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Milk products — Enumeration of presumptive bifidobacteria — Colony count technique at 37 °C

Produits laitiers — Dénombrement des bifidobacteria présumés — Technique par comptage des colonies à 37 °C

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

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 29981|IDF 220 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.

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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.

The main task of Standing Committees is to prepare International Standards. Draft International Standards adopted by the Standing Committees are circulated to the National Committees for endorsement prior to publication as an International Standard. Publication as an International Standard requires approval by at least 50 % of IDF National Committees casting a vote.

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All work was carried out by the Joint ISO-IDF Action Team on *Lactic acid bacteria and starters* of the Standing Committee on *Microbiology methods of analysis* under the aegis of its project leaders, Prof. W. Kneifel (AT) and Dr. U. Zitz (AT).

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Milk products — Enumeration of presumptive bifidobacteria — Colony count technique at 37 °C

1 Scope

This International Standard specifies a method for the selective enumeration of presumptive bifidobacteria in milk products by using a colony count technique at 37 °C under anaerobic conditions.

The method is applicable to milk products such as fermented and non-fermented milks, milk powders, infant formulae, and starter cultures where these microorganisms are present and viable, and in combination with other lactic acid bacteria. (For proposed quality criteria of dairy products, see, for example, Codex Stan 243:2003 [6].)

Bifidobacteria used in milk products usually belong to the species (e.g. see References [7][8][16]):

a) *Bifidobacterium adolescentis;*

b) *B. animalis* subsp. *animalis;*

c) *B. animalis* subsp. *lactis;*

d) *B. bifidum;*

e) *B. breve;*

f) *B. infantis;*

g) *B. longum.*

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2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cites applies. For undated references, the latest edition of the referenced documents (including any amendments) applies.

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 6887-5, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products*

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

ISO 7889|IDF 117, *Yogurt — Enumeration of characteristic microorganisms — Colony-count technique at 37 °C*

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ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

ISO 14461-1|IDF 169-1, *Milk and milk products — Quality control in microbiological laboratories — Part 1: Analyst performance assessment for colony counts*

ISO 14461-2|IDF 169-2, *Milk and milk products — Quality control in microbiological laboratories — Part 2: Determination of the reliability of colony counts of parallel plates and subsequent dilution steps*

3 Terms and definitions

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

3.1
bifidobacteria
anaerobic microorganisms that form lenticular or round whitish colonies, partially star shaped or trilobate of diameter 1 mm to 4 mm on transgalactosylated oligosaccharides-mupirocin lithium salt (TOS-MUP) medium under the conditions specified in this International Standard

4 Principle

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4.1 The antibiotic, mupirocin lithium salt (MUP), inhibits the growth of most lactic acid bacteria commonly used in fermented and non-fermented dairy products.

Owing to the proven selectivity of the MUP antibiotic when added to the medium, usually there is no growth of typical yogurt bacteria (*Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*), mesophilic cultures (e.g. *Lactococcus lactis*), *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus rhamnosus* on the medium specified.

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This property has been tested with a representative number of reference strains and isolates.

Additionally, TOS-agar enhances the growth of bifidobacteria used in dairy products (see Reference [17]).

NOTE 1 Examination under a microscope at a magnification of 100 times and oil immersion in contrast phase illumination shows rods of very varied shapes, usually curved and clubbed, often branched, arranged singly, in pairs, in V-shaped arrangements, in chains, in palisades of parallel cells, or in rosettes occasionally exhibiting swollen coccoid forms.

NOTE 2 Bifidobacteria are non-acid-fast, non-spore-forming, gram-positive, non-motile and catalase-negative chemoorganotrophs, which produce acetic acid and lactic acid. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate shunt in which fructose-6-phosphate phosphoketolase (F6PPK, EC 4.1.2.22) cleaves fructose-6-phosphate into acetyl phosphate and erythrose-4-phosphate.

NOTE 3 The optimum growth temperature is between 37 °C and 41 °C. For further details, see Reference [9].

4.2 Inoculation of appropriate decimal dilutions of the homogenized sample into TOS-agar containing MUP using the pour plate technique, is followed by anaerobic incubation at 37 °C for 72 h.

4.3 The colonies are counted.

NOTE Optionally, selected isolates from the plates can be confirmed by means of appropriate tests (e.g. F6PPK assay, see References [14][15]).

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

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5 Culture media, diluents and reagents

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

5.1 Basic materials

See ISO 6887-5 and ISO/TS 11133-1 for basic materials.

5.2 Diluent(s)

See ISO 6887-5 for the preparation of diluents.

To ensure comparability of the specified colony-forming unit (CFU) results, observe the following requirements.

a) Use quarter-strength Ringer's solution, or any other suitable diluent which is specified in ISO 6887-5 and proven to be equivalent.

b) Sterilize in bulk and use an adequate sterile dispenser unit.

c) Adjust the diluent to room temperature. Transfer the diluent by dripping, without incorporating air.

d) The uncertainty of measurement of volumes used shall be in accordance with the requirements of ISO 6887-1.

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5.3 Culture medium (TOS-MUP medium)

Use freshly prepared transgalactosylated oligosaccharides-mupirocin lithium salt (TOS-MUP) culture medium, which has not been exposed to direct sunlight.

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5.3.1 **Basic medium** (TOS-propionate agar medium, see Reference [10])

5.3.1.1 Composition

Trypticase peptone	10,0 g
Yeast extract	1,0 g
KH_2PO_4	3,0 g
K_2HPO_4	4,8 g
$(NH_4)_2SO_4$	3,0 g
$MgSO_4 \cdot 7H_2O$	0,2 g
(*R*)-Cysteine·HCl·H_2O	0,5 g
Sodium propionate	15,0 g
TOS (see 5.3.1.2)	10,0 g
Agar	15,0 g
Water	950 ml

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5.3.1.2 Transgalactosylated oligosaccharide mixture

A TOS mixture is obtained by enzymatic hydrolysis of lactose using *Aspergillus oryceae* β-galactosidase. The TOS mixture contains galactose (Gal) and glucose (Glc) units in accordance with the formula

$$[\mathrm{Gal}\overset{x}{-}(\mathrm{Gal})_n\overset{y}{-}\mathrm{Glc}]$$

where

$n = 1 \ldots 4$;

x = β-1,6 > β-1,4 and β-1,3;

y = β-1,4 ≫ β-1,3 and β-1,6.

The TOS mixture is purified by chromatography under defined conditions (see References [18][19]). The total sugar content (> 97 % mass fraction) includes a certain proportion of tri-, tetra-, penta- and hexasaccharides. (Modification of the ratio of oligosaccharides has no significant effect on the potential of the medium.)

5.3.1.3 Preparation

Suspend the ingredients in 950 ml water while heating carefully (e.g. using a hotplate or a water bath) with frequent agitation until completely dissolved.

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Distribute in portions of 190 ml into bottles of 250 ml capacity. Adjust the pH (6.6), if necessary, so that after autoclaving a final pH of 6,3 ± 0,2 pH-units is obtained at 25 °C.

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Autoclave the basic medium at 115 °C for 15 min.

If not used immediately, cool the prepared basic medium, unless otherwise specified. Store the medium between 2 °C and 4 °C for a maximum of 1 week under conditions not producing any change in its composition.

TOS-medium is sensitive to heat, thus excessive heat treatment can negatively influence the properties of the medium. Complete TOS-propionate media are commercially available and have a composition in accordance with this International Standard. However, if the medium is made up in the laboratory, the results can differ significantly from one preparation to another. Therefore media should be validated to ensure that growth performance of bifidobacteria, indicated by CFU results, are on a comparable level (see also ISO/TS 11133-1).

5.3.2 MUP supplement solution (see Reference [11])

Immediately before use, dissolve, for example, 50 mg MUP in 50 ml of water, or other amounts in the same proportion. Sterilize the solution obtained by filtration through a membrane (pore size 0,22 µm) as specified in 5.3.3.

5.3.3 Complete medium

Immediately before use, melt 190 ml portions of the prepared basic medium (5.3.1) under steam or equivalent. Cool it in a water bath (6.5) to 48 °C ± 1 °C. Add 10 ml of MUP supplement solution (5.3.2) to each portion by using a syringe equipped with a sterile filter unit of pore size 0,22 µm (6.11) shortly before pouring. Mix carefully while avoiding formation of air bubbles.

Put the completed medium back in the water bath (6.5) at 48 °C until it is ready to be poured.

The complete TOS-MUP medium shall have a final MUP concentration of 50 mg/l.

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6 Apparatus

Sterilization of equipment that comes into contact with the test sample, the diluent, the dilutions or the culture medium shall be carried out in accordance with the requirements of ISO 6887-5 as well as ISO/TS 11133-1. The glassware shall be resistant to repeated sterilization.

Use usual microbiological laboratory equipment (see ISO 7218) for the preparation of test samples and dilutions, as specified in ISO 6887-5. In particular, the following equipment is required.

6.1 Incubation equipment, conventional jars, or, alternatively, an anaerobic incubator.

6.1.1 Incubator, capable of maintaining a temperature of 37 °C ± 1 °C.

6.1.2 Anaerobic culture jars, providing an anaerobic atmosphere of volume fraction 10 % to 20 % of carbon dioxide; a volume fraction of approximately 70 % to 90 % of nitrogen; with a volume fraction of approximately 10 % of hydrogen (not obligatory). The gas mixture should not contain more than a volume fraction of 1 % of oxygen.

Other suitable and safety-proven low-temperature catalyst systems may be used.

6.1.3 Anaerobic incubator, capable of maintaining a temperature of 37 °C ± 1 °C, providing an anaerobic atmosphere (see 6.1.2).

6.2 Mechanical stirrer, capable of mixing or agitating the contents of test tubes, e.g. a vortex mixer.

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6.3 Colony-counting equipment, as specified in ISO 7218.

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6.4 Magnifying lens, of magnification 8 times to 10 times.

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6.5 Water baths, capable of maintaining temperatures of 20 °C ± 1 °C, 45 °C ± 1 °C, 48 °C ± 1 °C.

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

6.7 Flasks or bottles, of capacity 250 ml with suitable sealing caps or stoppers (to hold the culture medium as well as to prepare the initial dilution of the test sample).

6.8 Test tubes, of height about 150 mm and of diameter about 15 mm, equipped with suitable caps.

6.9 Graduated pipettes, for bacteriological use, sterilized and calibrated to the tip, capable of delivering 1 ml ± 0,02 ml and 10 ml ± 0,2 ml (see ISO 6887-1), respectively, ISO 835[20] class A.

6.10 Petri dishes, made of clear uncoloured glass or plastics, of diameter 90 mm and of minimum internal depth 10 mm. The bottom shall have no irregularities that may interfere with counting colonies.

6.11 Sterilization apparatus, for sterilization by filtration, 10 ml syringe equipped with a sterile filter unit of pore size 0,22 µm.

6.12 Autoclave, capable of maintaining a temperature of 115 °C ± 3 °C and equipped with short heating and cooling cycles.

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[1].

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