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Safety and efficacy of a feed additive consisting on *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) CECT 8350 and *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) CECT 8700 (AQ02) for suckling piglets (AQUILON CYL S.L.)

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Abstract

Following a request from the European Commission, the EFSA 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 a feed additive consisting on *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) CECT 8350 and *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) CECT 8700 (AQ02) for suckling piglets. The additive can be presumed safe for the target species, the consumer and the environment. In the absence of data, the Panel could not conclude on the potential of the additive to be a skin or eye irritant or a skin sensitiser. The additive should be considered a respiratory sensitiser. In the absence of adequate data, the Panel cannot conclude on the efficacy of AQ02 as a zootechnical feed additive for suckling piglets.

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Keywords: zootechnical additives, gut flora stabilisers, AQ02, *Lactiplantibacillus plantarum* CECT 8350, *Limosilactobacillus reuteri* CECT 8700, suckling piglets, efficacy

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Table of contents

Abstract.....	1
1. Introduction.....	4
1.1. Background and Terms of Reference.....	4
1.2. Additional information.....	4
2. Data and methodologies.....	4
2.1. Data.....	4
2.2. Methodologies.....	4
3. Assessment.....	5
3.1. Characterisation.....	5
3.1.1. Characterisation of the active agents.....	5
3.1.2. Characterisation of the additive.....	5
3.1.2.1. Manufacturing process.....	5
3.1.2.2. Stability and homogeneity.....	6
3.1.3. Conditions of use.....	6
3.2. Safety.....	6
3.2.1. Safety for user.....	6
3.2.1.1. Conclusions on safety for the user.....	7
3.3. Efficacy.....	7
3.3.1. Conclusions on efficacy.....	8
3.4. Post-market monitoring.....	9
4. Conclusions.....	9
5. Documentation as provided to EFSA/Chronology.....	9
References.....	9
Abbreviations.....	10
Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of the Analysis for <i>Lactobacillus plantarum</i> CECT 8350 and <i>Lactobacillus reuteri</i> CECT 8700.....	11

1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003¹ 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 AQUILON CYL S.L.² for authorisation of the product consisting of *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) CECT 8350 and *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) CECT 8700 (trade name AQ02), when used as a feed additive for suckling piglets (category: zootechnical additives; functional group: gut flora stabilisers).

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). The particulars and documents in support of the application were considered valid by EFSA as of 04 December 2019.

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, consumer, user and the environment and on the efficacy of the product consisting of *L. plantarum* CECT 8350 and *L. reuteri* CECT 8700 (AQ02), when used under the proposed conditions of use (see Section 3.1.3).

1.2. Additional information

The product, consisting of viable cells of *Lactiplantibacillus plantarum* CECT 8350 and *Limosilactobacillus reuteri* CECT 8700, has not been previously assessed, nor 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 dossier³ in support of the authorisation request for the use of the product consisting of *Lactiplantibacillus plantarum* CECT 8350 and *Limosilactobacillus reuteri* CECT 8700 (AQ02) as a feed additive.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA, to deliver the present output.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the AQ02 (*Lactobacillus plantarum* CECT 8350 and *Lactobacillus reuteri* CECT 8700) in animal feed. The Executive Summary of the EURL report can be found in Annex A.⁴

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of the product consisting of *Lactiplantibacillus plantarum* CECT 8350 and *Limosilactobacillus reuteri* CECT 8700 is in line with the principles laid down in Regulation (EC) No 429/2008⁵ and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance on the identity, characterisation and conditions of use of feed

¹ Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

² Facultad de Veterinaria, Campus de Vegazana, 24007, León, Spain.

³ FEED dossier reference: FAD-2019-0048.

⁴ The full report is available on the EURL website: <https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2019-0048-aq02.pdf>

⁵ Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

additives (EFSA FEEDAP Panel, 2017a), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c) and Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018b).

3. Assessment

The product under assessment is a preparation of viable cells of *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) and *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) intended for use as a zootechnical additive (functional group: gut flora stabiliser) in feed for suckling piglets. It will be referred thereof as AQ02, its trade name.

3.1. Characterisation

3.1.1. Characterisation of the active agents

Lactiplantibacillus plantarum and *Limosilactobacillus reuteri* strains were isolated from the gut of a wild boar and from the umbilicus of a newborn piglet, respectively. The strains are deposited in the Colección Española de Cultivos Tipo, with the accession numbers CECT 8350 and CECT 8700, respectively. The strains have not been genetically modified.

The taxonomic identification of the active agents was confirmed using OrthoANI (using the USEARCH algorithm (OrthoANIu)) and digital DNA–DNA hybridisation (dDDH). The reference genomes used in these *in silico* analyses were *L. plantarum* WCFS1 and *L. reuteri* DSM 20016^T. The OrthoANI values were 99.2% for *L. plantarum* CECT 8350 and 96.3% for *L. reuteri* CECT 8700. The dDDH estimation gave the values of 93.9% for the *L. plantarum* CECT 8350 and 71.8% for *L. reuteri* CECT 8700.


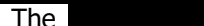

The susceptibility of the bacterial strains to the relevant antibiotics listed in the Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a) was tested with the broth microdilution method.⁶ All the minimum inhibitory concentration (MIC) values determined were equal or fell below the corresponding cut-off values. Therefore, the strains are susceptible to these antimicrobials.

The whole genome sequence (WGS) of the strains was interrogated for the presence of antimicrobial resistance (AMR) genes, using the ABRicate tool (more than 75% identity and default value coverage) against the following databases: Bacterial Antimicrobial Resistance Reference Gene Database (NCBI) AMRfinder Plus, ResFinder, ArgAnnot, CARD. No genes of concern were identified.

The WGS of both active agents was also interrogated for the presence of virulence determinants and results revealed no hits of concern.⁷

3.1.2. Characterisation of the additive

3.1.2.1. Manufacturing process

 The  cultures are  in a 1:1 ratio (*L. plantarum* CECT 8350 and *L. reuteri* CECT 8700) with dextrose to give a final concentration of 2×10^{10} colony forming units (CFU)/g additive.

Analysis of five batches showed average total lactic acid bacteria (LAB) counts of 2.2×10^{10} CFU/g additive (range of 1.89×10^{10} – 2.8×10^{10} CFU/g additive).⁹ No data on individual counts of each active agent were submitted.

The strains have been enumerated after plating in agar for 48h by visually identifying the growth of the colonies.¹⁰ However, the FEEDAP Panel notes that this would not allow to distinguish and count the single strains in the additive.

⁶ Technical Dossier/Section II/Supplementary Information November 2020/Annex1_ErytromycinEvaluation_UAB Report.pdf.

⁷ Technical Dossier/Section II/Supplementary Information November 2020/Question2_FINAL.pdf.

⁸ Technical Dossier/Section II/Supplementary Information November 2020/Question1_FINAL.pdf .

⁹ Technical Dossier/Section II/Annex_II_1_Internal Analysis.pdf.

¹⁰ Technical dossier/Section II/Annex_II_17_Method of Analysis_Colony count.pdf.

Microbiological quality was analysed in five production batches, and the results observed were: < 10 CFU of total coliforms/g, < 10 CFU of yeasts and filamentous fungi/g and absence of *Salmonella* spp. in 25 g.¹¹

The concentration of heavy metals and arsenic measured in three batches of the additive was below the limit of quantification (LOQ): < 0.25 mg cadmium/kg; < 0.010 mg mercury/kg; < 2.5 mg lead/kg; < 1 mg arsenic/kg.¹² Analyses of mycotoxins were not provided.

The additive is a white-yellow powder with a density of 791 kg/m³.¹³ Dusting potential of three batches of the additive (analysed according to DIN EN 481) ranged from 23.8 to 231 mg/g; with this value, the dustiness of the additive is classified as 'high'. Analytical data in the same batches showed that the particles with diameter < 10 µm ranged from 2.2% to 2.4%, those with diameter < 50 µm from 9.8% to 13.4% and those with diameter < 100 µm ranged from 57.8% to 77.0%.¹⁴

3.1.2.2. Stability and homogeneity

The applicant submitted stability studies based on the determination of total viable LAB counts, without further identification of the two strains.¹⁵

The shelf-life of the additive during storage was studied in three batches stored for 12 months at –20°C, 4°C or 30°C. Losses in total LAB counts were negligible on the overall experimental period when the additive was stored at –20°C and at 4°C (one log-unit), whereas considerable after two months at 30°C (4 log-unit losses).¹⁶

The stability of the additive in premixture (guar, gum dextrose and physiologic solution)¹⁷ and in aqueous media (physiological solution)¹⁸ was studied in one batch (10 subsamples) of the additive stored for three months at 4°C in sealed vials after dilution in 0.9% NaCl solution. After 90 days of storage the LAB counts losses were negligible (< 0.5 log-unit).

3.1.3. Conditions of use

The additive is intended to be administered directly to suckling piglets as a unique dose of 1×10^9 CFU after the first colostrum intake, within 24 h after farrowing. The additive should be mixed with feed materials to ensure the correct dosing.

3.2. Safety

The two lactobacilli species are considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2020). This approach requires the identity of the strains to be conclusively established and evidence that they do not harbour acquired antimicrobial genes to clinically relevant antibiotics. In the view of the FEEDAP Panel, the antibiotic resistance qualifications have been met and the identity of the strains established (see Section 3.1.1). Therefore, the strains are presumed safe for the target species, consumers and the environment. Since no concern is expected from the other component of the additive (dextrose), the additive can also be presumed as safe for the target species, the consumer and the environment.

3.2.1. Safety for user

The additive has a high dusting potential, therefore, exposure of users by inhalation is likely. Considering the proteinaceous nature of the active agents, the additive should be considered a respiratory sensitiser.

¹¹ Technical dossier/Section II and Annex_II_1_Internal Analysis.pdf.

¹² Technical dossier/Supplementary Information November 2020/Question 4/Annex1_Metales pesados_octubre 2020.pdf.

¹³ Technical dossier/Supplementary Information November 2020/Question 5.

¹⁴ Technical dossier/Section II/Annex_II_2_Physical state_AENOR.pdf and Supplementary Information November 2020/Question 5 and Question 6/Annex1_Dusting potential_clarifications_octubre 2020.pdf.

¹⁵ Technical Dossier/Section II/Annex_II_16_Final Report_LACYL_lacyl_and Supplementary Information November 2020/Answer to Q7.

¹⁶ Technical Dossier/Section II/Annex_II_14_Final Report Viability.pdf.

¹⁷ Technical Dossier/Section II/Annex_II_15_Stability feedingstuff_Final report.pdf.

¹⁸ Technical Dossier/Section II/Annex_II_18_Stability in aqueous media.pdf.

No studies were provided on the skin/eye irritancy or skin sensitisation potential of the additive.¹⁹ In the absence of data, no conclusions on skin or eye irritancy or on dermal sensitisation can be drawn.²⁰

3.2.1.1. Conclusions on safety for the user

In the absence of data, the Panel could not conclude on the potential of the additive to be a skin or eye irritant or skin sensitiser. Exposure of the users by inhalation is likely and the additive should be considered a respiratory sensitiser.

3.3. Efficacy

The applicant provided three studies conducted with the additive in two Member States, in three different locations. They all involved suckling piglets and shared a similar design.

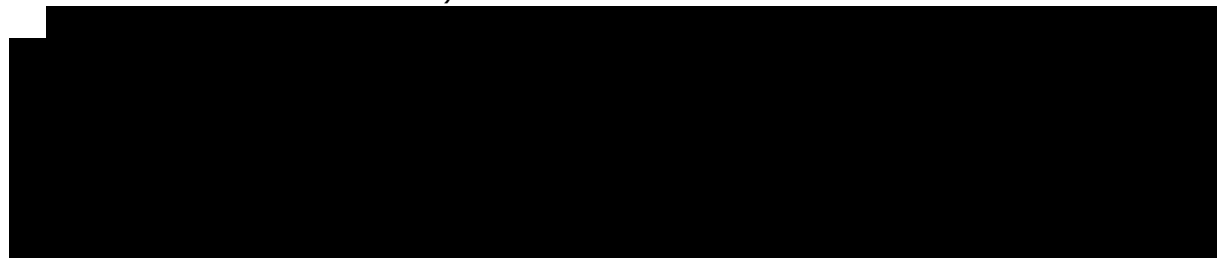
Study 1

A total of 373 piglets born from 30 hybrid sows (PIC maternal line, no information on sex, 1 day at start) in three consecutive batches was used.²¹ The sows were distributed into two homogeneous groups on the basis of parity and litter weight. Cross-fostering of the piglets was performed at the beginning of lactation. Supplemented piglets (184) born from 15 sows (on average parity 3.8) received one dose of 2 ml of complementary feed (dextrose, gums and physiologic solution) containing the additive (1×10^9 CFU/dose, not confirmed by analysis) after their first colostrum intake and the control piglets (189), born from the other 15 sows (on average parity order 3.6), received the same complementary feed without the additive. The trial lasted until weaning (day 28). Body weight was monitored on the day of birth (day 0) and at 14 and 28 days of age. Faeces were collected for microbiota analysis on days 0 and 28. Mortality and presence of diarrhoea were recorded daily for each animal. General health, bursitis, claw lesions, navel infections, unspecific traumatic lesions and welfare status were recorded from day 7 every other day. Behavioural patterns of piglets were assessed through direct observation on days 7, 14 and 21. Data were explored for normality and analysed with general linear model (GLM). Significance was set at $p < 0.05$. The statistical report was not provided.

No differences were observed between treatments at the end of the study in mortality (average 13%), final body weight (6.6 kg) and daily weight gain (198 g/day) of piglets. Frequency of diarrhoea and bursitis were significantly lower in supplemented piglets than in control piglets (diarrhoea 1.42 vs. 1.73 days ($p = 0.027$); bursitis 0.50 vs. 0.68 days occurrences ($p = 0.019$)). The results on the microbiota analysis and behavioural patterns were not considered since they did not provide evidence to support efficacy.

Study 2

Two different experiments were conducted in this study; however, the second experiment was not considered because the additive was not administered according to the conditions of use (additive administered before colostrum intake).²²



¹⁹ Technical Dossier/Supplementary Information November 2020/Answer to Q8.


²⁰ Technical Dossier/Section II/Annex_II_8_MSDS AQ02_Conf.pdf.

²¹ Technical Dossier/Section IV/Annex_IV_15_T13_TEAGASC_Final Report.pdf.

²² Technical Dossier/Section IV/Annex_IV_9_T12_PORTESA_Final Report.pdf; Supplementary Information October 2020/Q10; Sin Dec 2020 Clarifications_13_11_2020_Efficacy_FINAL.pdf.

²³ Technical Dossier/Section IV/Annex_IV_9_T12_PORTESA_Final Report.pdf; Annex_IV_6_ANNEX_C_PORTESA_T12_1.; Raw data: Annex_IV_12_T12_PORTESA_Raw_data.xlsx; Supplementary Information November 2020/RAW DATA T12_BAND1+2.xlsx.


²⁴ Technical Dossier/Supplementary Information November 2020/Clarifications_13_11_2020_Efficacy_FINAL.pdf and RAW DATA T12_BAND1+2.xlsx.



No significant difference was observed in the weight at weaning between the treated and control groups. Mortality data were not reported.

A significant reduction in the incidence of diarrhoea was observed in the treated groups at day 3 with respect to the control (55.6% of the litters of the control groups versus 25% of the treated groups; $p = 0.035$). At day 7, the values were 25.9% in the control and 5% in the treated group: $p = 0.06$).

Study 3



No significant differences were observed for weight loss during lactation and feed intake of sows and in the number of piglets in their litters (at birth and at weaning). No difference between treatments were recorded for mortality (average 7.84 and 5.57% in control and treated, respectively). No significant differences were observed for litter weight or average daily gain. Piglets intake of creep feed was not affected by the treatments. Faecal scores were not statistically different between treatments.

Other studies

The applicant provided a statistical analysis pooling data from different field studies performed in four different locations involving a total of 183 litters.²⁵ However, the Panel did not consider these data adequate to support efficacy given the deficiencies and limitations in the design and the reporting of the different studies (no clear information on how the additive was supplemented, additive not administered according to the conditions of use, farms with health issues or treatment with antibiotics, uncertainties on the test site, mortality above 10%).

Other studies (metagenomics for the piglets' gut microbiota²⁷ and qPCR for the faecal microbiota²⁸) were submitted that did not provide evidence to support the efficacy of the additive.

3.3.1. Conclusions on efficacy

The Panel notes that although some effects on the reduction in the incidence of diarrhoea of piglets treated with the additive was observed in two studies, the biological relevance of these observations is questionable. In addition, both studies showed limitations in the design/reporting which cast doubts on the acceptability of these studies to support the efficacy. In the absence of sufficient evidence, the Panel cannot conclude on the efficacy of AQ02 in suckling piglets.

²⁵ Technical Dossier/Section IV/Annex_IV_3_TR41_IRTA_Final Report.pdf /Supplementary Information November 2020/ Supplementary Information October 2020: Q9.

²⁶ Technical Dossier/Section IV/Annex_IV_23_Meta-analysis_Final report_24082018.pdf.

²⁷ Technical dossier/Section IV/Annex_IV_29_Summary Metagenomics studies.pdf.

²⁸ Technical dossier/Section IV/Annex_IV_34_Final Report_qPCR.pdf.

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 Regulation²⁹ and Good Manufacturing Practice.

4. Conclusions

The additive can be presumed as safe for the target species, the consumer and the environment.

In the absence of data, the Panel cannot conclude on the potential of the additive to be a skin or eye irritant or a skin sensitiser. The additive should be considered a respiratory sensitiser.

In the absence of adequate data, the Panel cannot conclude on the efficacy of AQ02 as a zootechnical feed additive for suckling piglets.

5. Documentation as provided to EFSA/Chronology

Date	Event
02/07/2019	Dossier received by EFSA. Dossier name. Submitted by AQUILON CYL S.L..
24/07/2019	Reception mandate from the European Commission
04/12/2019	Application validated by EFSA – Start of the scientific assessment
14/02/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: Characterisation of the additive: Characterisation of the production strain, purity, manufacturing; User safety</i>
04/03/2020	Comments received from Member States
04/03/2020	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
09/09/2020	Request of clarification to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: characterisation of the additive: characterisation of the production strain, purity, manufacturing; user safety, efficacy</i>
12/11/2020	Request of clarification to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: characterisation of the additive: characterisation of the production strain</i>
13/11/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: Characterisation of the additive: characterisation of the production strain, efficacy</i>
24/11/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
06/05/2021	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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²⁹ Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

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Abbreviations

AMR	antimicrobial resistance
CFU	colony forming unit
CECT	Colección Española de cultivos tipo
dDDH	DNA–DNA hybridisation
EURL	European Union Reference Laboratory
GLM	General Linear Model
LAB	lactic acid bacteria
LOQ	limit of quantification
MIC	minimum inhibitory concentration
QPS	qualified presumption of safety
WGS	whole genome sequence

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of the Analysis for *Lactobacillus plantarum* CECT 8350 and *Lactobacillus reuteri* CECT 8700

In the current application authorisation is sought under Article 4(1) for *Lactobacillus plantarum* CECT 8350 and *Lactobacillus reuteri* CECT 8700 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 suckling piglets. According to the Applicant, the product contains as active substance viable cells of the non-genetically modified strains *Lactobacillus plantarum* CECT 8350 and *Lactobacillus reuteri* CECT 8700. The product is to be marketed as a powder containing a minimum content of total active substances of 2×10^{10} Colony Forming Unit (CFU)/g and is intended to be administered directly in liquid feedingstuffs. For the identification of *Lactobacillus plantarum* CECT 8350 and *Lactobacillus reuteri* CECT 8700, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains. For the enumeration of *Lactobacillus plantarum* CECT 8350 and *Lactobacillus reuteri* CECT 8700 in the feed additive and in feedingstuffs the Applicant submitted an in-house plate counting method, which is neither single-laboratory validated nor further verified by another laboratory. The EURL is aware of a ring-trial validated spread plate method EN 15787 developed by CEN for the analysis of *Lactobacillus* spp. Furthermore, the Applicant confirmed, upon request of the EURL, the applicability of the EN 15787 to its product. Based on the performance characteristics available, the EURL recommends instead the ring-trial validated spread plate CEN method EN 15787 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.