SLOVENSKI STANDARD
oSIST prEN 15788:2020

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Krma: metode vzorčenja in analize - Izolacija in štetje domnevno prisotnih Enterococcus (E. faecium) spp

Animal feeding stuffs: Methods of sampling and analysis - Isolation and enumeration of Enterococcus (E. faecium) spp.

Futtermittel: Probenahme- und Untersuchungsverfahren - Trennung und Zählung von Enterococcus spp. (E. faecium)

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Isolement et dénombrement de l'entérocoque (E. faecium) spp.

Ta slovenski standard je istoveten z: **prEN 15788**

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Animal feeding stuffs: Methods of sampling and analysis - Isolation and enumeration of Enterococcus (E. faecium) spp.

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Isolement et dénombrement de l'entérocoque (E. faecium) spp.

Futtermittel: Probenahme- und Untersuchungsverfahren - Trennung und Zählung von Enterococcus spp. (E. faecium)

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

This document (prEN 15788:2019) 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 document is currently submitted to the CEN Enquiry.

This document will supersede EN 15788:2009.

Introduction

This methodology has been developed to enumerate enterococci (*E. faecium*) as feed additives to enable the European Commission to control proper label ling of animal feeding products (EU project SMT4-CT98-2235 “Methods for the official control of probiotics (microorganisms) used as animal feeds”) [1]. It was amended by a second medium and validation data from VDLUFA method 28.2.3 “Enumeration of Enterococcus faecium” [7]. The method is based on an extensive screening of 12 pre-selected, commercially available media for the detection and enumeration of enterococci. The described methodology was validated in interlaboratory studies for *Enterococcus faecium*. It may be assumed that the method is also suitable for other *Enterococcus* spp.

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

1 Scope

This document defines general rules for the enumeration of enterococci (E. faecium) in feedingstuffs (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 compound feeds with critical amounts of copper demands a special procedure (see Annex A). 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 R767/2009) [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;

The detection limit is defined in EN ISO 7218.

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)*

EN ISO 7218, *Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations (ISO 7218)*

EN ISO 6887-1, *Microbiology of the food chain - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 1: General rules for the preparation of the initial suspension and decimal dilutions (ISO 6887-1)*

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:

- IEC Electropedia: available at http://www.electropedia.org/
- ISO Online browsing platform: available at http://www.iso.org/obp

3.1
enterococcus faecium
gram-positive, catalase negative coccus, which usually occurs in pairs or short chains

Note1 to entry This description is based on their characteristics as used for this standard

Note 2 to entry *Enterococcus faecium* is classified as aerotolerant anaerobe with the ability to reduce 2,3,5-triphenyl tetrazolium chloride to formazan and capable of hydrolyzing aesculin at 44 °C ± 0,5 °C. It forms colonies fitting the description of this species on the specified selective 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 selective medium tempered at 44 °C to 47 °C.

b) A representative test sample is taken under aseptic conditions.

c) An initial suspension is prepared with a tempered diluent to obtain a homogeneous distribution of enterococci cells from the test portion.

d) The number of microorganisms per unit volume is reduced by the preparation of further decimal dilutions from the initial suspension to obtain a countable number of colonies on the selective enumeration media.

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

f) The inoculated plates are incubated for 24 h resp. 48 h at 37 °C ± 1 °C under aerobic conditions.

g) Counting of typical colonies, considering the specific properties of *Enterococcus faecium* as listed in 3.1.

h) Morphological verification of isolates by use of microscopy or biochemical confirmation if necessary.

i) Calculation of the colony forming units of *Entercoccus faecium* per g or kg of feed sample.

5 Diluents and selective media

5.1 Diluents

5.1.1 Diluent for initial suspension

This diluent is used for the preparation of the initial suspension and may also be used for the preparation of further decimal dilutions.

Table 1 — Phosphate buffered saline supplemented with Tween®[1] 80 (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
Polyoxyethylensorbitanmonooleate (Tween® 80)		1 ml
Water, distilled or deionized		1 000 ml

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

[1] Tween® 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.

Temper to 40 ± 1°C in a water bath or incubator immediately before usage.

NOTE The use of commercially available PBS buffer tablets is acceptable. However, 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 medium as specified in this International Standard.

5.1.2 Diluents for serial dilutions

For serial dilutions, the diluent for initial suspension (5.1.1) or alternatively peptone salt solution (PSS) according to EN ISO 6887-1 can be used.

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

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

Dissolve the components in the water in flasks, bottles or test tubes. 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 weight loss during autoclaving. Sterilize at 121 °C ± 1 °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 Enumeration media

5.2.1 General

Two different media are proposed:

a) Bile Aesculin Azide Agar;

b) Enterococcus selective medium according to Slanetz and Bartley.

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

WARNING — The selective 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 Aesculing Azide Agar

Table 3 — Composition of the agar

Casein peptone (tryptone)		17,0 g
Yeast extract		5,0 g
Peptone		3,0 g
Ox bile, dried		10,0 g
Sodium chloride	NaCl	5,0 g
Aesculin		1,0 g
Ammonium iron – III-citrate	$C_6H_8O_7FeNH_3$	0,5 g
Sodium azide	NaN_3	0,15 g
Agar agar		8 g to 18 g[a]
Water, distilled or deionized		1 000 ml
pH 7,1 ± 0,1 at 25 °C		
a Depending on the gel strength of the agar.		

5.2.2.2 Slanetz and Bartley medium

Table 4 — Composition of the agar

Tryptose		20,0 g
Yeast extract		5,0 g
D-(+)-Glucose		2,0 g
Di-Potassiumhydrogenphosphate	K_2HPO_4	4,0 g
Sodium azide	NaN_3	0,4 g
2,3,5-Triphenyltetrazolium chloride		0,1 g
Agar agar		8 g to 18 g[a]
Water, distilled or deionized		1 000ml
pH 7,2 ± 0,2 at 25 °C		
a Depending on the gel strength of the agar.		

5.2.3 Preparation

5.2.3.1 Bile Aesculin Azide Agar

Dispense the agar medium into suitable containers (bottles or flasks with non-toxic metal screw-caps may be used). Dissolve all components described in 5.2.2.1in water by boiling. If necessary adjust the pH so that after sterilization it is pH 7,1 ± 0,1 at 25 °C. Sterilize at 121 °C ± 1 °C for 15 min.

5.2.3.2 Slanetz and Bartley medium

Dissolve all components described in 5.2.2.2 in water by boiling in a steamer or on a heatable magnetic stirrer. If necessary, adjust the pH so that after heating it is pH 7,2 ± 1 °C at 25 °C. Excessive heating must be avoided. According to the manufacturer's instructions, this medium may not be autoclaved. It should not be remelted.

6 Apparatus and glassware

Usual microbiological laboratory equipment and, in particular, the following:

6.1 Equipment for dry sterilization (oven) and wet sterilization (autoclave)

According to EN ISO 7218.

6.2 Incubator

Capable of maintaining a temperature of 30 °C ± 1 °C. Optionally also capable of maintaining a temperature of 40 ± 1°C and/or 44 °C to 47 °C.

6.3 Water bath

Capable of maintaining a temperature of 44 °C to 47 °C and 40 °C ± 1 °C.

6.4 Blending equipment

The following apparatus may be used (EN ISO 7218):

— a rotary homogenizer (blender) with a notional variable speed of 3 000 rpm to 10 000 rpm, as well as aseptic glass or metals bowls equipped with covers; or

— a peristaltic blender with sterile bags (paddle homogenizer), possibly with the option to adjust blending speed and time; or

— a vibrational mixer with sterile bags; or

— any other homogenizing system with equivalent efficiency (e.g. a hand blender with aseptic beaker).

6.5 Mechanical stirrer

A mechanical stirrer e.g. Vortex Mixer (see EN ISO 7218), or equivalent.

6.6 Balance

Balances of the required range and accuracy according EN ISO 7218 for the different products to be weighed.

6.7 Flasks or screw-cap bottles of appropriate capacities

6.8 Test tubes of appropriate capacities

6.9 Pipettes or Pipettor and sterile tips to dispense 0.1 ml to 1 ml

6.10 Sterile 5 ml graduated pipettes

For full outlet with wide (approx. 3 mm) tips (e.g. serological pipette; alternative: 5 ml-graduated pipettes without tips).