EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

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

Opinion of the Scientific Committee on Animal Nutrition on the use of *Bacillus licheniformis* NCTC 13123 in feedingstuffs for pigs (Product AlCare™)

(Adopted on 18 April 2002)

1. BACKGROUND

The product AlCare™, based on a strain of *Bacillus licheniformis* (NCTC 13123), is intended for use as a feed additive. The Commission has received a request for provisional Community authorisation of this product under the conditions set out in the following table.

Additive	Chemical formula, description	Species or category of animal	Maximum Age	Minimum content	Maximum content
				CFU/kg of complete feedingstuff	
Bacillus licheniformis NCTC 13123	Preparation of *Bacillus licheniformis* containing a minimum of 2.5 x 10^{10} CFU/g additive	Piglets	4 months	10^9	10^{10}

The Company producing the product AlCare™ prepared a dossier that has been submitted through the national rapporteur (United-Kingdom) to the Commission. The dossier has been found by the Member States to be in compliance with the requirements of the Council Directive 87/153/EEC[1] fixing guidelines for the assessment of additives in animal nutrition as amended.

The authorisation procedure laid down in article 4 of Council Directive 70/524/EEC[2] as last amended by Council Directive 96/51/EC[3] includes a period of 320 days for the evaluation of the dossier submitted to the Commission. This procedure commenced on 29 February 2000.

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1 E.C. OJ n° L 64 of 07/03/1987, p. 19

2 E.C. OJ n° L270 of 14/12/1970, p. 1

3 E.C. OJ n° L235 of 17/09/1996, p.39

2. TERMS OF REFERENCE

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

2.1. Is the use of *Bacillus licheniformis* NCTC 13123 safe:
- for the target animal: pigs to four months of age?
- for the user?
- for the consumer?

In making its assessment, the Committee is requested to consider in particular toxin production by the strain used in the product AlCare™ and its resistance to antibiotics.

2.2. What is the nature and persistence of the excreted strain of *Bacillus licheniformis* (or products derived therefrom)? Can these be prejudicial to the environment?

3. OPINION OF THE COMMITTEE

3.1. Product description

AlCare™ is a granular product whose sole active ingredient is a strain of the endospore-forming bacterium *Bacillus licheniformis*. The fermentation broth containing both spent medium and bacterial cells is mixed with calcium carbonate as an inert carrier (approximately 70% of total weight) and spray dried to provide a final product containing 2.5×10^{10} spores/g dry matter. Analysis has shown the final product to contain trace amounts of arsenic, lead and other heavy metals in concentrations that do not exceed those considered by JECFA and other bodies as safe for human consumption. The product is routinely tested for the presence of *Aspergillus niger* and for contaminating coliforms with >30 cfu/g product leading to rejection. In addition, specific tests are made for *Salmonella* spp and *Escherichia coli* and the batch released for use only in their absence.

The strain of bacterium used was derived from *B. licheniformis* ATCC 10716 (originally isolated from soil) by conventional mutagenesis and is deposited with the UK National Collection of Type Cultures with the accession number NCTC 13123. It is not a product of recombinant DNA technology. Identification to the species level is based on morphological and biochemical characteristics and meets the criteria defined for *B. licheniformis.* A RAPD (randomly amplified polymorphic DNA) test was developed which, while not exhaustively tested, appears able to distinguish the product strain from other strains of the same species. It should be noted that strains of *B. licheniformis* from other sources are likely to be present in treated feed or in the digestive tract of animals fed treated feed only in low numbers compared to the production strain. The biochemical characteristics used for speciation, supplemented with the RAPD method also are adequate for monitoring the genetic stability of the product.

The absence of antibiotic activity was demonstrated by drying a concentrated methanolic extract of the product onto paper disks which were then incubated according to the conditions described in the "New Dutch Kidney Test" (Nouws *et al*., 1988) using *B. subtilis* BGA as test strain. Bacitracin activity, found in other strains of *B. licheniformis*, was not detected in treated feed or in faecal samples of animals fed feed treated with the maximum recommended dose or a ten-fold higher dose. However, since the original strain ATCC 10716 is known to produce bacitracin, the absence of this antibiotic from the culture medium also needs to be demonstrated.

3.2. Intended conditions of use

The product is intended for inclusion in feeds for pigs for fattening up to four months of age at a final concentration of 10^9 – 10^{10} spores/ kilo complete feedingstuff. As would be expected the product is stable with no loss of spore viability detected after six months storage as a premix at ambient temperatures and three months at 40^{o}C. Consequently, extended storage of the product is unlikely to affect the declared numbers and viability of spores and hence numbers of microorganisms reaching the target species. Effects of pelleting on spore viability are not documented although such experiments are reportedly planned.

3.3. Safety of the product for the target species

B. licheniformis occasionally is associated with bovine toxemia and abortions (Johnson *et al*., 1994). However, it is evident that this species is only weakly virulent and usually will multiply freely only in animals which, for various reasons, are immune compromised (Anon, 1997). The species is not associated with any disease of pigs.

A tolerance test with 18 piglets (7-8 kilo start weight, nine males and nine females) assigned to one of three groups – control, x1 maximum recommended dose or x10 maximum recommended dose - is reported. Animals were monitored daily for 14 days for signs of ill health, and bodyweights and rectal temperatures recorded. Blood samples were taken for haematology and clinical chemistry at the start of the experiment and on days 7 and 14. Food consumption was measured daily and feed conversion ratios calculated for the experimental period. On day 14 the animals were killed, organ weights determined and tissue samples taken for histological examination.

Administration of x10 the recommended dose resulted in no adverse effects in males but caused mild gastrointestinal disturbance in the three females, which, in the case of one animal, was sufficiently severe to adversely affect weight gain and the feed to gain ratio. Alterations to some clinical chemical parameters were also seen in this animal but it is reasonable to conclude that these were secondary to the observed diarrhoea. Organ weights were unaffected by treatment and there were no macroscopic or microscopic evidence of test article toxicity in any animals.

Because of the adverse effects observed in females the Company proposes to repeat this study using a larger number of animals and an extended duration of four weeks.

A microbiological examination of faeces collected from the eighteen pigs in the first tolerance test on days 0, 7 and 14 showed the expected increase in numbers of *B. licheniformis* present on days 7 and 14. Approximately 80% of the cells were present in a vegetative form indicating that *B. licheniformis* spores germinate in the pig digestive tract. Other than the expected increase in *B. licheniformis,* there were no significant changes in the total number of aerobes or anaerobes or in numbers of *Bacteroides* spp, enterococci, lactobacilli or *E.coli* between treatment groups.

In addition, in the course of developing data on efficacy, the product at doses between the minimum and maximum claimed has been fed to over 1000 pigs of various stages of development between weaning and reaching slaughter weight. No negative effects on the health and welfare have been observed to date.

Conclusion. Results from the single tolerance study reported are insufficient to conclude that the product when used in ten-fold excess of the recommended dose is well tolerated by piglets.

3.4. Operator and end-user safety

The product is granular in nature with <1% of the product passing a 100µm screen and has a low dusting potential as determined by the Stauber-Heubach test. A product of this physical structure would not be expected to pose a serious respiratory hazard. However the product is proteinaceous in nature and there remains the possibly of respiratory sensitisation in those mill operators and farmers handling the product on a regular basis.

A number of acute toxicity tests with laboratory animals were commissioned by the company to further explore the risk to those handling the product. These tests were made with a powdered form of the product to ensure maximum exposure to the bacterial endospores. In an *acute inhalation toxicity study*, ten rats were exposed to nose only inhalation of the product in an airborne concentration of 5.1mg/l, while a second matched group of animals were exposed only to the chamber air. The test involved a single exposure over a four-hour period during which the chamber air was replaced 60 times. Thereafter the animals were monitored for 14 days and then killed and the lungs examined for evidence of ill effects. Under these conditions the product was well tolerated by the rats and at subsequent necropsy, lung weights and macroscopic examination did not suggest an effect of exposure. It should also be noted that the mass median aerodynamic diameter of the airborne particles was 3.8µm in the powdered product, some 100-fold smaller than the mean diameter of the granulated form marketed.

An *eye irritation study* made with three rabbits given a single ocular instillation of the product indicated that the product was an irritant. Two of the three rabbits developed diffuse corneal opacifaction and all three

evidence of conjunctivitis. However symptoms were much reduced two days after treatment and had disappeared within four days. A comparable *skin irritation study* to determine whether the product was corrosive or an irritant to intact rabbit skin, failed to demonstrate any detectable effects in the three animals tested. This single exposure model study was backed by a second study in guinea pigs deigned to detect *skin sensitisation* (delayed contact hypersensitivity). A total of twenty females were assigned to a test group and further ten female guinea pigs to a control group. In the initial phase each animal was given intradermal injects of the adjuvant alone, the test substance alone and the test substance with adjuvant. Eight days after intradermal injection, lint pads containing Vaseline and the feed additive (test group) or Vaseline alone (control group) were applied to the injection sites for period of 48 hours. Finally, in the challenge phase of the study, all animals were exposed to the feed additive in Vaseline for 24 hours. Approximately 75% of test animals gave responses to this final challenge indicative of delayed contact hypersensitivity compared to 1/10 of the control animals.

Non-toxigenic strains of *B. licheniformis* are generally considered to have GRAS (generally recognised as safe) status and so accidental infection with the production organism via an oral route is not cause for concern. *B. licheniformis*, as a soil inhabitant, is often consumed by humans and is commonly encountered as a transient member of the flora of the GI tract.

Conclusion: The product has been demonstrated capable of inducing a delayed sensitivity reaction and thus to pose a risk for those repeatedly handling the product. However, this response is not unique to the product and is a well recognised characteristic of most proteinaceaous feed additives, particularly enzymes. The product has been formulated to reduce the risk of sensitisation by an inhalatory route and the remaining risk can be adequately managed by the precautions normally applied to products of this type (use of gloves and face masks) as detailed in the Safety Data Sheets. Provided these recommendations for handling are followed, SCAN is of the opinion that the product does not pose an undue risk for those handling or otherwise exposed to the product.

3.5. Consumer safety

3.5.1. Toxin production

Since all *Bacillus* spp. produce resistant endospores, the likelihood of survival in viable form is substantially increased compared to bacteria that exist only in a vegetative state. This property is likely to ensure that, in the event of carcass contamination, any organisms transferred to consumers are viable. Consequently, the capacity of the production strain to produce toxic agents is a particular cause for concern. A second concern is the possibility of any antibiotic resistance factors present being transferred to other members of the gastointestinal flora of the target species and/or consumers of products of the target species.

Experimental evidence is provided of the absence of any enterotoxigenic capacity in the strain used in their product. The strain of *B. licheniformis* was grown in broth and the concentrated supernatant extract tested for cytotoxicity using a Vero cell assay. No reduction in the incorporation of [^{14}C]-leucine was recorded compared to the negative control with extracts from either strain. A positive control was included which did demonstrate the expected reduction in ^{14}C incorporation. In addition, use of the commercial immunoassays for the non-haemolytic toxin (Tecra) and the BCET-RPLA kit (Oxoid) for a component of the haemolytic toxin proved negative. Genes encoding elements of the haemolytic and non-haemolytic enterotoxins and enterotoxin T (*hbl, nhe* and *ent T*) also could not be detected by PCR amplification.

Aqueous and methanolic extracts of cultures of the production strain were tested for sperm toxicity in comparison with a extracts of a positive control strain (*B. cereus* F-5881) known to produce an emetic-like toxin (cereulide). No reduction in sperm motility was observed with extract concentrations 2000-fold (aqueous) and 600-fold (methanol) greater than the concentration of equivalent extracts of the positive control strain at which motility was fully inhibited.

3.5.2. Resistance to antibiotics

The susceptibility of the production strain to therapeutic antibiotics was investigated by use of the E-test. MICs were determined only for chloramphenicol, penicillin, enrofloxacin, streptomycin, tetracycline and trimethoprim. The strain was found sensitive to all these drugs. The strain *B. licheniformis* NCTC 13123 is, however, resistant to sulfonamides, to erythromycin (MIC value <256 mg/l as determined by the E-test) and, as would be expected, to bacitracin.

Sulfonamide resistance, which is common to the large majority of *Bacillus* spp.(Kundrat, 1963), was identified by PCR to be encoded for by *sulII*. Use of a PCR fragment from *B. licheniformis* NCTC 13123 as a probe in a Southern blot indicated that this gene had a chromosomal location. A plasmid apparently present in the strain did not hybridise to the probe.

Evidence from the literature (Docherty *et al*., 1981) and from the study of a limited number of strains (18) undertaken by the Company shows that resistance to erythromycin and related antibiotics is prevalent, but not universal, amongst strains of *B. licheniformis*. This is also the case for strains deposited in culture collections before 1950 and thus before the widespread use of antibiotics in human and veterinary medicine. Two genes confering erythromycin resistance have been described for strains of *B. licheniformis* - *ermD* (Gryczan *et al*., 1984) and *ermK* (Kwak *et al*., 1991). Labelled oligonucleotides designed from the published sequences of both *ermD* and *ermK* were used to probe chromosomal DNA extracted from the product strain. Hybridisation occurred with the *ermD* probe and to DNA from a positive control strain. No hybridisation could be detected with the *ermK* probe. Unfortunately, the Company could not locate a positive control strain expressing *ermK*. Neither gene was associated with a transposon or

bordered by recognised insertion sequences in the original published descriptions, but this has not been specifically demonstrated for the strain NCTC 13123.

In the first experiment described by the Company in which mixed probes to erythromycin resistance determinants were used in a Southern blot experiment, no oligonucleotide specific for *ermD* was included, although one designed to detect *ermK* was present. *ErmK* has a high sequence and amino acid homology (97-99%) with *ermD* (Roberts *et al*., 1999) and would be expected to cross hybridise, but it remains a possibility that another *erm* resistance determinant in addition to *erm*D is present.

Conjugation experiments using the filter mating method with a single strain of the closely related *B. subtilis* and two strains of *Enterococcus faecalis* as recipients showed no transfer of erythromycin resistance.

3.5.3. Conclusion:.

Toxin production

Since primer pairs to the the haemolytic and non-haemolytic enterotoxins and enterotoxin T failed to generate PCR products, SCAN concludes that the production strain is unable to produce these toxins, at least in a functional form. This was confirmed by the negative reaction obtained with the two commercial immunoassays and the lack of general cytotoxicity to Vero cells. Similarly, no production of the emetic-like toxin (cereulide) could be detected under conditions that allowed detection for a known positive strain of *B. cereus*.

Transfer of antibiotic resistance

The presence of acquired genes conferring resistance to sulphonamide (*sulII*) and erythromycin (*ermD*) have been demonstrated. The available evidence suggests the absence of *ermK*, although this could not be firmly established in the absence of a positive control. However, since both *ermD* and *ermK* are closely related (Roberts et al., 1999), both genes coding for a 23rRNA methylase inducible only in the presence of erythromycin or similar antibiotic and regulated by transcriptional and translational attenuation, the additional presence of *ermK* would not alter SCAN's conclusions.

In the view of SCAN, the chromosomal location of *ermD* and *sulII* is not evidence of a lack of potential for transfer. However SCAN also recognises that *sulII* is found in all but a few species of bacilli and concludes that its presence alone does not warrant the exclusion of this industrially important genus of bacteria from use in agriculture.

The macrolide-lincosamide-streptogramin (MLS) group of antibiotics have far more clinical relevance than the sulphonamides and consequently resistance to this group of antibiotics is of greater concern. In estimating the risk associated with resistance to erythromycin SCAN took the following into consideration:

- The original published sequence data for ermD and ermK did not show any association with a transposon or other insertion sequences likely to promote gene transfer.

- *B. licheniformis* is transient in the gut and its natural habitat, soil, forms the significant reservoir of resistance genes for this species.

- Horizontal transfer of resistance is most likely to closely related species of the *B. subtilis* group, which are also transient in the gut and found only in low numbers compared to most species of the resident flora. Conjugative transfer to *B.subtilis* could not be detected under the experimental conditions used. Consequently transfer of resistance to other Bacillus species can be considered as a rare event. Even when this occurs the recipient strain would not colonise the gastro-intestinal tract, would not undergo clonal expansion and would be rapidly lost from the animal ecosystem.

- The probability of transfer of resistance to the more distantly related bacteria forming the permanent flora is likely to occur at a substantially lower rate than transfer to Bacillus spp. Although, in practice, this lower rate would be offset by the greater number and variety of potential recipients, the overall risk is probably no greater than that involving other bacilli.

The presence of *ermD* poses a hazard, although in the view of SCAN the low probability of transfer of resistance encoded by *ermD* to the resident flora would not constitute a measurable risk to the continuing clinical use of the MLS group of antibiotics *in the absence of any selective pressure*. Because of the nature of the regulation of the expression of the methylase enzyme conferring resistance, which is induced by erythromycin or the closely related oleandomycin, the greatest selective pressure would be found in the presence of these antibiotics. However, inducible *erm* genes also provide a lower level of resistance to a wide range of MLS antibiotics. Consequently, given the widespread and continuing use of MLS antibiotics in pigs for both therapeutic and prophylactic purposes it has to be assumed that transfer of *ermD* resistance, while a rare event, would provide a selective advantage to the recipient strain and would be propagated.

3.6. Safety for the wider environment

B. licheniformis is a commonly occurring soil saprophyte which has been isolated from a wide range of habitats and is probably universal in occurrence. There is no evidence that the production stain has been modified to an extent that would alter its capacity to colonise its natural habitats relative to the strain from which it derived (ATCC 10766) or to the type strain for the species. The natural response of vegetative forms of *Bacillus* spp. to adverse conditions is the formation of endospores which are considerable more resistant than the vegetative form and which can exist for considerable periods without loss of viability. This was confirmed for the production strain in which faecal material from animals fed the microbial product were mixed either with an equal amount of faeces from untreated

animals or with soil or water. The survival of *B. licheniformis* then was monitored over an 84 day period with no significant changes in numbers observed regardless of the conditions imposed, although a trend to falling numbers was observed.

Strains of *B.licheniformis* are not known as pathogens of plants or aquatic species. As a natural inhabitant of soil their presence would be expected in watercourses or other bodies of water subject to runoff from soil. As indicated above, although the organism would be expected to survive for a considerable period, no proliferation would be expected under these conditions.

Conclusion: The production strain derived originally from a soil isolate and is a member of a species widely distributed in nature. There is no reason to suppose that strain NCTC 13123 differs in any substantive way from the range of phenotypes exhibited within the species or that it would behave in a manner different to other strains of the same species. If anything it might be at a selective disadvantage compared to the original isolate because of its lost/reduced ability to produce bacitracin. Any localised concentration produced from faeces of treated animals is very unlikely to be of any significance or cause for concern. SCAN therefore concludes that the use of this organism as a feed additive will not adversely affect the wider environment.

3.7. Final conclusion

Because of its concerns about the antibiotic resistance genes present in the product strain, SCAN decided to conclude its review of the AlCare™ product before all of the issues related to safety had been fully resolved.

In the view of SCAN, use of *Bacillus licheniformis* NCTC 13123 as a feed additive would be unsafe because of the risk of dissemination of genes that confer resistance to clinically important antibiotics via the food chain. In reaching this decision SCAN recognises that the magnitude of the risk cannot as yet be quantified, but is probably low. However given the continuing widespread use of macrolide antibiotics in pig production, and the selective pressure this provides, SCAN has decided to adopt a precautionary approach consistent with its previously expressed Opinion on antibiotic resistance genes in microbial products[4].

4. REFERENCES

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