BRITISH STANDARD

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Animal feeding stuffs — Isolation and enumeration of Bifidobacterium 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 15785:2009) 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of an identical text or by endorsement, at the latest by March 2010, and conflicting national standards shall be withdrawn at the latest by March 2010.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 bifidobacteria, to enable the European Commission to control proper labelling of animal feed stuffs (EU project SMT4-CT98-2235 - "Methods for the official control of probiotics (microorganisms) used in animals feeds") [1]. The proposed enumeration method for probiotic bifidobacteris was validated in an interlaboratory study [2].

The method is not selective for probiotic bifidobacteria but can be applied to enumerate them in additives, premixtures and feeding stuffs assuming that they are present in higher numbers than any other bacteria.

1 Scope

This European Standard defines general rules for the enumeration of probiotic bifidobacteria in feed samples (additives, premixtures and feeding stuffs) that contain bifidobacteria 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
bifidobacteria (described by their characteristics as used for this standard)
bacteria which form colonies fitting the descriptions of the species, on the specified media of 36 h to 48 h at a temperature of 37°C under incubation; anaerobic conditions)

Morphology of colonies:

a) circular;

b) convex;

c) entire;

d) cream;

e) shiny surface;

f) opaque.

Colony size varies between 0,5 mm and 1 mm in diameter.

Phase contrast microscopical examination of selected colonies shows that cells are of various rod shapes.

4 Principle

a) Preparation of sterile and dry poured agar plates.

b) Drawing of 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 36 h to 48 h at 37 ºC ± 1 ºC, under anaerobic conditions.

g) Counting of typical colonies, considering the specific properties of bifidobacteria as listed above.

h) Morphological verification of isolates within the *Bifidobacterium* genus using microscope analysis.

i) Calculation of the colony count per g or kg of feed sample.

5 Diluent, 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 sample suspension of 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 is dissolved in 1 l of distilled water. Aliquote this saline is aliquoted into appropriate containers (e.g. universals, bottles or flasks). Autoclave all capped containers with the initial diluent are autoclaved 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.

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 agar;

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

c) AMRSA: Acidified MRS agar;

d) Selective medium: MRS medium supplemented with cysteine hydrochloride and mupirocin.

For routine enumeration of bifidobacteria 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, lactobacilli and bifidobacteria will tolerate a lower pH than enterococci (pH 5.0 to pH 6,5) and will grow on acidified MRS agar. When pediococci and lactobacilli are expected, MRS agar supplemented with TTC allows differentiation of bifidobacteria colonies by different translucent brown-red coloration after anaerobic incubation. The MRS medium supplemented with mupirocin is selective for bifidobacteria and should be used when other probiotic lactic acid bacteria are present in higher numbers than bifidobacteria.

5.2.2 Composition

5.2.2.1 MRS agar

The composition of the MRS agar per l of distilled water is as follows:

20,0 g dextrose, 10,0 g polypeptone, 10,0 g meat extract, 5,0 g yeast extract, 5,0 g sodium acetate 3 x H_20, 2,0 g sodium phosphate, 2,0 g tri-ammonium citrate, 1,0 g Tween 80, 0,2 g magnesium sulphate 7 x H_2O, 0,05 g manganese sulphate 4 x H_2O, agar 15,0 g, pH 6,5 ± 0,2.

NOTE Bacto™ Lactobacilli MRS agar from Difco Laboratories or from any other supplier producing a medium of same composition may be used.

5.2.2.2 MRS agar supplemented with TTC

Prepare 1 g Triphenyl Tetrazolium Chloride (TTC) in 100 ml water and filter sterilise. Add 1 ml per 100 ml MRS agar medium (see 5.2.3.1) which is temperated at 48 °C ± 1 °C after autoclaving.

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.

5.2.2.4 Selective medium

MRS agar (see 5.2.2.1) supplemented with 0,05 % cysteine hydrochloride (e.g. Sigma C-1276, or equivalent) and with a final concentration of 50 µg mupirocin (e.g. Oxoid CT0523B, or equivalent) per ml.

5.2.3 Preparation

5.2.3.1 MRS agar

Suspend all ingredients in distilled water and sterilise by autoclaving at 121 ºC ± 1 °C for 15 min.

5.2.3.2 MRS agar supplemented with TTC

Prepare 1 g Triphenyl Tetrazolium Chloride (TTC) in 100 ml water and filter sterilise. Add 1 ml per 100 ml MRS agar medium (see 5.2.3.1) which is temperated at 48 °C ± 1 °C after autoclaving.

NOTE TTC is destroyed by autoclaving.

5.2.3.3 AMRSA

Adjust the pH of MRS agar with HCl to 5,4 ± 0,1 prior to autoclaving. Sterilise at 121°C ± 1°C for 15 min.

5.2.3.4 Selective medium

Selective medium (see 5.2.2.4) supplemented with 0,05% (wt/vol) cysteine hydrochloride (e.g. Sigma C-1276, or equivalent).

In order to prepare a mupirocin solution add 50 disks (e.g. Oxoid CT0523B or equivalent with 200 µg per disk) to 10 ml of 37 °C warm sterile distilled water and shake for 30 min. Decant the solution from disks and add to 190 ml molten medium held at about 48 °C prior to plate pouring and drying. Final mupirocin concentration shall be 50 µg/ml.

5.3 Phenotypic characterisation

Characteristic appearance of the bifidobacteria under phase contrast microscopy is rod shaped. Cell morphology can be used to distinguish bifidobacteria from lactobacilli, enterococci, pediococci and bacilli.

6 Apparatus and glassware

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

6.1 Equipment for dry sterilisation (oven) and wet sterilisation (autoclave)

According to EN ISO 7218.

6.2 Incubator

Capable of maintaining a temperature of 37 °C ± 1 °C.

6.3 Water bath

Capable of maintaining a temperature of 48 °C ± 1 ºC.

6.4 Blending equipment

Two-speed or a variable adjustable blender (18 000 rotations per minute (rpm) and 22 000 rpm), with a one litre bowl that is 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

6.6 Balance

Capable of weighing to the nearest 0,01 g.

6.7 Microscope

Capable of phase-contrast microscopy at 600x to 1 000x magnification.

6.8 Flasks or screw-cap bottles of appropriate capacities

6.9 Test tubes of appropriate capacities

6.10 Pipettor and sterile tips to dispense 100 µl and 1 ml

Wide bore tips to pipette homogenised feed stuff for dilution

6.11 pH meter

6.12 Sterile Petri dishes, 90 mm in diameter

6.13 Bacterial Cell Spreaders

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

6.14 Equipment for anaerobic incubation

Appropriate anaerobic jars or chambers

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 microorganisms. Particularly after sampling additives and premixtures supplemented with microorganisms. If needed, clean and disinfect sampling equipment between each sample,

particularly after sampling additives and premixtures containing microorganisms. 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 Feeds with a single or multi-component probiotic microflora

If bifidobacteria are the only bacterial component in the feed use MRS agar. If they are dominant with additional microflora, enumeration can proceed on AMRSA or MRS + TTC or the selective antibiotic medium. If bifidobacteria are not dominant use the selective antibiotic medium.

9.2 Preparation of poured agar plates

Prepare MRS agar according to the manufacturer's directions.

Carry out acidification of MRS agar for preparation of AMRS agar before autoclaving.

For MRS + TTC agar add filter-sterilised TTC after autoclaving the MRS agar for 15 min at 121 °C ± 1 °C, when the medium has cooled down in a water bath to a temperature of 48 °C ± 1 °C.

Melt the MRS base medium supplemented with cysteine-hydrochloride the day before use and keep at 48°C ± 1°C. Add mupirocin (see 5.2.2.4) to the medium immediately before pouring.

Pour approximately 15 ml portions of medium into each Petri dish (6.12) under sterile conditions and spread to give a homogeneous layer.

Check the dried plates for sterility.

WARNING — Probiotics tend to settle quickly after blending. This shall be avoided by handling only properly homogenised samples.

Dried plates, if correctly protected against dehydration, may be stored for 2 weeks in a fridge. In this case bring the plates to room temperature about 30 min before use.

9.3 Preparation of the initial suspension and decimal dilutions

Weigh 20 g ± 0,1 g of additive or premixture and 50 g ± 0,5 g of feed sample into a sterile blender's bowl. Add 180 ml or 450 ml of buffered saline at room temperature.

NOTE 1 It is recommended to check the pH of the initial suspension and to correct it in a range of pH 7,3 – 8,1.

Blend additives and premixtures for 3 min at high speed (see 6.4) and dilute immediately.

WARNING — Probiotics tend to settle quickly after blending. This shall be avoided by handling only properly homogenised samples.

Blend feeding stuffs for 1 min. at high speed (see 6.4); start to blend at low speed to avoid splashing, then turn the switch to high speed. Let the sample stand for 30 min. It is important for pelleted feeds to absorb the liquid. Blend for 2 min at high speed (see 6.4) and immediately dilute using a wide bore pipette.

NOTE 2 Intense foaming in initial suspensions during blending may be avoided by using a feasible antifoaming agent (e.g. silicon antifoaming agent) in adequate concentrations.

In the case of encapsulated products the application of adequate additives has to be used (e.g. Polyoxyethylensorbitanmonooleat; or equivalent) with adequate temperatures (e.g. 40°C).

The weight of the withdrawn pipette volume has to be proved in order to receive a correction factor for the calculation of the counts of *Bifidobacterium* species.

Prepare a series of appropriate dilutions (serial dilutions) using a sterile pipette such as a microdispenser set at 1 ml. Transfer 1 ml of the initial suspension into a tube containing 9 ml of sterile peptone saline brought to room temperature, and mix with a mechanical stirrer for 5 s. Repeat this procedure using the 10^{-2} dilution and all further dilutions until the appropriate estimate for the number of cells is obtained. After this dilution procedure, inoculate the plates immediately as described in section 9.4

It is recommended to prove the content of the copper in the initial suspension by a pretest. Copper contents exceeding 200 mg/kg need the application of chelating agents e.g. iminodiacetic acid in a feasible concentration with regard to the pH-value.

9.4 Inoculation and incubation of 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.

Reserve plates, stack them and incubate them anaerobically at 37°C for 36 h to 48 h.

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

9.5 Counting of colonies

After the incubation under the above specified conditions, all plates containing more than 30 and no more than 300 colonies are used for the enumeration of colony-forming units (CFU).

Count presumptive bifidobacteria colonies, and calculate the number of colony-forming units per gram feed according to the weighted arithmetical mean value in accordance with EN ISO 7218.

9.6 Cell morphology – confirmation

Depending on the number of morphologically different colonies appearing after incubation, 2 to 5 colonies of each morphology type are selected at random.

Disperse cells from a colony in 20 µl PBS on a microscope slide, cover with a cover slip and view with oil immersion at a magnification of 600x to 1 000x times using phase contrast microscopy. If cells are rods of various shapes: short, regular, thin cells with pointed ends, coccoidal regular cells, long cells with slight bends or protuberances or with a large variety of branchings; pointed, slightly bifurcated club-shaped or spatulated extremities; single or in chains of many elements; in star-like aggregates or disposed in “v” or “palisade” arrangements, they are considered bifidobacteria.

If colonies with differing characteristics are detected in the course of confirmation, repeat analysis with more selective medium (MRS+TTC, AMRSA, selective antibiotic medium).

10 Expression of results

The weighed average of the number of colony forming units (CFU) per gram of feed for bacteria which produce typical colonies on the specified medium and exhibit the typical morphology is calculated in accordance with EN ISO 7218.

The number of bifidobacterium spp (*N*) per gram or per millilitre is equal to:

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

$\sum C$ is the sum of the colonies counted on all the plates;

V is the volume of inoculum applied to each dish, in millilitres;

n_1 is the number of plates counted at the first dilution;

n_2 is the number of plates counted at the second dilution;

d is the dilution from which the first counts were obtained.

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 micro organisms 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 suggested in terms of repeatability and reproducibility was determined in an interlaboratory study.

11.2 Interlaboratory study

Details of the interlaboratory study on the precision of the method are published (see [1], [2]) 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 bifidobacteria 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 bifidobacteria 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;

h) Medium (see 5.2.1).

Annex A
(informative)

Notes on procedures

MRS agar allows growth of all lactic acid bacteria but also of other microorganism species. The addition of TTC to MRS agar provides a useful tool to distinguish e.g. bifidobacteria by different red coloration after anaerobic incubation.

On Acidified MRS agar (AMRSA) other microorganisms than bifidobacteria used as feed additives such as enterococci or bacilli do not form colonies. Only some yeast, moulds or lactobacilli and pediococci may be able to produce colonies under the specified conditions.

The selective MRS medium supplemented with mupirocin should be used if bifidobacteria are present in lower concentrations than other probiotic microorganisms.

An anti-fungal agent like nystatin (50 U/ml) can be added to MRS agar, AMRSA or MRS+TTC to inhibit moulds and yeasts.

Annex B
(informative)

Results of the interlaboratory study

An interlaboratory study involving 17 laboratories in 11 European countries was carried out at two different levels of contamination (low and high). The test was organized in 2002 and coordinated by Central Science Laboratory [1]. For the interlaboratory study homogeneous test samples were prepared using feeding stuffs which contained bifidobacteria as a sole component in the high level samples (depending on the medium used for MRS 6.6 x 10^6 and for the selective bifidobacterium medium 9.5 x 10^7) and in combination with pediococci in the lower concentrated sample (depending on the medium used for MRS 1.3 x 10^5 and for the selective bifidobacterium medium 1.9 x 10^6). The precision data from the trial are summarised in Table 1.

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

parameters	Sample							
	MRSA*		MRSA + TTC*		AMRSA*		BSM*	
	Low level	High level	Low level	High level	Low level	High level	Low level	High level
Number of samples	2	2	2	2	2	2	2	2
Number of laboratories retained after eliminating outliers	5	5	7	9	6	9	8	10
Mean value, in $\log_{10}$ CFU/g)	5,30	7,69	5,22	7,49	5,08	7,53	5,27	7,81
Repeatability standard deviation, s_r , in $\log_{10}$ CFU/g	0,15	0,13	0,27	0,18	0,31	0,12	0,33	0,09
Coefficient of variation of repeatability, CV_r, in %	2,90	1,74	5,26	2,42	6,11	1,60	6,30	1,19
Repeatability limit, r	0,43	0,38	0,77	0,51	0,87	0,34	0,93	0,26
Reproducibility standard deviation, s_R , in $\log_{10}$ CFU/g	0,24	0,25	0,29	0,39	0,44	0,26	0,35	0,21
Coefficient of variation of reproducibility, CV_R, in %	4,52	3,30	5,51	5,21	8,74	3,50	6,55	2,62
Reproducibility limit, R	0,67	0,71	0,81	1,09	1,24	0,74	0,97	0,57

* media reference see clause 5

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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)'

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

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

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