SLOVENSKI STANDARD
SIST EN 15788:2022

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Nadomešča:
SIST EN 15788:2009

Krma: metode vzorčenja in analize - Določanje in štetje prisotnih Enterococcus (E. faecium) spp., uporabljenih kot krmni dodatek

Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of Enterococcus (E. faecium) spp. used as feed additive

Futtermittel: Probenahme- und Untersuchungs-verfahren - Nachweis und Zählung von Enterococcus spp. (E. faecium) als Futtermittelzusatzstoff

Aliments des animaux: Méthodes d'échantillonnage et d'analyse - Détection et dénombrement des souches de Enterococcus (E. faecium) spp. utilisées comme additifs pour l'alimentation animale

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Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of Enterococcus (E. faecium) spp. used as feed additive

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

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European foreword

This document (EN 15788:2021) 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.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 2022, and conflicting national standards shall be withdrawn at the latest by May 2022.

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 supersedes EN 15788:2009.

The main changes compared to the previous edition are as follows:

— Amendment of the title;

— Extension of the scope of application to all *Enterococci* used as feed additive;

— Updating of normative cross references;

— Supplement of phosphate buffered saline with Tween® 80;

— Addition of the option to use Tween® 80 supplemented phosphate buffered saline for the preparation of the initial suspension as well as diluent for serial dilutions;

— Addition of Slanetz and Bartley agar as selective detection medium;

— Replacement of the required laboratory mixer with a rotation speed of 18 000 min^{-1} to 22 000 min^{-1} by homogenization devices, for example according to EN ISO 7218, with a maximal requested rotation speed of 10 000 min^{-1};

— Unification of the homogenization time for the preparation of initial suspensions to five minutes for all feed matrices;

— Addition of the option to use a spiral plater for plating;

— Preparation of initial suspensions generally conducted with tempered tPBS;

— Addition of the pour plate method as an alternative cultivation technique;

— Addition of a procedure for the investigation of feeding stuffs containing high amounts of copper in the informative Annex A;

— Addition of validation data derived from VDLUFA ring trials of different feeding stuff matrices using Slanetz and Bartley agar as enumeration media;

— Adjustment of the range of accepted colony numbers for counting from '≥ 30 to ≤ 350' to '≥ 10 to ≤ 200' colonies per plate.

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.

According to the CEN-CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Republic of North Macedonia, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Introduction

This methodology has been developed to enumerate enterococci (*E. faecium*) as feed additives to enable the European Commission to control proper labelling of animal feeding products. It was compiled first during the EU project SMT4-CT98-2235 “Methods for the official control of probiotics used as feed additives” [1]. It was amended with a second medium from VDLUFA method 28.2.3 “Enumeration of *Enterococcus faecium*” [2]. The method is based on an extensive screening of 12 pre-selected, commercially available media for the detection and enumeration of enterococci. The specified methodology was validated in interlaboratory studies for *E. faecium* ([1], [2], [3]). It can be assumed that the method is also suitable for other *Enterococcus* spp.

This method is not selective for enterococci (*E. faecium*) used as feed additives, but can be applied to enumerate *Enterococcus* spp. in additives, premixtures and compound feeds assuming that the added enterococci (*E. faecium*) are present in far higher numbers than any other enterococci.

This method is not applicable for the detection of any ubiquitous or faecal contaminants of *Enterococcus* spp. in food and animal feeding stuffs.

1 Scope

This document specifies general rules for the enumeration of enterococci (*E. faecium*) in feeding stuffs (additives, premixtures and compound feeds excluding mineral feeds) that contain enterococci as a single microorganism component or in a mixture with other microorganisms. Applying the method to premixtures and compound feeds with critical amounts of copper demands a special procedure (see A.2). The document is not applicable to mineral feeds which are defined as complementary feeding stuffs composed mainly of minerals and containing at least 40 % crude ash (Regulation (EC) 767/2009) [4].

There are different categories of feed samples:

a) Additives containing about 10^{10} colony forming units (CFU)/g;

b) Premixtures containing 10^{11} CFU/kg;

c) Compound feeds, meal or pellets which contain about 10^{9} CFU/kg.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 6498, *Animal feeding stuffs - Guidelines for sample preparation (ISO 6498)*

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
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* are 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. *Enterococci* 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 (see 9.6).

4 Principle

a) Preparation of sterile and dry poured agar plates or preparation of sterile liquid culture media tempered at 44 °C to 47 °C;

b) Drawing a representative test sample under aseptic conditions;

c) Preparation of the initial suspension with a tempered diluent to obtain a homogeneous distribution of bacterial cells from the test portion;

d) Preparation of further decimal dilutions of the initial suspension in order to reduce the number of microorganisms per unit volume to allow, after incubation, the counting of colonies;

e) Inoculation of the prepared poured plates with an aliquot of the optimum dilutions and dispersion of the inoculum using a sterile spreader or inoculation of blank plates with an aliquot of the optimum dilutions and pouring of the molten agar medium into each plate, mixing and solidification;

f) Incubation of inverted plates for 24 h resp. 48 h at 37 °C ± 1 °C under aerobic conditions;

g) Counting of typical colonies, considering the specific properties of enterococci;

h) Morphological verification of isolates though the use of microscopy or biochemical confirmation if necessary;

i) Calculation of the colony forming units of enterococci per gram or kilogram of feed sample.

5 Diluents and culture media

5.1 Diluents

5.1.1 Diluents for initial suspension

The diluent is used for the preparation of the initial suspension and may also be used for the preparation of further decimal dilutions. The composition of the diluent is given in Table 1.

Table 1 — Phosphate buffered saline with Polysorbate 80 (Tween® 80)[1] (tPBS)

Sodium chloride	NaCl	8,00 g
Potassium chloride	KCl	0,20 g
Disodium hydrogen phosphate anhydrous	Na_2HPO_4	1,15 g
Potassium dihydrogen phosphate anhydrous	KH_2PO_4	0,20 g
Polyoxyethylene (20) sorbitan monooleate (Tween® 80)[1]	$C_{64}H_{124}O_{26}$	1 ml
Water, distilled or deionized	H_2O	1 000 ml

Dissolve the components (see Table 1) in water. If necessary, adjust to a final pH of 7,3 ± 0,2 at 25 °C after sterilization. Fill the solution into appropriate containers (e.g. bottles, flasks, or test tubes) and sterilize at 121 °C ± 3 °C for 15 min. To avoid loss during autoclaving, screw cap bottles are recommended.

For immediate use, hold at 40 °C ± 1 °C in a water bath or incubator.

NOTE When using commercially available PBS buffer tablets, please take note that variations in composition and pH can occur between products from different manufacturers and could therefore give results different from the ones obtained with the buffer as specified in this document.

[1] Tween® 80 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.

5.1.2 Diluents for serial dilutions

For serial dilutions, the diluent for initial suspension (5.1.1) or alternatively a peptone salt solution (PSS) according to EN ISO 6887-1 [5] can be used. The composition of PSS is given in Table 2.

Table 2 — Peptone salt solution (PSS) according to EN ISO 6887-1

Enzymatic digest casein		1,0 g
Sodium chloride	NaCl	8,5 g
Water, distilled or deionized	H_2O	1 000 ml

Dissolve the components (see Table 2) in water in flasks or bottles. Adjust the pH if necessary so that, after sterilization, it is 7,0 ± 0,2 at 25 °C. For decimal dilutions, prepare test tubes containing 9,0 ml ± 0,1 ml after sterilization or use screw cap bottles to avoid volume loss during autoclaving.

Sterilize at 121 °C ± 3 °C for 15 min. Bring the diluent to room temperature before use.

NOTE Commercially available, ready-to-use PSS tubes of 9 ml are suitable.

5.2 Culture media

5.2.1 General

Two different culture media are proposed:

a) Bile aesculin azide agar;

b) Enterococcus selective medium according to Slanetz and Bartley (Slanetz and Bartley agar).

NOTE Both media can be used for the spread plate method as well as for the pour plate method.

WARNING — The culture media described contain sodium azide. As this substance is highly toxic and mutagenic, precautions shall be taken to avoid contact with it, especially by inhalation of fine dust during the preparation of commercially available dehydrated complete media.

For uniformity of results, in the preparation of media, either use a dehydrated complete medium or use constituents of uniform quality and chemicals of recognized analytical grade.

5.2.2 Composition

5.2.2.1 Bile aesculin azide agar

The composition of the bile aesculin azide agar is given in Table 3. The resulting pH value at 25 °C is 7,1 ± 0,2.

Table 3 — Composition of the bile aesculin azide agar

Casein peptone (tryptone)	17,0 g
Yeast extract	5,0 g
Peptone	3,0 g
Ox bile, dried	10,0 g
Sodium chloride	5,0 g
Aesculin	1,0 g
Ammonium iron(III) citrate	0,5 g
Sodium azide	0,15 g
Agar	8 g to 18 g[a]
Water, distilled or deionized	1 000 ml
[a] Depending on the gel strength of the agar.	

5.2.2.2 Slanetz and Bartley agar

The composition of the Slanetz and Bartley agar is given in Table 4. The resulting pH value at 25 °C is 7,2 ± 0,2.

Table 4 — Composition of the Slanetz and Bartley agar

Tryptose	20,0 g
Yeast extract	5,0 g
D-(+)-glucose	2,0 g
Di-potassium hydrogen phosphate	4,0 g
Sodium azide	0,4 g
2,3,5-triphenyl tetrazolium chloride (TTC)	0,1 g
Agar	8 g to 18 g[a]
Water, distilled or deionized	1 000 ml
[a] Depending on the gel strength of the agar.	

5.2.3 Preparation

5.2.3.1 Bile aesculin azide agar

Dispense the medium (Table 3) into suitable containers, e.g. bottles or flasks with non-toxic metal screw-caps. Dissolve all components specified in Table 3 in water by boiling. If necessary, adjust to a final pH of 7,1 ± 0,2 at 25 °C. Sterilize at 121 °C ± 3 °C for 15 min.