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Microbiology of food and animal feeding stuffs — Horizontal method for the emumeration of mesophilic lactic acid bacteria — Colony-count technique at 30 °C

Microbiologie des aliments — Méthode horizontale pour le dénombrement des bactéries lactiques mésophiles — Techniques par comptage des colonies à 30 °C

Contents

		Page
1	Scope	**1**
2	Normative references	**1**
3	Definitions	**1**
4	Principle	**2**
5	Diluent and culture medium	**2**
6	Apparatus and glassware	**3**
7	Sampling	**3**
8	Preparation of test sample	**4**
9	Procedure	**4**
10	Expression of results	**5**
11	Confidence limits	**6**
12	Test report	**6**
Annex A (informative) **Bibliography**		**7**

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 15214 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 9, *Microbiology*.

Annex A of this International Standard is for information only.

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods which are specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this International Standard is next reviewed, account will be taken of all information then available regarding to extent to which this horizontal method has been followed and the reasons for deviations from this method in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this International Standard so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of mesophilic lactic acid bacteria — Colony-count technique at 30 °C

1 Scope

This International Standard specifies a horizontal method for the enumeration of viable mesophilic lactic acid bacteria by counting the colonies growing in a solid medium after incubation at 30 °C for 3 days.

NOTE In some food products, there exist psychotrophic or thermophilic lactic acid bacteria necessitating culture temperatures different from 30 °C. Moreover, not all lactic acid bacteria grow on MRS agar at pH 5,7 and some grow only weakly.

Subject to the limitations discussed in the introduction and in the note above, this International Standard is applicable to products intended for human consumption or animal foodstuffs.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of the publication, the editions indicated were valid. All standards are subject to revision, and parties to agreement based on the International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards inidicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 6887-1:—[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 intial suspension and decimal dilutions.*

ISO 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations.*

3 Definitions

For the purposes of this International Standard, the following definition applies.

3.1
mesophilic lactic acid bacteria
bacteria which form colonies at 30 °C in a solid selective medium (MRS at pH 5,7) under the test conditions specified in this International Standard

1) To be published. (Revision of ISO 6887:1983)

4 Principle

4.1 Preparation of two dishes using MRS agar at pH 5,7 contained in Petri dishes. Poured-plate or possibly surface[2] inoculation of the dishes with a specified quantity of the test sample if the initial product is liquid, or with a specified quantity of the initial suspension in the case of other products.

4.2 Inoculation of other pairs of dishes, under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

Incubation of the dishes at 30 °C for 72 h.

4.3 Calculation of the number of mesophilic lactic acid bacteria (3.1) per gram or per millilitre of test sample from the number of colonies obtained in 4.2 in the dishes selected, and possibly confirmed[3].

5 Diluent and culture medium

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Diluent

See ISO 6887-1.

NOTE The buffered peptone water does not always allow satisfactory resuscitation of lactic acid bacteria (see annex A, references [1], [2], [3]).

5.3 Culture medium: MRS medium (de Man, Rogosa and Sharpe) at pH 5,7 (see reference [4])

NOTE The use of commercially available ready-to-use media is acceptable. However, attention is drawn to the fact that variations in composition and pH may occur between products from different manufacturers and could therefore give results different from the ones obtained with the medium as specified in this International Standard.

5.3.1 Composition

Enzymatic digest of casein	10,0 g
Meat extract	10,0 g
Yeast extract	4,0 g
Triammonium citrate [$(NH_4)_3C_6H_5O_7$]	2,0 g
Sodium acetate (CH_3COONa)	5,0 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0,2 g
Manganese sulfate tetrahydrate ($MnSO_4 \cdot 4H_2O$)	0,05 g
Dipotassium hydrogen phosphate (K_2HPO_4)	2,0 g
Glucose ($C_6H_{12}O_6$)	20,0 g
Polyoxyethylenesorbitan monooleate (Tween 80)	1,08 g
Agar	12 g to 18 g[1]
Water	1 000 ml

1) Depending on the gel strength of the agar.

[2] See note 1 in 9.2.

[3] See note 2 in 9.3.

5.3.2 Preparation

5.3.2.1 Dissolve the components or the dehydrated complete medium in the water by boiling.

Using the pH-meter (6.7), adjust the pH so that after sterilization it is 5,7 ± 0,1[4)] at 25 °C.

Transfer the medium to bottles of appropriate capacity.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

If the medium is to be used immediately, cool it before use to appoximately 47 °C in the water bath (6.5), or by any other technique giving equivalent results (see ISO 7218).

If not, in order to avoid any delay when pouring the medium before beginning the microbiological examination, completely melt the medium, in a boiling water bath (6.6), then cool it to approximately 47 °C in the water bath (6.5).

5.3.2.2 If there is a risk of extensive yeast contamination (e.g. in dried sausage), add sorbic acid to the MRS medium as follows.

Dissolve 1,4 g of sorbic acid in about 10 ml of a 1 mol/l solution of sodium hydroxide. Sterilize by filtration. Add this solution to 1 000 ml of sterilized MRS agar, previously cooled to approximately 47 °C. The final pH of the medium shall be 5,7 ± 0,1 at 25 °C.

6 Apparatus and glassware

Usual microbiological laboratory apparatus (see ISO 7218) and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**

See ISO 7218.

6.2 Incubator, capable of operating at 30 °C ± 1 °C.

6.3 Petri dishes, made of glass or plastic, of diameter 90 mm to 100 mm.

6.4 Total-delivery graduated pipettes, of nominal capacity 10 ml and 1 ml, graduated respectively in 0,5 ml and 0,1 ml divisions.

6.5 Water bath, or similar apparatus, capable of operating at 47 °C ± 2 °C.

6.6 Boiling water bath.

6.7 pH-meter, capable of being read to the nearest 0,01 pH unit at 25 °C, enabling measurements to be made which are accurate to ± 0,1 pH unit.

7 Sampling

Sampling is not part of the method specified in this International Standard. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

[4)] In order that the pH-value does not fall below 5,6, the tolerance here is ±0,1 instead of ±0,2 as usual.

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage (see ISO 7218).

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilutions

Prepare the initial suspension and dilutions in accordance with ISO 6887-1.

9.2 Inoculation and incubation

NOTE 1 Surface plating in combination with incubation under anaerobic or microaerobic conditions can be applied instead of the pour-plating procedure described. Candle jars may be used to obtain appropriate conditions.

NOTE 2 It is also possible to use a double-layer MRS medium.

9.2.1 Take two sterile Petri dishes (6.3). Using a sterile pipette (6.4), transfer to each dish 1 ml of the test sample if the product is liquid, or 1 ml of the initial suspension in the case of other products.

Take two other sterile Petri dishes. Using a fresh sterile pipette, transfer to each dish 1 ml of the first decimal dilution of the test sample if the product is liquid, or 1 ml of the first decimal dilution of the initial suspension in the case of other products.

Repeat the procedure described with the further dilutions, using a fresh sterile pipette for each decimal dilution.

NOTE If high numbers of lactic acid bacteria are expected, it is possible to inoculate only those dilutions necessary to be able to enumerate according to the general case (see 10.1).

9.2.2 Pour into each Petri dish approximately 15 ml of the MRS medium (5.3) which has been prepared then cooled to approximately 47 °C in the water bath (6.5).

Carefully mix the inoculum with the medium and allow the mixture to solidify.

9.2.3 Invert the prepared dishes and incubate them in the incubator (6.2) set at 30 °C for 72 h ± 3 h.

Avoid desiccation of the agar during incubation so that the medium does not become too inhibitory.

9.3 Counting of colonies

After the specified period of time (see 9.2.3), count the colonies in each dish (see notes 1 and 2).

Retain dishes containing fewer than 300 colonies at two successive dilutions, and more than 15 colonies on at least one dish.

NOTE 1 Some *Leuconostoc* spp. may form large slimy colonies which may hinder the development of other colonies, thus causing an underestimation of the number of lactic acid bacteria.

NOTE 2 Due to the possible development of microorganisms other than lactic acid bacteria on MRS medium, as described in 9.2, it may be necessary in some cases and for some products to confirm the colonies obtained in 9.2 by simple techniques (such as Gram staining, or the test for catalase). Such a procedure, if conducted, should be mentioned in the test report.

10 Expression of results

10.1 General case

Calculate the number N of mesophilic lactic acid bacteria present in the test sample, as the weighted mean from two successive dilutions, using the equation:

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

where

$\sum C$ is the sum of the colonies counted on all the dishes from two successive dilutions, at least one of which contains at least 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 off the results calculated to two significant figures (see ISO 7218).

Take as the result the number of mesophilic lactic acid bacteria per millilitre (liquid products) or per gram (other products), expressed as a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE

$$N = \frac{\sum C}{V(n_1 + 0{,}1n_2)\, d} = \frac{168 + 215 + 14 + 25}{1(2 + 0{,}1\times 2)10^{-2}} = \frac{422}{0{,}022} = 19\,182$$

Rounding off to two significant figures gives 19 000 or $1{,}9 \times 10^4$ mesophilic lactic acid bacteria per gram of product.

10.2 Estimation of low numbers

10.2.1 If the two dishes, at the level of the test sample (liquid products) or of the initial suspension (other products) contain less than 15 colonies, calculate the arithmetical mean y of the colonies counted on two dishes.

Express the results as follows:

— for liquid products: estimated number of mesophilic acid bacteria per millilitre $N_E = y$;

— for liquid products: estimated number of mesophilic lactic acid bacteria per gram $N_E = y/d$;

where d is the dilution factor of the initial suspension.