

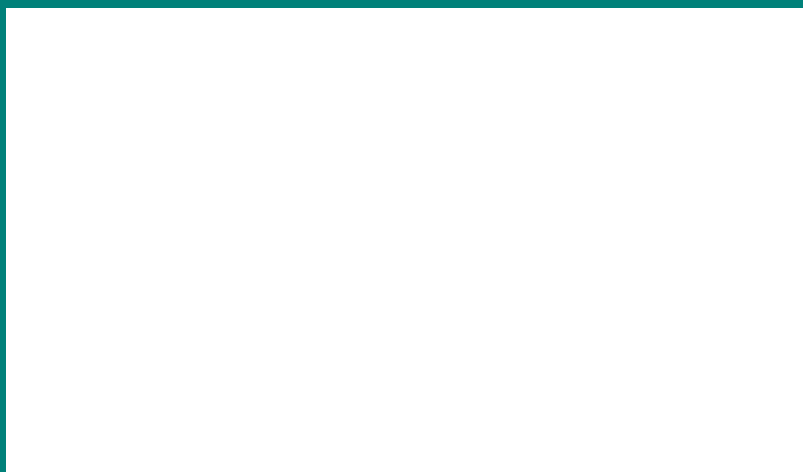
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Djurfoder – Isolering och kvantifiering av *Pediococcus* spp.

**Animal feeding stuffs – Isolation and enumeration of
Pediococcus spp.**



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EUROPEAN STANDARD
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EN 15786

September 2009

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English Version

Animal feeding stuffs - Isolation and enumeration of *Pediococcus* spp.

Aliments des animaux - Isolement et dénombrement du
Pediococcus spp.

Futtermittel - Keimzählung von *Pediococcus* spp.

This European Standard was approved by CEN on 1 August 2009.

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Foreword

This document (EN 15786:2009) 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of an identical text or by endorsement, at the latest by March 2010, and conflicting national standards shall be withdrawn at the latest by March 2010.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 pediococci 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 pediococci in feed samples (additives, premixtures and feeding stuffs) that contain pediococci as a single bacterial component or in a mixture with other microorganisms. This standard is not applicable for 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

pediococci (described by their characteristics as used for this standard)

pediococci are characterised as bacteria which form colonies fitting the description of these species on the specified media after incubation of 24 h to 48 h for MRSA, AMRSA and MRSA+TTC and 24 h for *Pediococcus* selective medium at a temperature of 37 °C under anaerobic conditions [4].

Morphology of colonies on agar plates:

- a) circular;
- b) convex;
- c) entire;

- d) white;
- e) glistening surface;
- f) opaque.

Colony size varies between 1 mm and 3 mm in diameter.

Phase contrast microscopical examination of selected colonies shows that cells are spherical arranged in pairs or tetrads.

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 24 h to 48 h at $37\text{ °C} \pm 1\text{ °C}$, under anaerobic conditions.
- g) Counting of typical colonies, considering the specific properties of pediococci as listed above.
- h) Morphological verification of isolates within the *Pediococcus* genus through the use of microscope analysis.
- i) Calculation of the colony count per g or kg of feed sample.

5 Diluent, selective media and phenotypic characterisation

5.1 Diluents

5.1.1 Diluent for initial suspension

This diluent is used to decimally dilute the sample to prepare an initial decimally diluted 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 $7,3 \pm 0,2$ 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 $121\text{ °C} \pm 1\text{ °C}$ 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 $7,0 \pm 0,2$ at $25 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$. For decimal dilutions, prepare test tubes containing $9,0 \text{ ml} \pm 0,1 \text{ ml}$ after sterilisation or use screw cap bottles to avoid weight loss during autoclaving.

Sterilise in the autoclave for 15 min at $121 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$. Bring the diluent to room temperature before use.

5.2 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, novobiocin and nystatin.

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 incubation. The MRS medium supplemented with three 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.

5.2.2 Composition

5.2.2.1 MRS agar

The composition of the MRS 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 $3\text{xH}_2\text{O}$, 2,0 g sodium phosphate, 2,0 g tri-ammonium citrate, 1,0 g Tween 80, 0,2 g magnesium sulphate $7\text{xH}_2\text{O}$, 0,05 g manganese sulphate $4\text{xH}_2\text{O}$, agar 15,0 g, pH $6,2 \pm 0,2$.

5.2.2.2 MRS agar supplemented with TTC

Sterilise MRS agar (5.2.2.1) by autoclaving at $121 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ 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.