SLOVENSKI STANDARD
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Krma: metode vzorčenja in analize - Izolacija in štetje prisotnih Pediococcus spp

Animal feeding stuffs: Methods of sampling and analysis - Isolation and enumeration of Pediococcus spp.

Futtermittel: Probenahme□ und Untersuchungsverfahren - Trennung und Zählung von Pediococcus spp.

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Isolement et dénombrement des souches de Pediococcus spp.

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Animal feeding stuffs: Methods of sampling and analysis - Isolation and enumeration of Pediococcus spp.

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Isolement et dénombrement des souches de Pediococcus spp.

Futtermittel: Probenahme- und Untersuchungsverfahren - Trennung und Zählung von Pediococcus spp.

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

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European foreword

This document (prEN 15786:2019) 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 15786:2009.

Introduction

This methodology has been developed to enumerate *pediococci* used as feed additives to enable the European Commission to control proper labelling of animal feeding products (EU project SMT4-CT98-2235 – "Methods for the official control of probiotics (microorganisms) used as feed additives") [1]. The described methodology was validated in an interlaboratory study [2]. The method is validated in this project for one strain of *Pediococcus acidilactici.* It may be assumed that the method is suitable also for other *Pediococcus* strains used as feed additives.

1 Scope

This document defines general rules for the enumeration of *pediococci* in feeding stuffs (additives, premixtures and compound feeds excluding mineral feeds) that contain *pediococci* 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) [3].

There are different categories of feed samples:

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

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

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

The detection limit is as 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
pediococci
gram-positive catalase negative immobile cocci that grow under aerobic as well as under anaerobic conditions

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

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 [6] (see 9.7).

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 *Pediococcus* 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 48 h to 72 h at 37 °C ± 1 °C under aerobic or anaerobic conditions;

g) Counting of typical colonies, considering the specific properties of *Pediococcus spp.* as listed in 3.1;

h) Morphological verification of isolates by use of microscopy;

i) Calculation of the colony forming units of *Pediococcus* spp. 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 °C ± 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	1,0 g
Sodium chloride (NaCl)	8,5 g
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

Four different media are proposed:

a) MRS agar;

b) MRS agar supplemented with Triphenyl Tetrazolium Chloride (TTC) ;

c) AMRSA: Acidified MRS agar;

d) Selective media: MRS medium supplemented with cysteine hydrochloride, vancomycin and novobiocin.

For routine enumeration of pediococci the use of MRS agar will be sufficient assuming that the probiotic strain is present in far higher numbers than any other microorganism. The medium is designed to encourage the growth of 'lactic acid bacteria' such as pediococci, enterococci and lactobacilli. Selection can be made by pH adjustment, as pediococci and lactobacilli will tolerate a lower pH than enterococci (pH 5,0 to 6,5). When enterococci are expected to be present in similar concentrations as pediococci, acidified MRS agar (AMRSA) should be used. When pediococci in combination with lactobacilli are expected, MRS agar supplemented with TTC allows differentiation of colonies by different colouration after anaerobic incubationbut can exert a negative influence on the colony amount. The MRS medium supplemented with two antibiotics is selective for pediococci and should be used when the probiotic lactobacilli colony count exceeds the pediococci colony count by a factor of 50 or more.

NOTE An antifungal agent like nystatin (50 U/ml) can be added to MRS agar, AMRSA or MRS+TTC to inhibit moulds and yeasts.

5.2.2 Composition

5.2.2.1 MRS agar

Table 3 — Composition of the MRS agar

Glucose	20 g
Peptone	10 g
Meat extract	8 g
Yeast extract	4 g
Sodium acetate 3 H_2O	5 g
Dipotassium hydrogen phosphate	2 g
Triammonium citrate	2 g
Sorbitan mono-oleate (Tween® 80)	1 ml
Magnesium sulphate 7 H_2O	0,2 g
Manganese sulphate 4 H_2O	0,05 g
Agar agar	10–15 g [a]
Water, distilled or deionized	1 000 ml
pH 6,2 ± 0,2 at 25 °C	
a Depending on the gel strength of the agar.	

NOTE The use of commercially available, ready-to-use media is acceptable. However, please take note that variations in composition and pH can occur between products from different manufacturers and could therefore given results different from the ones obtain with the medium as specified in this International Standard.

5.2.2.2 MRS agar supplemented with TTC (0,01 %)

Sterilize MRS agar (5.2.2.1) by autoclaving at 121 °C ± 1 °C for 15 min. Supplement with 1 ml of a filter sterilized 1 g/100 ml water solution of Triphenyl Tetrazolium Chloride (TTC) per 100 ml MRS agar.

5.2.2.3 AMRSA

Acidified MRS agar can be obtained by adjusting the pH of MRS agar (see 5.2.2.1) to 5,4 ± 0,1 with HCl prior to autoclaving.

5.2.2.4 Selective medium

MRS agar supplemented with 0,05 % cysteine hydrochloride. The medium is supplemented with 10 µg/ml vancomycin and 0,1 µg/ml novobiocin.

5.2.3 Preparation

5.2.3.1 MRS 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 5.2.2.2 in water by boiling. If necessary adjust the pH so that after sterilization it is pH 6,2 ± 0,2 or 5,4 ± 0,1. Sterilize at 121 °C ± 1 °C for 15 min. Excessive heating shall be avoided.

5.2.3.2 MRS agar supplemented with TTC

Prepare 1 g Triphenyl Tetrazolium Chloride (TTC) in 100 ml water and filter sterilize. Add 1 ml per 100 ml MRS agar medium which is tempered at 44 °C to 47 °C after autoclaving.

NOTE TTC is destroyed by autoclaving. Protect the solution from light, and discard it if a pink tinge develops.

5.2.3.3 AMRSA

Prepare the medium as described in 5.2.3.1. Adjust the pH of MRS agar with HCl to 5,4 ± 0,1 prior to autoclaving. Sterilize at 121 °C ± 1 °C for 15 min.

5.2.3.4 Selective medium

Prepare MRS medium with 500 mg/l cysteine hydrochloride as described in 5.2.3.1. Autoclave the medium for 15 min at 121 °C ± 1 °C and temper it at 44 °C - 47 °C in a water bath or incubator.

Prepare 100 mg Vancomycin in 100 ml water and sterilize by sterile filtration. Add 1 ml per 100 ml MRS medium after cooling. This procedure results in a final vancomycin concentration of 10 mg/l.

Prepare a solution of 10 µg/ml of Novobiocin through a series of ten-fold dilutions from an initial 1 mg/ml stock and sterilize by sterile filtration. Add 1 ml Novobiocin per 100 ml MRS medium after cooling. This procedure results in a final Novobiocin concentration of 100 µg/l.

WARNING — Care is needed in preparing the novobiocin stock as levels above 100 µg/l may be inhibitory to pediococci.

Add the selective agents from sterile-filtered stocks to the medium immediately before pouring.

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).