

**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)**

TERMS OF REFERENCE (November 1991)

The Scientific Committee for Animal Nutrition (SCAN) is requested to give an opinion on the following questions:

1. Has the use of Toyocerin (a product containing 6% w/w of dried spores of *Bacillus toyoi*, giving a concentration of 10^{10} viable spores per g), a favourable effect on the characteristics of the feedingstuffs or livestock production when incorporated under the conditions proposed for its use as an additive in feedingstuffs for Calves (milk replacers), Cattle for fattening, Chickens for fattening, Laying hens, Piglets, Pigs, Rabbits and Sows for breeding (see background)?
2. Is this use safe to Calves (milk replacers), Cattle for fattening, Chickens for fattening, Laying hens, Piglets, Pigs, Rabbits and Sows for breeding?
3. Can it be monitored in animal feedingstuffs?
4. Can it result in development of resistance in bacteria to prophylactic or therapeutic preparations?
5. What is the metabolic fate of *Bacillus toyoi* in Calves (milk replacers), Cattle for fattening, Chickens for fattening, Laying hens, Piglets, Pigs, Rabbits and Sows for breeding? Does the proposed use result in residues in animal tissues? If so, what are the qualitative and quantitative composition and persistence of these residues?
6. Do the toxicology studies allow to conclude that the proposed use does not present risks
 - for the consumer?
 - for the user?
7. What are the nature and the persistence of the excreted products derived from *Bacillus toyoi*? Can these products be prejudicial to the environment?
8. In the light of the answer to the above questions, are the proposed conditions of use acceptable?

BACKGROUND

The use of Toyocerine (Dried product containing 6% of spores of *Bacillus toyoi*, equivalent to 10^{10} Colony Forming Units per gram) as additive to feedingstuffs for Calves (milk replacers), Cattle for fattening, Chickens for fattening, Laying hens, Piglets, Pigs, Rabbits and Sows for breeding was subjected of a submission for inclusion in Annex II of Council Directive

70/524/EEC of 23 November 1970 concerning additives in feedingstuffs¹ as amended by Council Directive 84/587/EEC of 29 November 1984², according to the following conditions:

J. Growth Promoters

Species or category of animals	Maximum age	Minimum Content mg/kg of complete feedingstuff	Maximum content	Other provisions
Calves	3 mois	50	100	Milk replacers
Cattle for fattening	6 mois	20	100	
Cattle for fattening		20	100	
Chickens for fattening		20	100	
Laying hens		20	100	
Piglets Weaning		50	100	Milk replacers only
Piglets	2 mois		100	
Piglets	4 mois	50	100	
Pigs		20	50	
Rabbits, breeding doe		100	200	From service
Rabbits for fattening		100	200	
Sows for breeding		100	200	From service

The above indicated quantities correspond to the following CFU per g of complete feedingstuff :

Species or category of animals	Maximum age	Minimum content mg/kg of complete feedingstuff	Maximum content	Other provisions
Calves	3 mois	0,5 x 10 ⁶	1 x 10 ⁶	Milk replacers
Cattle for fattening	6 mois	0,2 x 10 ⁶	1 x 10 ⁶	
Cattle for fattening		0,2 x 10 ⁶	1 x 10 ⁶	
Chickens for fattening		0,2 x 10 ⁶	1 x 10 ⁶	
Laying hens		0,2 x 10 ⁶	1 x 10 ⁶	
Piglets Weaning		0,5 x 10 ⁶	1 x 10 ⁶	Milk replacers only
Piglets	2 mois		1 x 10 ⁶	
Piglets	4 mois	0,5 x 10 ⁶	1 x 10 ⁶	
Pigs		0,2x 10 ⁶	0,5 x 10 ⁶	
Rabbits, breeding doe		1 x 10 ⁶	2 x 10 ⁶	From service
Rabbits for fattening		1 x 10 ⁶	2 x 10 ⁶	
Sows for breeding		1 x 10 ⁶	2 x 10 ⁶	From service

OPINION OF THE COMMITTEE

1 O.J. No. L270, 14.12.70 p.1

2 O.J. No. L319, 08.12.84, p.13

Toyocerin, a feed additive containing some 10^{10} viable *Bacillus toyoi* spores/g in a corn flour (4%) Ca carbonate (90%) carrier, was studied in 4 different animal species although the SCAN limits its present opinion to data related to swine.

1. The number of pigs per trial and per treatment was rather small. Studies involving piglets from the weaning stage showed that Toyocerin improved feed intake, weight gain and feed conversion ratios. However, data on performance was not always statistically analyzed making it difficult to extract a conclusion. When incorporated in feedingstuffs and at higher levels, a dose-related improvement in feed intake (4%), weight gain (9%) and feed conversion ratio (6%) were observed. These parameters were also evaluated in comparison with four well-established antibiotic feed additives (Zn-Bacitracin, Virginiamycin, Tylosin and Flavomycin) and comparable results were obtained. An explanation for the gained improvement in homogeneity of body weight is not given but it is said that this is retained within the Toyocerin fed group for at least 70 days after withdrawal of toyocerin from the feed. A few European trails have also assessed the benefits of Toyocerin (2×10^9 *B. toyoi* CFU:sow/day) in breeding gilts and sows. It seems that the administration of Toyocerin does not affect total number of piglets born, the number of piglets born alive or mortality from birth to weaning.

The incidence and severity of diarrhoea was noticeably less in piglets from Toyocerin-fed sows, on a subjective assessment made by the farmer and justified as due to fewer undesirable bacteria (e.g. enterobacteriaceae) in the faeces of treated sows. In conclusion, the efficacy of Toyocerin both in growing pigs and in breeding stock, judged by the limited data, appeared somewhat equivocal.

However, results are reasonably promising and Toyocerin needs further evaluation under different housing conditions and larger number of animals. A comparison with other growth promoters under European conditions should be provided. There is a need for more efficacy dose-range studies in order to define better the recommended level of usage and the resultant benefits.

Great care was taken to distinguish between the probiotic *B. toyoi* strain and its potentially toxin-producing relatives. A possible weak point is the use of corn flour of "feed-grade" quality, as cereals are known for their contents of *Bacillus* species. The problem with *B. toyoi* is that it belongs to a group of well-known food-poisoning bacteria, *B. cereus*.

2. *Bacillus toyoi* proved to be very sensitive to the antibacterial effects of the macrolide antibiotic Kitasamycin (Mic = 0,2 µg/ml). It is difficult to explain the finding that a concomitant administration of spores of a *Bacillus* strain sensitive to an antibiotic with this antibiotic provides better results than the administration of the spores alone. It is difficult to interpret this phenomenon by a synergetic action since enterobacteriaceae, as Gram-negative microorganisms, are not sensitive to the antibacterial effects of a macrolide antibiotic.

Furthermore, the effects of *B. toyoi* on gut microflora showed inconsistent results as reflected by volatile fatty acid production and acetic acid-propionic acid ratios. In acute toxicity tests (e.g. 3 days) *B. toyoi* showed no adverse effect on clinical signs and caused no mortality in mice and rats.

3. The manufacturer supports his shelf-life claim with a stability period of 18 months with 80-89% live spores in CFU. The spores in premixes and mixed feeds resist the temperature and pressure of pelleting, with not more than 2-10% losses, and a 3 month in feed shelf life is judged acceptable. Stability may be affected in presence of high levels of certain metals in feedingstuffs (e.g. FeSO₄ and CuSO₄).
4. There is no indication that *B. toyoi* colonises the alimentary tract; it has disappeared within a few days after closing. There is no evidence for absorption/invasion from alimentary canal into the body and it does not result in the development of resistance in bacteria to prophylactic or therapeutic antibiotics.
5. *B. toyoi* not being absorbed has no metabolic fate. Spores are not affected by gastric acidity and in pigs they can be isolated in the stomach, duodenum, ileum, caecum, colon and rectum. The excretion of *B. toyoi* is faecal and it is completely eliminated after 3 weeks in the pig. Therefore the proposed use does not result in residues in animal tissues.
6. *B. toyoi* spores have been demonstrated to be a safe organism by toxicological studies in various species of laboratory animals as well as by safety tests in swine. *B. toyoi* does not colonise the alimentary tract of animals with normal, destroyed or suppressed intestinal flora. Orally administered *B. toyoi* spores germinate and establish in high numbers throughout the gut but disappear from the intestinal tract within 120 hours after application. There is no evidence for invasion of host mucosa or spreading to organs.

B. toyoi can be differentiated from food poisoning *B.cereus* strains. Genetic and biochemical data underline that *B.toyoi* is taxonomically distinct from food poisoning *B.cereus* strains. When ribotyping is used, *B.toyoi* can be distinguished from emetic or diarrhoeogenic *Bacillus* strains.

Toxicity tests (rabbit ileal loop test cytotoxicity test, erythrocyte lysis assay, egg yolk agar diffusion turbidity assay) were conducted not only with *B.toyoi* spores but also with viable vegetative cells, culture filtrates resp. supernatants and cell lysates. From these results it can be concluded that viable cells, culture filtrates resp. supernatants and cell lysates have no signs of in vivo toxicity. Supernatants and lysates displayed low haemolytic and lecithinase activity as "hemolysin" are not primarily enterotoxic these toxic activities may not be related to the risk of any intestinal disorder.

Feed intoxications should not be expected, because there is not any significant germination or multiplication of *B-toyoi* in the animal feed. Spores were stable during a 3 months storage period in e.g. milk replacer, starter and mash feed (room temperature, 30°C).

B.toyoi is genetically stable. Isolates obtained from either the standard strain or from all the production lots showed identical plasmid DNA-patterns. There were not plasmids found carrying determinants for pathogenicity as has been demonstrated e.g. in *B.anthraxis*.

Plasmid transfer or acquisition is not very probable, because a minimum growth level is an essential condition for transfer or acquisition of any plasmid. The necessary high number of bacteria (10^7 - 10^8 CFU/ml) is not available (the inclusion rate of *B.toyoi* in the feed is in the range of 10^5 - 10^6 per g and the bacterial count of Bacillus strains in soil is about 5×10^5 /g of soil). As *B.toyoi* does not have a significant multiplication in feed or intestinal tract, it can be assumed that *B.toyoi* will not achieve the necessary activity required for any plasmid transfer.

B.toyoi is naturally resistant to several antibiotics such as chloramphenicol, tetracyclines and sulphonamides. Antibiotic resistance determinants are coded on the chromosomal DNA level and are not based on plasmid determined resistance. Plasmid DNA from *B.toyoi* does not carry antibiotic determinants.

It can be concluded from the results of genetic studies (transconjugation test and transformation test) that transfer of resistance genetic factors from *B.toyoi* should not be expected.

7. The products excreted through the faeces are spores of *B.toyoi* and since this is originally isolated from soils, it is not likely to be prejudicial to the environment.
8. In the light of the answers to the above questions, the committee is of the opinion that Toyocerin, having characteristics of a growth promotant feed additive, should be assessed as such. For Toyocerin to be admitted without risks, it would be desirable to obtain information on various aspects such as impurities, iron sulphate and copper sensitivity of *B.toyoi* spores, safety dust formation in premix, observation period in assessing toxicity, the efficacy mechanism (synergism with a macrolide antibiotic), the potential effect on the quality of the animal products and sensitisation potency.

In order for the SCAN to be able to answer if the use of spores of *Bacillus toyoi* has favourable effects on animal performances, the dossier submitted for examination needs to be supplemented with the results of new performance-experiments, from weaning stage, conducted under varied European conditions, and under extended dose-range in order to best define the optimal dose. These performance experiments should be carried out with an appropriate number of animals supporting the conclusions drawn from relevant statistical analysis.