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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 THE USE OF TREATED MYCELIUM, LIQUID CO-PRODUCT OF PENICILLIN PRODUCTION, BY *PENICILLIUM CHRYSOGENUM*, HEAT TREATED AND ACIDIFIED, AS FEED ADDITIVE

(adopted on 25 June 2001)

1. TERMS OF REFERENCE (MARCH 1998)

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

(1) Does the treated mycelium, liquid co-product of penicillin production, by *Penicillium chrysogenum*, heat treated and acidified have a nutritional value for ruminants and pigs?

(2) Can the use in animal nutrition of the treated mycelium, liquid co-product of penicillin production, by *P. chrysogenum*, heat treated and acidified result in risks for humans (consumer or user), or animal health, or be prejudicial to the environment?

(3) Does the use of the treated mycelium, liquid co-product of penicillin production, by *P. chrysogenum,* heat treated and acidified impair the distinctive features of animal products?

(4) Can the above mentioned product be monitored in feedingstuffs?

2. BACKGROUND

On December 1996, a request was made to authorise a treated mycelium, liquid co-product of penicillin production by *P. chrysogenum* (CBS 307.98) heat treated and acidified, as a source of protein in ruminants and pigs.

3. INTRODUCTION

Vevocel® is a mixture of inactivated mycelium and fermentation broth derived from the cultivation of *Penicillium chrysogenum* Thom for the production of penicillin. The product is marketed as a liquid feed containing 88 – 130 g dry matter (DM)/kg product.

The production strain is deposited in The Netherlands as CBS 307.48. The organism has not been subject to genetic modification. Vevocel® has been used since 1960 for feeding of dairy cows and pigs for fattening in farms near the product plant. Production quantities of the mycelium increased from 34,000 tons in 1980 to more than 60,000 in the middle of the nineties. Vevocel® is now intended to be more widely used as a protein/nitrogen-rich source for pigs and beef cattle, mainly in liquid feeding. The proposed amount and alternatively concentrations of Vevocel® in the complete feedingstuffs are:

- For pigs of 25-50 kg: 2 l/d up to 100g DM/kg feed;
- For pigs over 50 kg and pregnant sows: 3-5 l/d up to 150-200g DM/kg feed;
- For lactating sows: 4-8 l/d up to 100-200 g DM/kg feed;
- For ruminants (beef cattle and dairy cows) 20-40 l/d up to 100-200 g DM/kg feed, with a maximum of 4 to 5 % of the body weight.

4. CHARACTERISTICS OF THE PRODUCT

Vevocel® is an aqueous suspension of the washed filter cake. It consists of the residual fermentation substrates (carbohydrate, hydrolysed protein, inorganic salts and mycelium residues) and the mycelium. Penicillin is inactivated by heating at 80 °C for 10 hours, and then the mycelium is acidified to a pH value of 3.5 - 4.2 to prevent further bacterial growth. The company recommends that the product be fed to the animals promptly and while still hot, keeping the temperature of at least 50 ºC and up to 80 ºC.

4.1. Chemical composition

Variations in the content of dry matter (88 to 130 g/kg) for different batches of the raw product used in the experiments have been described in the dossier for analysis performed in 1988, 1992, 1993 and 1996.

The composition on a DM basis includes per kilo: 560 g of crude protein, 45 g of ether extract, 70 g of ash. The remainder (325 g) largely consists of compounds of fungal cell wall origin which is rich in β-glucan, chitin, cellulose and manno-proteins. The nitrogen fraction is formed by 75% amino acids, 10-15 % aminosugars, 10 % ammonia plus amides, nitrites (NO_2) <10 mg/kg and <0.1 % nitrates. Data on the content of amino acids are available for 3 batches used in digestibility experiments showing in particular the following content: 21.1-23.3 g lysine/kg DM, 6.2-6.8 g methionine/kg, 11.4-

12.8 g cystine/kg, 2-20.5 g threonine/kg. No data on the presence of D amino acids and on the content of nucleotides are given.

Some 40% of the ether extract fraction consists of esterified fatty acids, mainly linoleic acid, palmitic acid and oleic acid, while the remaining part is represented by non-saponifiable substances and phospholipids. Fat contains a high level of unsaturated fatty acids (50 % C18:2; 17 % C18:1; 12 % C16:1 compared to 12 % C16:0). No odd chain fatty acids (C15 and C17) were identified.

Qualitative and quantitative analyses are also given for minerals and heavy metals. Mineral content is high, in particular phosphorus (12.3 –14.6 g/kg DM). The product could consequently cover up to 30 % of the phosphorus requirements of animals (pigs) if recommended at the maximum level of introduction. Data available for trace elements indicate: iron 141 mg/kg; zinc 89 mg/kg; manganese 79 mg/kg and copper 3 mg/kg. For heavy metals, levels are arsenic < 1 mg/kg DM, cadmium < 0.1 mg/kg DM, mercury < 0.4 mg/kg DM, and lead < 0.2 mg/kg. Vevocel® is rich in chloride (26 g/kg DM) and potassium (23 g/kg DM).

No solvents were used during production process.

Three batches of Vevocel® (1995, 1996 and 1997) were examined for the presence of mycotoxins. Aflatoxins, T2 toxin, ochratoxin and zearalenone could not be detected (consistent with the fact that *P. chrysogenum* is not known to produce these toxins). Citrinine, Roquefortine C, meleagrin, xantocillin, chrysogine and 2pab identified in other strains of *P. chrysogenum*, were not included in the analysis.

4.2. Stability of the product, and degradation of penicillin

The heat treatment of the mycelium of *P. chrysogenum* at 60 °C for 30 min led to less than 10 cfu/g. Keeping the temperature of Vevocel® above 50 °C before use would certainly be very unfavourable to the development of micro-organisms during storage. However should this temperature not be maintained, it has been established that the pH of Vevocel® kept for 21d at the room temperature in laboratory conditions decreased from 4.5 (initial value) to 3.85, and it was observed that yeast counts raised from <10 to $3.5x10^2$ cfu/g, mould count remained at <10 cfu/g and lactobacilli increased from <10 to $2.2x10^2$ cfu/g. No data were given for the presence of penicillin in the product.

The benzylpenicillin content of the raw product determined using HPLC method showed considerable batch to batch variation (100-2000 mg/kg). The inactivation of the antibiotic has been assessed by measuring the benzylpenicillin content of a sample containing an even higher initial concentration (3900 mg/kg) and subjected to a heat treatment at 80 °C for 10 hours, *i.e.* the treatment used to prepare Vevocel®. Data (Table 1) show that benzylpenicillin disappears after five hours treatment, but that the low sensitivity of the HPLC method (limit of detection: 60 mg/kg fresh matter) does not allow to establish whether significant amounts of residues in terms of antibiotic activity are still present. Other data have been obtained using a more

sensitive microbiological method (Delvotest, limit of detection: 0.004 IU/ml or 0.0024 µg/ml). On 48 samples analysed in 1997, benzylpenicillin content was found to be less than 0.05 IU/ml or 0.03 µg/ml (limit of detection in the product).

The degradation of penicillin is a complex process well described by Lipsczinski (1988). In his experimental model of the degradation of benzylpenicillin, 50 % was converted into penilloic acid, 40 % into phenaceturic acid/N-formylpenicillamine and 5 % into benzylpenilloaldehyde/penicillamine. Benzylpenicillin may react covalently with protein or polysaccharides. Penicilloyl conjugates, formed naturally and spontaneously, are the major determinants in penicillin allergy. They arise from the linkage of penicillin to the epsilon- amino group of lysine. A penicillin degradation product, penicillenic acid, is formed by a proton-catalysed reaction proceeding via an intermediate tautomeric oxazol-thiazolidine structure. Minute amounts appear during the production of penicillin, but significant formation has been shown to occur in the acidic conditions prevaling in the stomach. Therefore, it can be expected that this compound would appear following the acidification of the mycelium cake filtrate. The covalent binding of penicillenic acid at C-7, as well as the rearrangement of the penicilloyl determinant give rise to another allergenic determinant called penamaldyl. Once formed, penicillenic acid is rapidly hydrolysed under pH 4 mainly to penamaldic acid, and penillic acid. Benzylpenilloic acid, the major degradation product of benzylpenicillin through the intermediary benzylpenillic acid, has a poor ability to react covalently to proteins and gives rise to (D)-penicillamine and benzylpenilloaldhehyde (Bundgaard, 1983; De Weck, 1983; Sneider,1983).

Table 1. Evaluation of the of breakdown rate of residual benzylpenicillin in mycelium samples of *P. chrysogenum* submitted to a 80 °C heat treatment (HPLC method, limit of detection: 60 mg/kg fresh matter, average of three batches)

Heating time (hours, 80 °C)	Benzylpenicillin (mg/kg)
0	3900
5	100
10	<60
24	<60

Although the degradation rate and degradation products of penicillin under various *in vitro* and *in vivo* conditions are well known and widely reported in the literature, it appears also as a complex matter, not occurring in a uniform manner and depending on a number of factors (*e.g.* pH, temperature, buffers). Numerous data on the degradation products of penicillin in Vevocel® have been supplied. Determination of penilloic acid in successive Vevocel® batches produced during 1992, indicates that penilloic acid content is highly variable, ranging from <20 to 1800 mg/kg. A model experiment was used where the concentration of benzylpenicillin and several degradation products, *e.g.* penilloic acid, benzylpenilloaldehyde, penicillamine, phenaturic acid and N-formylpenicillamine were measured. The latter compound was found at

concentrations ranging from 50 to 90 mg/kg. No data are available on the content of either benzylpenicillin or degradation products after 2-3 weeks of storage in particular with respect to a significant decrease in temperature over this period (estimated to only 40 °C after 6 days of storage). These conditions might allow the outgrowth from any surviving *P. chrysogenum* spores.

5. EFFICACY STUDIES

5.1. Nutritional trials

The following results have been recorded on farm animals:

- Two long term feeding trials on bulls in three groups in which Vevocel® supplied 0; 10 and 20 percent of the DM for 70 days leading to a daily intake of Vevocel® of 18 to 35 kg/day did not show any difference on performance when compared to controls. Data extended to a field trial without control group but on 400 bulls confirmed similar tendencies in the results.

- Two feeding trials (1977 and 1988) using 36 pigs in three groups from 32 to 105 kg body weight and from 35 to 102 kg body weight, respectively, and receiving either 0; 1.6 and 3.2 kg fresh product per day in a first experiment or 0; 2.2 kg (7.5 %) and 4.3 kg (15 % of dietary dry matter) of fresh product per day in a second experiment have been performed. No difference on performance expressed as daily body weight gain and carcass quality were recorded between the experimental and the control group.

- One feeding trial (1998) using 3 groups of pregnant sows receiving 0; 3.75 and 7.5 kg/ day of fresh product for 112 days show neither differences for the number and the average birth weight of piglets at farrowing, nor difference in the postnatal survival rate of their piglets.

- There were no data on nutritional benefits for dairy cows and lactating sows.

5.2. Digestibility and feeding value of the product

Digestibility trials have been conducted in 1992 and 1993 using pigs cannulated at the ileo-cecal junction for the measurement of ileal digestibility of nitrogen and amino acids. The level of intake (restricted and pair-feeding) represented 28-32 % of energy and dry matter intake in experiment 1 and 31-36 % of energy and dry matter in experiment 2 respectively. The experiments conducted during a period of less than one month lead to values of 73.6 and 80.3 % for apparent total tract digestibility and 73.4 and 66.5 % for ileal digestibility for nitrogen, respectively. While ileal digestibility of amino acid was higher than that of nitrogen, except for valine and cystine, the ileal digestibility of amino acid is highly variable, in particular in the case of cystine for which the average ileal digestibility varied between experiments from 22.7 to 46.2 percent. This must be taken in account in the case of recommendations used in feed tables.

In addition, no data on nitrogen retention and on the composition of urinary nitrogen (creatinine) are available to clarify the potential consequences of the presence of nucleotides in the Vevocel® on the nitrogen metabolism in the pig.

6. SAFETY FOR THE TARGET SPECIES

Since the composition and the pH value of the final product vary from batch to batch the animal performance as well as potential adverse effects can not be accurately predicted.

No standard tolerance tests on target species were performed. However, SCAN recognises that tolerance tests (with overdoses) can hardly be conducted with feedingstuffs, in particular, withthe recommended highest dose amounts to 20 % of the ration (on a dry matter basis).

A feeding experiment on pregnant sows is presented, in which the highest Vevocel® concentration exceeded the maximum recommended dose by 50 %. No adverse effects on general performance (litter size and survival rate) were observed.

A previous experiment on growing pigs at the same level, but with the dehydrated product, resulted in severe digestive disturbances.

For both experiments, no data on gross pathological examination as well as haematological, enzymatic and metabolic parameters were provided.

Also feeding bulls with up to 40 liters of Vevocel® (highest recommended dose) per day corresponding to 200 g Vevocel®/kg diet DM did not affect daily feed intake and body weight gain.

However, it has led to a daily protein intake of 2.24 kg crude protein/per animal and day representing almost 200 % of their protein requirement. The effects on rumen fermentation, nitrogen metabolism and water balance were not assessed. Also the high intake of acid equivalents (rumen acidosis) and of electrolytes (Na, K, Cl) were not considered. Thus, the Company recommends to mix the product with roughage when fed to cattle and to supply large amounts of water, and even to add 100 g of calcium carbonate per cow.

The consequences of the use of Vevocel® on the digestive flora of either pigs or ruminants were not investigated.

7. WORKERS' SAFETY

The product must be delivered at high temperature (50-80 °C) and for this could be a risk for the workers handling the product.

Warnings have been specifically recommended to avoid dusting of the product when spilled and allowed to dry.

The only studies supplied concern skin sensitisation on guinea pigs, namely the Magnusson-Kligman (MK) and the Buehler tests. The product is a skin sensitiser for

the guinea pig. As a consequence, the warning label R43 is included on the packaging.

It was noted that no tests with Vevocel® on mucosa, skin or eye irritation on guinea pigs have been performed.

8. SAFETY FOR THE CONSUMER

An acute oral toxicity has been addressed in a limit test without adverse effects; however, this test is of no relevance for consumer safety. The absence of studies of most aspects of the toxicology of Vevocel® (including genotoxicity assay and repeat dose toxicity, *e.g.* 90 day toxicity) is noted.

The residual penicillin content of the untreated (not heated) mycelium cake filtrate are quantitatively important and highly variable. Following heat treatment the penicillin concentration falls below 0.03 mg/kg (limit of detection of the method), that would correspond to the theoretical maximum concentration of only 0.01 ppm in the animal diets. This level would not be sufficient to select resistant micro-organisms but induction of mechanisms of resistance could not be totally excluded in strains having the resistance gene.

The link between penicillin residues in food, especially milk, and food allergy in humans has been documented. It is well established that the allergenic potential of benzylpenicillin is due to the hapten benzylpenicilloyl, but also to the allergenic determinant penamaldylprotein. Since significant amounts of benzylpenilloic acid are present in Vevocel® thus implying that the penicillenic degradation pathway occurred, it can therefore be anticipated that penamaldylprotein adducts would be present. As it has been shown that such haptens can be absorbed at low rate (2 %) retaining their allergenic property (Wal, 1985), demonstration of absence of allergenic compounds in animal produce is needed. It must be noted that no attempt was made to identify either penicilloyl products or penicillin degradation end products in animal tissues and milk.

No evidence of carcinogenicity (Castagnero and McGregor, 1998), immunotoxicity (Oswald and Comera,1998), haemotoxicity (Parent-Massin and Parchement,1998), oestrogenicity (Shier, 1998) or mutagenicity (Dierheimer, 1998) has been reported for the range of specific mycotoxins produced by *Penicillium chrysogenum* (benzylpenicillin producing strains or not). The limit of detection of mercury (0.4 mg/kg) does not permit to assess the safety of the product according to the maximum limit (0.1 mg/kg) fixed by Council Directive 1999/29/CE.

9. SAFETY FOR THE ENVIRONMENT

There are no specific studies concerning the environment as the Company considered that these were not necessary.

On the basis of the composition of the residue of Vevocel®, consisting mainly in organic matter, and in the absence of antimicrobial activity, the product does not seem to be of primary environmental concern. Penilloic acid and N-formylpenicillamine have not been measured in the slurry. However as different

mould strains produce penicillin and release penicillin degradation products in the environment, no further assessment of these metabolites is considered necessary.

10. CONCLUSIONS

10.1. Nutritional value of the product

(1) Vevocel® constitutes a potential protein source for livestock, although limited by the considerable variations observed between batches in dry matter content, low pH value and digestibility of specific amino acids (cysteine).

(2) The nutritional value for pigs, gestating sows and bulls has been demonstrated. In the absence of data on the nutritional potential for the dairy cow and to a lesser extent for the lactating sow, the nutritional value of the product has not been demonstrated for these particular target animal categories.

(3) Excess of protein supply in case of fattening bulls should be prevented and the recommended levels in the complete feedingstuff (diet) for ruminants should be reconsidered.

10.2. Safety

The inherent microbiological instability of the product is evident from the need to maintain it at a temperature at which most micro-organisms are unable to grow. However this heat-chain cannot be guaranteed and insufficient data are available to judge the consequences for target animals and consumers of animal products of any failure to sustain this elevated temperature during distribution and subsequent storage.

(1) For target animals

Due to lack of data, safety for the target animals cannot be recognised. No attempt was made to gain data for dairy cows and lactating sows. Data for bulls, pigs and pregnant sows were lacking gross pathological, heamatological, enzymatic and metabolic parameters.

(2) For workers

The sensitisation potential of Vevocel® and its high temperature at delivery represent risks for the workers in handling the product. No data on irritation potential are available.

(3) For consumers

Vevocel® has not been adequately tested for toxicity.

The levels of contaminants identified in the product do not comply with the requirements of the Council Directive 1999/29/EC on undesirable substances in feed.

Despite wide variations of penicillin residues in Vevocel® before the acidification and heat processing steps are applied, it appears that penicillin levels are constantly very low or absent (below detection limit). However, the potential of eventual amounts in terms of induction of resistance cannot be assessed.

Vevocel® contains considerable amounts of penicillin degradation products, some of which are able to react with proteins to produce highly antigenic determinants. As such compounds are known to be absorbed to some extent and to keep their allergenic property, it cannot be excluded that products coming from animals fed Vevocel® could contain them.

Therefore, the safety for human consumers cannot be ensured.

(4) For the environment

As different mould strains produce penicillin and release penicillin degradation products in the environment, no further assessment of penicillin metabolites present in animal manure is considered necessary.

Indirect consequences on the environment of spreading slurry from animals fed high amount of nitrogen and phosphorus from Vevocel® should also be considered.

10.3. Animal products

As data on products of animal origin were not provided, the impact of the use of Vevocel® in feed on the organoleptic properties of products of animal origin cannot be evaluated.

10.4. Monitoring

No method is provided for the identification of Vevocel® itself, or as feed material in feedingstuffs.

11. REFERENCES

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