SLOVENSKI STANDARD
SIST EN 17697:2023

01-september-2023

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

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

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

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

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Ta slovenski standard je istoveten z: **CEN/TS 17697:2023**

ICS:

65.120	Krmila	Animal feeding stuffs

SIST EN 17697:2023 **en,fr,de**

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TECHNICAL SPECIFICATION

SPÉCIFICATION TECHNIQUE

TECHNISCHE SPEZIFIKATION

CEN/TS 17697

June 2023

ICS 65.120

English Version

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

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

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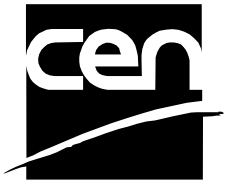

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European foreword

This document (CEN/TS 17697:2023) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs: Methods of sampling and analysis”, the secretariat of which is held by NEN.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

This document has been prepared under a Standardization Request given to CEN by the European Commission and the European Free Trade Association.

Any feedback and questions on this document should be directed to the users’ national standards body. A complete listing of these bodies can be found on the CEN website.

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Introduction

DNA fingerprinting by pulsed field gel electrophoresis (PFGE) allows the comparison of large restriction fragments greater than 50 kbp. 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.

This protocol describes the preparation of genomic DNA for Pulsed Field Gel Electrophoresis and further details of the PFGE typing procedure.

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1 Scope

This document specifies a Pulsed Field Gel Electrophoresis (PFGE) methodology for the identification of authorized probiotic strains of Lactobacillus, Pediococcus, Enterococcus and Bacillus. The method can be applied to purified colonies obtained from cultured premixtures and feeds. The method can be used, even in the presence of a significant microbiological background, to verify the presence of microorganisms (strains and declared concentrations) used as feed additives in animal feeding stuffs.

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 terminology 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

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

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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.

Note 3 to entry: See EN 15788:2021, paragraph 9.6 for the characteristics of the colonies after incubation of 24 h to 48 h at a temperature of 37 °C under aerobic conditions with an appropriate media.

[SOURCE: EN 15788:2021, 3.1, modified – Added Note 3 to entry.]

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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.

Note 3 to entry: See EN 15787:2021, paragraph 9.7 for the characteristics of the colonies after incubation of 48 h to 72 h at a temperature of 37 °C under anaerobic conditions with an appropriate media.

[SOURCE: EN 15787:2021, 3.1, modified – Added Note 3 to entry.]

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.

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Note 3 to entry: See EN 15786:2021, paragraph 9.7 for the characteristics of the colonies after incubation of 48 h to 72 h at a temperature of 37 °C under anaerobic and aerobic conditions with an appropriate media.

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[SOURCE: EN 15786:2021, 3.1, modified – Added Note 3 to entry.]

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4 Principle

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Single colonies are selected after culture on the appropriate media (see the enumeration method described in CEN methods 7, 8, 9, 10). These colonies are cultured overnight in 10 ml of liquid cultures at 37 °C. After incubation, the liquid culture is centrifuged. The bacterial pellet is re-suspended (TE), the cells are lysed and the chromosomal DNA are placed in agar plugs.

The chromosomal DNA is the digested to specific fragments by the action of restriction enzymes and the obtained fragments are separated and visualized on gel after PFGE.

Genomic DNA was prepared for PFGE by modifying and adapting well known procedures described previously for Lactobacilli and Pediococci (1, 2, 3, 5) and for Enterococci and Bacilli (4,6).

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 "Results of the collaborative ring trial".

5 Reagents

5.1 Tris (hydroxymethyl)aminomethane hydrochloride (Tris-HCl)

5.2 Ethylenediaminetetraacetic acid (EDTA)

5.3 Tris-HCl TE 10 mmol Tris-Hcl, 1 mmol EDTA, adjust to pH 8,0 and autoclave

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5.4 Lysozyme (≥20 000 U/mg)

5.4.1 Lysozyme stock solution

3 000000 U lysozyme (approximately 150 mg/ml – 200 mg/ml with most commercial lysozyme preparations) per ml TE-buffer (5.3). Distribute in aliquots (100 µl to 200 µl), store at - 20 °C, thaw at ambient temperature, use immediately after thawing.

5.5 Mutanolysin (≥4 000 U/mg)

5.5.1 Mutanolysin stock solution

2 500 U mutanolysin per ml TE-buffer (5,3). Distribute in aliquotes (100 µl to 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 µl to 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 1g/100 ml in 100 mmol Tris-HCl, 100 mmol EDTA, pH8.

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5.8 Proteinase K- inhibitor solution

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

5.9 10 x TBE

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1 M Tris-HCl, 0,9 M Boric acid, 0,01 mol 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 to 47 °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 to 47 °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 kpb to 250 kbp)

5.14 Ethidium bromide

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6 Media

6.1 MRS (Lactobacilli, Pedicocci)

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

Peptone 10 g/l

Sodium acetate trihydrate 5 g/l

Yeast extract, 4 g/l

Distilled water up to 1 l

pH 6,2 ± 0,2

Dissolve in boiling water, distribute into suitable portions and sterilize by autoclaving at 121 °C for 15 min. Store refrigerated (4 °C to 6 °C) for up to six months

6.2 Tryptic soy broth (Enterococci, Bacilli)

Casein peptone 17 g/l

Dipossaium hydrogen phosphate 2,5 g/l

Glucose 2,5 g/l

Soya peptone 3 g/l

Distilled water up to 1 l

Final pH 7,3 ± 0,2

Dissolve in boiling water, distribute into suitable portions and sterilize by autoclaving at 121 °C for 15 min. Store refrigerated (4 °C to 6 °C) for up to six months.

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