European Food Safety Authority

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SCIENTIFIC OPINION

Safety and efficacy of the product ColiCure (*Escherichia coli*) as a feed additive for horses[1]

Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed

(Question No EFSA-Q-2005-167)

Adopted on 3 March 2009

PANEL MEMBERS

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SUMMARY

Following a request from the European Commission, the European Food Safety Authority (EFSA) was asked to deliver a scientific opinion on the safety and efficacy of the product ColiCure, a preparation of *Escherichia coli*, as a feed additive for horses. The applicant proposes the use of a strain of *E. coli* to 'stabilise' the intestinal flora of horses exposed to stress situations (e.g. following a change of feed or during transport). The contents of one 100 mL bottle containing a minimum of 1 x 10^{11} CFU is mixed with a small amount of feed and given immediately to an individual horse once a day for three consecutive days.

The administration of ColiCure to horses with abnormal faeces for three days significantly improved the rate of return to normal consistency in three studies. In the view of the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) this establishes the potential of ColiCure to reduce the risk of gastrointestinal disturbances in horses.

Doses up to 100-fold the minimum guaranteed dose did not adversely affect the health of horses given ColiCure for three consecutive days and monitored for a further month. Consequently, the FEEDAP Panel considers the product safe for adult horses when used as proposed. However, as the youngest horse exposed to the product was three years old, the conclusion on safety for young/mature horses should not be extended to the use of the product in horses of less than one year of age.

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The FEEDAP Panel considers that any hazard for the consumer and the primary hazard for the user would arise mainly from the inadvertent exposure to the live organism. The use of PCR methods and a specific microarray designed to pathotype *E. coli* showed the absence of the virulence determinants associated with human pathogenic strains in the *E. coli* strain used in ColiCure. Given the lack of virulence determinants, the limited duration of application of the product to horses and the intended market, the FEEDAP Panel considers any risk to the consumers and the users to be remote.

Since *E. coli* are ubiquitous and since the limited use of the product proposed with horses would not measurably alter the occurrence or concentration of *E. coli* in the environment, use of ColiCure would not pose a risk for the environment.

Key words: zootechnical additive, gut flora stabiliser, horses, gastrointestinal disturbances ColiCure, *Escherichia coli*, safety, virulence, efficacy

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BACKGROUND

Regulation (EC) No 1831/2003[2] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from the company NordVacc[3] for authorisation of the product ColiCure, *Escherichia coli*, to be used as a feed additive for horses (category: zootechnical additives; functional group: gut flora stabilisers) under the conditions mentioned in Table 1.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). EFSA received directly from the applicant the technical dossier in support of this application.[4] According to Article 8 of that Regulation, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. The particulars and documents in support of the application were considered valid by EFSA as of 21 June 2006.

The additive ColiCure is a preparation of *Escherichia coli* (LMG S-17146). This product has not been previously authorised in the European Union.

TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the efficacy and the safety for the target animal(s), user and consumer and the environment of the product ColiCure, *Escherichia coli*, when used under the conditions described in Table 1.

ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank the members of the Working Group on Micro-organisms and Dr Alfredo Caprioli for the preparation of this opinion.

[2] OJ L 268, 18.10.2003, p.29
[3] NordVacc Läkemedel AB. Västertorpsvägen 135, Hägersten, Sweden
[4] Dossier reference: FAD-2005-0019

Table 1. **Register entry as proposed by the applicant**

Additive	ColiCure
Registration number/EC No/No (if appropriate)	
Category of additive	Zootechnical additives
Functional group of additive	Gut flora stabilisers

Description			
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)
Composition: 1 bottle (100 mL) contains $>10^9$ cfu mL^{-1} of viable organism of the *E. coli* bacteria suspended in phosphate buffered saline q.s. pH 7.0 Description: Colicure is a suspension of a purified and stabilized *E. coli* strain originally isolated from the intestine of a healthy horse. The bacterial suspension is intended to be given in a small amount of feed to horses with disturbances in the intestinal microflora.	-	*Escherichia coli* bacteria confirmed by phenotyping system (API)	CFU. 10-fold serial dilution in physiological saline and then inoculated onto agar plates

Trade name	ColiCure
Name of the holder of authorisation	

Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period
		CFU kg^{-1} of complete feedingstuffs		
Horses	-	Not relevant	Not relevant	None

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use (if appropriate)	None
Specific conditions or restrictions for handling (if appropriate)	None
Post market monitoring (if appropriate)	Questionnaire to be filled in by the user. Reason of usage. Eventual results/improvements/adverse reactions after usage, smell and consistency of excrements
Specific conditions for use in complementary feedingstuffs	None

Maximum Residue Limit (MRL)

Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues
None	None	None	None

ASSESSMENT

1. Introduction

Oral administration of non-pathogenic strains of *Escherichia coli* as a means of controlling some forms of gastrointestinal disturbance was first proposed in the early 1900s (Nissle, 1918). Although interest subsequently turned to other commensal members of the gut microbiota, there has remained a continuing interest in the deliberate introduction of selected *E. coli* preparations for the prevention/treatment of a variety of conditions in both animals (Schamberger et al., 2004; von Buenau et al., 2005) and humans (Boudeau et al., 2003; Lodinová-Zádniková et al., 2003). Non-pathogenic *E. coli* are presumed to compete with and displace potential pathogenic bacterial strains from specific binding sites on the surface of epithelial cells, limiting overgrowth and invasion. This may occur by direct competition or possibly through the effects of intermediaries, such as the stimulation of β-defensins or mucin production (Wehkamp et al., 2004). Often, type 1 fimbriated strains are used. These show a high specificity for mannosyl residues in surface glycoproteins, a property shared with many pathogenic *E. coli* and other members of the enterobacteriaceae, notably *Salmonella*.

In the present case, the applicant proposes the use of a strain of *E. coli* to 'stabilise' the intestinal flora of horses exposed to stress situations (e.g. following a change of feed or during transport) or when there is already evidence of a 'disturbed' intestinal flora.[5]

2. Characterisation of the product

2.1. Production and composition of the product

The additive consists of viable cells of a specific strain of *E. coli* suspended in phosphate buffered saline (pH 7.0) with a minimum count of 1 x 10^9 CFU mL^{-1} (range 1–6 x 10^9 CFU mL^{-1}). Bacteria are grown in a sterilised laboratory medium (tryptone, soytone, glucose and mineral salts) as batch cultures. Cells are harvested by centrifugation and then washed twice before suspension in the buffered saline and packaging in 100 mL plastic bottles. Residual material derived from the fermentation is said to represent < 0.1 % of total contents.

The additive is monitored at all stages of production for contaminating yeasts, other fungi and bacterial species other than *E. coli*.[6] None are permitted in the final product. An analysis of three batches of the product (ICP atomic emission spectrometry) for cadmium, lead, arsenic and mercury showed that the concentrations were, in each case, below the LOQ.[7]

2.2. Characterisation of the active agent

The strain of *E. coli* was originally isolated from faeces of a healthy horse and is deposited at the culture collection of the University of Gent, with accession number LMG S-17146.[8] Its identity was established based on cell and colony morphology, typing of somatic, flagellar and capsular antigens, biochemistry and molecular methods.[9] The later included amplified fragment length polymorphism (AFLP) DNA fingerprints and pulsed field gel electrophoresis. The strain appears free of plasmids and is not genetically modified.

[5] Tecnical dossier/Section II
[6] Technical dossier/Section II/Enclosure 7.10
[7] Technical dossier/Section II/Enclosure 7.11
[8] Technical dossier/Section II/Enclosure 7.1
[9] Technical dossier/Section II/Enclosure 7.4

The serotype of the production strain is not fully determined since the O-antigen could not be established.[10] However, the strain was typed to H25. The genetic determinants for the capsular and fimbrial adhesins K88, K99, F41, 987P and intimin could not be detected by PCR.

The capacity to produce the heat-labile enterotoxin (LT), the heat-stable enterotoxins (STa and STb and the related EAST) and the verotoxins VT1 and VT2 (now known as STX1 and STX2; Gyles, 2007) was excluded by PCR.[11] The strain is haemolytic.

Heamolytic strains of *E. coli* serotyped as O untypeable and H25 occur widely in healthy cattle; they have, in some cases, proved to be phenotypically similar to enterohemorrhagic *E. coli* (Sheng *et al*., 2005) and have been isolated from human clinical samples. To ensure that the ColiCure strain did not exhibit any of the known virulence determinants associated with enterohemorrhagic strains, further data was provided using a DNA microarray developed to pathotype strains of *E. coli* isolated from clinical samples (Anjum et al., 2007). The array carried 60 oligonucleotide gene probes, including 39 directed towards virulence determinants and seven targeting bacteriocins.[12] The remaining probes acted as positive controls. None of the genes coding for the virulence determinants included in the array, among which *hylA* and *hylE*, proved to be positive.

2.2.1. Genetic stability

Genetic stability was indicated by a comparison of biochemistry and AFLP fingerprints from the original deposit (1998) with a single production batch. No differences of significance were found.

2.2.2. Antibiotic resistance

The production strain was tested against the list of antibiotics (or acceptable alternatives) included in the latest revision of the Opinion on antibiotic resistance criteria (EFSA, 2008). Using the breakpoints presented in the opinion, the strain was judged susceptible to all of the 14 antibiotics tested.[13]

2.2.3. Stability and homogeneity

The manufacturer recommends refrigerated storage between 4–8 ^{o}C. Under those conditions, the minimum declared content was retained for five months in the three batches tested. The applicant states that no loss in viability occurs over a period of ten days at ambient temperatures up to 37 ^{o}C.

As the product is intended for immediate use, stability in feed was measured only over a two-hour period.[14] Data from two batches of the product showed that loss in viability in the region of 2.5 % would be expected.

2.3. Proposed conditions of use

The contents of one 100 mL bottle containing a minimum of 1 x 10^{11} CFU is mixed with a small amount of feed and given immediately to an individual horse. The amount of feed used

[10] Technical dossier/Section II/Enclosure 7.2
[11] Technical dossier/Section II/Enclosures 7.3 and 7.7
[12] Technical dossier/Supplementary information January 09/Enclosure IV 7.28
[13] Technical dossier/Section II/Enclosure 7.8
[14] Technical dossier/Section II/Enclosure 7.14

should be that consumed immediately. This is repeated daily for two further days (the product is sold as a pack of three 100 mL plastic bottles).

2.4. Evaluation of the analytical methods by the Community Reference Laboratory (CRL)

EFSA has verified the CRL report as it relates to the methods used for the control of the *Escherichia coli* in animal feed. The Executive Summary of the CRL report can be found in the Appendix.

3. Efficacy

A total of five studies made with horses of various ages are described, backed by a report on the field use of the product. With the exception of one trial, all the data originates from a single European country.

Four of the five studies were carried out with horses with signs of faecal abnormalities (poor faecal consistency and smell), and in one of them the microbiology of the faeces was monitored. As the faecal abnormalities were naturally occurring, the start date for treatment and observation differed for individual animals within the same study. In addition, where true negative controls were included (three of the five studies), the total study period was limited to seven days, the longest period considered acceptable for the horses given a placebo to remain untreated. In all the studies, the minimum dose given to the animals was confirmed by analysis.

In the first study,[15] nine of 11 horses (trotters) with poor faecal consistency were given ColiCure and the remaining two a placebo (buffered saline). Faecal consistency was reported to have returned to 'normal' within a week of the start of treatment in the nine treated horses but to have persisted in the two controls. The numbers of coliforms in faeces increased, as would be expected, during treatment and in horses with initially high numbers of clostridia (four horses) or *Bacillus* spp. (four horses); they were significantly reduced after treatment. However, as the two control animals were housed in a different location than most of the treated animals and in the absence of any semi-quantitative assessment of faecal quality, it is not possible to ascribe with certainty any observed beneficial changes to treatment.

The second study[16] consisted of only five apparently healthy horses, four treated and one given a placebo. Observations were limited to faecal counts of coliforms, clostridia, bacilli and fungi (moulds) before treatment, immediately after treatment and seven days thereafter. No other effects than an increase in coliforms after treatment with ColiCure were discernable.

The remaining three studies had a common protocol. Animals, described as 'mature trotters', were divided into two equal groups: one given ColiCure and the other buffered saline as control. Faecal consistency was scored (scale from 1 [normal] to 5 [watery consistency]) from the start of treatment to four days after the treatment period was completed. For the purpose of faecal scoring, each experiment was blinded. The results of the three studies are summarised in Table 2.

[15] Technical dossier/Section III/Enclosure III 7.16
[16] Technical dossier/Section III/Enclosure III 7.17

Table 2. **Summary of mean faecal scores for treated and control horses**

Study number	Treatment	No of animals	Faecal score		
			Start of treatment	End of treatment	Final observation
3[17]	Control	10	2.7	2.6	2.6
	ColiCure	10	2.9	1.9*	1.4**
4[18]	Control	10	3.4	2.4	2.3
	ColiCure	10	3.5	1.9	1.4**
5[19]	Control	5	3.8	3.8	2.6
	ColiCure	5	4.8*	2.8	1.0**

*, ** Significantly different from controls at $P < 0.05$ or $P < 0.01$, respectively.

Although in two of the three studies faecal consistency was improving over time in the control group, in all three studies improvement was significantly faster in the treated group, essentially returning to normal within one week of the start of treatment.

For two years, 96 user reports were returned to the company, describing the results of practical experience. Loose to watery faeces was the reason for treatment in 41 of those reports, of which 31 showed normal faecal consistency within one week of the start of treatment.

3.1. Conclusions on efficacy

In mature horses displaying abnormal faecal consistency, treatment with ColiCure improved the rate of return to normal consistency in three studies, establishing the potential of ColiCure to reduce the risk of gastrointestinal disturbances. This potential was supported by reports of field use.

4. Safety for the target species

4.1. Tolerance study

Three horses (two males, aged three and 13 years, and one mare, aged six years) were dosed with ColiCure at over 100 times the recommended dose (confirmed by analysis).[20] To achieve the high dose, the product was slightly reformulated to contain 2.6×10^{10} CFU mL^{-1} and 500 mL was given via a stomach tube for the three successive days. The horses were separately housed and monitored from the start of the experiment and for 28 days following the last treatment. Appetite, rectal temperature, faecal appearance and other clinical signs were checked daily. In addition, faecal samples were taken on the first and last day of the study and examined for numbers of coliforms, *Bacillus* spp., clostridia and fungi (moulds), and blood samples were taken for haematology and blood chemistry.

There was no indication of an adverse response in any of the horses at any time. Haematology and blood chemistry values in all cases were within normal ranges.

Two other studies involving an overdose of ColiCure are described. The first study[21] was carried out with three horses (two females, aged three and six years, and one male, aged three years) given approximately 70 times the normal dose with a small amount of feed for three consecutive days. In addition of clinical observations, faecal samples were taken for microbiological examination and blood samples for haematology and blood chemistry.

[17] Technical dossier/Section III/Enclosure III 7.23 E-4
[18] Technical dossier/Section III/Enclosure III 7.24 E-5
[19] Technical dossier/Section III/Enclosure III 7.25 E-6
[20] Technical dossier/Section IV/Enclosure IV 7.22
[21] Technical dossier/Section IV/Enclosure IV 7.19

Although the period of observation (11 days) was considered too short to provide complete assurance about the safety of the product, the absence of treatment-related adverse responses in the study supports the finding of the full tolerance study described above.

In the second study,[22] five horses (four females and one male between three and 11 years of age) were dosed with 100 mL of a ColiCure preparation said to contain 15 x 10^9 CFU mL^{-1} through feed for three consecutive days. This dose was apparently not confirmed by analysis and is described in the study report as a three-fold overdose. The total duration of the study was one month, during which the horses were observed and faecal samples taken for microbiological analysis. No changes to rectal temperature, appearance of faeces or general health were reported during the post-treatment period of observation.

4.2. Influence on the microflora of the digestive tract

A limited microbiological analysis of faecal samples collected in the three overdose studies was made. This consisted of counts of total coliforms, clostridia, *Bacillus* spp. and 'moulds' performed before and at various times after treatment. There were insufficient samples for statistical treatment of the data collected and so only limited conclusions can be drawn from those studies. In general, where low numbers of coliforms were seen prior to treatment they tended to increase after treatment, while in animals with relatively high counts of clostridia and bacilli they tended to decrease in the period following completion of the treatment.

4.3. Conclusions on target animals safety

The FEEDAP Panel considers the product safe for adult horses when used as proposed by the applicant in the Register entry. The youngest horse tested was three years old and the conclusion on safety for young/mature horses should not be extended to the use of the product with animals of less than one year of age.

5. Safety for the consumer

The product is evidently primarily intended for leisure or working horses with no consequences for consumers. However, in the event that the product would be used with horses intended for meat production, any hazard for the consumer would be restricted to the inadvertent exposure to the live organism via carcass contamination. The organism has been shown to lack the adhesion and other virulence factors commonly associated with human enteropathogenic strains of *E. coli*. Consequently, given this lack of virulence factors, the limited duration of application of the product to horses and the intended market, the FEEDAP Panel considers any risk to the consumers to be remote.

5.1. Safety for the user

The primary hazard for users handling the product would be accidental contamination with the live organism. Similarly to consumers, the risk associated with this hazard appears remote in the absence of known virulence determinants.

The product has a neutral pH and is sold in small volumes to be poured onto a limited quantity of feed. Exposure is thus likely to be via dermal route with little or no exposure via respiration. Suspensions of micro-organisms are generally not considered to be dermal irritants and, since it is intended to be used intermittently for short periods, it is unlikely to lead to dermal sensitisation.

[22] Technical dossier/Section IV/Enclosure IV 7.20

5.2. Safety for the environment

E. coli is a commensal organism common to the gastrointestinal tract of most mammals, where it occurs as part of the dominant microbiota. Consequently, the organism is already widely distributed in the environment. The limited use proposed with horses would not measurably alter the local or general occurrence or concentration of *E. coli* in the environment.

6. Post-market monitoring

Any risk associated with the use of the product is negligible. It is considered that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[23] and Good Manufacturing Practice.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

The evidence provided establishes the potential of ColiCure to reduce the risk of gastrointestinal disturbances in horses.

The FEEDAP Panel considers the product safe for adult horses when used as proposed. However, as the youngest horse exposed to the product was three years old, the conclusion on safety for young/mature horses should not be extended to the use of the product in horses of less than one year of age.

The FEEDAP Panel considers that any hazard for the consumer and the primary hazard for the user would arise mainly from the inadvertent exposure to the live organism. As the organism has been shown to lack the adhesion and other virulence factors commonly associated with human enteropathogenic strains of *E. coli*, and given the limited duration of application of the product to horses and the intended market, the FEEDAP Panel considers any risk to the consumers and the users to be remote.

The use of the product as proposed by the applicant would not pose a risk for the environment.

RECOMMENDATIONS

The strain of *Escherichia coli* should be specified in the Register entry by inclusion of the culture collection accession number.

The FEEDAP Panel recommends that the use of the product should be restricted to animals older than one year of age.

The conditions of use should be specified under "other provisions" and should state: *contents of one 100 mL bottle containing a minimum of 1 x 10^{11} CFU should be mixed with a small amount of feed and given immediately to an individual horse once a day for three consecutive days.*

DOCUMENTATION PROVIDED TO EFSA

1. Dossier on ColiCure for horses. August 2005. Submitted by NordVacc Läkemedel AB.
2. Supplementary dossier on ColiCure for horses. July 2008 and January 2009. Submitted by NordVacc Läkemedel AB.

[23] OJ L 35, 8.2.2005, p.1

3. Evaluation report of the Community Reference Laboratory for Feed Additives on the methods(s) of analysis for ColiCure.
4. Comments from Member States received through the ScienceNet.

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APPENDIX

Executive Summary of the Evaluation Report of the Community Reference Laboratory for Feed Additives on the Method(s) of Analysis for ColiCure

In the current application authorisation is sought for the microbial feed additive Colicure under the category "zootechnical additives", functional group "gut flora stabiliser" according to Annex I of Regulation (EC) No 1831/2003. The active agent in the additive is *Escherichia coli* E-101-88, LMG S-1746. Specifically, authorization is sought to use Colicure for horses. It is proposed that one bottle of 100 ml Colicure which contains at least $1x10^9$ colony forming units c.f.u/ml of the additive agent *Escherichia coli* E-101-88, LMG S-1746 suspended in phosphate buffered saline is added to a small amount of feedingstuffs for horses for immediate consumption. For the quantification of the active agent (*Escherichia coli* E-101-88, LMG S-1746) of Colicure in the feed additive and feedingstuffs, an appropriate non-selective surface plate count method based on well-known principles using nutrient agar supplemented with 5% bovine blood and an incubation temperature of 37 ºC was proposed by the applicant. The method iand if required of *feedignstuffs* supplemented with Colicure corresponding officially recognised standard methods such as ISO and/or CEN methods for example ISO 4832, ISO 16649-2 or ISO 21528-2 are recommended. The identity of the microbial strain was analysed by a range of techniques including microscopy, serology, biochemistry, polymerase chain reaction (PCR) and restriction enzyme analysis using three enzymes. The applicant used amplified fragment length polymorphism (AFLP) and pulsed field gel electrophoresis (PFGE). PFGE is a generally recognised standard methodology for microbial identification and is considered suitable for official controls.

On the basis of the supplied documentation, no supplementary work (testing or method validation) is required.