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Milk products — Enumeration of presumptive *Lactobacillus acidophilus* on a selective medium — Colony-count technique at 37 °C

Produits laitiers — Dénombrement de Lactobacillus acidophilus *présomptifs sur un milieu sélectif — Technique de comptage des colonies à 37 °C*

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Foreword

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ISO 20128|IDF 192 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of IDF National Committees casting a vote.

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ISO 20128|IDF 192 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO-IDF Action Team on *Lactic acid bacteria and starters*, of the Standing Committee on *Microbiological methods of analysis*, under the aegis of its project leader, Mrs D. Ellekaer (DK).

Introduction

Because of the large variety of fermented and non-fermented milks, this method may not be appropriate in every detail for certain products.

This could be the case where the number of presumptive *Lactobacillus acidophilus* is very much lower than the number of other microorganisms such as *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus helveticus* and yeasts.

Milk products — Enumeration of presumptive *Lactobacillus acidophilus* on a selective medium — Colony-count technique at 37 °C

1 Scope

This International Standard specifies a method for the enumeration of presumptive *Lactobacillus acidophilus* in milk products on a selective medium by using a colony-count technique at 37 °C.

The method is applicable to fermented and non-fermented milks, milk powders and infant formulae where presumptive *L. acidophilus* is present and in combination with other lactic acid bacteria and bifidobacteria.

The method is not applicable when the number of presumptive *L. acidophilus* is less than 10^4 CFU/g and the numbers of *Lactobacillus rhamnosus, Lactobacillus reuteri* and *Lactobacillus paracasei* subsp. *paracasei* are greater than 10^6 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.

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

ISO 8261|IDF 122, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
presumptive *Lactobacillus acidophilus*
microorganism forming flat, mat, rough, grey to whitish colonies with more or less irregular edges and a diameter of 1 mm to 3 mm depending on the number of colonies when grown on a solid selective medium under the conditions specified in this International Standard

NOTE *L. acidophilus* is closely related to *Lactobacillus johnsonii*, *Lactobacillus gasseri* and *Lactobacillus crispatus*. The method specified in this International Standard cannot distinguish between these four species and, therefore, only presumptive *L. acidophilus* is mentioned.

4 Principle

4.1 The antibiotics clindamycin and ciprofloxacin both inhibit the growth of the most common microorganisms used in fermented milks, non-fermented milks and infant formulae, such as *Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus delbrueckii* subsp. *lactis, Streptococcus thermophilus*, bifidobacteria, lactococci, *Lactobacillus casei, Lactobacillus paracasei* subsp. *paracasei, Lactobacillus rhamnosus*, *Lactobacillus reuteri* and *Leuconostoc* species.

4.2 A known amount of sample is homogenized with diluent and decimal dilutions are prepared.

4.3 Appropriate dilutions are spread plated on MRS-agar with the addition of clindamycin and ciprofloxacin.

4.4 The plates are incubated anaerobically at 37 °C for 72 h ± 3 h.

4.5 Typical colonies are counted.

4.6 The number of characteristic microorganisms per gram of sample is calculated from the number of colonies obtained on plates chosen at dilution levels so as to give a significant result.

5 Diluents, culture media and reagents

5.1 Basic materials

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity. See ISO 7218.

5.2 Diluent

See ISO 8261|IDF 122.

5.3 Culture media

5.3.1 MRS/clindamycin/ciprofloxacin agar (MRS/CL/CIP agar)

MRS/CL/CIP agar consists of MRS agar (5.3.2) with the addition of 0,1 mg of clindamycin and 10,0 mg of ciprofloxacin per litre of medium (see 5.3.4).

5.3.2 Basic medium: MRS agar

5.3.2.1 Composition

Peptone 1 (enzymatic digest of casein)	10,0 g
Meat extract	10,0 g
Yeast extract (dried)	5,0 g
Glucose	20,0 g
Tween 80 (sorbitan mono-oleate)	1,0 ml
Dipotassium hydrogen phosphate (K_2HPO_4)	2,0 g
Sodium acetate trihydrate ($NaCH_3CO_2{\cdot}3H_2O$)	5,0 g
Triammonium citrate ($(NH_4)_3HC_6H_5O_7$)	2,0 g
Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$)	0,2 g
Manganese sulfate tetrahydrate ($MnSO_4{\cdot}4H_2O$)	0,05 g
Agar	12 g to 18 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

5.3.2.2 Preparation

Suspend the ingredients in the water. Heat the suspension to boiling with frequent agitation until a complete solution is obtained. Distribute the medium in portions of 100 ml ± 1 ml into bottles (6.9) of 150 ml capacity or in portions of 200 ml ± 2 ml into bottles (6.9) of 250 ml capacity.

If needed, adjust the pH (6.8) so that, after sterilization, it is 6,2 ± 0,2. Sterilize in the autoclave (6.6) set at 121 °C for 15 min. If the medium is to be used immediately, cool it in a water bath (6.7) to between 44 °C and 47 °C. Do not expose the medium to direct sunlight.

The thus-prepared MRS agar may be stored in the dark at 1 °C to 5 °C for 6 months.

NOTE The complete MRS agar is commercially available but the results obtained may differ significantly from one supplier to another (See also ISO/TS 11133-1 and ISO/TS 11133-2.)

5.3.3 Clindamycin stock solution

5.3.3.1 Composition

Clindamycin hydrochloride	2,0 mg
Water up to	10,0 ml

5.3.3.2 Preparation

Dissolve the clindamycin hydrochloride in the water. Filter the solution then sterilize through a 0,22 µm filter (6.13) into a sterile test tube (6.14).

If the solution is not to be used immediately, distribute it in small sterile cryotubes (6.17) and keep the tubes at –20 °C. The frozen solution may be stored for 6 weeks.

5.3.4 Ciprofloxacin stock solution

5.3.4.1 Composition

Ciprofloxacin hydrochloride	20,0 mg
Distilled water up to	10,0 ml

5.3.4.2 Preparation

Dissolve the ciprofloxacin hydrochloride in the water and sterilize by filtration through a 0,22 µm filter (6.13) into a sterile test tube (6.14).

If the solution is not to be used immediately then keep it at –20 °C. The frozen solution may be stored for 8 weeks.

5.3.5 Complete medium: Preparation of plates

5.3.5.1 Composition

MRS agar (5.3.2)	100 ml	or	MRS agar (5.3.2)	200 ml
Clindamycin stock solution (5.3.3)	0,05 ml		Clindamycin stock solution (5.3.3)	0,1 ml
Ciprofloxacin stock solution (5.3.4)	0,5 ml		Ciprofloxacin stock solution (5.3.4)	1,0 ml

5.3.5.2 Preparation

Immediately before use, melt the MRS agar (5.3.2) in a boiling water bath (6.7). Cool it in a water bath to between 44 °C and 47 °C.

Add 0,05 ml of the clindamycin stock solution (5.3.3) and 0,5 ml of ciprofloxacin stock solution (5.3.4) to 100 ml of MRS agar (5.3.2), or add 0,1 ml of clindamycin stock solution (5.3.3) and 1,0 ml of ciprofloxacin stock solution (5.3.4) to 200 ml of MRS agar (5.3.2) and mix very carefully. Avoid gas bubbles.

Pour the medium into Petri dishes (6.11) with 12 ml to 15 ml in each. Allow the medium to cool and solidify by placing the Petri dishes with the lids in place on a cool horizontal surface.

The prepared MRS/CL/CIP plates may be stored in the dark at 4 °C to 7 °C for 10 days.

Before use, dry the agar surface according to ISO 7218.

6 Apparatus and glassware

Usual microbiological laboratory equipment required for the preparation of test samples and dilutions as specified in ISO 7218 and, in particular, the following.

6.1 Glassware

Sterilize all equipment that will come into contact with the test sample, the diluent, the dilutions or the culture medium, as specified in ISO 7218. The glassware shall be resistant to repeated sterilization.

6.2 Incubator, capable of operating at 37 °C ± 1 °C.

6.3 Anaerobic incubator, capable of being controlled at 37 °C ± 1 °C, or **anaerobic culture jars**, providing an atmosphere of approx. 9 % to 13 % carbon dioxide.

6.4 Blender, either a peristaltic-type blender (Stomacher) with sterile plastic bags or a rotary blender, capable of operating at a minimum rotational frequency of 20 000 min^{-1}, with sterile glass or metal containers of appropriate capacity.

6.5 Colony-counting equipment, consisting of an illuminated base with a dark background, fitted with a magnifying lens to be used at a magnification of 1,5 × and a mechanical or electronic digital counter.

6.6 Autoclave, capable of operating at 121 °C ± 1 °C.

6.7 Water baths, capable of maintaining a temperature of between 44 °C and 47 °C and of boiling.

6.8 pH meter, with temperature compensation, accurate to ± 0,1 pH unit at 25 °C.

6.9 Bottles or **flasks**, of capacity 150 ml or 250 ml, and with suitable sealing caps or stoppers (to hold the culture medium).

6.10 Pipettes, sterile, calibrated for bacteriological use, capable of delivering 0,05 ml ± 0,002 ml, 0,1 ml ± 0,02 ml, 1,0 ml ± 0,02 ml and 10 ml ± 0,2 ml.

6.11 Petri dishes, sterile, made of clear colourless glass or plastic, of diameter 90 mm, of internal depth 10 mm minimum. The bottom shall have no irregularities that may interfere with counting colonies.

6.12 Spatula, sterile, made of glass or metal.

6.13 Filter, sterile, with cellulose acetate membrane of 0,22 µm pore size.

6.14 Test tubes, sterile, of capacity 20 ml, with suitable sealing caps.

6.15 Spreader, sterile, made of glass or metal.

6.16 Drying cabinet, see ISO 7218.

6.17 Cryotubes, sterile, of capacity 2 ml.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707|IDF 50.

8 Procedure

8.1 Sample preparation and decimal dilutions

Prepare the test samples, test portion, initial suspension and further dilutions in accordance with ISO 8261|IDF 122.

The samples shall be plated in a minimum of three serial dilutions in order to verify growth of presumptive *L. acidophilus* and growth of other lactic acid bacteria.

8.2 Inoculation and incubation

8.2.1 By use of a sterile pipette (6.10), transfer 0,1 ml of the appropriate dilution(s) onto the surface of two Petri dishes containing the MRS/CL/CIP agar (5.3.5).

8.2.2 Spread the sample over the entire surface of the medium using of a sterile spreader (6.15). Use one spreader for each dilution.

8.2.3 Allow the medium to absorb the sample before the dishes are inverted and incubated anaerobically in the anaerobic incubator (6.3) set at 37 °C for 72 h ± 3 h.

8.2.4 Use an uninoculated Petri dish with medium as media sterility control.

8.3 Enumeration of colonies

After the specified period of incubation (8.2.3), enumerate those colonies showing the features of characteristic microorganisms (see 3.1) on plates having between 10 and 300 colonies. To facilitate counting, suitable counting equipment (6.5) may be used.

9 Calculation and expression of results

9.1 Calculation

9.1.1 Use counts from plates containing between 10 and 300 colonies as obtained under 8.3.

9.1.2 Calculate the number N of presumptive *L. acidophilus* in the test sample per gram, using the following equation:

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

where

$\sum c$ is the sum of characteristic colonies (3) counted on all dishes retained;

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

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

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

9.2 Expression of results

9.2.1 Round the calculated result to two significant digits. For a three-digit number, round the third digit to the nearest zero. If the third digit is 5, round to the digit below if the first two digits are an even number, and to the digit above if the first two digits are an odd number.

9.2.2 If there are only counts less than 10 colonies, report the number of microorganisms per gram as "less than $10 \times 1/d$" where d is the value corresponding to the lowest dilution.

9.2.3 If there are only counts exceeding 300 colonies, calculate an estimated count from dishes having a count nearest to 300 colonies and multiply with the reciprocal of the value corresponding with the highest dilution. Report as the "estimated minimum number of microorganisms per gram".

9.2.4 Express the result as a number from 1,0 to 9,9 multiplied by the appropriate power of 10.

9.3 Example

A count of colonies gave the following results (two Petri dishes per dilution):

a) first dilution (10^{-5}) 280 colonies and 299 colonies;

b) second dilution (10^{-6}) 31 colonies and 36 colonies:

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

$$N = \frac{280 + 299 + 31 + 36}{(2 + 0{,}1 \times 2) \times 10^{-5}}$$

$$N = \frac{646}{2{,}2 \times 10^{-5}} = 294 \times 10^5 \text{ CFU/g}$$

In accordance with 9.2, this is equal to 290×10^5. The estimated number is therefore, $2{,}9 \times 10^7$ CFU/g.

10 Precision

10.1 Interlaboratory test

Details of the interlaboratory test on the precision of the method are summarized in Annex A. The repeatability and reproducibility limits were determined using six types of fermented milk products containing various levels of presumptive *L. acidophilus* and one reference.

The values derived from the interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

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

As an indication of repeatability limit (r), Table 1 shows the estimations of the repeatability of the different products as their standard deviations (s_r) for fermented milk products.

NOTE The repeatability standard deviations vary between 0,01 and 0,16 (product A). In most cases, the repeatability standard deviation is smaller than 0,1.

Table 1 — Repeatability standard deviations, s_r

Sample	Product	s_r
A	Commercial product containing *L. acidophilus, L. johnsonii* and bifidobacteria	0,16
B	Commercial product containing *L. acidophilus*	0,01
C	Artificial product containing medium level of *L. gasseri* and high levels of *L. rhamnosus, L. reuteri, L. paracasei* subsp. *paracasei* and yoghurt bacteria	0,13
D	Artificial product containing low level of *L. acidophilus* and high levels of bifidobacteria, *L. rhamnosus, L. reuteri, L. paracasei* subsp. *paracasei* and yoghurt bacteria	0,05
E	Artificial product containing medium level of *L. acidophilus* and high levels of bifidobacteria *L. rhamnosus, L. reuteri, L. paracasei* subsp. *paracasei* and yoghurt bacteria	0,05
F	Commercial product containing *L. acidophilus,* yoghurt bacteria, *L. rhamnosus* and bifidobacteria	0,06
G	Reference culture: *L. acidophilus*	0,05

10.3 Reproducibility

The absolute difference between two single ($\log_{10}$-transformed) test results (number of presumptive *L. acidophilus* per gram or per millilitre) 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 by different operators using different equipment, will in not more than 5 % of cases exceed the reproducibility limit, R.

As an indication of reproducibility limit (R), Table 2 shows the estimations of the reproducibility of the different products as their standard deviations (s_R) for fermented milk products.

NOTE The reproducibility is slightly higher with values about 0,15, but for the products C and D it is $>$ 0,5 which indicates that the method is not robust when the contents of competing microorganisms are high compared to those of the presumptive *L. acidophilus*.

Table 2 — Reproducibility standard deviations, s_R

Sample	Product	s_R
A	Commercial product containing *L. acidophilus, L. johnsonii* and bifidobacteria	0,19
B	Commercial product containing *L. acidophilus*	0,14
C	Artificial product containing medium level of *L. gasseri* and high levels of *L. rhamnosus, L. reuteri, L. paracasei* subsp. *paracasei* and yoghurt bacteria	0,53
D	Artificial product containing low level of *L. acidophilus* and high levels of bifidobacteria, *L. rhamnosus, L. reuteri, L. paracasei* subsp. *paracasei* and yoghurt bacteria	1,26
E	Artificial product containing medium level of *L. acidophilus* and high levels of bifidobacteria *L. rhamnosus, L. reuteri, L. paracasei* subsp. *paracasei* and yoghurt bacteria	0,07
F	Commercial product containing *L. acidophilus,* yoghurt bacteria, *L. rhamnosus* and bifidobacteria	0,06
G	Reference culture: *L. acidophilus*	0,17