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Safety and efficacy of *Lactobacillus reuteri* NBF-1 (DSM 32203) as a feed additive for dogs

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Abstract

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of *Lactobacillus reuteri* NBF-1 when used in feed for dogs at a minimum dose of 6×10^9 colony forming units (CFU) per animal and day. The additive is a preparation of viable cells of *L. reuteri* DSM 32203. This species is considered by the European Food Safety Authority to be suitable for the qualified presumption of safety (QPS) approach establishing safety for the target species and the environment. The active agent fulfils the requirements of the QPS approach to the assessment of safety. Consequently, in the absence of concerns from other components of the additive, *Lactobacillus reuteri* NBF-1 is presumed safe for the target animals and the environment. *Lactobacillus reuteri* NBF-1 should be considered a potential respiratory sensitiser. In the absence of data, the FEEDAP Panel cannot conclude on the irritancy of the additive to skin and eyes or on its dermal sensitisation. The FEEDAP Panel is not in the position to conclude on the efficacy of *Lactobacillus reuteri* NBF-1 for dogs.

Keywords: zootechnical additives, *Lactobacillus reuteri* NBF-1, *Lactobacillus reuteri* DSM 32203, safety, QPS, efficacy, dogs

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003 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 NBF Lanes s.r.l. for authorisation of *Lactobacillus reuteri* NBF-1 (*Lactobacillus reuteri* DSM 32203) when used as a feed additive for dogs (category: zootechnical additives; functional group: gut flora stabilisers).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the applications to the European Food Safety Authority (EFSA) as applications under Article 4(1) (authorisation of a feed additive or new use of a feed additive). The particulars and documents in support of this application were considered valid by EFSA as of 31 May 2017.

According to Article 8 of Regulation (EC) No 1831/2003, 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. EFSA shall deliver an opinion on the safety for the target animals, user and the environment and on the efficacy of the product *Lactobacillus reuteri* NBF-1 (*L. reuteri* DSM 32203), when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

Lactobacillus reuteri NBF-1 is a preparation containing viable cells of *L. reuteri* DSM 32203. It has not been previously authorised as a feed additive in the European Union.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[1] in support of the authorisation request for the use of *Lactobacillus reuteri* NBF-as a feed additive.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active agent in animal feed. The Executive Summary of the EURL report can be found in Annex A.[2]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of *Lactobacillus reuteri* NBF-1 is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on zootechnical additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011, 2012b), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b), Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012c) and Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018).

3. Assessment

The additive is a preparation of viable cells of *L. reuteri* DSM 32203 intended for use as a zootechnical additive (gut flora stabilisers) in feed for dogs to exert beneficial effects in their gastrointestinal tract leading to an increase in faecal consistency.

[1] FEED dossiers reference: FAD-2017-0001.

[2] The full report is available on the EURL website: https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports/fad-2017-0001?search&form-return

3.1. Characterisation

3.1.1. Characterisation of the active substance

L. reuteri DSM 32203 was isolated from dog faeces and is deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) with the accession numbers DSM 32203.[3] The applicant declares that it has not been genetically modified.

Taxonomical identification of the product strain as *L. reuteri* was established by morphological, and biochemical properties (sugar fermentation pattern) and by analysing the partial sequence of the 16S rRNA gene.[4] For strain-specific identification, randomly amplified polymorphic DNA (RAPD) was used.

The bacterial strain was tested for antibiotic susceptibility using broth microdilution techniques.[5] The battery of antibiotics used included those recommended by EFSA (EFSA FEEDAP Panel, 2012c, 2018). The minimum inhibitory concentration (MIC) values for erythromcyin, clindamycin, tetracycline and chloramphenicol were below or equal to the corresponding EFSA cut-off values, while those for the remaining antibiotics were exceeded by one or more dilutions (i.e. ampicillin by 1 dilution (4 vs 2 μg/mL), gentamicin and streptomycin by 2 dilutions (32 vs 8 μg/mL) and kanamycin by 3 dilutions (512 vs 64 μg/mL)). Exceedance of the cut-off values by one dilution is considered to fall within the normal variation around the mean, and thus, does not raise concerns for safety.

To elucidate the nature of the resistance to gentamicin, streptomycin and kanamycin, the applicant performed a whole genome sequence analysis.[6] The calculated genome size is 2.5 Mb. The genome was interrogated for the presence of known genes coding for antimicrobial resistances by comparing with antimicrobial resistance genes deposited in comprehensive antibiotic resistance database (CARD), antibiotic resistance gene annotation database (ARG-ANNOT), antibiotic resistance genes database (ARDB) and MEGARes database. No significant matches were detected for genes coding for aminoglycoside resistance or for other acquired genes coding for resistance to antimicrobial of clinical relevance. Consequently, the resistance is assumed to be caused by mechanisms other than known acquired genes, and therefore has minimal potential for horizontal spread. Consequently, it is not considered to be a hazard.

3.1.2. Characterisation of the additive

The active agent is grown in a sterilised medium, typical of those used for lactic acid bacteria, then separated from the growth medium by centrifugation. Cryoprotectants (maltodextrin and L-cysteine), are added and the cell mix is freeze-dried and ground. The active agent (10%) is then mixed with maltodextrin and corn starch (90%) to meet the minimum specified concentration of 1×10^{11} colony forming units (CFU)/g additive.

Analysis of five batches showed a mean value of 1.6×10^{11} CFU/g (range 1.3–2.1 $\times 10^{11}$ CFU/g).[7]

Microbial contamination is routinely monitored at various points in the manufacturing process and in the final product. Limits are set for yeasts and filamentous fungi (< 10 CFU/g), *Escherichia coli* (absent in 1 g), Enterobacteriaceae (< 10 CFU/g), *Salmonella* spp. (absent in 25 g), *Staphylococcus aureus* (absent in 1 g) and anaerobic sulfite reducers (< 10 CFU/g). Levels of aflatoxin M1 (< 0.5 μg/kg), lead (< 3 ppm), mercury (< 0.1 ppm) and cadmium (< 1 ppm) are measured in raw materials once a year. Compliance with action levels for all the mentioned impurities was confirmed in three production batches.[8]

The additive is a powder whose particle size distribution was determined using laser diffraction, based on three batches.[9] Results showed that (at 2 bars) 30% (v/v) of the additive consist of particles with diameter lower than 50 μm and 7% lower than 10 μm.[10] The dusting potential of the same three

[3] Technical dossier/Section II/Annex II.1 and Supplementary information May 2018/Question 3_Annex NBF-1 – Characterisation of the active agent (3c).
[4] Technical dossiers/Supplementary information May 2018/Question 3_Annexes NBF-1 – Characterisation of the active agent (3).
[5] Technical dossier FAD-2017-0001/Supplementary information May 2018/Question 4_Annexes NBF-1 – Characterisation of the active agent (4).
[6] Technical dossier FAD-2017-0001//Supplementary information May 2018/Question 4 – Annexes 1 (report 2018.TE.2249.1.2) and 2 (raw data).
[7] Technical dossier/Supplementary information May 2018/Question 6_Annex NBF-1 – Characterisation of the additive (6).
[8] Technical dossier/Section II/Annex II.7 and Supplementary information May 2018/Question 7_Annex NBF-1 – Characterisation of the active additive (7).
[9] Technical dossier/Supplementary information May 2018/Question 8_Annex NBF-1 – Characterisation of the active additive (8).
[10] Technical dossier/Section II/Annex II.1.5a.

batches, tested with a Heubach dustometer, showed a mean value of 13.6 g/m^3 for NBF-1,[11] which is considered high.

3.1.3. Stability and homogeneity

The stability of the additive was tested when stored in aluminium packaging at 4°C and 25°C for a period of 14 months and at 40°C for a period of 6 months.[12] No losses were observed at 4°C, while losses of 1 log or greater were observed after 11 months at 25°C and after 1 month at 40°C.

In the same experiment the stability of the additive (three batches) was investigated when mixed with a complementary dog feed (described as consisting of microencapsulated tributyrate and red orange polyphenols) at 9 × 10^{11} CFU/kg feed.[13] Samples were stored at 25°C and 40°C for a period of 6 months. Losses of 1 log were observed after 3 months at 25°C while at 40°C losses of 2 log were observed after 1 month.

To test the capacity of the additive (three batches) to be homogeneously incorporated into a dry dog feed (not described) according to the proposed conditions of use of 6 × 10^9 CFU/head per day (i.e. mixing approximately 0.05 g of additive in 100 g of feed), 10 subsamples were collected and subjected to lactobacilli determination.[14] Counts showed a coefficient of variation of 10%.

3.1.4. Conditions of use

Lactobacillus reuteri NBF-1 is intended for use in complete and complementary feed for dogs (with a moisture content < 14%) at a daily dose of 6 × 10^9 CFU/head per animal, which would approximately equate to a minimum of 8.5 × 10^9 and a maximum of 3 × 10^{10} CFU of *L. reuteri* DSM 32203/kg complete feedingstuffs for dogs.[13,15]

3.2. Safety

3.2.1. Safety for the target species and the environment

Two studies were presented to support the safety of *Lactobacillus reuteri* NBF-1 for dogs, but none could be further considered due to inadequate study design and reporting (e.g. in one case no control was included[16] and in the other the end-points measured were only subjective faecal scores and frequency of illness[17]). Therefore, no conclusions can be drawn from these studies.

The bacterial species *L. reuteri* is considered by EFSA to be potentially suitable for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2017). This approach requires the identity of the strain to be conclusively established and evidence that it does not show acquired resistance to antibiotics of human and veterinary importance. In the view of the FEEDAP Panel, the identity of the active agent is established as *L. reuteri*. *L. reuteri* DSM 32203 showed phenotypic resistance to gentamicin, streptomycin and kanamycin. Interrogation of the whole genome sequence did not evidence the presence of any known gene coding for resistance to antimicrobials of clinical relevance. Consequently, the resistance is assumed to be caused by mechanisms other than known acquired genes, with a minimal potential for horizontal spread. Therefore, the antibiotic resistance qualification has been met and the strains can be presumed safe for the target species and the environment. Since no concerns are expected from the other components of the additives, *Lactobacillus reuteri* NBF-1 is also considered safe for target animals and the environment.

3.2.2. Safety for the user

No data on skin/eye irritation, skin sensitisation or inhalation toxicity have been provided. The dustiness of the preparation tested indicated a potential for users to be exposed via inhalation. A significant fraction of the product (30%) consists of fine particles that have the potential to reach the alveoli when inhaled. Given the proteinaceous nature of the active agent, the additive should be

[11] Technical dossier/Supplementary information May 2018/Question 8_Annexes NBF-1 – Characterisation of the active additive (8a, b and c).
[12] Technical dossier/Supplementary information May 2018/Question 9_Annex NBF-1 – Characterisation of the active additive (9).
[13] Technical dossier/Supplementary information May 2018/Questions 10 and 9.
[14] Technical dossier/Supplementary information May 2018/Question 10_ Annex_ NBF-1 – Characterisation of the active additive (10).
[15] Technical dossier/Supplementary information May 2018/Question 12.
[16] Technical dossier/Section III/Annex III.1.
[17] Technical dossier/Supplementary information May 2018/Question 13_Annex NBF-1 – Efficacy (13) Efficacy and tolerance study (3).

considered a potential respiratory sensitiser. In the absence of data, the FEEDAP Panel cannot conclude on the irritancy of *Lactobacillus reuteri* NBF-1 to skin and eyes or on its dermal sensitisation.

3.3. Efficacy for dogs

The additive is a preparation of viable cells of *L. reuteri* DSM 32203 intended for use as a zootechnical additive (gut flora stabilisers) in feed for dogs to exert beneficial effects in their gastrointestinal tract, leading to an increase in faecal consistency.

Four efficacy studies were performed to support the efficacy of *Lactobacillus reuteri* NBF-1 for dogs. However, none could be further considered due to weaknesses in the experimental design and/or reporting. In the first case, due to its short duration (15 days) and insufficient reporting.[18] In the remaining three cases[19] ,[20] ,[21] the experiments involved dogs kept in individual houses with their owners/caretakers who were trained for evaluating subjectively faecal scores (the only end-point). The studies were described as double-blind experiments, declaring that the experimental foods were the only source of nutrients and energy for dogs. Additionally, the study reports did not include any information, among other, regarding the methodology applied for the randomisation of the treatments, the blinding and its maintenance during the whole experiment, the details regarding food ingestion by dogs and any other source of feed they might have had, the potential deviations from the protocol. Therefore, no conclusions can be drawn from these studies.

3.3.1. Conclusions on efficacy for dogs

The FEEDAP Panel is not in the position to conclude on the efficacy of *Lactobacillus reuteri* NBF-1 for dogs.

3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[22] and Good Manufacturing Practice.

4. Conclusions

The active agent fulfils the requirements of the QPS approach to the assessment of safety. Consequently, in the absence of concerns from other components of the additives, *Lactobacillus reuteri* NBF-1 is presumed safe for the target animals and the environment.

Lactobacillus reuteri NBF-1 should be considered a potential respiratory sensitiser. In the absence of data, the FEEDAP Panel cannot conclude on the irritancy potential of the additive to skin and eyes or on its dermal sensitisation potential.

The FEEDAP the Panel is not in the position to conclude on the efficacy of *Lactobacillus reuteri* NBF-1 for dogs.

Documentation provided to EFSA

1) *Lactobacillus reuteri* NBF-1. January 2017. Submitted by NBF Lanes s.r.l.
2) *Lactobacillus reuteri* NBF-1. Supplementary information. January 2018. Submitted by NBF Lanes s.r.l.
3) *Lactobacillus reuteri* NBF-1. Supplementary information. May 2018. Submitted by NBF Lanes s.r.l.
4) *Lactobacillus reuteri* NBF-1. Supplementary information. July 2018. Submitted by NBF Lanes s.r.l.
5) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for *Lactobacillus reuteri* NBF-1.
6) Comments from Member States.

[18] Technical dossier FAD-2017-0001/Section III/Annex IV.1.
[19] Technical dossier FAD-2017-0001/Supplementary information May 2018/Question 13/Annexes NBF-1 – Efficacy (13)/Efficacy and tolerance study (3) and 5 and Supplementary information July 2018/Efficacy and Annex 1c.
[20] Technical dossier FAD-2017-0001/Supplementary information May 2018/Question 13_Annexes NBF-1 – Efficacy (13)/Efficacy study (2) and 4 and Supplementary information July 2018/Efficacy and Annex 1b.
[21] Technical dossier FAD-2017-0001/Supplementary information May 2018/Question 13/Annexes 3 and Supplementary information July 2018/Efficacy and Annex 1a.
[22] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 May 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

Chronology

Date	Event
3/1/2017	Dossier received by EFSA
19/1/2017	Reception mandate from the European Commission
31/5/2017	Application validated by EFSA – Start of the scientific assessment
4/7/2017	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation, safety and efficacy*
18/9/2017	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
13/2/2018	Clarification teleconference during risk assessment with the applicant according to the "EFSA's Catalogue of support initiatives during the life-cycle of applications for regulated products"
14/5/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
7/6/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended *Issues: efficacy*
13/07/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
24/10/2018	Comments received from Member States
27/11/2018	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011. Technical guidance: tolerance and efficacy studies in target animals. EFSA Journal 2011;9(5):2175, 15 pp. https://doi.org/10.2903/j.efsa.2011.2175

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012a. Guidance for the preparation of dossiers for zootechnical additives. EFSA Journal 2012;10(1):2536, 19 pp. https://doi.org/10.2903/j.efsa.2012.2536

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012b. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. https://doi.org/10.2903/j.efsa.2012.2539

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012c. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal 2012;10(6):2740, 10 pp. https://doi.org/10.2903/j.efsa.2012.2740

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances usedin Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, Lopez-Alonso M, Lopez Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Glandorf B, Herman L, K€arenlampi S, Aguilera J, Anguita M, Brozzi R and Galobart J, 2018. Guidance on the characterisation of microorganismsused as feed additives or as production organisms. EFSA Journal 2018;16(3):5206, 24 pp. https://doi.org/10.2903/j.efsa.2018.5206

Abbreviations

ARDB	antibiotic resistance genes database
ARG-ANNOT	antibiotic resistance gene annotation database
CARD	comprehensive antibiotic resistance database
CFU	colony forming unit
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EURL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
MIC	minimum inhibitory concentration

PFGE	pulsed field gel electrophoresis
QPS	qualified presumption of safety
RAPD	randomly amplified polymorphic DNA

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for *Lactobacillus reuteri* NBF-1

In the current application authorisation is sought under Article 4(1) for *Lactobacillus reuteri NBF-1* (DSM 32203) under the category/functional group 4(b) 'zootechnical additives'/'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for dogs.

According to the Applicant, the feed additive contains as active substance viable cells of the strain *Lactobacillus reuteri NBF-1* (DSM 32203). The *feed additive* is to be marketed as a lyophilised powder containing a minimum *Lactobacillus reuteri NBF-1* (DSM 32203) content of 1,011 Colony Forming Unit (CFU)/g. The *feed additive* is intended to be administered as complementary *feedingstuffs* with a maximum content of 6×10^{10} CFU/kg or to be mixed with usual feed.

For the identification of *Lactobacillus reuteri NBF-1* (DSM 32203) the Applicant applied 16S rRNA gene sequence analysis and random amplified polymorphic DNA analysis. The EURL recommends instead for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains.

For the enumeration of *Lactobacillus reuteri* NBF-1 (DSM 32203) in the feed additive and feedingstuffs, the Applicant submitted the ring-trial validated spread plate method EN 15787. Based on the performance characteristics available, the EURL recommends this method for official control.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.