International Standard

ISO 29981

IDF 220

Milk products — Enumeration of bifidobacteria — Colony-count technique

Produits laitiers — Dénombrement des bifidobacteria présumés — Technique par comptage des colonies

Second edition
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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

ISO draws attention to the possibility that the implementation of this document may involve the use of (a) patent(s). ISO takes no position concerning the evidence, validity or applicability of any claimed patent rights in respect thereof. As of the date of publication of this document, ISO had not received notice of (a) patent(s) which may be required to implement this document. However, implementers are cautioned that this may not represent the latest information, which may be obtained from the patent database available at www.iso.org/patents. ISO shall not be held responsible for identifying any or all such patent rights.

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document 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.

This second edition cancels and replaces the first edition (ISO 29981 | IDF 220:2010), which has been technically revised.

The main changes are as follows:

— diluents which can be used have been added;

— preparation of the test portion and primary dilution in cases of dried milk products has been added;

— a new culture medium, TOS agar, has been introduced;

— storage of incubated plates has been included;

— expression of results has been changed to be in accordance with ISO 7218;

— performance testing of the culture media has been introduced;

— performance characteristics, with the results of an interlaboratory study, which are based on the method of this second edition, have been included as Annex C.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

IDF (the International Dairy Federation) is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/ food control authorities.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

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This document was prepared by IDF *Standing Committee on Methods for Dairy Microbiology* and ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by ISO and IDF.

The work was carried out by the IDF/ISO Action Team (D09) of the *Standing Committee on Methods for Dairy Microbiology* under the aegis of its project leader Masamichi Muto (JP).

Introduction

Bifidobacteria are non-acid-fast, non-spore-forming, Gram-positive, non-motile and catalase-negative chemoorganotrophs bacilli, which produce acetic acid, lactic acid and formic 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.

Many reports show that bifidobacteria have various physiological functions and bifidobacteria are widely applied to foods in milk products such as yoghurt, infant formula and milk powders, and also in non-milk products such as starter and probiotic cultures. Many bifidobacteria-containing products describe the bacterial cell counts on the product label which is an important indicator of the functionality. An accurate bifidobacteria enumeration method, such as the one given in this document, is important to guarantee the bacterial cell counts.

The main technical changes listed in the Foreword, introduced in this document compared to ISO 29981 | IDF 220:2010, are considered as major (see ISO 17468). These technical changes have a major impact on the performance characteristics of the method.

Milk products — Enumeration of bifidobacteria — Colony-count technique

1 Scope

This document specifies a method for the selective enumeration of 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 (e.g. yoghurts) and non-fermented milks (e.g. pasteurized milks, skim milks, whey protein concentrates), milk powders and formulae (e.g. infant formulae, follow-up formulae for older infants, products for young children) where these microorganisms are present and viable, in combination with other lactic acid bacteria or alone. The method is also applicable to starter and probiotic cultures. For proposed quality criteria of dairy products, see, for example, CXS 243-2003[6].

Bifidobacteria used in milk products usually belong to the following species (e.g. References [7] and [10]):

— *Bifidobacterium adolescentis*;

— *B. animalis* subsp. *animalis*;

— *B. animalis* subsp. *lactis*;

— *B. bifidum*;

— *B. breve*;

— *B. longum* subsp. *infantis*;

— *B. longum* subsp. *longum*.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 6887-1, *Microbiology of the food chain — 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 the food chain — 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 the food chain — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

ISO 19036:2019, *Microbiology of the food chain — Estimation of measurement uncertainty for quantitative determinations*

3 Terms and definitions

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

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
bifidobacteria
anaerobic microorganisms of the family *Bifidobacteriaceae*, usually capable of growth in/on TOS-MUP agar or TOS agar by forming typical colonies and displaying certain characteristics in microscopic examination

Note 1 to entry: The morphology of typical colonies of bifidobacteria in/on TOS-MUP agar and TOS agar is described in 9.6. The microscopic examination and other confirmation test are described in 9.7.

4 Principle

4.1 General

The enumeration of bifidobacteria requires three successive stages as specified in Annex A.

TOS-MUP agar contains the antibiotic mupirocin lithium salt (MUP), which inhibits the growth of most lactic acid bacteria commonly used in products, such as fermented and non-fermented milks (e.g. pasteurized milks, skim milk, whey protein concentrate), milk powders and infant formulae, as well as starter and probiotic cultures (see Reference [8]).

Owing to the proven selectivity of the MUP antibiotic when added to the base medium, usually there is no growth of typical yoghurt bacteria (*Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus*), mesophilic cultures (e.g. *Lactococcus lactis*), *Lactobacillus acidophilus, Lacticaseibacillus casei* and *Lacticaseibacillus rhamnosus* on the medium specified. This property has been tested with a representative number of reference strains and isolates.

For the enumeration of bifidobacteria from samples containing only bifidobacteria, TOS agar with or without the antibiotic MUP can be used.

4.2 Preparation of initial suspension and decimal dilutions

An initial dilution and decimal dilutions are prepared from the test sample.

4.3 Isolation and selection for confirmation

TOS-MUP agar or TOS agar is inoculated with a specified quantity of the test sample if the product is liquid, or of the initial suspension in the case of other products. Other plates are prepared under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

The dishes are incubated anaerobically at 37 °C for 72 h. Alternatively, if the colony size is large enough to count accurately, the dishes can be examined after 48 h incubation.

4.4 Confirmation

Colonies of presumptive bifidobacteria can be confirmed by microscopic examination and/or appropriate tests (e.g. F6PPK-assay, see References [11] and [14]).

Confirmation of presumptive bifidobacteria by microscopic examination is required, but is optional in the case of test samples containing only bifidobacteria.

4.5 Calculation

The number of bifidobacteria per millilitre or gram of the test sample is calculated from the number of confirmed typical colonies per dish.

5 Culture media and reagents

Follow current laboratory practices in accordance with ISO 7218. The composition of culture media and reagents and their preparation are specified in Annex B. For performance testing of culture media, follow the procedures in accordance with Clause B.5 and ISO 11133.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications. The usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following shall be used.

6.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave), as specified in ISO 7218.

6.2 Autoclave, capable of operating at a temperature of 115 °C ± 3 °C and equipped with short heating and cooling cycles.

6.3 Equipment for culture in an anaerobic atmosphere, as specified in ISO 7218, capable of operating at a temperature of 37 °C ± 1 °C, providing an anaerobic atmosphere of volume fraction 5 % 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.

6.4 Refrigerator (optional), capable of operating at 5 °C ± 3 °C.

6.5 Water baths, one capable of being maintained at 37 °C ± 1 °C and another capable of being maintained between 44 °C and 47 °C.

6.6 Sterile test tubes or flasks, of appropriate capacity. Bottles or flasks with non-toxic metallic or plastic screwcaps may be used.

6.7 pH meter, accuracy to within ±0,1 pH unit at 25 °C.

6.8 Sterile graduated pipettes or automatic pipettes, of nominal capacities 25 ml, 10 ml, 1 ml and 0,1 ml.

6.9 Sterile Petri dishes, vented, with a diameter of approximately 90 mm.

6.10 Peristaltic blender (stomacher), with sterile bags, possibly with a device for adjusting speed and time, as specified in ISO 7218.

6.11 Microscope (optional), preferably with phase-contrast, and with slides and cover slips, as specified by ISO 7218.

6.12 Colony-counting equipment with a magnifying lens (optional), e.g. 8 times to 10 times, as specified in ISO 7218.

7 Sampling

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

Recommended sampling techniques are given in ISO 707 | IDF 50 for milk and milk products.

It is important that the laboratory receives a sample that is representative of the product under consideration. The sample should not have been damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned: follow the procedures specified in ISO 6887-1 and ISO 6887-5. If there is no specific International Standard available, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 General

Take all necessary precautions to ensure sample preparation and examination in the laboratory are conducted under aseptic conditions (see ISO 7218).

Perform the procedures specified in 9.2 to 9.5 by gentle mixing, to limit exposure to aerobic conditions such as air bubbles.

9.2 Preparation of the test portion and primary dilution

9.2.1 Dried milk products (e.g. infant milk formulae) including dehydrated starter and probiotic cultures

Follow the procedures specified in ISO 6887-5 and the mentioned steps below.

Warm the bottles of diluent in the water bath (6.5) at 37 °C.

Massage the bag (by hand) to dissolve the test portion. Then mix in a peristaltic blender (6.10) for 2 min.

9.2.2 Non-dried fermented (e.g. yoghurt) and non-fermented milk-based products (e.g. pasteurized milks)

Follow the procedures specified in ISO 6887-5 and the mentioned steps below.

Thoroughly mix the contents of the closed sample container by repeatedly shaking and inverting it (preferably 10 times, with a movement of about 300 mm, for about approximately 7 s). If not possible, thoroughly mix the content with a sterile spatula or similar after opening the packaging to obtain homogeneous samples.

Take the test portion required using a sterile spatula or a pipette.

Prepare the test portion and initial suspension (primary dilution) as described by ISO 6887-1, using a diluent listed in ISO 6887-5 which is adjusted to laboratory ambient temperature.

Mix the content as described above or use peristaltic blender (6.10).

9.3 Microscopic examination of initial suspension or primary dilution (optional)

Carry out a preliminary microscopic examination of several fields of a smear of the liquid or the primary dilution (9.2) of the dried and solid samples to select the proper range of dilutions to be used, especially in those cases where the manufacturer gives no product information.

Alternatively, phase contrast microscopy (6.11) can be applied with staining.

9.4 Preparation of decimal dilution series

Follow the procedures specified in ISO 6887-1.

9.5 Inoculation and incubation

9.5.1 Take one sterile Petri dish (6.9). Using a sterile pipette (6.8), transfer to the dish 1 ml of the test sample or 1 ml of the appropriate dilution step. Repeat the procedure described with further dilutions if necessary, always using a fresh sterile pipette for each dilution.

If only the initial suspension is used, then inoculate two plates of this dilution (see ISO 7218).

For laboratories that do not operate under quality assurance principles, two plates per dilution shall be used in accordance with ISO 7218, to improve reliability of the results.

NOTE In order to restrict the range of enumeration to a given interval, especially if high numbers of microorganisms are foreseen (see CXS 243-2003[6]), it is possible to inoculate only the necessary decimal dilutions needed to facilitate proper enumeration (see Clause 10 and ISO 7218).

9.5.2 Add into each Petri dish approximately 12 to 15 ml of the agar medium (see Clause B.3 or Clause B.4) which has been prepared then cooled to 44 °C to 47 °C in the water bath (6.5). The time elapsing between the inoculation of the Petri dishes and the moment when the medium is poured into the dishes shall not exceed 15 min.

Mix the molten culture medium and the inoculum carefully to obtain a homogeneous distribution of the microorganisms within the medium. Allow the agar medium to solidify, with the Petri dishes standing on a cool surface.

9.5.3 The time between ending the preparation of the primary dilution (initial dilution ready-made) until addition of the agar medium shall not exceed 45 min.

9.5.4 After plates have solidified, invert the prepared dishes and transfer them into the equipment for culture in an anaerobic atmosphere (6.3). Incubate them at 37 °C for 72 h ± 3 h.

Alternatively, 48 h ± 3 h incubation can be applied for the test sample if the colony size is large enough to count accurately.

9.6 Counting of colonies

After 72 h ± 3 h or 48 h ± 3 h incubation, count the typical colonies (see ISO 7218 for general guidance on counting of typical colonies). Alternatively, store the dishes in the refrigerator for a maximum of 48 h (see ISO 7218). Typical colonies of bifidobacteria are lenticular or round whitish colonies, partially star shaped or trilobate of diameter 1 mm to 4 mm in/on TOS-MUP agar or TOS agar under the conditions specified in this document.

Count all plates of the selected dilutions by considering all colonies on the plate directly after completion of the incubation.

To facilitate counting, use the suitable colony-counting equipment (6.12). Avoid mistaken particles of undissolved sample or precipitated matter in dishes for pinpoint colonies. Examine doubtful objects carefully, using a lens of higher magnification (6.12) if required, to distinguish colonies from foreign matter.

9.7 Confirmation

Confirmation of presumptive bifidobacteria by microscope observation is required, but optional in the case of test samples containing only bifidobacteria.

Identify colonies of presumptive bifidobacteria by their whitish colour. Select at random five typical colonies from the plates used for microscopic confirmation. Optionally, a F6PPK-assay can be performed to confirm the results (see References [11] and [14]).

If shown to be reliable, miniaturized galleries for the biochemical identification of presumptive bifidobacteria may be used (see ISO 16140-4 or ISO 16140-6).

NOTE 1 Some strains of bifidobacteria can show differing colony sizes and appearances on the same plate. Most colonies of bifidobacteria give off an acetic acid odour.

NOTE 2 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.[13]

NOTE 3 Alternative procedures (see ISO 7218) can be used to confirm the isolate as bifidobacteria, provided that the suitability of the alternative procedure has been validated (see ISO 16140-4 or ISO 16140-6).

10 Expression of results

For the calculation of the results, follow the procedure(s) in accordance with ISO 7218. Calculate and report the results as the number of bifidobacteria in cfu per gram or per millilitre.

In cases where no colonies of the target organism have been detected, follow ISO 7218 for the expression of results for special cases.

11 Validation of the method

11.1 Validation in accordance with ISO 17468

This standardized reference method was validated in accordance with ISO 17468.

The performance characteristics of the method as derived from the interlaboratory study are described in 11.2.

11.2 Performance characteristics

The performance characteristics of the method (repeatability and reproducibility standard deviations) were determined in an interlaboratory study. It is possible that the values derived from the interlaboratory study are not applicable to concentration ranges and food categories other than those used in the study. All data are given in Annex C.

A summary of the interlaboratory repeatability standard deviations (s_r) is given in Table 1.

Table 1 — Summary of s_r values from the interlaboratory study

Food category	Food item	s_r values from the interlaboratory study			
		Low inoculation level	Intermediate inoculation level	High inoculation level	Median value of three inoculation levels
Heat-processed milk and dairy products	Yoghurt	0,03	0,04	0,03	0,03
Infant formula and infant cereals	Powdered formula	0,21	0,12	0,04	0,12
Infant formula and infant cereals	Starter and probiotic culture	0,06	0,07	0,08	0,07

A summary of the interlaboratory reproducibility standard deviations (s_R) is given in Table 2.

Table 2 — Summary of s_R values from the interlaboratory study

Food category	Food item	s_R values from the interlaboratory study			
		Low inoculation level	Intermediate inoculation level	High inoculation level	Median value of three inoculation levels
Heat-processed milk and dairy products	Yoghurt	0,05	0,08	0,12	0,08
Infant formula and infant cereals	Powdered formula	0,24	0,13	0,10	0,13
Infant formula and infant cereals	Starter and probiotic culture	0,30	0,12	0,12	0,12

12 Test report

The test report shall specify the following:

— the test method used, with reference to this document, i.e. ISO 29981 | IDF 220:2024;

— the sampling method used, if known;

— all operating conditions not specified in this document, or regarded as optional, together with details of any incidents which can have influenced the test result(s);

— any deviations from this document;

— all information necessary for the complete identification of the sample;

— the incubation time and culture medium used;

— the test result(s) obtained;

— the date of the test;

— when necessary, or if requested by the client, an estimate of the measurement uncertainty of quantitative test results, in accordance with ISO 19036:2019, Clause 9.

13 Quality assurance

The laboratory should have a quality control system to ensure that the equipment, reagents and techniques are suitable for the method. The use of positive controls, negative controls and blanks are part of the method. Performance testing of culture media is specified in Annex B and described in ISO 11133.

Annex A
(normative)

Flow diagram of the procedure

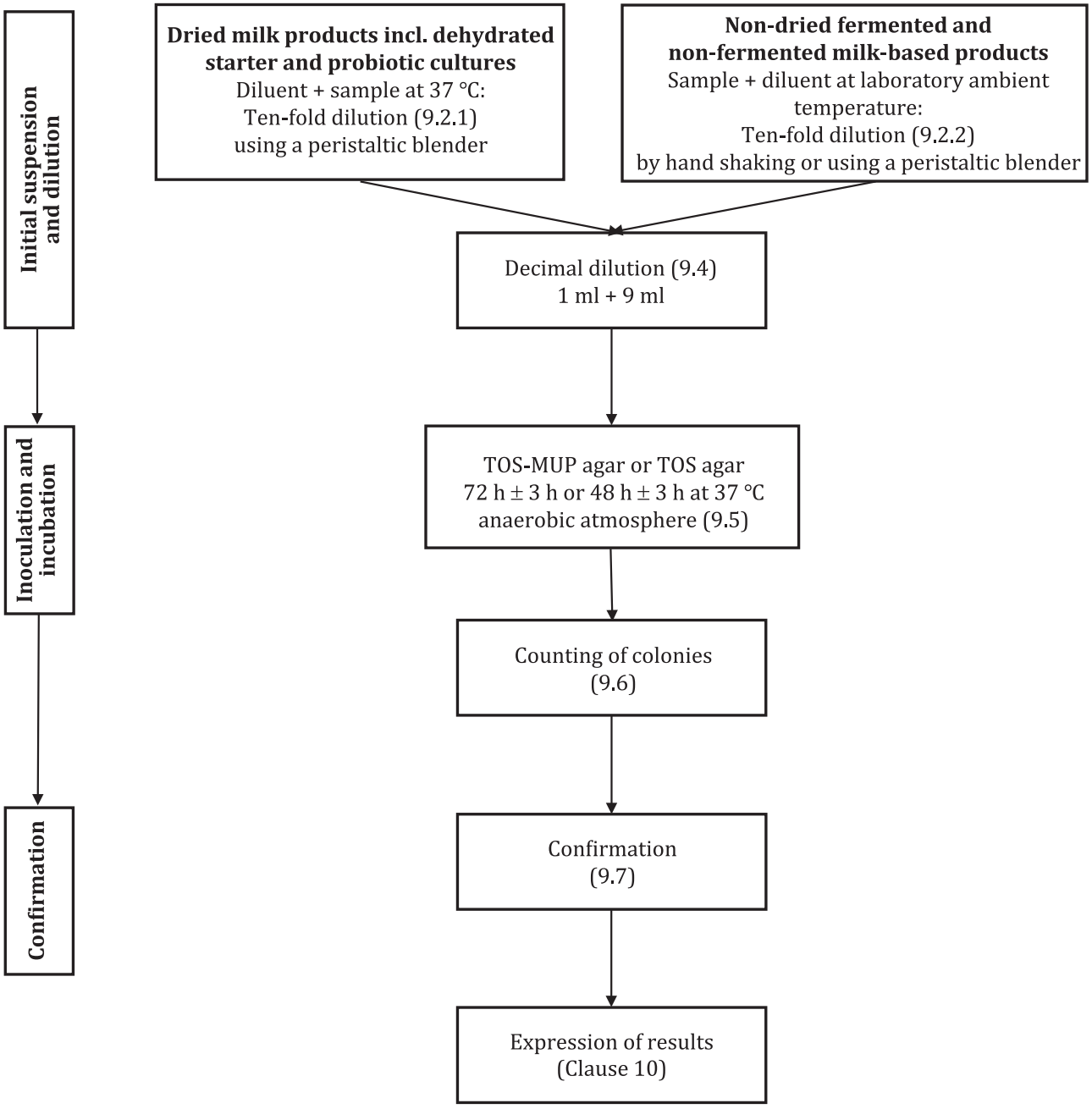

Figure A.1 — Flow diagram of the procedure for enumeration of bifidobacteria

Annex B
(normative)

Culture media and reagents

B.1 General

The general specifications of ISO 11133 are applicable to the preparation and performance testing of the culture media described in this annex. If culture media or reagents are prepared from dehydrated complete media/reagents or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf life of the media indicated in this annex has been determined in some studies. The user shall verify these under their own storage conditions (in accordance with ISO 11133).

Performance testing of culture media is described in Clause B.5.

B.2 Diluent(s)

Follow the procedures as specified in ISO 6887-5.

B.3 TOS-MUP agar (transgalactosylated oligosaccharides-mupirocin lithium salt agar)

B.3.1 Base medium

NOTE See References [5] and [12].

B.3.1.1 Composition

Enzymatic digest of casein		10,0 g
Yeast extract		1,0 g
KH_2PO_4	CAS[b] RN® 7778-77-0	3,0 g
K_2HPO_4	CAS RN® 7758-11-4	4,8 g
$(NH_4)_2SO_4$	CAS RN® 7783-20-2	3,0 g
$MgSO_4 \cdot 7H_2O$	CAS RN® 0034-99-8	0,2 g
(*R*)-cysteine·HCl·H_2O	CAS RN® 7048-04-6	0,5 g
Sodium propionate	CAS RN® 137-40-6	15,0 g
TOS (see B.3.1.2)		10,0 g
Agar		9 g to 15 g[a]
Water		950 ml

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

[b] CAS Registry Number® is a trademark of the American Chemical Society (ACS). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

B.3.1.2 Transgalactosylated oligosaccharide (TOS) mixture

A TOS mixture is obtained by enzymatic hydrolysis of lactose using Aspergillus oryzae β-galactosidase. The TOS mixture contains galactose (Gal) and glucose (Glc) units in accordance with Formula (B.1):

$$[\mathrm{Gal}\overset{x}{-}(\mathrm{Gal})_n\overset{y}{-}\mathrm{Glc}] \quad \text{(B.1)}$$

where

n 1 … 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 [15] and [16]). 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 TOS-MUP agar or TOS agar.

B.3.1.3 Preparation of the base medium

Dissolve the components or the dehydrated complete base in 950 ml water by boiling carefully (e.g. using a water bath or flowing steam) with frequent agitation until completely solved.

Adjust the pH, if necessary, so that after autoclaving it is 6,7 ± 0,2 at 25 °C.

Dispense the medium in quantities of 190 ml or another appropriate volume into flasks or bottles (6.6) of appropriate capacity.

Sterilize for 15 min in the autoclave (6.2) set at 115 °C.

If the base medium is used immediately, cool it in a water bath (6.5) maintained at 44 °C to 47 °C before adding MUP supplement solution, see B.3.3.

If not, allow the base medium to solidify in the flask or bottle (6.6) and store it in the dark at a temperature of 5 °C (6.4) for no longer than four weeks, under conditions that do not allow any changes in its composition and properties. Before use, melt the base medium completely in a boiling water bath, then cool it in the water bath (6.5) maintained at 44 °C to 47 °C.

B.3.2 MUP solution

Immediately before use, dissolve, for example, 50 mg MUP (lithium mupirocin; CAS RN® 73346-79-9) in 50 ml of water, or other amounts in the same proportion. Mix and sterilize by filtration through filter pore size 0,2 µm.

B.3.3 Complete TOS-MUP agar medium

B.3.3.1 Composition

Base medium (B.3.1)	190 ml
MUP solution (B.3.2)	10 ml

If not freshly prepared, melt the base medium, then let it cool down to 44 °C to 47 °C in a water bath (6.5).

Under aseptic conditions, add 10 ml of the MUP supplement solution (B.3.2) to each portion of 190 ml of base medium. Mix well by rotation to minimize foaming. The final concentration of MUP in the complete medium is 50 mg/l. Other volumes can be used when the final concentration is 50 mg/l.

TOS-MUP agar is sensitive to heat, thus excessive heat treatment can negatively influence the properties of the medium. Use the molten medium within 4 h of its preparation.

B.4 TOS agar (transgalactosylated oligosaccharides agar)

NOTE See Reference [5].

B.4.1 Composition

Enzymatic digest of casein		10,0 g
Yeast extract		1,0 g
KH_2PO_4	CAS RN® 7778-77-0	3,0 g
K_2HPO_4	CAS RN® 7758-11-4	4,8 g
$(NH_4)_2SO_4$	CAS RN® 7783-20-2	3,0 g
$MgSO_4 \cdot 7H_2O$	CAS RN® 0034-99-8	0,2 g
(*R*)-cysteine·HCl·H_2O	CAS RN® 7048-04-6	0,5 g
Sodium propionate	CAS RN® 137-40-6	15,0 g
TOS (see B.3.1.2)		10,0 g
Agar		9 g to 15 g[a]
Water		1 000 ml

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

B.4.2 Preparation

Dissolve the components or the dehydrated complete base in 1 000 ml water by boiling carefully (e.g. using a water bath or flowing steam) with frequent agitation until completely solved.

Adjust the pH, if necessary, so that after autoclaving it is 6,7 ± 0,2 at 25 °C.

Dispense the medium in appropriate flasks or bottles (6.6) of appropriate capacity.

Sterilize for 15 min in the autoclave (6.2) set at 115 °C.

If the base medium is used immediately, cool it in a water bath (6.5) maintained at 44 °C to 47 °C.

If not, allow the base medium to solidify in the flask or bottle (6.6) and store it in the dark at a temperature of 5 °C (6.4) for no longer than four weeks, under conditions that do not allow any changes in its composition and properties. Before use, melt the base medium completely in a boiling water bath, then cool it in the water bath (6.5) maintained at 44 °C to 47 °C.

TOS agar is sensitive to heat, thus excessive heat treatment can negatively influence the properties of the medium. Use the molten medium within 4 h of its preparation.

B.4.3 Preparation using TOS-MUP agar base medium (B.3.1)

Prepare TOS-MUP agar base medium as described in B.3.1. Under aseptic conditions, add 10 ml of sterile water (instead of MUP solution) to each portion of 190 ml of base medium as described in B.3.3. Mix well by rotation to minimize foaming. Other volumes can be used when the final composition is as given by B.4.1.

B.5 Performance testing

The definition of selectivity and productivity is specified in ISO 11133. Test the performance of the culture media in accordance with the methods and criteria as described in ISO 11133. Table B.1 provides the performance testing for the quality assurance of TOS-MUP agar and TOS agar.

Table B.1 — Performance testing for the quality assurance of TOS-MUP agar and TOS agar

Medium	Function	Incubation	Control strains	WDCM numbers[a]	Reference medium	Method of control	Criteria[c]
TOS-MUP agar	Productivity	72 h ± 3 h or 48 h ± 3 h/ 37 °C ± 1 °C anaerobic atmosphere	*Bifidobacterium animalis* subsp. *lactis*[b]	00223	Media batch TOS-MUP agar already validated	Quantitative	$P_R \geq 0,70$
			Bifidobacterium breve[b]	00224			
			Bifidobacterium longum subsp. *longum*[b]	00225			
	Selectivity		*Lactobacillus delbrueckii* subsp. *bulgaricus*[d] or *Lacticaseibacillus casei*[d]	00102 or 00100	—	Qualitative	Total inhibition (0)
TOS agar	Productivity	72 h ± 3 h or 48 h ± 3 h/ 37 °C ± 1 °C anaerobic atmosphere	*Bifidobacterium animalis* subsp. *lactis*[b]	00223	Media batch TOS agar already validated	Quantitative	$P_R \geq 0,70$
			Bifidobacterium breve[b]	00224			
			Bifidobacterium longum subsp. *longum*[b]	00225			

[a] Refer to the reference strain catalogue on http://www.wfcc.info for information on culture collection strain numbers and contact details. WDCM: World Data Centre for Microorganisms.

[b] Strains to be used as a minimum.

[c] Growth is categorized as 0: no growth; 1: weak growth (partial inhibition); 2: good growth. P_R = productivity ratio (see ISO 11133).

[d] Strain free of choice; one of the strains shall be used as a minimum.

Annex C
(informative)

Performance characteristics of the method

An interlaboratory study involving 15 laboratories in 7 countries was carried out. The following food items were included in the study: yoghurt, infant formula in powder form, as well as starter and probiotic culture. The food items were each tested at three different concentration levels. The study was organized in 2023 by ADRIA (France) and Morinaga Milk Industry (Japan) as part of the IDF/ISO Action Team (D09) of the *Standing Committee on Methods for Dairy Microbiology*. The full report is available in the Bulletin of the IDF n°530[9].

The method submitted to the interlaboratory study was that of this document (i.e. ISO 29981 | IDF 220:2024).

Data obtained by some collaborators have been excluded from the calculations only on the basis of clearly identified technical reasons (e.g. deviations from the protocol).

The values of the performance characteristics, for each food item and category, derived from this interlaboratory study are shown in Tables C.1 to C.3, and were calculated in accordance with ISO 17468.

Table C.1 — Results of data analysis obtained with yoghurt (food category: heat-processed milk and dairy products)

Parameter	Concentration level		
	Low	Medium	High
Number of participating collaborators	21	21	21
Number of collaborators retained after evaluation of the data	19	19	19
Number of samples	42	42	42
Number of sample results retained after evaluation of the data	38	38	38
Mean value Σa ($\log_{10}$ cfu/g)	4,11	6,10	8,13
Interlaboratory repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,03	0,04	0,03
Interlaboratory reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,05	0,08	0,12
NOTE Strain used for inoculation: *Bifidobacterium animalis* subsp. *lactis* (CNCM I-2494).			

Table C.2 — Results of data analysis obtained with powdered formula (food category: infant formula and infant cereals)

Parameter	Concentration level		
	Low	Medium	High
Number of participating collaborators	20	20	20
Number of collaborators retained after evaluation of the data	17	17	17
Number of samples	40	40	40
Number of sample results retained after evaluation of the data	34	34	34
Mean value Σa ($\log_{10}$ cfu/g)	5,99	7,04	9,02
Interlaboratory repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,21	0,12	0,04
Interlaboratory reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,24	0,13	0,10
NOTE Strain used for inoculation: *Bifidobacterium breve* M-16V (MCC-1851).			

Table C.3 — Results of data analysis obtained with starter and probiotic culture (food category: infant formula and infant cereals)

Parameter	Concentration level		
	Low	Medium	High
Number of participating collaborators	19	19	19
Number of collaborators retained after evaluation of the data	13	13	13
Number of samples	38	38	38
Number of sample results retained after evaluation of the data	26	26	26
Mean value Σa ($\log_{10}$ cfu/g)	8,10	10,29	11,31
Interlaboratory repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,06	0,07	0,08
Interlaboratory reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,30	0,12	0,12
NOTE Strain used for inoculation: *Bifidobacterium animalis* subsp. *lactis.*			

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