BRITISH STANDARD

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

ICS 65.120

BSI British Standards

National foreword

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A list of organizations represented on this committee can be obtained on request to its secretary.

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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of an identical text or by endorsement, at the latest by March 2010, and conflicting national standards shall be withdrawn at the latest by March 2010.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

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

6.6 Balance

A balance capable of weighing to two decimal places.

6.7 Screw-cap bottles

6.8 Pipettor and tips

Total-delivery graduated pipettes or automatic pipettes and sterile tips to dispense 100 µl and 1 ml. Wide bore tips to pipette homogenised feed stuff for dilution.

6.9 Spreaders

Sterile L- or triangular-shaped spreaders from glass or metal or sterile disposable plastic spreaders.

6.10 Sterile Petri dishes, triple vent, 90 mm in diameter

6.11 Laminar flow cabinet

6.12 Microscope for use with 100x oil immersion lens

6.13 pH meter

7 Sampling

Carry out the sampling procedure in accordance with the specific standard appropriate to the product concerned. If such a specific standard is not available, it is recommended that agreement be reached on this subject among the parties concerned. Apply community rules [1] for official control sampling of animal feeds.

NOTE Sampling can be done according to ISO 6497 [4]. Although ISO 6497 is not applicable for microorganisms, due to the lack of other reference, it seems it is the most suitable protocol to be taken into account

WARNING — Take precautions to avoid potential cross-contamination of samples with bacilli. Particularly after sampling additives and premixtures supplemented with bacilli. If needed, clean and disinfect sampling equipment between each sample, particularly after sampling additives and premixtures containing bacilli. Put the sample in a sterile container.

8 Preparation of test sample

The test sample preparation shall be done in accordance with ISO 6498 and the congruent product standard. If such a specific standard is not available, it is recommended that agreement be reached on this subject among the parties concerned. ISO 6498 gives general guidelines on test sample preparation.

9 Procedure

9.1 Preparation of poured agar plates

Prepare the medium according to the manufacturer's directions. Autoclave the medium for 15 min at 121 °C ± 1°C and then cool in a water bath to a temperature of 48 °C ± 1 °C, subsequently pour portions of 12 ml to 15 ml medium into each Petri dish (6.10) under sterile conditions and spread to give a homogeneous layer.

When the medium has solidified, pile quantities of four plates reversed on each other and dry at room temperature or in an incubator at 37°C ± 1°C for approximately 12 h or over night. Alternatively spread the plates out in a lamina flow cabinet and dry the agar surface with the lids partially removed for about 30 min.

Check the dried plates for sterility.

WARNING — If the surface of the agar plates is not dry or the portions of media are more than 15 ml per Petri dish, the *Bacillus* species strains will very often make swarming colonies on the entire plate and the detection of viable cell count will not be possible.

Dried plates, if correctly protected from dehydration, may be stored for two weeks in a fridge. In this case bring the plates to room temperature about 30 min before use.

9.2 Preparation of the initial suspension and decimal dilutions

9.2.1 Additives

Weigh 20 g ± 0,1 g of plain additive. Add 180 g ± 0,1 g of diluent phosphate buffered saline (5.1) to plain additives. Homogenise the mixture with a suitable blender (6.4). Start blending at a low speed to avoid splashing and turn to the high speed for 2 min at high speed and dilute immediately.

NOTE Intense foaming in initial suspensions during blending may be avoided by using a feasible antifoaming agent (e.g. silicon antifoaming agent) in adequate concentrations.

WARNING — Probiotics tend to settle with sample particles quickly therefore sub-samples have to be taken immediately after mixing when the suspension is still homogeneous

Transfer 1,0 ml of the initial suspension into a tube with 9,0 ml of sterile diluent 'Polysorbate 80' peptone salt solution (10^{-2}) (5.1.2). Mix the contents using a whirl mixer at maximum speed 3 times for 1 s. Heat treat the tube in a water bath at 80 °C ± 1 °C for 10 min. Cool the tubes in cold water (of approximately 10 °C), and dilute when the tubes have reached room temperature.

The weight of the withdrawn pipette volume has to be calibrated to receive a correction factor for the calculation of the counts of *Bacillus* species

9.2.2 Feeding stuffs and premixtures

Weigh 50 ± 0,5 g of sample. Add 450 g ± 1 g of 0,2% sodium hydroxide diluent (5.2) to feeding stuffs or premixtures. Homogenise the mixture with a suitable blender (6.4). Start blending at low speed to avoid splashing and then turn to the high speed (see 6.4). Blend feeding stuffs and premixes for 1 min at high speed.

NOTE Intense foaming in initial suspensions during blending may be avoided by using a feasible antifoaming agent (e.g. silicon antifoaming agent) in adequate concentrations.

Place the sample in a cool place of approx. + 5 °C for 30 min. Blend again for 1 min at high speed. Dilute immediately using wide bore pipettes tips.

WARNING — Probiotics tend to settle with sample particles quickly therefore sub-samples have to be taken immediately after mixing when the suspension is still homogeneous.

Transfer 1,0 ml of the initial suspension using wide bore pipettes tips into a tube with 9,0 ml of sterile diluent 'Polysorbate 80' peptone salt solution (10^{-2}) (5.1.2). Mix the solution by using a whirl mixer at maximum speed for 3 x 1 s. Heat treat the tube in a water bath at 80°C ± 1°C for 10 min. Cool the tubes in cold water (about 10°C) and dilute when the tubes have reached room temperature.

Prepare (from the heat treated diluent 10^{-2}) series of decimal dilutions by pipetting 1 ml from the heat treated diluent 10^{-2} mixture into the 9 ml sterile diluent 'Polysorbate 80' peptone salt solution (5.1.2) and mix with a mechanical stirrer (6.5). Repeat this procedure using the 10^{-3} dilution and all further dilutions until the

appropriate estimate for the number of cells is obtained. After this diluent procedure, inoculate the plates immediately as described in 9.3.

The time elapsing between the end of the preparation of the initial suspension and the moment when medium is inoculated shall not exceed more than 30 min. Prepare a poured and spread control plate to check sterility.

The weight of the withdrawn pipette volume has to be calibrated to receive a correction factor for the calculation of the counts of *Bacillus* species.

9.3 Inoculation and incubation of the plates

Use from each of the chosen dilutions 0,1 ml to inoculate two separate agar plates. Spread it uniformly as quickly as possible on the surface of the medium using a sterile spreader.

WARNING — Selected appropriate dilutions must be homogeneous prior to transferring a subsample onto plates.

Reverse four plates, pile them on top of each other and incubate them at 37 °C ± 1 °C for 16 - 24 h of incubation under aerobic conditions.

9.4 Counting of colonies

After the incubation under above specified conditions, all plates containing more than 30 and not more than 300 presumptive bacilli (*Bacillus* species) colonies are used for the enumeration of colony-forming units (CFU). If possible, count 4 dishes (two successive dilutions). If the mean of colonies on the plates of a particular dilution is below 20, the test should be repeated. If a result is achieved with less than 20 colonies, the counts are taken into the calculation of the weighed mean. In the test report (12) this result should be provided with the following remark: This result is below the limit of detection.

Bacteria which form on the specified media typical colonies with morphology as described in 3.1, are detected and counted as *Bacillus* species. The number of colony forming units per gram of feed is calculated according to the procedure described in section 10.

10 Expression of results

The number of colony-forming units per gram of feed is calculated according to EN ISO 7218.

The number of bacilli per gram or ml is equal to:

$$N = \frac{\sum C}{V\left(n_1 + 0{,}1\, n_2\right) d} \qquad (1)$$

$\sum C$ is the sum of colonies counted on all the dishes from one or two successive dilutions, with at least one which contains 15 colonies;

V is the volume of inoculum applied to each dish, in ml (usually 0,1 ml);

n_1 is the number of dishes retained at the first countable dilution;

n_2 is the number of dishes retained at the second countable dilution;

d is the dilution factor corresponding to the first dilution.

Round off the result obtained to two significant figures. For a three-figure number, round off the third to the nearest 0. If the third is 5, round off to the figure below if the first two figures are even numbers, and to the figure above if the first two figures are odd numbers.

The result is the number of microorganisms per gram product, expressed as a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

11 Precision

11.1 General

The precision of the method using different media in terms of repeatability and reproducibility was determined in an interlaboratory study [2].

11.2 Interlaboratory study

Details of the interlaboratory study on the precision of the method are published (see [1]) and are summarized in Annex B. Repeatability and reproducibility limits were determined using three types of feed samples contaminated at two levels of contamination. The values derived from the interlaboratory study may not be applicable to concentration ranges and matrices other than those given.

11.3 Repeatability

The absolute difference between two independent single ($\log_{10}$-transformed) test results (number of Bacilli per g or per ml) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in the same laboratory by the same operator using the same apparatus within the shortest feasible time interval, will in not more than 5% of the cases exceed the repeatability limit *r*.

11.4 Reproducibility

The absolute difference between two single ($\log_{10}$-transformed) test results (number of Bacilli per g or per ml) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5% of cases exceed the reproducibility limit *R*.

12 Test report

The test report shall specify:

a) information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this European Standard;

d) the temperature of incubation;

e) operating details not specified in this European Standard, or regarded as optional, together with

f) details of any incidents which may have influenced the test result(s);

g) the test result(s) obtained, or, if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Notes on procedure

Most vegetative cells from microorganisms will be inactivated by the heat treatment used in this method.

In the case where feeding stuff is “contaminated” with a high level of non-probiotic *Bacillus* species. It can be recommended to add appropriate antibiotics to the agar that are able to suppress background flora.

Annex B
(informative)

Results of the interlaboratory study

A interlaboratory study involving 17 laboratories in 12 European countries was carried out on three different sample types which included bacilli at two levels and a third sample represented a blank. The low level sample represented a feed sample and contained bacilli only. The higher concentrated sample represented a premixture containing bacilli and as background the flora pediococci and yeast were added. The blank sample contained lactobacilli, pediococci, enterococci and yeast however no bacilli spores. The results of the blank evaluation are not presented in Table 1 and can be found in the cited publications.

The test was organized in 2002 and coordinated by the Central Science Laboratory (UK) [1], [2]

Table B.1 — Precision data obtained from the collaborative study [2].

Parameters	**sample (contamination level)**	
	low level 10^5 CFU/g	**high level 10^9 CFU/g**
Number of samples	2	2
Number of laboratories retained after eliminating outliers	11	11
Mean value, in $\log_{10}$ cfu/g	5,95	9,53
Repeatability standard deviation, s_r, in $\log_{10}$ CFU/g	0,07	0,09
Coefficient of variation of Repeatability, CV_r, in %	1,13	0,99
Repeatability limit r (=2,8 x s_r)	0,19	0,26
Reproducibility standard deviation, s_R, in $\log_{10}$ CFU /g	0,35	0,32
Coefficient of variation of reproducibility, CV_R, in %	5,80	3,40
Reproducibility limit R (=2,8 x s_R)	0,97	0,91

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[1] European Community Project SMT4-CT98-2235. 'Methods for the official control of probiotics used as feed additives (vol. 1-3). 2002. Report EUR 20873/1-3. Office for Official Publications of the European Communities. ISBN 92-894-6249-3 (set)'

[2] Leuschner R.G.K., J. Bew, A. Cruc. 2003. Enumeration of probiotic bacilli spores in animal feed: A collaborative study. J. AOAC 86, 568-575

[3] Council Directive (79/373/EEC) of 2 April 1979 on the marketing of compound feeding stuffs (OJ No L 86, 6.4.1979, p.30)

[4] ISO 6497, *Animal feeding stuffs – Sampling*.

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