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 EVALUATION OF THE SATEFY OF CALFMIX®, A MICRO-ORGANISM PRODUCT

(Adopted on 26 March 2003)

1. BACKGROUND :

The product "Calfmix", a mixture of two Lactobacillus strains (*Lactobacillus fermentum* DSM 12476 *and Lactobacillus fermentum* DSM 12477), is intended for the use as feed additive. The Commission received a request for provisional Community authorisation of this product under the conditions set out in the following table:

Additive (Calfmix)	Chemical formula, description	Species or category of animal	Maximum age	Minimum content	Maximum content	Other provisions
				CFU/kg of complete feedingstuff		
Micro-organisms						
Lactobacillus fermentum (DSM 12476), Lactobacillus fermentum (DSM 12477) (In a 1,1/1 ratio)	Mixture of *Lactobacillus fermentum* (DSM 12476) and *Lactobacillus fermentum* (DSM 12477) containing a minimum of $1x10^{11}$ CFU/g of the additive	Calves	11 weeks	1×10^9	1×10^{11}	In the directions for use of the additive and the premixture, indicate the storage temperature and storage life. Recommended daily dosage of 5×10^8 CFU/calf

The company producing Calfmix prepared a dossier that has been submitted through the national rapporteur (Finland) to the Commission. The dossier was checked by the Member States for its compliance with the requirements of Council Directive 87/153/EEC fixing guidelines for the assessment of additives in animal nutrition. The Member States concluded in the Standing Committee of Animal Nutrition on 27 April 2001 that the dossier fulfilled these requirements.

The authorisation procedure laid down in article 4 of Council Directive 70/524/EEC as last amended by Council Directive 96/51/EC includes a period of 320 days for the evaluation of the dossier submitted to the Commission. The Standing Committee of Animal Nutrition started the evaluation of the product on 27 April 2001.

2. TERMS OR REFERENCE

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

Is the use of *Lactobacillus fermentum* DSM 12476 and *Lactobacillus fermentum* DSM 12477 safe:

- for the target animal: calves up to 11 weeks
- for the user?
- for the consumer?
- for the environment?

3. OPINION OF SCAN

3.1. Product description and intended use

The active agents in Calfmix are two strains of *Lactobacillus fermentum*, both isolated from the digestive tract of healthy calves and deposited with the German Culture Collection as DSM 12476 and 12477. They are products of separate fermentations and are mixed in a ratio of 1.1:1.0 to give a total of 10^{11} cfu/g additive. Three formulations are produced, all intended for use with calves to 11 weeks of age and to deliver 5 x10^{8}cfu/calf/day via the milk replacer.

Freeze dried culture: essentially the mixed fermentation products with skimmed milk powder (approx. 35%) as cryoprotectant to give 10^{11} cfu/g dry product. This form of the product is intended to be used by the producer of milk replacers.

Premix for milk replacer: A mixture of the freeze dried culture further diluted with a carrier of lactose and sweet whey powder to give 10^{9} - 10^{10} cfu/g dry product. This formulation also is intended to be used by the producer of milk replacers.

Probiotic for farm use: As above but with a higher lactose content to give around 10^{8} cfu/g dry product. This product is intended for on-farm use and to mixed with milk replacer immediately prior to feeding.

The formulations are routinely monitored for heavy metals and aflatoxin M_1 (relevant to whey content) and limits of detection are provided. Acceptable levels are substantially below those permitted for other complete feedstuffs and would be further diluted in the milk replacer. The product is also monitored for microbial contamination (coliforms, *Clostridium* spp, *Salmonella* spp., yeasts and other fungi) and numbers leading to product rejection stated (including the complete absence of *Salmonella*).

Strain identification is based essentially on the API-50 CHL and PFGE. The latter is also used to match the production cultures with the deposited organism to confirm the lack of any genetic drift. Both strains carry a single plasmid of 15-20 kb.

3.2. Effects on target animal

A tolerance test was made with Holstein bull calves in which groups of 24 animals were fed a control diet or the same diet supplemented with x1 or x100 the maximum recommended dose of the additive. The duration of the trial was 11 weeks, the total period claimed. Animals were monitored for weight gain, faecal and general condition. No negative effects were noted with the treated groups but both had an average daily weight gain significantly higher than the control group. No significant differences were noted between test groups.

The total number of lactobacilli in the faeces of calves fed the additive were 10 to 100-fold greater than was found in the faeces of control animals. Biochemical tests and PFGE confirmed the presence in viable form of the added strains and that they comprised the bulk of the lactobacilli present. The lactobacilli in the faeces of control calves were either of a different species or were strains of *L. fermentum* that could be readily distinguished by PFGE patterns from the added strains.

3.3. Effects on intestinal flora

The number of lactobacilli, coliforms, enterococci and clostridia were determined in faecal samples taken on a single occasion from three calves all given feed supplemented with Calfmix. Lactobacilli were the most numerous, followed by enterococci, coliforms and clostridia. Since no comparison was made with control calves not fed the additive, it is not possible to determine directly the effect exerted by the product on the intestinal microflora. High numbers of lactobacilli would be expected and these were found in both test groups in the tolerance study described above when compared to the untreated control. Numbers of enterococci, coliforms and clostridia were similar to those typically reported for dairy calves. Consequently, it appears that no obvious negative effects are exerted by the product on the intestinal microflora.

3.4. Antibiotic resistance

Both strains were sensitive to erythromycin, gentamicin, amoxicillin, cefuroxim and chloramphenicol and, as would be expected, resistant to vancomycin. No *vanA*, *vanB* or *vanC1-3* could be detected using enterococcal probes and so the intrinsic resistance appears not to mask the presence of any acquired resistance to vancomycin. Strain DSM 12477 had a high MIC of doxycycline (a tetracycline) (24 mg/L) when the MICs were determined using the Epsilon test (E-test). The company subsequently presented data on MICs obtained by micro and macrodilution according to NCCLS standards. In these tests the MIC of tetracycline is within normal ranges. The MICs of

trimethoprim, gentamicin and enrofloxacin are higher than the threshold values set by SCAN[1] denoting a need for further investigation of resistance.

In view of the scarcity of data on MICs of various antibiotics for lactobacilli and the heterogeneity among these species the MICs need to be assessed on a case by case basis. High MICs against aminoglycosides are common among lactobacilli and the gentamicin MIC of 16 and 32 mg/L are not higher than those of streptomycin and neomycin and accumulating data indicate that a higher breakpoint than 1 mg/L should be accepted for most lactobacilli. Lactobacilli often have decreased susceptibility to trimethoprim because of less sensitive dehydrofolate reductases than many other species. However, most *Lactobacillus* spp. have an MIC of no more than 32 mg/L of trimethoprim, considerably lower than the 1024 mg/L seen here. The MIC of enrofloxacin is also relatively high for both strains. MICs of linezolid are not reported.

3.5. Worker safety

The particle size distribution of the formulations was determined by dry sieving. In the freeze-dried culture virtually all the product is retained by a 100 µm screen and poses little respiratory hazard. In the probiotic for farm use, the proportion of lactose carrier is much increased and the freeze-dried mixture proportionally reduced. In this formulation at least 25% passed a 53 µm screen and is potentially inhalable. Most of the material passing the screen is lactose and of little concern. However, the Company subsequently showed that more than 10^8 viable organisms were associated with the material passing a 45 µm screen. As a consequence the company has amended the material safety data sheet. Use of an inhalation mask and safety glasses, amongst else, is recommended to protect personnel from exposure when handling the product.

3.6. Safety for the wider environment

L. fermentum is a common component of the bacterial flora of the digestive tract occurring in relatively large numbers in many animals and humans. It is also found in many spontaneous fermentation's and those adapted for the production of some human foods. Strains are not known as pathogens of plants or aquatic species. As *L. fermentum* is regularly deposited on soil via faeces and slurry, its presence would be expected in watercourses or other bodies of water subject to runoff from soil. However, the organism is a facultative anaerobe requiring acidic conditions and would not be expected to thrive under most environmental conditions.

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[1] 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

4. CONCLUSIONS

The product is evidently well tolerated by the target animal (calves to 11 weeks of age). There were no indications of any adverse responses by the calves when the product was fed at 100x the maximum recommended dose for the entire treatment period. As the two active strains are natural inhabitants of the digestive tract of the target species (and humans), routine dosing with the additive would be expected to increase numbers of *L. fermentum,* but to have little other effects on the gut flora. However, attempts to demonstrate this experimentally were not wholly adequate.

The product can be assumed capable of inducing a delayed sensitivity reaction and thus to pose a risk for those repeatedly handling the product. This is not unique to the product but is a recognised characteristic of most proteinaceaous feed additives. The form of the product intended for on-farm use presents the greatest risk of sensitisation by an inhalatory route but the 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 directly exposed to the product.

The two bacterial strains in the product are resistant to a number of antibiotics, most importantly trimethoprin and the fluoroquinolone enrofloxacin. Resistance to these antibiotics is common amongst lactobacilli and in many cases can be shown to be due to a structural modification. In these cases resistance is not transferable and would offer no particular selective advantage to the organism under the proposed conditions of use. Although it is likely that the resistance shown by the two production strains is also due to a structural modification this has not been demonstrated. Consequently, it would be unsafe to assume that the resistance is not transferable and does not contribute to the pool of resistance genes. In order to rule out that linezolid resistance does not pose a threat to human or animal health, MICs of that drug should be provided.

L. fermentum is a normal inhabitant of the human digestive tract not associated with any pathology. Consequently, contamination of animal products by the production strains would not pose a risk to consumers.

Any localised concentration of *L. fermentum* 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 these organisms as a feed additive will not adversely affect the wider environment.

In the view of SCAN this product has been demonstrated safe for the target animal under the conditions of use proposed and does not pose a hazard for the wider environment. In addition the risks to those handling to product are no greater than for comparable products for which adequate risk management exists.

5. RECOMMENDATION

The only hazard associated with the product is a possible transfer of resistance to antibiotics currently used as therapeutic agents. SCAN recognises that this is probably more theoretical than real, but would wish to see this issue resolved before

finally concluding on the safety of the product. Consequently SCAN recommends that the necessary studies to exclude the presence of acquired resistance and to establish that resistance is due to structural modification are completed.