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**Evaluation Report on the Analytical Methods submitted
in connection with the Application for Authorisation of a
Feed Additive according to Regulation (EC) No 1831/2003**

Biomim[®] C5
(FEED-2022-3870; CRL/220000)



**Evaluation Report on the Analytical Methods submitted
in connection with the Application for Authorisation of a
Feed Additive according to Regulation (EC) No 1831/2003**

Dossier related to: **FEED-2022-3870 - CRL/220000**

Name of Product: ***Biomim[®] C5***

Active Agent (s): **Enterococcus faecium DSM 33761,
Pediococcus acidilactici DSM 33758,
Bifidobacterium animalis DSM 16284,
Limosilactobacillus reuteri DSM 33751,
Ligilactobacillus salivarius DSM 16351**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
JRC Geel, Belgium**

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Date: **09/01/2023**

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Date: **09/01/2023**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4 for *Biomini® C5* under the category/functional group 4(b) "zootechnical additives"/"gut flora stabilisers", according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the feed additive for chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding and minor avian species other than laying species.

According to the Applicant, the *feed additive* contains as *active substances* *Enterococcus faecium* DSM 33761, *Pediococcus acidilactici* DSM 33758, *Bifidobacterium animalis* DSM 16284, *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351. The *feed additive* is intended to be marketed in non-coated and coated formulations that have a total contain of viable cells of the microorganisms respectively of 1.0×10^{11} and 4.0×10^{10} Colony Forming Unit (CFU) / g product. The *feed additive* is intended to be used in *complete feed* (coated formulation) or in *water* for drinking (non-coated formulation). The Applicant proposed a minimum dosage of the product corresponding to 1.0×10^8 CFU / kg *complete feed* and 5.0×10^7 CFU / L *water* for drinking.

For the enumeration of *Enterococcus faecium* DSM 33761 in the *feed additive*, *premixtures*, *compound feed* and *water* the Applicant proposed for official control to apply the ring-trial validated method EN 15788. The standard, recently revised by CEN, describes a spread plate (or pour plate) method on bile esculin azide agar or, alternatively, on enterococcus selective medium Slanetz and Bartley agar. For the enumeration of *Pediococcus acidilactici* DSM 33758 in the *feed additive*, *premixtures*, *compound feed* and *water* the Applicant proposed for official control to apply the ring-trial validated method EN 15786. The standard, recently revised by CEN, describes a spread plate (or pour plate) method on MRS (de Man, Rogosa, Sharp) agar. For the enumeration of *Bifidobacterium animalis* DSM 16284 in the *feed additive*, *premixtures*, *compound feed* and *water* the Applicant proposed for official control to apply the ring-trial validated spread plate (or pour plate) method on MRS agar EN 15785. For the enumeration of *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351 in the *feed additive*, *premixtures*, *compound feed* and *water* the Applicant proposed for official control to apply the ring-trial validated method EN 15787. The standard, recently revised by CEN, describes a spread plate (or pour plate) method on MRS agar.

Based on the available performance characteristics, the EURL recommends for official control the ring-trial validated EN 15788, EN 15786, EN 15785 and EN 15787 methods for the enumeration of *Enterococcus faecium* DSM 33761, *Pediococcus acidilactici* DSM 33758, *Bifidobacterium animalis* DSM 16284, *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351, respectively, in the *feed additive*, *premixtures*, *compound feed* and *water*.

For the taxonomic identification of the five bacterial strains contained in the product, the Applicant did not propose any analytical method suitable for official control. However, in former reports for similar dossiers, the EURL recommended for official control DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE). The EURL considers that the above-mentioned methodologies are fit-for-purpose for the bacterial identification of authorised additives at a strain level.

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.

KEYWORDS

BioMin[®] C5, *Enterococcus faecium* DSM 33761, *Pediococcus acidilactici* DSM 33758, *Bifidobacterium animalis* DSM 16284, *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351, zootechnical additives, gut flora stabilisers, chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding and minor avian species other than laying species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for a formulation named *BioMin*[®] C5 containing pure cultures of five bacterial strains under the category/functional group 4(b) "zootechnical additives"/"gut flora stabilisers", according to Annex I of Regulation (EC) No 1831/2003 [1-2]. The authorisation is sought for the use of the *feed additive* for chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding and minor avian species other than laying species [2].

The *feed additive* is intended to be marketed in two different formulations (i.e. non-coated and coated) containing as *active substances*: *Enterococcus faecium* DSM 33761, *Pediococcus acidilactici* DSM 33758, *Bifidobacterium animalis* DSM 16284, *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351 [2]. The non-coated and coated formulations have a total content of viable cells of the microorganisms respectively of 1.0×10^{11} and 4.0×10^{10} Colony Forming Unit (CFU) / g product. Furthermore, the Applicant indicated minimum contents of viable cells for the different bacterial strains [3].

The bacterial strains and the corresponding concentrations are presented in Table 1.

Table 1: Biomim[®] C5 bacterial strains and the corresponding concentrations as presented by the Applicant for non-coated and coated formulations [3].

Biomim [®] C5		non-coated	coated
Strain code	Bacterial strain	concentration min. (CFU/g)	
DSM 33761	Enterococcus faecium	3.25 x 10 ¹⁰	1.3 x 10 ¹⁰
DSM 33758	Pediococcus acidilactici	3.25 x 10 ¹⁰	1.3 x 10 ¹⁰
DSM 16284	Bifidobacterium animalis	3 x 10 ¹⁰	1.2 x 10 ¹⁰
DSM 33751	Limosilactobacillus reuteri	2.5 x 10 ⁹	1 x 10 ⁹
DSM 16351	Ligilactobacillus salivarius	2.5 x 10 ⁹	1 x 10 ⁹

The five bacterial strains contained in the product are all deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) [3].

The *feed additive* is intended to be used in *complete feed* (coated formulation) or in *water* for drinking (non-coated formulation) [4]. The Applicant proposed a minimum dosage of the product corresponding to 1.0 x 10⁸ CFU / kg *complete feed* and 5.0 x 10⁷ CFU / L *water* for drinking [5].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *Biomim[®] C5* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the enumeration of the different bacterial strains (i.e. *Enterococcus faecium* DSM 33761, *Pediococcus acidilactici* DSM 33758, *Bifidobacterium animalis* DSM 16284, *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351) in the *feed additive, premixtures, compound feed* and *water*, the Applicant proposed a series of ring-trial validated dedicated EN methods [6].

For the enumeration of *Enterococcus faecium* DSM 33761 in the *feed additive, premixtures, compound feed* and *water* the Applicant proposed to apply the ring-trial validated method EN 15788 [6,7]. The method has been recently revised by CEN resulting in an updated method dedicated for the enumeration of enterococci spp. in *feed additives, premixtures* and *compound feeds* (excluding mineral feeds). The method is suitable for matrices that contain the enterococci as a single microorganism component or in a mixture with other microorganisms [8]. The levels of *Enterococcus faecium*, as proposed by the Applicant, is similar to the concentration levels of CFU as indicated in the scope of the corresponding EN standard for the various target matrices [3,5,8].

Following the updated method's protocol, the sample (5 to 50 g) is suspended in phosphate buffered saline containing Polysorbate 80 (Tween® 80) (tPBS). For serial dilutions, the peptone salt solution (PSS) is used. The appropriate dilutions are then spread on Petri plates using spread plate (or pour plate) methods on bile esculin azide agar or, alternatively, on enterococcus selective medium according to Slanetz and Bartley (Slanetz and Bartley agar). The agar plates are incubated at 37 °C for 24 hours (bile esculin azide) or 48 hours (Slanetz and Bartley agar) before colony counting. The following performance characteristics, expressed in terms of precision, are reported in the frame of the ring-trial validation studies after logarithmic transformation of the CFU values ranging from 1.38 to 8.87 log₁₀ CFU/g [8]:

- a standard deviation for *repeatability* (S_r) ranging from 0.13 to 1.43 log₁₀ CFU/g and
- a standard deviation for *reproducibility* (S_R) ranging from 0.23 to 2.64 log₁₀ CFU/g

For the enumeration of *Enterococcus faecium* in *water*, the Applicant did not present any study aiming to extend the scope of the EN 15788 [6]. However, in the frame of a former report, the EURL considered the method suitable for the analysis of *enterococci* in *water* [9]. Furthermore, in the frame of the stability tests, the Applicant obtained satisfactory results applying the standard method to the analysis of *Enterococcus faecium* in *water* [10].

Based on the performance characteristics and the available information, the EURL recommends for official control the ring-trial validated EN 15788 method for the enumeration of *Enterococcus faecium* DSM 33761 in the *feed additive, premixtures, compound feed* and *water*.

For the enumeration of *Pediococcus acidilactici* DSM 33758 in the *feed additive, premixtures, compound feed* and *water* the Applicant proposed to apply the ring-trial validated method EN 15786 [6,11]. The method has been recently revised by CEN resulting in an updated method dedicated for the enumeration of pediococci spp. in *feed additives, premixtures* and *compound feeds* (excluding mineral feeds). The method is suitable for matrices that contain the pediococci as a single microorganism component or in a mixture with other microorganisms [12]. The levels of *Pediococcus acidilactici*, as proposed by the Applicant, is

similar to the concentration levels of CFU as indicated in the scope of the corresponding EN standard for the various target matrices [3,5,12].

Following the updated method's protocol, the sample (5 to 50 g) is suspended in phosphate buffered saline containing Polysorbate 80 (Tween® 80) (tPBS). For serial dilutions, the peptone salt solution (PSS) is used. The appropriate dilutions are then mixed on Petri plates using spread plate (or pour plate) methods. Different culture media are indicated. For routine purposes, the analysis can be carried out with MRS (de Man, Rogosa, Sharp) agar. However, since enterococci are expected to be present in similar concentrations as pediococci, acidified MRS agar (AMRSA) could be also used. Furthermore, since the presence of pediococci in combination with lactobacilli is expected, MRS agar could incorporate TTC in order to allow differentiation of colonies. The agar plates are incubated anaerobically at 37 °C for 48 to 72 h. The following performance characteristics, expressed in terms of precision, are reported in the frame of the ring-trial validation studies after logarithmic transformation of the CFU values ranging from 4.98 to 9.65 log₁₀ CFU/g [12]:

- a S_r ranging from 0.01 to 0.17 log₁₀ CFU/g and
- a S_R ranging from 0.07 to 1.72 log₁₀ CFU/g

For the enumeration of *Pediococcus acidilactici* in water, the Applicant did not present any study aiming to extend the scope of the EN 15786 [6]. However, in the frame of the stability tests, the Applicant obtained satisfactory results applying the standard method to the analysis of *Enterococcus faecium* in water [10].

Based on the performance characteristics and the available information, the EURL recommends for official control the ring-trial validated EN 15786 method for the enumeration of *Pediococcus acidilactici* DSM 33758 in the feed additive, premixtures, compound feed and water.

For the enumeration of *Bifidobacterium animalis* DSM 16284 in the feed additive, premixtures, compound feed and water the Applicant proposed to apply the ring-trial validated method EN 15785 [6,13]. The method is suitable for matrices that contain the bifidobacteria as a single microorganism component or in a mixture with other microorganisms. The levels of *Bifidobacterium animalis*, as proposed by the Applicant, is similar to the concentration levels of CFU as indicated in the scope of the corresponding EN standard for the various target matrices [3,5,13].

The sample (20 to 50 g) is suspended in phosphate buffered saline (PBS). For serial dilutions, the PSS is used. The appropriate dilutions are then mixed on Petri plates using spread plate (or pour plate) methods. Different culture media are indicated. For routine purposes, the analysis can be carried out with MRS agar. However, since pediococci and lactobacilli are

expected to be present, it is possible to differentiate the bifidobacteria colonies using MRS agar supplemented with TTC. The agar plates are incubated anaerobically at 37 °C for 36 to 48 h. The following performance characteristics, expressed in terms of precision, are reported in the frame of the ring-trial validation studies after logarithmic transformation of the CFU values ranging from 5.22 to 7.81 log₁₀ CFU/g [13]:

- a S_r ranging from 0.09 to 0.33 log₁₀ CFU/g and
- a S_R ranging from 0.21 to 0.39 log₁₀ CFU/g.

For the enumeration of *Bifidobacterium animalis* in *water*, the Applicant did not present any study aiming to extend the scope of the EN 15785 [6]. However, in the frame of the stability tests, the Applicant obtained satisfactory results applying the standard method to the analysis of *Bifidobacterium animalis* in *water* [10].

Based on the performance characteristics and the available information, the EURL recommends for official control the ring-trial validated EN 15785 method for the enumeration of *Bifidobacterium animalis* DSM 16284 in the *feed additive, premixtures, compound feed and water*.

For the enumeration of *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351 in the *feed additive, premixtures, compound feed and water* the Applicant proposed to apply the ring-trial validated method EN 15787 [6,14]. The method has been recently revised by CEN resulting in an updated method dedicated for the enumeration of lactobacilli spp. in feed additives, premixtures and compound feeds (excluding mineral feeds). The method is suitable for matrices that contain the lactobacilli as a single microorganism component or in a mixture with other microorganisms [15]. The levels of lactobacilli, as proposed by the Applicant, is similar to the concentration levels of CFU as indicated in the scope of the corresponding EN standard for the various target matrices [3,5,15].

Following the updated method's protocol, the sample (5 to 50 g) is suspended in phosphate buffered saline containing tPBS. For serial dilutions, the PSS is used. The appropriate dilutions are then mixed on Petri plates using spread plate (or pour plate) methods. Different culture media are indicated. For routine purposes, the analysis can be carried out with MRS agar. However, since lactobacilli are expected to be present in similar concentrations as enterococci, AMRSA could be used. Furthermore, since the presence of lactobacilli in combination with pediococci is expected, MRS agar could incorporate TTC in order to allow differentiation of colonies. The agar plates are incubated anaerobically at 37 °C for 48 to 72 h [15]. The following performance characteristics, expressed in terms of precision, are reported in the frame of the ring-trial validation studies after logarithmic transformation of the CFU values ranging from 7.40 to 8.03 log₁₀ CFU/g [15]:

- a S_R ranging from 0.10 to 0.26 log₁₀ CFU/g and
- a S_R ranging from 0.18 to 0.39 log₁₀ CFU/g.

For the enumeration of *Limosilactobacillus reuteri* and *Ligilactobacillus salivarius* in water, the Applicant did not present any study aiming to extend the scope of the EN 15787 [6]. However, in the frame of the stability tests, the Applicant obtained satisfactory results applying the standard method to the analysis of *Limosilactobacillus reuteri* and *Ligilactobacillus salivarius* in water [10].

Based on the performance characteristics and the available information, the EURL recommends for official control the ring-trial validated EN 15787 method for the enumeration of *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351 in the feed additive, premixtures, compound feed and water.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

An evaluation of corresponding methods of analysis is not relevant for the present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the taxonomic identification of *Enterococcus faecium* DSM 33761, *Pediococcus acidilactici* DSM 33758, *Bifidobacterium animalis* DSM 16284, *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351, the Applicant did not proposed any analytical method suitable for official control [6].

However, in former reports for similar dossiers, the EURL recommended for official control DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE) [16]. PFGE is a generally recognised methodology for the genetic identification of bacterial strains. The method has been recently ring trial validated and is supposed to become a CEN Technical specification [17].

The EURL considers that the above-mentioned methodologies are fit-for-purpose for the bacterial identification of authorised additives at a strain level.

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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control (i) DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE) for the identification of *Enterococcus faecium* DSM 33761, *Pediococcus acidilactici* DSM 33758, *Bifidobacterium*

animalis DSM 16284, *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351; and (ii) the ring-trial validated spread plate (or pour plate) CEN methods: - EN 15788 for the enumeration of *Enterococcus faecium* DSM 33761 in the *feed additive, premixtures, compound feed and water*; - EN 15786 for the enumeration of *Pediococcus acidilactici* DSM 33758 in the *feed additive, premixtures, compound feed and water*; - EN 15785 for the enumeration of *Bifidobacterium animalis* DSM 16284 in the *feed additive, premixtures, compound feed and water*; - EN 15787 for the enumeration of *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351 in the *feed additive, premixtures, compound feed and water*.

Recommended text for the register entry (analytical method)

For the identification of *Enterococcus faecium* DSM 33761, *Pediococcus acidilactici* DSM 33758, *Bifidobacterium animalis* DSM 16284, *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351:

- DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE)

For the enumeration of *Enterococcus faecium* DSM 33761 in the *feed additive, premixtures, compound feed and water*:

- Spread plate (or pour plate) method on bile esculin azide agar or on Slanetz and Bartley agar (EN 15788)

For the enumeration of *Pediococcus acidilactici* DSM 33758 in the *feed additive, premixtures, compound feed and water*:

- Spread plate (or pour plate) method on MRS agar (EN 15786)

For the enumeration of *Bifidobacterium animalis* DSM 16284 in the *feed additive, premixtures, compound feed and water*:

- Spread plate (or pour plate) method on MRS agar (EN 15785)

For the enumeration of *Limosilactobacillus reuteri* DSM 33751 and *Ligilactobacillus salivarius* DSM 16351 in the *feed additive, premixtures, compound feed and water*:

- Spread plate (or pour plate) method on MRS agar (EN 15787)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Biom[®] C5* have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

- [1] Forwarding of applications for authorisation of feed additives in accordance with Regulation (EC) No 1831/2003 – E-Submission Food Chain platform – <https://webgate.ec.europa.eu/esfc/#/applications/5950>
<https://open.efsa.europa.eu/questions/EFSA-Q-2022-00321>
 - [2] *Technical dossier, Section II: 2_1 and 2_2 Sect_II Identity_Characterisation_MIP
 - [3] *Technical dossier, Section II: 2_1 and 2_2 Sect_II Identity_Characterisation_MIP/2.1.3 Qualitative and quantitative composition
 - [4] *Scientific Summary, Section I: 2.4 Physico-chemical and technological properties of the additive
 - [5] *Technical dossier, Section II: 2.5 Conditions of use of the additive
 - [6] *Technical dossier, Section II: 2.6 Methods of analysis and reference samples
 - [7] EN 15788:2009 – Animal feeding stuffs: Isolation and enumeration of Enterococcus (*E. faecium*) spp.
 - [8] EN 15788:2021 – Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of Enterococcus (*E. faecium*) spp. used as feed additive
 - [9] EURL report: https://joint-research-centre.ec.europa.eu/system/files/2020-12/finrep_fad-2020-0058_bio-three.pdf
 - [10] *Technical dossier, Section II: Annex II_71 Stability of Biomim C5 in water_boxmarked_con
 - [11] EN 15786:2009 – Isolation and enumeration of *Pediococcus* spp.
 - [12] EN 15786:2021 – Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of *Pediococcus* spp. used as feed additive
 - [13] EN 15785:2009 – Animal feeding stuffs - Isolation and enumeration of *Bifidobacterium* spp.
 - [14] EN 15787:2009 – Animal feeding stuffs: Isolation and enumeration of *Lactobacillus* spp.
 - [15] EN 15787:2021 – Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of *Lactobacillus* spp. used as feed additive
 - [16] EURL reports: https://joint-research-centre.ec.europa.eu/eurl-fa-eurl-feed-additives/eurl-fa-authorisation/eurl-fa-evaluation-reports_en
 - [17] CEN project TC 327 WI00327127 (2020): DNA fingerprinting of lactobacilli, pediococci, enterococci and bacilli in animal feeds by pulsed field gel electrophoresis (PFGE) Draft Report of a validation trial
- *Refers to Dossier no: FEED-2022-3870

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, Geel, Belgium. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
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