SLOVENSKI STANDARD
SIST EN 15784:2009

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Krma - Izolacija in štetje domnevno prisotnih Bacillus spp

Animal feeding stuffs - Isolation and enumeration of presumptive Bacillus spp

Futtermittel - Keimzählung von vermutlichen Bacillus spp

Aliments des animaux - Isolement et dénombrement des souches probiotiques de Bacillus

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Animal feeding stuffs - Isolation and enumeration of presumptive Bacillus spp.

Aliments des animaux - Isolement et dénombrement des souches probiotiques de Bacillus spp.

Futtermittel - Keimzählung von Bacillus spp.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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EUROPÄISCHES KOMITEE FÜR NORMUNG

Management Centre: Avenue Marnix 17, B-1000 Brussels

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Foreword

This document (EN 15784:2009) 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of an identical text or by endorsement, at the latest by March 2010, and conflicting national standards shall be withdrawn at the latest by March 2010.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 and differentiate probiotic bacilli spores capable of germinating, 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 in animals feeds“) [1]. Vegetative cells are not taken into account in this method, as all approved *Bacillus* species products at present are spores.

Spores of *Bacillus* species survive a heat-treatment at 80 °C for 10 minutes and the *Bacillus* species characteristic colony morphology of the individually authorised strains is examined using the proposed method [2].

This method is not selective for probiotic bacilli but can be applied to enumerate bacilli in additives, premixtures and feeding stuffs assuming that the probiotic bacilli are present in far higher numbers than any other bacilli.

If the feeding stuffs are “contaminated” with a high level of non-probiotic *Bacillus* species it can be recommended to use a procedure based on antibiotics for more specific selective counting, taking the antibiotic resistance profile of the different *Bacillus* strains into account.

1 Scope

This European Standard defines general rules for the enumeration of probiotic bacilli in feeds containing bacilli (*Bacillus* species) as a single microorganism, component or mixed with other microorganisms. This method 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 limits are 500 (5×10^2) colony forming units per gram (CFU/g). The limits of determination are 2×10^4 CFU/g.

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
bacilli (described by their characteristics as used for this standard)
bacteria of the genus *Bacillus* which form colonies fitting the descriptions of these species, on the surface of Tryptone Soy Agar (TSA) after heat treatment and incubation at 37°C for 16 h to 24 h under aerobic conditions

Morphology of colonies on TSA of four *Bacillus* species:

a) *Bacillus subtilis*: 3 mm to 8 mm in diameter, round, surface dull, opaque, wrinkled and cream or brown coloured;

b) *Bacillus cereus* and *Bacillus coagulans*: 3 mm to 8 mm in diameter, dull or of frosted glass appearance and undulate shaped;

c) *Bacillus licheniformis*: 4 mm to 8 mm diameter, convex, from matt, chondroid, to very mucous, lobbulated, shape surrounded by smaller sub-colonies. The central mucous part of a colony may dry up and become flat, white and opaque while the colonies are still surrounded by small, mucous sub-colonies. Some parts of a colony will adhere more strongly to the substrate than others.

3.2
colony count of presumptive probiotic *Bacillus* species
number of colony-forming units (CFU) which is counted and calculated according to the procedure outlined in this standard

4 Principle

An initial suspension of the sample is prepared in a diluent using a suitable homogeniser. From this one new dilution is prepared and heat-treated at 80 °C for 10 min. Decimal dilutions are prepared from the heat treated sample and are spread plated on TSA agar and incubated at 37 °C for 16 h to 24 h aerobically. Colonies of *Bacillus* species are counted and the number of colony forming units per g or kg is calculated.

5 Diluents and selective medium

5.1 Diluents

5.1.1 Diluent for initial suspension

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

5.1.1.1 Initial diluent for additives

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.

5.1.1.2 Initial diluent for feeding stuffs and premixes

0,2% sodium hydroxide solution:

Dissolve 2 g sodium hydroxide in one litre of distilled water, Dispense aliquots of this solution appropriate to the initial dilution of feed pellets into capped flasks. Autoclave all capped flasks containing the diluent at 121 °C ± 1 °C for 15 min.

Bring the diluent to room temperature before use.

5.1.2 Diluent for serial dilutions, polysorbate peptone salt solution

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 Medium

Use Tryptone Soy Agar[1)] as a culture medium.

Composition in g/l:

a) tryptone 15,0 g

b) sodium chloride 5,0 g

c) soya peptone 5,0 g

d) agar 15,0 g

e) water 1 000 ml

Final pH 7,3 ± 0,2

6 Apparatus and glassware

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

6.1 Equipment for autoclaving

According to EN ISO 7218.

6.2 Incubator

Incubator capable of maintaining an incubation temperature 37 °C ± 1 °C.

6.3 Water bath

Water bath capable of keeping a temperature of 48 °C ± 1 °C and 80 °C ± 1 °C.

6.4 Blending equipment

A two-speed or a variable adjustable blender (18 000 rotations per minute (rpm) and 22 000 rpm), with a one litre bowl that has been sterilised in an oven for 1 h at 170 °C to 180 °C.

6.5 Mechanical stirrer

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

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1) The medium is commercially ready made available from various suppliers