SLOVENSKI STANDARD
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Nadomešča:
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Krma: metode vzorčenja in analize - Določanje in štetje prisotnih Lactobacillus spp., uporabljenih kot krmni dodatek

Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of Lactobacillus spp. used as feed additive

Futtermittel: Probenahme- und Untersuchungs-verfahren - Nachweis und Zählung von Lactobacillus spp. als Futtermittelzusatzstoff

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

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Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of Lactobacillus spp. used as feed additive

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

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European foreword

This document (EN 15787:2021) 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of an identical text or by endorsement, at the latest by May 2022, and conflicting national standards shall be withdrawn at the latest by May 2022.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 15787:2009.

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

— Amendment of the title;

— Extension of the scope of application to all *Lactobacilli* 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;

— Adjustment of the composition of the MRS agar to commercially available formulations;

— Relocation of the use of LAMVAB media from the normative part of the document to the informative Annex A;

— 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;

— 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;

— 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 lactobacilli used 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]. The specified methodology was validated in an interlaboratory study [2]. The method is validated in this project for one strain of *Lactobacillus acidophilus* and one strain of *Lactobacillus rhamnosus*. It can be assumed that the method is suitable also for other *Lactobacillus* strains used as feed additives.

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

This method is not applicable for the detection of ubiquitous contaminants of *Lactobacillus* spp. and any other lactic acid bacteria in food and animal feeding stuffs.

1 Scope

This document specifies general rules for the enumeration of lactobacilli in feeding stuffs (additives, premixtures and compound feeds excluding mineral feeds) that contain lactobacilli 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) No 767/2009) [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.

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
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).

4 Principle

a) Preparation of sterile and dry poured agar plates or preparation of sterile liquid culture medium 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 by 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 48 h to 72 h at 37 °C ± 1 °C under anaerobic conditions;

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

h) Morphological verification of isolates within the *Lactobacillus* genus through the use of microscope analysis if necessary;

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

5 Diluents and culture media

5.1 Diluents

5.1.1 Diluent 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
Polyoxyethylen (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 [4] 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

Three different culture media are proposed:

a) De Man, Rogosa and Sharpe (MRS) agar;

b) MRS agar supplemented with triphenyl tetrazolium chloride (TTC) (MRS+TTC);

c) Acidified MRS agar (AMRSA).

For routine enumeration of lactobacilli the use of MRS agar will be sufficient assuming that the added strain is present in far higher numbers than any other microorganism. The medium is designed to encourage the growth of the lactic acid bacteria such as pediococci, enterococci and lactobacilli. Selection can be made by pH adjustment, as lactobacilli will tolerate a lower pH than enterococci (pH 5,0 to pH 6,5), with pediococci growing best in this range. When enterococci are expected to be present in similar concentrations as lactobacilli, acidified MRS agar (AMRSA) should be used. When lactobacilli in combination with pediococci are expected, MRS agar supplemented with TTC allows differentiation of colonies by different colouration after anaerobic incubation but can exert a negative influence on the colony amount.

NOTE An antifungal agent like nystatin (50 IU/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

The composition of the MRS agar is given in Table 3. The resulting pH value at 25 °C is 6,2 ± 0,2.

Table 3 — Composition of the MRS agar

Glucose	20,0 g
Peptone	10,0 g
Meat extract	8,0 g
Yeast extract	4,0 g
Sodium acetate trihydrate	5,0 g
Dipotassium hydrogen phosphate	2,0 g
Triammonium citrate	2,0 g
Polyoxyethylen (20) sorbitan monooleate (Tween® 80)	1,0 ml
Magnesium sulphate heptahydrate	0,2 g
Manganese sulphate tetrahydrate	0,05 g
Agar	10 g to 15 g[a]
Water, distilled or deionized	1 000 ml
[a] Depending on the gel strength of the agar.	

NOTE When using commercially available, ready-to-use media, 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 obtain with the medium as specified in this document.

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

Sterilize MRS agar (5.2.2.1) by autoclaving at 121 °C ± 3 °C for 15 min. Supplement with 1 ml of a filter sterilized 1 g/100 ml water solution of 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.

5.2.3 Preparation

5.2.3.1 MRS 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 6,2 ± 0,2 after sterilization. Sterilize at 121 °C ± 3 °C for 15 min. Excessive heating during sterilization shall be avoided.

5.2.3.2 MRS agar supplemented with TTC

Prepare 1 g TTC in 100 ml water and filter sterilize. Add 1 ml per 100 ml MRS agar medium (5.2.2.1) which is tempered at 44 °C to 47 °C after autoclaving.

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 specified 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 ± 3 °C for 15 min.