria found in the digestive tract of the target species or in the wider environment;
- is safe for those handling the product provided that the precautions stipulated on the Safety Data Sheet are followed and for consumers of products derived from livestock fed Toyocerin®
- is safe for use with the target animals (piglets, sows, pigs for fattening, chicken for fattening, laying hens, rabbits for fattening and breeding does, calves and cattle for fattening);

SCAN also concludes that, on the basis of the data provided, the addition of Toyocerin® to the diets of piglets to two months of age (approximately 25 kg live weight) can significantly improve growth performance. Further the addition of Toyocerin® in the diet of the reproductive sow can result in a significant improvement in the survival rate and the weaning weight of piglets. Accordingly, SCAN considers that the efficacy of the product is demonstrated for these pig categories when it is used at the recommended level of application.

7. ANNEX

Table 2 Summary of the results on the efficacy of Toyocerin® in the piglets (up to 2 months of age).

Number of animals (Total)	Duration of experiment – Age of the animals (in days)		Dose level cfu/kg	Average daily gain (ADG)	Feed / gain ratio (FGR)	Diarrhoea score (DS)	Morbidity (m) Mortality (M)	Statistical significance (P value)	Reference in the dossier submitted by the applicant
	Start	Finish							
12	21	56	0 10^9	-	1.94 1.79	13.8-34.3 7.8-19.6	m (-)	NS	Annex 2
216 (Field)	23	63	0 10^9 10^9	336 [b] 376 [a] 370 [a]	1.84 [a] 1.55 [b] 1.51 [b]	0.575 [a] 0.358 [b] 0.400 [b]	M (-)	<0.05 (ADG, FGR, DS) NS (M)	Annex 3
50 (Field)	21	54	0 10^9	588 600	2.07 1.77	-	M (-)	<0.05 (ADG, FGR)	Annex 4
40	26	68	0 10^9	489 495	1.70 1.67	-	-	NS	Annex 5
60	21(1)	58(1)	0 10^9	384 410	1.73 1.67	-	-	<0.10 (ADG) 0.05 (FGR)	Annex 6
72	28	65	0 10^9	292 324	1.75 1.70	0.207 0.122	-	<0.10 (DS)	Annex 7
365	44(2)	72(2)	0 10^9	315 336	- -	DS (-)	-	0.07 (ADG)	Annex 8
48	21	65	0 0.25×10^9 0.50×10^9 10^9	370 380 406 394	1.84 1.80 1.77 1.77	DS (-)	-	NS	Annex 9
60	21	49	0 10^9	172 222	1.57 1.52	-	-	<0.05 (ADG)	Annex 10

(1) age at start extrapolated from the weight of the animals (7 kg at start) and age at finish from the indicated duration of the experiment (37 days)

(2) age at start extrapolated from the weight of the animals (10 kg at start) and age at finish from the indicated duration of the experiment (28 days)

NS not significant
m (-) reduced morbidity
M (-) reduced mortality
DS (-) reduced diarrhoea score
a,b average values bearing the same superscripts in the same column do not differ significantly at the indicated probability (P)

Table 3 Summary of data on the efficacy of Toyocerin® in growing pigs

Number of animals (Total)	Duration of experiment – Age of the animals (in days)				Dose level (CFU/kg)		Average daily gain (ADG)			Feed / gain ratio (FGR)			Reference in the dossier submitted by the applicant
	Period I		Period II		Period I	Period II	Period I	Period II	Total period	Period I	Period II	Total period	
	Start	Finish	Start	Finish									
72	60(1) (22 kg)	116(2) (71 kg)	116(3) (71 kg)	144(4) (~=98 kg)	0	0	887	932	901	2.34	2.58	2.55	Annex 12
					0.2×10^9	0.2×10^9	892	825	868	2.25	3.03	2.48	
					10^9	0.5×10^9	923	908	918	2.33	2.94	2.52	
48	70(1) (27 kg)	110(2) (54 kg)	110(3) (54 kg)	170(4)(5) (~=107 kg) 180***(2)	0	0	650 b	798 B	-	2.59 a	3.00 A	-	Annex 13
					0.5×10^9	0.2×10^9	763 a	876 A	-	2.27 b	2.73 B	-	
					10^9	0.5×10^8	663 b	800 B	-	2.64 a	2.91 A	-	
108 (101 at the end)	70(1) (26 kg)	98(2) (45 kg)	98(3) (45 kg)	172(4)(1) (~=107 kg) 177(4)(6)	0	0	592 b	847	772	3.05 a	3.55	3.42 a	Annex 14
					0.5×10^9	0.2×10^9	729 a	842	772	2.65 b	3.47	3.25 b	
					0.2×10^9	0.2×10^9	712 a	807	781	2.63 b	3.57	3.34 ab	

(1) age of start estimated on the basis of the weight of animals at start (indicated between brackets)
(2) age of finish of period I calculated on the basis of the indicated duration of period I and based on age of start of period I
(3) age of start of period II equal to age of finish of period I
(4) age of finish of period II calculated on the basis of the indicated duration of period II and based on age of start of period II
(5) tested animals
(6) control animals
a,b average values bearing the same superscripts in the same column do not differ significantly at the probability $P<0.05$
A,B average values bearing the same superscripts in the same column do not differ significantly at the probability $P<0.1$

Table 4 Summary of trials on the efficacy of Toyocerin® in sow feed – All test included unsupplemented controls and animals tested with one to two levels of Toyocerin® supplementation.

Number of animals (Total)	Duration of treatment	Level of supply (cfu/kg)	Statistical significance of various factors – P value					Reference in the dossier submitted by the applicant
			Survival rate (Birth to weaning)	Number of weaned per litter	Litter weight at weaning	Weaning to service	Digestibility of Dry matter/Nitrogen Diarrhoea (piglets)	
30	2 cycles	0.5×10^9	P<0.05	0.05	(+) NS			Annex 15
	Continuously	10^9	P<0.05	0.05	(+) NS	(-) NS	-	
27	2 cycles, Continuously	0.5×10^9	NS	NS				Annex 16
		10^9	P<0.10	0.05	(+) NS	-	-	
79 + 24[3]	F-8[1] to W	10^9	NS/NS	NS/NS	0.05/0.05	-	P<0.05	Annex 17
20	Continuously	0.5×10^9	P<0.05		(+) NS	NS		Annex 18
		10^9	NS	0.05 (at birth)	(+) NS	(-)NS	-	
30	F-8[1] to W	0.3×10^9	NS	NS	(+) NS	-	Less diarrhoea in piglets	Annex 19
178	F-10[2] to W	0.5×10^9	P<0.01	(+) NS	(+) not analysed	-	Less diarrhoea in piglets (not analysed)	Annex 20

F: Farrowing
W: Weaning
[1]: 8 days before farrowing
[2]: 10 days before farrowing
[3]: Two successive experiments
(-) NS or (+) NS indicates respectively reduction or improvement but not significant
-: Not recorded