EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate B - Scientific Health Opinions
Unit B3 - Management of scientific committees II

REPORT OF THE SCIENTIFIC COMMITTEE ON ANIMAL NUTRITION ON PRODUCT BIOPLUS 2B ® FOR USE AS FEED ADDITIVE

(Adopted on 22 June 2000)

1. TERMS OF REFERENCE

The additive Bioplus 2B® is a product whose active ingredients consist of two strains of microorganisms : *Bacillus licheniformis* (DSM 5749) and *Bacillus subtilis* (DSM 5750) in a 1:1 ratio.

It has been authorised provisionally for five years in piglets (until 17 July 1999)

The producer, Chr. Hansen BioSystems A/S, Denmark, is now requesting:

- a permanent Community authorisation of the product for use as a feed additive in piglets for fattening to two months of age at the recommended levels mentioned in Table I hereafter
- a provisional Community authorisation for use with other animal species or categories as summarised in Table I below.

(1) The Scientific Committee on Animal Nutrition is requested to assess the safety of product Bioplus 2B ®.

(2) In addition, as the permanent Community authorisation of a feed additive requires that its efficacy be demonstrated, the Scientific Committee is requested to assess the efficacy of the product for use in piglets up to two months of age and at the levels recommended by the Company.

Table I

N°.	Additive	Chemical formula, description	Species or category of animal	Maximum age	Minimum content	Maximum content	Other provisions	Period of authorisation
					CFU/kg of complete feedingstuff			
E 1700	Bioplus 2B® *Bacillus licheniformis* (DSM 5749) *Bacillus subtilis* (DSM 5750) (In a 1/1 ratio)	Mixture of *Bacillus licheniformis* and *Bacillus subtilis* containing a minimum of 3.2×10^9 CFU/g of the additive ($1,6 \times 10^9$ CFU/g of each bacterium)	Piglet	2 months	1.28×10^9	3.2×10^9	In the directions for use of the additive and premixture, indicate the storage temperature, storage life and stability to pelleting.	Without a time limit
20	Bioplus 2B® *Bacillus licheniformis* (DSM 5749) *Bacillus subtilis* (DSM 5750) (In a 1/1 ratio)	Mixture of *Bacillus licheniformis* and *Bacillus subtilis* containing a minimum of 3.2×10^9 CFU/g of the additive ($1,6 \times 10^9$ CFU/g of each bacterium)	Sows	15 days pre partum and during lactation period	0.96×10^9	$1,92 \times 10^9$	In the directions for use of the additive and premixture, indicate the storage temperature, life and stability to pelleting.	Maximum four years
			Pigs for fattening	-	0.48×10^9	1.28×10^9		
			Broilers	-	3.2×10^9	3.2×10^9		
			Turkeys for fattening		1.28×10^9	3.2×10^9		
			Calves	6 months	1.28×10^9	1.6×10^9		

2. GENERAL DESCRIPTION

The active constituent of the product is a mixture of two strains of *Bacillus, Bacillus subtilis* (DSM 5750) isolated from a soybean fermentation and *B. licheniformis* (DSM 5749) isolated from soil. The product is intended for use with sows, pigs for fattening, chickens for fattening, turkeys for fattening and calves up to 6 months for fattening for which provisional authorisation is sought. Permanent authorisation is sought for use only with piglets up to two months of age.

2.1. Identity, characterisation and conditions of use of the additives

The product contains at least 1.6 x 10^9 spores/g of *B. subtilis* and 1.6 x 10^9 spores/g of *B. licheniformis* and thus not less than 3.2 x 10^9 spores/g final product. The carrier substances are sodium aluminium silicate (1%) and whey permeate (98%). Particles below 10 μm (1% of total) consisted only of whey. Since sensitisation to proteinaceous material represents a hazard for those handling the product, the use of a dust mask to prevent inhalation is recommended. Contaminating heavy metals, micro-organisms and mycotoxins are either absent or fall within normal and acceptable limits.

2.2. Specifications of the active substance

The two *Bacillus* strains are deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH with the accession numbers DSM 5750 and DSM 5749. They are identified according to criteria given in Bergey's Manual of Systematic Bacteriology and are not genetically modified.

The product strains have been characterised biochemically and by molecular methods and shown to be genetically stable. They do not carry plasmids. Both strains are resistant to flavomycin and zinc-bacitracin and *B. licheniformis* is, in addition, resistant to clindamycin (MIC >32 mg/l). Bacitracin resistance was shown not to be transferable *in vitro* or *in vivo*. The strains do not produce enterotoxins (see 3.3).

2.3. Physico-chemical, technological and biological properties of the additive

The bacterial strains are incorporated into the final product as spores. As such, both strains demonstrate the typical resistance of bacterial endospores to desiccation and other environmental factors likely to kill or reduce the viability of the vegetative forms.

Storage stability of at least one year under recommended conditions as well as product survival after normal single pelleting has been satisfactorily demonstrated.

2.4. Conditions of use of the additive

For piglets to two months of age the additive is intended to be mixed with feed at a level of 0.4 – 1.0 kg/tonne feed to provide a minimum of 1.28×10^9 and a maximum of 3.2×10^9 cfu/kg complete feed. For other categories of livestock a minimum of 0.64×10^9 to 3.2×10^9 cfu/kg and a maximum of 1.6×10^9 to 3.2 cfu/kg of complete feed is proposed

3. STUDIES CONCERNING THE SAFETY OF USE OF THE ADDITIVE

3.1. Tolerance test

Tolerance tests have been done in all target species at 40 to 100 times the recommended maximum dose of the product in the feed. The duration of tests varied: 21 days (turkeys), 9 days (broilers), 37 days (calves), 6 weeks (piglets), and 7 days (sows). No effect on general health and growth rate were observed in any case.

3.2. Effect on the gastro-intestinal microflora

Effects of Bioplus 2B ® on the microbial flora of each target species was examined when given at the recommended dose or at higher doses. In all cases, numbers of *Bacillus* spp. recovered from the digestive tract of treated animals reflected the dose given and were significantly greater than was found in control animals. No significant effects on numbers of coliforms and enterococci or other lactic acid bacteria were observed.

3.3. Toxin production

Following the adoption by SCAN of the Opinion on the use of *Bacillus* products as feed additives, 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 Manufacturers of BioPlus 2B® anticipated the requirement for further testing and provided additional experimental evidence of the absence of any toxigenic capacity in the two strains used in their product. The two strains were individually grown in broth and the concentrated supernatant extracts 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 immunoassay for the non-haemolytic toxin (Tecra) and the BCET-RPLA kit (Oxoid) for a component of the haemolytic toxin proved negative. Finally genes encoding elements of the haemolytic, non-haemolytic and enterotoxin T (*hbl, nhe* and *ent T*) could not be detected by PCR amplification in either strain.

The absence of an emetic-like toxins, not detectable by the methods described above, was confirmed by the use of a published test based on the

inhibition of boar spermatozoan motility. A detection limit for this assay for the emetic toxin of *Bacillus cereus* was established at 0.5 ng cereulide ml^{-1} (equivalent to a water extract of 4 mg cells), and for the emetic-like toxin produced by another strain of *B. licheniformis* equivalent to a water-extract of 2 mg bacterial cells. Methanol and water extracts of 100 mg of the two test strains did not inhibit sperm motility but a positive control (an emetic strain of *B. cereus*) inhibited mobility within one day of exposure.

Two other non-specific tests for toxin production and toxicity were also described, a mouse assay for vascular permeability/necrotic effects and an ileal loop test in pigs. Neither test showed signs associated with the production of toxic metabolites.

4. EFFICACY

In its opinion (expressed on 18th February 2000) on how the efficacy of microbial products seeking permanent authorisation should be established, SCAN recommended that, for pigs for fattening, two post-weaning growth periods should be recognised. These were the period from weaning to approximately two months of age or around 25kg live weight, and from the end of this period until slaughter weight was reached, usually around six months of age. Company data predates this Opinion in some cases by over ten years and consequently the experimental periods selected do not always precisely match this requirement, but reflected industry practice at the time of experimentation. SCAN has taken this into account when reaching its opinion on the efficacy of this product.

The Company provided experimental data on 15 trials with ”piglets” covering or overlapping with this period. Of these 15 trials, four were disregarded because of the average age (42 and 43 days) or weight (>25kg) of the pigs entering the trials. In addition, data emanating from the USA in 1986 was presented as two short-term trials, but was in essence a single trial with data reported at two intervals. In the remaining nine trials, animals entered at 21-35 days of age representing the various weaning practices that have been seen in the industry. The trials were made over a 14-year period in five countries and the results of all but one were subject to statistical analysis (Table II).

Table II. Summary of trials made with piglets from weaning to approximately two months of age

Country & Year	Dose (g/tonne)	No of piglets	Age (days) Start-finish	Weight (kg) Start-finish	Percentage change compared to controls		P value (ADWG)
					ADWG	F/G	
USA 1986	480	96	21-35	6-8	0	-7.7	ns
Ditto	480	96	35- 56	8-15	8.6	-0.2	P<0.05
Denmark 1988	1000	647	28-70	7-28	13.7	-1.2	P<0.01
Denmark 1988	1000	640	21-48	7-28	11.0	-9.1	nd
Denmark 1989	400	1,426	23-71	7-26	8.4	-0.2	P<0.01
Germany 1990	400	48	21-56	7-22	16.4	-6.0	P<0.1
Germany 1990	400	198	34-55	8-14	5.0	-1.0	P<0.1
Germany 1992	400	36	24-52	7-19	13.6	2.1	P<0.1
Italy 1994	400*	200	0 -60	1-23	18.2	-9.3	P<0.05
Denmark 1997	400	36	28-42	8-12	12.0	-6.2	P<0.1
UK 1998	400	256	24-64	8-25	5.9	-4.9	P<0.05

*Study also included dose levels of 200 and 300 without significant effects

Four of the nine trials showed significant (P<0.05 or better) improvements in average daily weight gain (ADWG) and/or feed to gain ratio (F/G) when compared to control animals. The American trial also showed significant improvements in the second half of the trial but not in the first half. As it was not possible, in the absence of primary data, to combine the two parts of the study, it is not known whether the observed improvement to performance would retain statistical significance when averaged over the total period. In the remaining trials a consistent numerical improvement in performance (P<0.1), gives support to the studies in which an acceptable level of statistical significance was achieved.

Three of the statistically significant studies were made with a dose (400 g/tonne) calculated to provide the minimum content of 1.28×10^9 cfu/kg complete feed and one (1000g/tonne) the maximum of 3.2×10^9 cfu/kg complete feed claimed by the Company.

5. CONCLUSION

SCAN concludes, on the evidence provided and knowing of no other information to the contrary, that the product BioPlus 2B® is safe for use with the target animals (pigs, broilers, turkeys and calves) and for those handling the product provided simple precautions are taken. Further, no deleterious effects on consumers of products derived from livestock fed BioPlus 2B® are to be expected.

SCAN also concludes that, on the basis of the data provided, the addition of BioPlus 2B® to the diets of piglets for fattening from weaning to around two months of age or to a weight of approximately 25 kg live weight can significantly improve growth performance. Accordingly, SCAN considers that the efficacy of the product is demonstrated for pigs of this age, when used at the minimum proposed level of 1.28×10^9 cfu/kg of complete feedingstuff.