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Krma: Metode analize - Tipizacija laktobacilov, pediokokov, enterokokov in bacilov z metodo PFGE v krmi

Animal feeding stuffs: Methods of analysis - PFGE typing of Lactobacilli, Pediococci, Enterococci and Bacilli in animal feeds

Futtermittel: Probenahme- und Untersuchungsverfahren - PFGE Typisierung von Laktobazillen, Pediokokken, Enterokokken und Bazillen

Aliments des animaux : Méthodes d'analyse - Typage EGCP des lactobacilles, pédiocoques, entérocoques et bacilles dans les aliments des animaux

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Animal feeding stuffs: Methods of analysis - PFGE typing of Lactobacilli, Pediococci, Enterococci and Bacilli in animal feeds

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European foreword

This document (prEN 17697:2021) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs: Methods of analysis”, the secretariat of which is held by NEN.

This document is currently submitted to the CEN Enquiry.

This document has been prepared under a standardization request given to CEN by the European Commission.

Introduction

DNA fingerprinting by pulsed field gel electrophoresis (PFGE) allows the comparison of large restriction fragments greater than 50 Kb. This technique combined with restriction of the DNA molecule by rare cutting endonucleases (which recognize 6 or 8 base pair sequences) has been successfully applied to strain typing of various lactic acid bacteria including Lactobacilli and Pediococci.

The method described in this document defines the preparation of genomic DNA for Pulsed Field Gel Electrophoresis and further details of the PFGE typing procedure.

1 Scope

This document defines a Pulsed Field Gel Electrophoresis (PFGE) methodology for the identification of authorized probiotic Lactobacillus, Pediococcus, Enterococcus and Bacillus strains. The method can be applied to purified colonies obtained from cultured premixtures and feeds, in order to verify the presence of strains used as feed additives in declared concentrations, even against eventual microbial background resulting from nonsterile matrices.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
bacilli
any of various rodlike spore-producing bacteria constituting the family Bacillaceae

3.2
enterococci
gram-positive, catalase-negative cocci, which usually occurs in pairs or short chains

Note 1 to entry This description is based on their characteristics as used for this document

Note 2 to entry Enterococci classified as aerotolerant anaerobes with the ability to reduce 2,3,5-triphenyl tetrazolium chloride to formazan and capable of hydrolyzing aesculin at 44 °C ± 0,5 °C. They form colonies fitting the description of this species on the specified culture media after incubation at a temperature of 37 °C under aerobic conditions for 24 h resp. 48 h

[SOURCE: EN 15788:2020, 3.1]

3.3
lactobacilli
gram-positive, catalase negative, rod-shaped bacteria in chains

Note 1 to entry This description is based on their characteristics as used for this document

Note 2 to entry Lactobacilli form colonies fitting the description of these species on the specified selective media after incubation of 48 h to 72 h at a temperature of 37 °C under anaerobic conditions (see 9.7)

[SOURCE: EN 15787:2020, 3.1]

3.4
pediococci
gram-positive catalase negative immobile cocci that grow under aerobic as well as under anaerobic conditions

Note 1 to entry: This description is based on their characteristics as used for this document.

Note 2 to entry: Pediococci usually occur in pairs or tetrads, rarely in chains or singly, and divide along two planes of symmetry. They form colonies fitting the description of these species on the specified selective media after incubation of 48 h to 72 h at a temperature of 37 °C under aerobic or anaerobic conditions (see 9.7).

[SOURCE: EN 15786:2020, 3.1]

4 Principle

Single colonies can be selected from the appropriate media used, obtained by using the best available specific enumeration method, as for example described in CEN methods for the enumeration of probiotics Lactobacilli, Pediococci and Enterococci [7, 8, 9,10]. These are cultured overnight in 10 ml liquid cultures at 37 °C. Cells are harvested by centrifugation and included in agar plugs, where they are then submitted to lysis, restriction of chromosomal DNA, and PFGE to separate fragments of DNA so obtained.

Genomic DNA from pediococci or lactobacilli was prepared for PFGE by modifying and adapting weIl known procedures described previously (Tynkkynen et al. 1999; Luchansky et al., 1992, Smith and Cantor, 1987). The methods described by Turabelidze et al. (2002) and Zhang et al. (2016) were adapted for enterococci and bacilli, respectively.

The method has been developed and assessed in the course of the EU research Contract N° SMT4-CT98-2235 (“Methods for the Official Control of Probiotics Used as Feed Additives”).

The method has been further optimized and fully validated during the collaborative study. A summary of the collaborative study can be found in Annex A “Summary of the Validation study”.

5 Reagents

5.1 Tris (hydroxymet yl) aminomethane hydrochloride (Tris-HCl)

5.2 Ethylenediaminetetraacetic acid (EDTA)

5.3 TE-buffer: TE 10 mM Tris-Hcl, 1 mM EDTA, adjust to pH 8,0 and autoclave

5.4 Lysozyme (≥20 000 U/mg)

5.4.1 Lysozyme stock solution

3 00,000 U lysozyme (appr 150 – 200 mg/ml with most commercial lysozyme preparation) per mL TE-buffer (5.3). Distribute in aliquots (100 – 200 µL), store at - 20 °C, thaw at ambient temperature, use immediately after thawing.

5.5 Mutanolysin (≥4000 U/mg)

5.5.1 Mutanolysin stock solution

2 500 U mutanolysin per mL TE-buffer (5.3). Distribute in aliquotes (100 – 200 µL), store at - 20 °C, thaw at ambient temperature, use immediately after thawing.

5.6 Proteinase K (≥2.5 U/mg)

5.6.1 Proteinase K stock solution

50 U Proteinase K per mL TE-buffer (5.3) (50 U/mL). Distribute in aliquotes (100 – 200 µL), store at - 20 °C, thaw at ambient temperature, use immediately after thawing.

5.7 N-Lauroylsarcosine sodium salt (Sarcosyl)

5.7.1 Sarcosyl solution: Sarcosyl 1 % (w/v) in 100mM Tris-HCl, 100 mM EDTA, pH8

5.8 Proteinase K-inhibitor solution

Phenylmethylsulfonyl fluoride (PMSF) 17,5 mg/ml in isopropanol

5.9 10 x TBE

1 M Tris-HCl, 0,9 M Boric acid, 0,01 M EDTA, pH 8,4.

5.10 Low melting point agarose

2 % in 0,5 × TBE buffer (mix and melt in a microwave oven, keep at 45 °C before use)

5.11 Pulsed field certified agarose

1 % in 0,5 × TBE buffer (mix and melt in a microwave oven, keep at 45 °C before use)

5.12 Restriction enzymes (and respective restriction buffers)

5.12.1 SfiI (Lactobacilli)

5.12.2 SmaI (Lactobacilli, Pediococci and Enterococci)

5.12.3 SpeI (Bacilli)

5.13 Molecular weight markers (e.g. 10 – 250 kbp)

5.14 Ethidium bromide

6 Media

6.1 MRS (Lactobacilli, Pediococci)

— Dipotassium hydrogen phosphate 2 g/L

— Magnesium sulphate heptahydrate 0,2 g/L

— Manganous sulphate tetrahydrate 0,05 g/L

— Meat extract 8 g/L