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

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



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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.

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.

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).

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*.

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 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*

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

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.

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.

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.

- Use quarter-strength Ringer's solution, or any other suitable diluent which is specified in ISO 6887-5 and proven to be equivalent.
- Sterilize in bulk and use an adequate sterile dispenser unit.
- Adjust the diluent to room temperature. Transfer the diluent by dripping, without incorporating air.
- The uncertainty of measurement of volumes used shall be in accordance with the requirements of ISO 6887-1.

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.

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 ₂ PO ₄	3,0 g
K ₂ HPO ₄	4,8 g
(NH ₄) ₂ SO ₄	3,0 g
MgSO ₄ ·7H ₂ O	0,2 g
(<i>R</i>)-Cysteine·HCl·H ₂ O	0,5 g
Sodium propionate	15,0 g
TOS (see 5.3.1.2)	10,0 g
Agar	15,0 g
Water	950 ml

5.3.1.2 Transgalactosylated oligosaccharide 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 the formula

$$[\text{Gal} \xrightarrow{x} (\text{Gal})_n \xrightarrow{y} \text{Glc}]$$

where

$$n = 1 \dots 4;$$

$$x = \beta\text{-}1,6 > \beta\text{-}1,4 \text{ and } \beta\text{-}1,3;$$

$$y = \beta\text{-}1,4 \gg \beta\text{-}1,3 \text{ and } \beta\text{-}1,6.$$

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.

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 $6,3 \pm 0,2$ pH-units is obtained at 25 °C.

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 $48 \text{ }^{\circ}\text{C} \pm 1 \text{ }^{\circ}\text{C}$. 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.

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 $37\text{ °C} \pm 1\text{ °C}$.

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 $37\text{ °C} \pm 1\text{ °C}$, 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.

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

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

6.5 Water baths, capable of maintaining temperatures of $20\text{ °C} \pm 1\text{ °C}$, $45\text{ °C} \pm 1\text{ °C}$, $48\text{ °C} \pm 1\text{ °C}$.

6.6 pH meter, with temperature compensation, accurate to $\pm 0,1$ 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 $1\text{ ml} \pm 0,02\text{ ml}$ and $10\text{ ml} \pm 0,2\text{ ml}$ (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 $0,22\text{ }\mu\text{m}$.

6.12 Autoclave, capable of maintaining a temperature of $115\text{ °C} \pm 3\text{ °C}$ 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].

8 Procedure

8.1 General

The following procedures are based on corresponding standards taking into account the recommendations given, for example, in References [8][12].

Perform the procedures specified in 8.2 to 8.5 by gentle mixing, avoiding air formation or inclusion of gas bubbles, and not under direct sunlight.

Before opening the sample container, clean the external surface surrounding of the area from which the test sample is to be taken, in order to remove any material that might contaminate the sample. Swab the area with 70 % (volume fraction) ethanol, for example, to prevent further contamination. Open the container aseptically.

8.2 Preparation of the test portion and primary dilution

8.2.1 Dried milk products, e.g. infant milk formulae

Proceed as described in steps a) to i) (see also ISO 6887-5).

- a) Thoroughly mix the content of the closed packaging by repeatedly shaking and inverting it.
- b) Open the packaging, withdraw the test sample required with a sterile spatula and proceed as indicated in the following. Immediately reclose the bag. It is recommended to use an airtight clip and to put the bag inside a tight glass jar to enable storage at 4 °C.
- c) Weigh $90 \text{ g} \pm 0,1 \text{ g}$ of diluent in each of the 250 ml pre-sterilized bottles (6.7). Close the bottles.
- d) Warm the 250 ml bottles containing the 90 g of diluent in the water bath (6.5) at 45 °C.
- e) Weigh, to the nearest 0,05 g, $10 \text{ g} \pm 0,1 \text{ g}$ of test sample. Add the weighed test portion to the diluent in each bottle at 45 °C. Alternatively, weigh 10 g of the test sample directly into the bottle with the diluent at 45 °C.
- f) To dissolve the test portion, swirl slowly to wet the powder. Then shake the bottle 10 times, with a movement of about 300 mm, for approximately 7 s.
- g) Place the bottles in the water bath (6.5) at 45 °C for 5 min while shaking occasionally.
- h) Immediately cool under running tap water while shaking for 2 min. Rapidly adjust to room temperature, e.g. by using a water bath (6.5) at 20 °C.
- i) Start the examination as quickly as possible (see also 8.5).

IMPORTANT — To obtain acceptable repeatability of the method, observe strictly the procedures specified in steps a) to i).

8.2.2 Probiotic yogurt or yogurt-like products

Proceed as described in the following steps a) to g) (see also ISO 7889|IDF 117).

- a) Adjust the diluent to room temperature.
- b) Weigh $90 \text{ g} \pm 0,1 \text{ g}$ of the diluent in each of the 250 ml pre-sterilized bottles (6.7). Close the bottles.

- c) Thoroughly mix the contents of the closed sample package by repeatedly shaking and inverting it (preferably 10 times, with a movement of about 300 mm, for approximately 7 s) — or, if not possible, thoroughly mix the content with a sterile spatula or similar after opening the packaging to get homogeneous samples.
- d) Open the packaging, withdraw the test sample required using a sterile spatula or a pipette and proceed as indicated in the following.
- e) Weigh, to the nearest 0,05 g, 10 g \pm 0,1 g, of test sample. Add the weighed sample to the diluent in each bottle.
- f) Shake the bottle 10 times, with a movement of about 300 mm, for approximately 7 s.
- g) Start the examination as quickly as possible (see also 8.5).

After the preparation of the primary dilution (sample suspension = 1st decimal dilution, D1; 100 ml), prepare the dilution steps immediately.

8.3 Microscopic examination

Carry out a preliminary microscopic examination of several fields of a smear of the liquid or the primary dilution (8.2) 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 can be applied without staining.

8.4 Preparation of decimal dilutions

For general requirements, see ISO 6887-1. For special requirements of bifidobacterial growth, take into account the following.

The described operation shall be carried out by gentle mixing under the standardized conditions specified below.

Prepare the dilutions as follows.

- a) Shake the primary dilution (8.2) preferably 10 times, with a manual movement of about 300 mm for approximately 7 s to obtain homogeneity.
- b) Pipette (6.9) 1 ml of the primary dilution (bacterial initial suspension) into a test tube (6.8) containing 9 ml of the sterile diluent at the appropriate temperature (see also 5.2).
- c) Thoroughly mix the dilution for 3 s by using a vortex mixer (6.2). For further dilution steps proceed in the same manner until the required working density of 200 CFU/ml to 500 CFU/ml is obtained. Always mix in the same manner, e.g. one period of 3 s. For each dilution step use a fresh sterile pipette (6.9).

Avoid filling the pipette with air bubbles. Take care to drain the pipettes completely, especially at higher sample concentrations.

8.5 Inoculation

Transfer by dripping, 1 ml of each of the appropriate dilution steps (4 decimal dilution steps within the countable area are recommended) into each empty Petri dish with two replicate plates per dilution. Pour 12 ml to 15 ml of the medium (5.3.3) into the Petri dishes. Mix the medium gently with the diluent by moving the Petri dishes in circular movements without incorporating air.

NOTE In order to restrict the range of enumeration to a given interval, especially if high numbers of microorganisms are foreseen (see Codex Stan 243:2003 [6]), it is possible to inoculate only the necessary decimal dilutions (at least two successive dilutions) needed to facilitate proper enumeration (see 9.1 and ISO 7218).

Alternatively, apply automated spread preparation techniques, if validated with reference to this International Standard.

8.6 Duration of the procedure

The time between ending the preparation of the primary dilution (initial dilution ready-made) until addition of culture medium shall not exceed 15 min (see Reference [8]).

8.7 Incubation

Immediately after solidification of the medium, invert all Petri dishes in the anaerobic culture jar or anaerobic incubator (6.1) and incubate at 37 °C for 72 h ± 3 h.

8.8 Counting of the colonies

Count the colonies after incubation by considering only the dilution steps within the countable area [i.e. dilutions for which the expected average count per plate, $\bar{x} \leq 300$ CFU (see also ISO 7218)].

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

Examine the plates under subdued light. To facilitate counting, use the suitable colony-counting equipment (6.3). Avoid mistaken particles of undissolved sample or precipitated matter in dishes for pinpoint colonies. Examine doubtful objects carefully, using a lens of higher magnification if required, to distinguish colonies from foreign matter.

After incubation, immediately examine the dishes, if possible. Alternatively, store the dishes in the refrigerator for 48 h maximum (see ISO 7218).

8.9 Reading of the Petri dishes — confirmation

Identify bifidobacterial colonies by their whitish colour. Select typical colonies (see 3.1) from the plates used for counting and examine microscopically.

Optionally, a F6PPK-assay can be performed to confirm the results (see References [14][15]).

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

9 Calculation and expression of results

9.1 Calculation

Use all counts from plates originating from the dilution steps within the countable area as obtained in 8.7. The countable area includes all dilutions for which the expected average count per plate, $\bar{x} \leq 300$ CFU.

Calculate the number of CFU of presumptive bifidobacteria per gram of product, N , using the equation

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

where

$\sum x_i$ is the sum of colonies counted on all dishes retained (8.8);

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

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

n_3 is the number of dishes retained in the third dilution;

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

If there are only two countable dilutions, modify the equation to

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

Determine the reliability of the colony counts obtained from parallel plates and subsequent dilution steps according to ISO 14461-2|IDF 169-2. For the calculation of the result, use only reliable counts (see also ISO 7218).

9.2 Expression of results

Express the results to two significant figures as the number of CFU of bifidobacteria per gram of product, representing a number between 1,0 and 9,9 multiplied by the appropriate power of 10. For the validity of results see 10.4.

If the last figure is below 5, the preceding figure is not modified. If the last figure is 5 or more, increase the preceding figure by one unit. Proceed stepwise until two significant figures are obtained (see ISO 7218).

EXAMPLE 1 Assuming that a count of bifidobacteria on the medium gave the results shown below (two Petri dishes per dilution incubated), the final result can be calculated as shown.

Table 1 — Example 1

Dilution	Plate 1	Plate 2
D4 (decimal dilution 10^{-4})	300	298
D5 (decimal dilution 10^{-5})	30	25
D6 (decimal dilution 10^{-6})	2	3

Insertion of the figures into Equation (1) gives

$$N = \frac{\sum x_i}{(n_1 + 0,1n_2 + 0,01n_3)d} = \frac{300 + 298 + 30 + 25 + 2 + 3}{(2 + 0,1 \times 2 + 0,01 \times 2) \times 10^{-4}} =$$

$$N = \frac{658}{2,22 \times 10^{-4}} = 296 \times 10^4 = 3,0 \times 10^6$$

EXAMPLE 2 Assuming that a count of bifidobacteria on the medium gave the results shown below (two Petri dishes per dilution incubated), the final result can be calculated as shown.

Table 2 — Example 2

Dilution	Plate 1	Plate 2
D5 (decimal dilution 10^{-5})	311	286
D6 (decimal dilution 10^{-6})	27	21
D7 (decimal dilution 10^{-7})	2	0

Insertion of the figures into Equation (1) gives

$$N = \frac{\sum x_i}{(n_1 + 0,1n_2 + 0,01n_3)d} = \frac{311 + 286 + 27 + 21 + 2 + 0}{(2 + 0,1 \times 2 + 0,01 \times 2) \times 10^{-5}} =$$

$$N = \frac{647}{2,22 \times 10^{-5}} = 291 \times 10^5 = 2,9 \times 10^7$$

10 Precision

10.1 Interlaboratory test

Details of an interlaboratory test done in 2006 on the precision of the method are summarized in Annex A (see also References [12][13]). The repeatability and the reproducibility limits were determined by using a powdered probiotic infant milk formula as well as six different probiotic yogurt products containing various bifidobacterial strains commercially used both in Europe and Japan.

The values derived from the interlaboratory test may not be applicable to concentration ranges and matrices other than those given. The tested concentration ranges of the bifidobacterial strains within the selected products are representative for the worldwide market and in accordance with Codex Stan 243:2003 [6].

10.2 Repeatability

The repeatability is the closeness of agreement between successive and independent results obtained by the same method on identical test material, under the same conditions (apparatus, operator, laboratory and short intervals of time); i.e. *repeatability conditions*.

The repeatability limit, r , is the value less than or equal to which the absolute difference between two test results (presumptive bifidobacteria per gram, converted to logarithms to the base 10) obtained under repeatability conditions is expected to lie with a probability of 95 % (see ISO 3534-1 [2], ISO 5725-1 [3], ISO 5725-2 [4] and ISO 16140 [5]).

Table 3, second column from the right, shows the repeatability of different milk products obtained in the ring trial in 2006, expressed as repeatability limit, r . These results were calculated by applying the robust analyses in ISO 16140 [5] taking into account all variations (deviations) reflecting usual and practical conditions.

10.3 Reproducibility

The reproducibility is the closeness of agreement between single test results on identical test material using the same method and obtained by operators in different laboratories using different equipment; i.e. *reproducibility conditions*.

The reproducibility limit, R , is the value less than or equal to which the absolute difference between two test results (presumptive bifidobacteria CFU per gram, converted to logarithms to the base 10) obtained under reproducibility conditions is expected to lie with a probability of 95 % (see ISO 3534-1 [2], ISO 5725-1 [3], ISO 5725-2 [4] and ISO 16140 [5]).

Table 3, rightmost column, shows the reproducibility of different milk products obtained in the ring trial in 2006, expressed as reproducibility limit, R . These results were calculated by applying the robust analyses in ISO 16140 [5] taking into account all variations (deviations) reflecting usual and practical conditions.

Table 3 — Repeatability limits, r robust, and reproducibility limits, R robust

Product	Description	Type	r lg(CFU/g)	R lg(CFU/g)
Yogurt 1	Commercial European probiotic yogurt product containing <i>B. animalis</i> subsp. <i>lactis</i> , <i>L. acidophilus</i> , <i>S. thermophilus</i>	Liquid	0,115	0,227
Yogurt 2	Commercial European probiotic yogurt product containing <i>B. animalis</i> subsp. <i>lactis</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>S. thermophilus</i>	Firm	0,182	0,389
Yogurt 3	Commercial European probiotic yogurt product containing <i>B. animalis</i> subsp. <i>lactis</i> , <i>L. casei</i> , <i>S. thermophilus</i>	Liquid	0,123	0,538
Yogurt 4	Commercial Asian probiotic yogurt product containing <i>B. breve</i> , <i>L. casei</i> , <i>S. thermophilus</i>	Liquid	0,118	0,400
Yogurt 5	Commercial Asian probiotic yogurt product containing <i>B. longum</i> , <i>L. gasseri</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>S. thermophilus</i>	Firm	0,543	0,543
Yogurt 6	Commercial Asian probiotic yogurt product containing <i>B. animalis</i> subsp. <i>lactis</i> , <i>L. acidophilus</i> , <i>S. thermophilus</i>	Firm	0,213	0,291
Infant milk	Commercial probiotic infant milk product containing <i>B. animalis</i> subsp. <i>lactis</i>	Powder	0,221	0,529

10.4 Precision data collectively defined for dairy products

Based on the similarities of the results obtained for some of the products, the precision data of the method were defined, thereby distinguishing between three different types of products (see Table 4).

The results obtained for yogurt 5 exhibited irregularities possibly due to inhomogeneity and were excluded from the calculation (see References [12][13] for more details).

Depending on the type of product, apply the following precision data (repeatability limits as well as reproducibility limits, Table 4):

- a) liquid yogurts, including all yogurt drinks or similar;
- b) firm and creamy yogurts (including set-style yogurts);
- c) powder products, including infant milk formulae or similar.

Table 4 — Precision data collectively defined for the product types

Product type	s_r collective lg(CFU/g)	s_R collective lg(CFU/g)	r lg(CFU/g)	R lg(CFU/g)
Liquid	0,042	0,139	0,12	0,39
Firm	0,071	0,144	0,20	0,40
Powder	0,079	0,189	0,22	0,53

Further explanations with reference to practical applications are given in the example.

EXAMPLE Examination of a yogurt product:

Table 5 — 1st examination

Dilution	Plate 1	Plate 2
D5 (decimal dilution 10^{-5})	128	145
D6 (decimal dilution 10^{-6})	9	11
D7 (decimal dilution 10^{-7})	0	0

Insertion of the figures into Equation (1) gives

$$N_1 = \frac{\sum x_i}{(n_1 + 0,1n_2 + 0,01n_3)d} = \frac{128 + 145 + 9 + 11}{(2 + 0,1 \times 2) \times 10^{-5}} = 133,2 \times 10^5 = 1,332 \times 10^7$$

$$\lg N_1 = \lg(1,332 \times 10^7) = 7,13$$

Table 6 — 2nd examination

Dilution	Plate 1	Plate 2
D5 (decimal dilution 10^{-5})	186	171
D6 (decimal dilution 10^{-6})	17	21
D7 (decimal dilution 10^{-7})	1	0

Insertion of the figures into Equation (1) gives

$$N_2 = \frac{\sum x_i}{(n_1 + 0,1n_2 + 0,01n_3)d} = \frac{186 + 171 + 17 + 21 + 1 + 0}{(2 + 0,1 \times 2 + 0,01 \times 2) \times 10^{-5}} = 178,4 \times 10^5 = 1,784 \times 10^7$$

$$\lg N_2 = \lg 1,784 \times 10^7 = 7,25$$

In order to verify whether the repeatability is in agreement with the defined precision data of this International Standard, proceed as follows.

a) Calculate the difference of the logarithms of the two individual examination results (N_1 and N_2) obtained:

$$|\lg N_1 - \lg N_2| = |7,12 - 7,25| = 0,13$$

- b) Compare the absolute value calculated with the precision limit, r , listed in Table 4. Under repeatability conditions the following requirement should be met:

$$|\lg N_1 - \lg N_2| \leq r$$

Depending on the type of product the following decision shall be made:

- 1) For liquid yogurt: $0,13 > r_{\text{Tab4}} = 0,12 \quad \blacktriangleright \text{not valid !!}$

If possible, repeat the examination after having considered possible reasons of exceptional variations.

- 2) For firm or creamy yogurt: $0,13 \leq r_{\text{Tab4}} = 0,20 \quad \blacktriangleright \text{valid !!}$

- c) Express the test results as follows.

- 1) For valid results (agreement with specified precision data is confirmed), quote the final result(s) by calculating the arithmetical mean from the test results.
- 2) If the requirement as shown above is not met, this examination should preferably be repeated. If a repetition is not possible, report the individual test results, with an indication "data not validated".

11 Knowledge of use of the method

To fulfil good laboratory practice requirements, demonstration is recommended of the knowledge of use of this International Standard by assessing the performance of each analyst when applying the pour plate technique (e.g. see ISO 14461-1).

12 Test report

The test report shall contain at least the following information:

- a) all information required for the complete identification of the sample;
- b) the sampling method used, if known;
- c) the test method used, with reference to this International Standard;
- d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incident which may have influenced the result(s);
- e) the individual test result(s) obtained or, if the repeatability is in agreement with the precision data, the final quoted result(s) obtained.

Annex A (informative)

Interlaboratory trial – A 'bifido' ring trial

An international collaborative study involving 23 laboratories in 11 countries was carried out to evaluate commercially available probiotic milk products (fermented and non-fermented). The trial was subdivided into: phase A, the assessment of the analyst performance of all participating partners (international); phase B, the examination of infant milk formulae; and phase C, the examination of selected probiotic yogurt products. Phase C was carried out as a two-centre study by considering typical commercial yogurt products representative of the European and Asian markets.

The ring trial was organized by the Department of Food Science and Technology, University of Natural Resources and Applied Life Sciences (BOKU), Vienna, Austria in 2005 and 2006. The method including all relevant instructions was submitted to all participating partners. After collection and preparation of the resulting data, statistical analyses were performed by BOKU, in close co-operation with the Institute of Biometrics and Data Processing as well as the Institute of Statistics and Econometrics, Free University of Berlin, Berlin, Germany (for more details see References [12][13]).

Table A.1 — Interlaboratory results

Parameter	Sample						
	International	Europe			Asia		
	Infant Milk K, L, N	Yogurt 1 A, B	Yogurt 2 C, D	Yogurt 3 E, F	Yogurt 4 A, B	Yogurt 5 C, D	Yogurt 6 E, F
Number of participants	23	17	17	17	6	6	6
Sample size	3	2	2	2	2	2	2
Total examinations per participant	12	4	4	4	4	4	4
Mean value [lg(CFU/g)]	7,344	6,337	7,813	6,723	7,262	7,674	7,824
Datasets retained	23	17	16	17	6	6	6
Results used to estimate r , R	69	34	32	34	12	12	12
Standard deviation of repeatability, $s_{r \text{ robust}}^a$	0,079	0,041	0,065	0,044	0,042	0,194	0,076
Repeatability limit, r_{robust}^a	0,221	0,115	0,182	0,123	0,118	0,543	0,213
Standard deviation of reproducibility, $s_{R \text{ robust}}^a$	0,189	0,081	0,139	0,192	0,143	0,194	0,104
Reproducibility limit, R_{robust}^a	0,529	0,227	0,389	0,538	0,400	0,543	0,291
<p>^a Based on robust analyses in accordance with ISO 16140 [5], none of the datasets had to be excluded.</p> <p>Description of test samples used:</p> <p>Infant milk formula Commercial probiotic infant milk product containing <i>B. animalis</i> subsp. <i>lactis</i></p> <p>Yogurt 1 Commercial European probiotic yogurt product containing <i>B. animalis</i> subsp. <i>lactis</i>, <i>L. acidophilus</i>, <i>S. thermophilus</i></p> <p>Yogurt 2 Commercial European probiotic yogurt product containing <i>B. animalis</i> subsp. <i>lactis</i>, <i>L. delbrueckii</i> subsp. <i>bulgaricus</i>, <i>S. thermophilus</i></p> <p>Yogurt 3 Commercial European probiotic yogurt product containing <i>B. animalis</i> subsp. <i>lactis</i>, <i>L. casei</i>, <i>S. thermophilus</i></p> <p>Yogurt 4 Commercial Asian probiotic yogurt product containing <i>B. breve</i>, <i>L. casei</i>, <i>S. thermophilus</i></p> <p>Yogurt 5 Commercial Asian probiotic yogurt product containing <i>B. longum</i>, <i>L. gasseri</i>, <i>L. delbrueckii</i> subsp. <i>bulgaricus</i>, <i>S. thermophilus</i></p> <p>Yogurt 6 Commercial Asian probiotic yogurt product containing <i>B. animalis</i> subsp. <i>lactis</i>, <i>L. acidophilus</i>, <i>S. thermophilus</i></p>							

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