SLOVENSKI STANDARD
SIST EN 15787:2009

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Krma - Izolacija in štetje domnevno prisotnih Lactobacillus spp

Animal feeding stuffs - Isolation and enumeration of Lactobacillus spp

Futtermittel - Keimzählung von Lactobacillus spp

Aliments des animaux - Isolement et dénombrement du Lactobacillus spp

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Animal feeding stuffs - Isolation and enumeration of Lactobacillus spp.

Aliments des animaux - Isolement et dénombrement du Lactobacillus spp.

Futtermittel - Keimzählung von Lactobacillus spp.

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Foreword

This document (EN 15787:2009) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs”, 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of an identical text or by endorsement, at the latest by March 2010, and conflicting national standards shall be withdrawn at the latest by March 2010.

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Introduction

This methodology has been developed to enumerate probiotic lactobacilli 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].

1 Scope

This European Standard defines general rules for the enumeration of probiotic lactobacilli in feed samples (additives, premixtures and feeding stuffs) that contain lactobacilli as a single bacterial component or in a mixture with other microorganisms. This standard is not applicable to mineral feeds, which are defined as complementary feeding stuffs composed mainly of minerals and containing at least 40% crude ash (Council Directive 79/373/EEC [3]).

There are different categories of feed samples:

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

b) Premixtures containing about 10^{8} CFU/g;

c) Feeds, meal or pellets, which contain about 10^{6} CFU/g and include complete feeding stuffs and milk replacers.

The detection limit is as defined in EN ISO 7218.

2 Normative references

The following referenced documents are indispensable for the application 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 6887-1, *Microbiology of food and animal feeding stuffs - 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 6887-1:1999)

EN ISO 7218, *Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations (ISO 7218:2007)*

ISO 6498, *Animal feeding stuffs – Preparation of test samples*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
lactobacilli (described by their characteristics as used for this standard)
lactobacilli are bacteria which form colonies fitting the description of these species on the specified selective medium after incubation of 48 h to 72 h at a temperature of 37 °C under anaerobic conditions [4]:

Morpholoy of colonies:

a) circular;

b) regular or irregular (starry) surrounding;

c) convex or conical;

d) dull or glistening surface;

e) translucent, white, pale green, dark green.

Colony size varies between 0,5 mm and 3 mm in diameter.

Phase contrast microscopic a examination of selected colonies shows that cells are varying from long and slender sometimes bent rods, to short, often coryneform coccobacilli and chain formation is common.

NOTE For a detailed account of morphology see [4].

4 Principle

a) Preparation of sterile and dry poured agar plates.

b) Drawing a representative test sample under sterile conditions.

c) Preparation of the initial suspension 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 plates with an aliquot of the optimum dilutions and dispersion of the inoculum by using a sterile spreader.

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.

i) Calculation of the colony count per gram or kilogram of feed sample.

5 Diluent, selective media and phenotypic characterisation

5.1 Diluents

5.1.1 Diluent for initial suspension of premixtures, additives and feeding stuffs

This diluent is used to decimally dilute the sample to prepare an initial decimally sample suspension (10^{-1}) in appropriate containers (e.g. universals, bottles or flasks).

Phosphate buffered saline (PBS):

Dissolve 8 g sodium chloride, 0,2 g potassium chloride, 1,15 g disodium hydrogen phosphate, 0,2 g potassium dihydrogen phosphate, pH 7,3 ± 0,2 in 1 l of distilled water. Aliquote this saline into appropriate containers (e.g. universals, bottles or flasks). Autoclave all capped containers with the initial diluent at 121 °C ± 1 °C for 10 min. To avoid loss during autoclaving, screw cap bottles are recommended.

Bring the diluent to room temperature before use.

Measure the pH of the diluent to ensure the suitable buffer capacity.

5.1.2 Diluent for serial dilutions

This diluent is used to decimally dilute the initial sample suspension and subsequent dilutions.

Peptone salt solution:

A peptone salt solution is made complying with EN ISO 6887-1.

Compose the solution of enzymatic digest of 1 g casein such as pancreatic peptone of casein (or peptone of same quality) and 8,5 g sodium chloride) per liter (l) distilled water. Dissolve the ingredients in water. Adjust the pH to 7,0 ± 0,2 at 25 °C ± 1 °C. For decimal dilutions, prepare test tubes containing 9,0 ml ± 0,1 ml after sterilisation or use screw cap bottles to avoid weight loss during autoclaving.

Sterilise in the autoclave for 15 min at 121 °C ± 1 °C. Bring the diluent to room temperature before use.

5.2 Media

5.2.1 General

Four different media are proposed:

a) MRS medium;

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

c) AMRSA: Acidified MRS agar;

d) LAMVAB: Lactobacillus Anaerobic MRS with vancomycin and bromocresol green.

For routine enumeration of lactobacilli 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 the 'lactic acid bacteria' such as lactobacilli, enterococci and pediococci. 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 coloration after anaerobic incubation. LAMVAB is a selective medium for lactobacilli.

5.2.2 Composition

5.2.2.1 MRS agar

The composition of the agar per l of distilled water is as follows [5]:

20,0 g dextrose, 10,0 g polypeptone, 10,0 g meat extract, 5,0 g yeast extract, 5,0 g sodium acetate $3xH_20$, 2,0 g sodium phosphate, 2,0 g tri-ammonium citrate, 1,0 g Tween 80, 0,2 g magnesium sulphate $7xH_2O$, 0,05 g manganese sulphate $4xH_2O$, agar 15,0 g, pH 6,2 ± 0,2.

5.2.2.2 MRS agar supplemented with TTC

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