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Milk, milk products and mesophilic starter cultures — Enumeration of citrate-fermenting lactic acid bacteria — Colony-count technique at 25 °C

Lait, produits laitiers et ferments mésophiles — Dénombrement des bactéries lactiques fermentant le citrate — Technique de comptage des colonies à 25 °C

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Foreword

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The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 17792 | IDF 180 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 17792 | IDF 180 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 starters*, of the Standing Committee on *Microbiological methods of analysis*, under the aegis of its project leader, Prof. B. Bianchi-Salvadori (IT).

This edition of ISO 17792 | IDF 180 cancels and replaces IDF 180:1997, which has been technically revised.

Milk, milk products and mesophilic starter cultures — Enumeration of citrate-fermenting lactic acid bacteria — Colony-count technique at 25 °C

1 Scope

This International Standard specifies methods for the enumeration of citrate-fermenting lactic acid bacteria using a colony-count technique at 25 °C.

The methods are applicable to dairy starter cultures and dairy products where these characteristic microorganisms are present.

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 565, *Test sieves — Metal wire cloth, perforated metal, plate and electroformed sheet — Nominal sizes of openings*

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
citrate-fermenting bacteria
homofermentative and heterofermentative lactic acid bacteria that form lenticular colonies with a diameter between 0,5 mm and 1,2 mm in selective media containing citrate and special indicators as specified in this International Standard

NOTE The most important citrate-fermenting mesophilic lactic acid bacteria belong to the following species.

a) For lactococci:

— *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*;

microscopic appearance: lactococci are normally spherical or ovoid cells (0,4 µm to 1,0 µm), occurring in pairs or chains; they are non-spore-forming, Gram-positive, non-motile and catalase-negative.

b) For leuconostocs:

— *Leuconostoc mesenteroides* subsp. *cremoris*;

— *Leuconostoc mesenteroides* subsp. *mesenteroides*;

— *Leuconostoc mesenteroides* subsp. *dextranicum*;

— *Leuconostoc lactis*;

microscopic appearance: leuconostocs are generally spherical or lenticular cells (0,4 µm to 0,5 µm), occurring in pairs or chains; they are non-spore-forming, Gram-positive, non-motile and catalase-negative.

4 Principle

4.1 Decimal dilutions of the sample are inoculated into:

a) Nickels and Leesment medium [4], modified [3], followed by aerobic incubation at 25 °C for 72 h, for the count of citrate (zones) and non-citrate (no zones) fermenting bacteria. Then X-gal is added followed by aerobic incubation at room temperature for 24 h to differentiate between *L. lactis* subsp. *lactis* biovar *diacetylactis* (white colonies with zones) and leuconostoc species (blue colonies with or without zones).

b) Nickels and Leesment medium [4] plus vancomycin, followed by aerobic incubation at 25 °C for 3 days to 5 days, for the enumeration of leuconostocs.

4.2 Colonies are counted and confirmed by means of appropriate tests.

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

5 Diluents, culture media and reagents

5.1 General

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

Tests to determine the suitability of water for microbiological applications have been published [2].

CAUTION — Some reagents are toxic and/or dangerous and can cause allergy through inhalation and skin contact.

5.2 Basic materials

See ISO 8261 | IDF 122 and ISO 7218.

5.3 Diluent

See ISO 8261 | IDF 122.

5.4 Culture media

5.4.1 Nickels and Leesment medium + X-gal [3]

NOTE The main problem with the use of modified Nickels and Leesment medium is the poor growth of non-fermenting bacteria. These bacteria grow abundantly but are so small that they can be mistaken for the particles of insoluble calcium citrate.

5.4.1.1 Basic medium

5.4.1.1.1 Composition

Tryptic digest of casein	20,0 g
Yeast extract	5,0 g
Gelatine	2,5 g
Glucose ($C_6H_{12}O_6$)	5,0 g
Lactose ($C_{12}H_{22}O_{11}$)	5,0 g
Sodium chloride (NaCl)	4,0 g
Trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$)	2,0 g
Agar	(12,0 to 15,0) g[a]
Water up to	1 000 ml

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

5.4.1.1.2 Preparation

Suspend separately each of the above-mentioned components in the water. Heat the suspension to boiling under frequent agitation to dissolve all components completely. Mix the thus-dissolved components well. Add water to a final volume of 1 000 ml.

Distribute the medium into bottles and sterilize in an autoclave set at 121 °C ± 1 °C for 15 min. If necessary, adjust the pH by using the required pH-adjusting reagent (5.5) and pH meter (6.7), so that after sterilization the pH is between 6,6 and 6,7.

5.4.1.2 Calcium lactate solution

5.4.1.2.1 Composition

Calcium lactate pentahydrate ($C_6H_{10}CaO_6 \cdot 5H_2O$)	8,0 g
Water	100 ml

5.4.1.2.2 Preparation

Dissolve the calcium lactate pentahydrate in the water by heating. Sterilize in an autoclave set at 121 °C ± 1 °C for 15 min.

5.4.1.3 Calcium citrate suspension

5.4.1.3.1 Composition

Tricalcium dicitrate tetrahydrate ($C_{12}H_{10}Ca_3O_{14}{\cdot}4H_2O$)	13,3 g
Carboxymethylcellulose (CMC)	0,8 g
Water up to	100 ml

5.4.1.3.2 Preparation

Grind the calcium citrate tetrahydrate, which has previously been sifted through a sieve of 0,8 mm nominal aperture size (see ISO 565), and the CMC together in a mortar.

Slowly add prewarmed water at approximately 45 °C to a final volume of 100 ml. Blend the obtained mixture for 10 min and vacuum filter through cotton cloth. Sterilize the filtered suspension in an autoclave set at 121 °C ± 1 °C for 15 min.

NOTE Approximately 30 % of the calcium citrate is lost during filtration.

5.4.1.4 Starter culture serum

Prepare the starter culture medium by growing a L-, D- or LD-starter culture in autoclaved skimmed or autoclaved recombined skimmed milk at 25 °C ± 1 °C for 24 h.

Filter through filter paper and sterilize the filtrate at 115 °C ± 1 °C for 15 min. Remove any sediment by decantation and sterilize 200 ml of filtrate once again.

5.4.1.5 X-gal solution

CAUTION — X-gal and NMP are toxic and must be handled in a fume cupboard.

5.4.1.5.1 Composition

5-Bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside (X-gal)	400 mg
N-Methyl-2-pyrrolidone (NMP)	100 ml

5.4.1.5.2 Preparation

Dissolve the X-gal in NMP and sterilize by filtration (see 6.13) through a 0,45 µm Durapore filter (e.g. from Millipore)[1)]. Store the solution at 20 °C.

1) Millipore is the name of the producer of a product available commercially. This information is given for the convenience of the users of this International Standard and does not constitute an endorsement by either ISO or IDF of this product.

5.4.1.6 Complete medium

5.4.1.6.1 Composition

Basic medium (5.4.1.1)	900 ml
Calcium lactate solution (5.4.1.2)	100 ml
Calcium citrate suspension (5.4.1.3)	50 ml
Starter culture serum (5.4.1.4)	100 ml

5.4.1.6.2 Preparation

Just before use, melt the basic medium (5.4.1.1) in a boiling water bath. When melted, cool the basic medium to between 48 °C and 50 °C.

Prewarm the calcium lactate solution (5.4.1.2), the calcium citrate suspension (5.4.1.3) and the starter culture serum (5.4.1.4) in a water bath (6.6) set at between 48 °C and 50 °C. Aseptically, add each of them to the melted basic medium and mix.

5.4.2 Nickels and Leesment medium [4] plus vancomycin

5.4.2.1 Basic medium

See 5.4.1.1.

5.4.2.2 Calcium lactate solution

See 5.4.1.2.

5.4.2.3 Calcium citrate suspension

See 5.4.1.3.

5.4.2.4 Starter culture serum

See 5.4.1.4.

5.4.2.5 Vancomycin solution (of volume fraction 2 %)

CAUTION — Vancomycin can cause allergy through inhalation and skin contact. Pregnant and breastfeeding women must not work with this agent.

5.4.2.5.1 Composition

Vancomycin	360 mg to 440 mg
Water	20 ml

5.4.2.5.2 Preparation

Dissolve the vancomycin in the distilled water and sterilize by filtration.

The vancomycin solution may be stored at between 4 °C and 7 °C for 1 week or at −20 °C for 4 weeks.

5.4.2.6 Complete medium

5.4.2.6.1 Composition

Basic medium (5.4.1.1)	900 ml
Calcium lactate solution (5.4.1.2)	100 ml
Calcium citrate suspension (5.4.1.3)	50 ml
Starter culture serum (5.4.1.4)	100 ml
Vancomycin solution (5.4.2.5)	11,5 ml

5.4.2.6.2 Preparation

Before use, melt the basic medium (5.4.1.1) in a boiling water bath and cool to between 48 °C and 50 °C.

Prewarm the calcium lactate solution (5.4.1.2), the calcium citrate suspension (5.4.1.3), and the starter culture serum (5.4.1.4) respectively, to between 48 °C and 50 °C. Aseptically, add each of them to the melted basic medium.

Immediately before use, add 11,5 ml of vancomycin solution (5.4.2.5). Mix carefully and use the thus-prepared medium within 5 min.

5.5 Reagents for pH adjustment

5.5.1 **Sodium hydroxide** (NaOH), approximately 0,1 mol/l solution.

5.5.2 **Hydrochloric acid** (HCl), approximately 0,1 mol/l solution.

5.5.3 **Acetic acid** (CH_3COOH), glacial.

6 Apparatus and glassware

Usual microbiological laboratory apparatus, the apparatus required for the preparation of test samples and dilutions as specified in ISO 8261 | IDF 122 and, in particular, the following.

6.1 **Incubator**, capable of operating at 25 °C ± 1 °C.

6.2 **Blender**, either a peristaltic-type blender (stomacher) with sterile plastic containers, 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.

6.3 **Test tube agitator**, for example, a vortex mixer.

6.4 **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.

6.5 **Lens**, of magnification 8× to 10×.

6.6 **Water bath**, capable of operating at between 48 °C and 50 °C.

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

6.8 **Dilution bottles**, of capacity 150 ml to 250 ml, or **test tubes**, of diameter 18 mm and length 180 mm, with suitable seal cap or stopper made of rubber or synthetic material.

6.9 Flasks or bottles, of capacity 150 ml to 250 ml, and **test tubes**, of capacity about 20 ml, to hold the culture medium.

6.10 Automatic pipettes, with sterile tips, or **graduated pipettes**, calibrated to the tip, both capable of delivering 1 ml ± 0,02 ml, 10 ml ± 0,2 ml and 11 ml ± 0,2 ml respectively.

Presterilized pipettes made of synthetic materials may be used instead of glass pipettes.

6.11 Petri dishes, of diameter 90 mm and 140 mm respectively, of clear uncoloured glass or plastic, with minimal internal depth of 10 mm. The bottom shall have no irregularities that may interfere with the counting of colonies.

6.12 Glass spreader.

6.13 Apparatus for sterilization by filtration.

Sterilize equipment that will come into contact with the test sample, the diluent, the dilutions or the culture medium, in accordance with ISO 8261 | IDF 122.

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 Preparation and inoculation

8.1 Preparation of the test sample and test portion

See ISO 8261 | IDF 122.

SAFETY PRECAUTIONS — Before opening the container of the starter culture or the milk products, clean the external surface immediately surrounding the area from which the sample is to be taken, in order to remove any material that might contaminate the sample. The area may be swabbed with 70 % (volume fraction) ethanol to prevent further contamination. Open the container aseptically.

During the preparation stage, it is important to obtain not only a homogeneous dilution (see 6.2 and 6.3), but also a fragmentation of the chains of microorganisms into individual cells or short chains, so that the results, expressed as a total viable count per gram of product, are reproducible and representative.

8.2 Microscopic examination

Carry out a preliminary microscopic examination of several fields of a smear of the liquid or the first dilution of the dried and solid samples (see ISO 8261 | IDF 122), to select the proper range of dilutions to be used.

8.3 Preparation of primary dilution

See ISO 8261 | IDF 122.

Take care that the time lapse between the inoculation of the dilutions and pouring them into Petri dishes does not exceed 15 min (see ISO 7218).

8.4 Preparation of decimal dilutions

See ISO 8261 | IDF 122.

8.5 Inoculation

Transfer, using a sterile pipette (6.10), 1 ml of each appropriate dilution into each of two Petri dishes (6.11).

For the enumeration of different species of citrate-fermenting cocci (9.1), use the Petri dishes with a diameter of 140 mm. For the enumeration of leuconostocs (9.2), use the Petri dishes with a diameter of 90 mm.

9 Procedure

9.1 Enumeration of different species of citrate-fermenting cocci

9.1.1 Incubation

Pour 15 ml to 20 ml of complete Nickels and Leesment medium (5.4.2.6) into each 140 mm Petri dish (6.11). Immediately after pouring, carefully mix the inoculum with the medium by rotating the Petri dishes. Allow the mixture to solidify by leaving the Petri dishes to stand on a cool horizontal surface.

After solidification, add a further 4 ml to 5 ml of complete medium as a top layer in order to prevent spreading of colonies when X-gal is added (9.1.2).

Incubate the prepared dishes, in an inverted position, in an aerobic incubator (6.1) set at 25 °C for 72 h. In the case of non-citrate-fermenting lactic acid bacteria (O-type cultures), incubate the Petri dishes at 25 °C for 5 days. Stack not more than six high. Stacks of dishes should be separated from one another and from the walls and the top of the incubator.

9.1.2 Reading of the Petri dishes and counting of colonies

First reading: after incubation, choose Petri dishes with between 30 colonies and 300 colonies and examine all dishes carefully using the appropriate equipment (6.4 and 6.5). Sort out dishes showing large clear zones in which several colonies are represented. In these cases, it will not be possible to determine which colonies have produced the clear zone.

After sorting, count all colonies with or without clear zones. Separately count and mark all colonies with clear zones. After the counting, spread using a sterile glass spreader (6.12), 1,5 ml of X-gal solution (5.4.1.5) on the surface of each Petri dish. Incubate the dishes at room temperature inside a fume cupboard for 24 h.

Second reading: after the second incubation, count all blue colonies, with or without a clear zone.

9.1.3 Diagnostic characteristics

Leuconostoc species are blue, with or without a clear zone. *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* colonies are white with a clear zone. *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* colonies are white without a clear zone.

NOTE Some lactobacilli, pediococci and strains of *Streptococcus thermophilus* can also form blue colonies. Many lactobacilli and probably pediococci can utilize citrate and produce zones. Some of them can also produce sufficient acid around the colony to solubilize the citrate and give rise to false positives. Thus caution should be used for cheese samples. Microscopic examination or further testing can be used for confirmation of this.

9.2 Enumeration of leuconostocs

9.2.1 Incubation

Pour 12 ml to 15 ml of complete Nickels and Leesment medium plus vancomycin (5.4.2.6) into each of the 90 mm Petri dish (6.11). Immediately after pouring, carefully mix the inoculum with the medium by rotating the Petri dishes. Allow the mixture to solidify by leaving the Petri dishes to stand on a cool horizontal surface.

Incubate the prepared dishes, in an inverted position; in an aerobic incubator (6.1) set at 25 °C for 5 days. Stack not more than six high. Separate stacks of dishes from one another, from the walls and form the top of the incubator (see ISO 7218).

9.2.2 Reading of the Petri dishes

After incubation, choose Petri dishes having between 30 colonies and 150 colonies and count all colonies.

9.3 Confirmation

Select colonies from the plates used for counting so that the number taken is equal to the square root of the total colony count. Stain these colonies using the Gram method and confirm that they are non-spore-forming, Gram-positive, catalase-negative chains of cocci or diplococci.

NOTE Most Group 2 lactobacilli and pediococci are vancomycin resistant.

10 Calculation and expression of results

10.1 Calculation

10.1.1 Use counts from normal plates (of diameter 90 mm) containing between 30 colonies and 150 colonies and from large plates (of diameter 140 mm) containing between 30 colonies and 300 colonies.

10.1.2 Calculate the number of each characteristic microorganism, N, per gram, by using the following equation:

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

where

ΣC is the numerical value of the sum of colonies counted on the plates, as in 10.1.1;

n_1 is the numerical value of the number of plates counted at the lower dilution;

n_2 is the numerical value of the number of plates counted at the higher dilution;

d is the numerical value of the value corresponding to the dilution from which the higher counts were obtained.

In the case where there are more than two countable dilutions, modify the equation by taking the further dilution into account.

Thus for three dilutions, calculate N by using the following equation:

$$N = \frac{\sum C}{(n_1 + 0{,}1n_2 + 0{,}01n_3)d}$$

where n_3 is the numerical value of the number of plates counted in the third dilution.

10.2 Expression of results

10.2.1 Round the result obtained in 10.1.2 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.

EXAMPLE Round

— 234 to 230,

— 235 to 240,

— 225 to 220, and

— 245 to 240.

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

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

10.2.4 Express the test results as a number from 1,0 to 9,9 multiplied by the appropriate power of 10.

10.2.5 The total number of characteristic citrate fermenting lactic acid bacteria, N_b, per gram of product is equal to:

$$N_b = N_L + N_l$$

where

N_L is the numerical value of the number of *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* per gram, calculated in 10.1.2;

N_l is the numerical value of the number of leuconostocs per gram, calculated in 10.1.2.

10.3 Example of calculation

Assume that a count of citrate-fermenting lactic acid bacteria on the medium gave the following results (two Petri dishes per dilution were incubated):

— 10^{-5} dilution: 295 and 245 colonies

— 10^{-6} dilution: 33 and 40 colonies