BRITISH STANDARD

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Animal feeding stuffs — Isolation and enumeration of Enterococcus (E. faecium) 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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Contents

Page

Foreword

This document (EN 15788:2009) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs”, the secretariat of which is held by NEN.

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Introduction

This methodology has been developed to enumerate enterococci (*E. faecium*) 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 animal feeds") [1]. The method is based on an extensive screening of 12 pre-selected, commercially available media for the detection and enumeration of enterococci. The described methodology was validated in an interlaboratory study [2].

This method is not selective for probiotic enterococci (*E. faecium*) but can be applied to enumerate enterococci in additives, premixtures and feeding stuffs assuming that the probiotic enterococci (*E. faecium*) is present in far higher numbers than any other enterococci.

1 Scope

This European Standard defines general rules for the enumeration of enterococci in feed samples (additives, premixtures and feeding stuffs) that contain enterococci (*E. faecium*) as a single microorganism component or in a mixture with other microorganisms. This standard is not applicable to mineral feeds which are defined as complementary feedingstuffs 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 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
***enterococcus faecium* (described by their characteristics as used for this standard)**
enterococcus faecium is charaterised as a bacterium which forms colonies fitting the description of the species on the specified selective medium after incubation of 24 h at a temperature of 37 °C under aerobic conditions:

a) morphology of colonies on selective medium;

b) circular;

c) convex to dome-shaped;

d) entire;

e) white;

f) glistening surface;

g) opaque.

Colony size varies between 1 mm and 2 mm in diameter

The medium surrounding the colonies shows a dark brown to black coloration, due to the hydrolysis of esculin

Phase contrast microscopical examination of selected colonies typically shows spherical cells arranged in pairs.

4 Principle

An initial suspension of the sample is prepared in a diluent with suitable buffer capacity using a suitable homogeniser. Dilutions of the initial suspension have to be immediately prepared before the suspension settles. Spread plates (Bile Esculin Azide Agar) are inoculated with the chosen dilutions. The plates are incubated aerobically for 24 h ± 2 h at 37 °C ± 1 °C.

Presumptive enterococci (*E. faecium*) colonies are counted and the number of colony forming units per g or kg is calculated.

NOTE To verify the colony count, a phenotypic characterisation and confirmation of a selection of colonies may be done by means of an identification kit.

5 Diluents, selective medium and test kit for 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 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 ± 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 Selective medium

Bile Esculin Azide Agar[1)] is used as a selective medium.

Composition in g/l final medium:

a)	Peptone 1 (pancreatic digest of casein)	17,0 g
b)	Peptone 2 (peptic digest of meat)	3,0 g
c)	Yeast extract	5,0 g
d)	Ox bile (dehydrated)	10,0 g
e)	Sodium chloride	5,0 g
f)	Esculin	1,0 g
g)	Ferric ammonium citrate	0,5 g
h)	Sodium azide	0,25 g
i)	Agar	13,5 g

Final pH 7,1 ± 0,2.

5.3 Phenotypic characterization and confirmation

Check selected colonies microscopically for enterococci-like morphology.

NOTE A phenotypic kit may be used for phenotypic characterisation of isolated colonies if required.

6 Apparatus and glassware

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

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.

1) the agar is commercially available from various suppliers.

6.3 Water bath

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

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 which 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 two decimal places.

6.7 Screw-cap bottles

Appropriate capacities, 25 ml universals, bottles, test tubes, flasks and 1 000 ml Duran bottles

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

Wide bore tips to pipette homogenised feed stuff for dilution.

6.9 Bacterial Cell 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

With phase-contrast-imaging (400x) and for use with oil immersion (1 000x).

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 precaution to avoid potential cross-contamination of samples with enterococci, particularly after sampling additives and premixtures supplemented with enterococci.

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

The selective medium is prepared according to the manufacturer's directions. Pour after autoclaving and cooling in a water bath to a temperature of approximately 48 °C ± 1 °C, portions of approximately 20 ml into each Petri dish (6.10) under sterile conditions and spread to give a homogeneous layer.

WARNING — Sodium azide is a heat sensitive ingredient and does not allow repeated liquefaction by heating!

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.

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

Weigh 20 g ± 0,1 g of plain additive or premixture, or 2 g ± 0,01 g of a microgranule (encapsulated) additive or 50 g ± 0,5 g of feed. Add 180 g ± 0,1 g of diluent to plain additives or premixtures, 198 g ± 0,1 ml to microgranule additives or 450 g ± 1 g to feeding stuffs. Homogenise the mixture with a suitable homogeniser such as a blender.

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

The numbers of capsules should be more than 25, to obtain a SD (Standard Deviation) of less than 20% for a good repeat of analysis.

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

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 or equivalent) 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 calibrated in order to receive a correction factor for the calculation of the counts of E*nterococcus faecium*.

WARNING — Suspensions containing probiotics tend to settle quickly after blending. This shall be avoided by rapid pipetting subsamples of only properly homogenised samples for further dilutions.

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. 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 are inoculated immediately as described in section 9.3.

NOTE 3 It is recommended to examine 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.

NOTE 4 For samples with high yeast count (additives, premixes), better precision is obtained by using 1/100 and/or 1/1 000 dilutions instead of decimal dilutions. Change the pipette tip between each dilution.

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 plates, stack them and incubate them at 37 °C ± 1 °C for 24 h ± 2 h 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 250 presumptively enterococci (*E. faecium*)-positive (dark brown to black coloration of the medium surrounding the colonies) colonies should be counted. If possible, count four dishes (two successive dilutions). In the case that no feasible incubated plates with colony counts between ≥ 15 and < 150 are available the analysis has to be repeated with appropriate dilutions.

9.5 Phenotypic characterisation – confirmation

Bacteria that produce typical colonies on the specified medium (see clause 3) and exhibit the typical phenotypic profile are detected and counted as enterococci (*E. faecium*).

These results form the basis for calculating the number of colony-forming units (CFU) per gram of feed.

10 Expression of results

The number of colony-forming units per gram feed is calculated according to the weighted arithmetical mean value (follow the formula from EN ISO 7218).

The number of enterococci (*E. faecium*) (*N*) per gram or per ml 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 dishes retained from two successive dilutions, at least one of which contains 15 colonies;

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

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

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

d is the dilution factor corresponding to the first dilution retained.

Round the result obtained to two significant figures. For a three-figure number, round the third to the nearest zero. If the third is 5, round 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 and 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 [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 enterococci 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 enterococci 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 of the other microorganisms used as feed additives do not form colonies under the specified conditions.

Some pediococci and lactobacilli may form pinpoint colonies on the Bile Esculin Azide Agar under the specified conditions. These colonies can easily be differentiated and are not used for colony counting.

Annex B
(informative)

Results of the interlaboratory study

An interlaboratory study involving 20 laboratories in 12 European countries was carried out on two different levels of contamination. The study was organized in 2002 and coordinated by Central Science Laboratory (UK) [1], [2]. For the interlaboratory study homogeneous test samples using feeding stuffs were prepared which contained *E. faecium* as a sole component in the low level samples ($4x10^5$ CFU/g) and in combination with lactobacilli and yeast at equivalent numbers in the higher concentrated ($4x10^7$ CFU/g) sample.

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

	BEAA (Bile Esculine Agar Azide)*	
	Low	high
Number of samples	2	2
Number of laboratories retained after eliminating outliers	14	12
Mean value (log_{10} CFU/g)	5,52	7,57
Repeatability standard deviation, s_r (log_{10} CFU/g)	0,20	0,12
Repeatability relative standard deviation, RSD_r (%)	3,60	1,49
Repeatability limit, r	0,56	0,32
Reproducibility standard deviation, s_R (log_{10} CFU/g)	0,41	0,23
Reproducibility relative standard deviation, RSD_R (%)	7,37	2,91
Reproducibility limit, R	1,14	0,63
* 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)'

[2] Leuschner et al. J.App.Microbio 93 (2002) 781

[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*

[5] Commission Directive 76/371/EEC, 1 March 1976, Community methods of sampling for the official control of feeding stuffs

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