DRAFT INTERNATIONAL STANDARD ISO/DIS 29981
IDF 220

ISO/TC **34**/SC **5** Secretariat: **NEN**

Voting begins on: **2008-01-25**

Voting terminates on: **2008-06-25**

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION • МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ • ORGANISATION INTERNATIONALE DE NORMALISATION

MERCK KGaA Darmstadt

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

ICS 67.100.01

In accordance with the provisions of Council Resolution 15/1993 this document is circulated in the English language only.

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This draft is submitted to a parallel enquiry vote in ISO and IDF.

To expedite distribution, this document is circulated as received from the committee secretariat. ISO Central Secretariat work of editing and text composition will be undertaken at publication stage.

Pour accélérer la distribution, le présent document est distribué tel qu'il est parvenu du secrétariat du comité. Le travail de rédaction et de composition de texte sera effectué au Secrétariat central de l'ISO au stade de publication.

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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 International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 29981 | IDF 202 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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All work was carried out by the Joint ISO-IDF Action Team on *Lactic acid bacteria* of the Standing Committee on *Microbiology methods of analysis* under the aegis of its project leader, Prof. W. Kneifel (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, e.g., see [1]).

Bifidobacteria used in milk products usually belong to the following species [see ref.: 2, 3, 14]:

a) *Bifidobacterium adolescentis;*

b) *Bifidobacterium animalis* subspecies *animalis;*

c) *Bifidobacterium animalis* subspecies *lactis;*

d) *Bifidobacterium bifidum;*

e) *Bifidobacterium breve;*

f) *Bifidobacterium infantis;*

g) *Bifidobacterium longum.*

Microscopic appearance with magnification 100*x* and oil immersion in contrast phase illumination: Rods of very varied shapes, usually curved and clubbed, often branched, arranged singly, in pairs, in V arrangements, in chains, in palisades of parallel cells, or in rosettes, occasionally exhibit swollen coccoid forms.

They are non-acid-fast, non-sporeforming, gram-positive, non-motile and catalase-negative. chemoorganotrophs, with the production of acetic and lactic acid. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate shunt in which fructose-6-phosphate phosphoketolase (F6PPK - EC 4.1.2.11) cleaves fructose-6-phospate into acetyl phosphate and erythrose-4-phosphate.

The optimum growth temperature is at between 37 °C and 41°C. For further details, see reference [4].

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 7218, *Microbiology of food and animal stuffs – General rules for microbiological examinations.*

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

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

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 purpose of this International Standard, the following terms and definitions apply.

3.1
Bifidobacteria
anaerobic microorganisms that form lenticular or round colonies, partially star-shaped or trilobate of diameter 1 mm to 4 mm on TOS-MUP medium under the conditions specified in this International Standard.

4 Principle

4.1 The antibiotic Li-Mupirocin (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 antibiotic supplement Li-Mupirocin 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 important criterion is tested with a representative number of reference strains and isolates.

Additionally, TOS-Agar enhances the growth of bifidobacteria used in dairy products [15].

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 could be confirmed by means of appropriate tests (e.g. F6PPK assay [12, 13]).

4.4 **T**he number of bifidobacteria per gram of sample are 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 demineralised water or water of equivalent purity.

5.1 Basic materials

See ISO 8261 | IDF 122 and ISO/TS 11133-1

5.2 Diluent(s)

See ISO 8261 | IDF 122.

The following recommendations are made.

a) The use of Quarter-strength Ringer`s solution.

b) The sterilization in bulk and the use of an adequate sterile dispensing unit during dispensing.

Adjust the diluent to room temperature, preferably by using a water bath at 20 °C ± 1°C. Transfer the diluent by dripping, without incorporating air.

The uncertainty of measurement of the used volumes shall not exceed ± 2 %.

5.3 Culture medium - TOS-MUP medium (TOS-agar + Li-MUP-solution)

Use freshly prepared culture medium (TOS-MUP medium) not being exposed to direct sunlight.

5.3.1 **Basic medium** (TOS-propionate agar medium [5])

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
$MgSO_4.7H_2O$	0,2 g
L-cysteine $HCl.H_2O$	0,5 g
Sodium propionate	15,0 g
Galactooligosaccharides (TOS-S, see 5.3.1.2)	10,0 g
Agar	(12 – 18) g[a]
Water	950 ml
[a] Depending on the gel strength of the agar.	

5.3.1.2 TOS-S

TOS-S is a transgalactosylated oligosaccharide-mixture derived from lactose containing galactose- and glucose-molecules according to [Gal $\overset{x}{—}$ $(Gal)_n$ $\overset{y}{—}$ Glc]* of the following proportion: Trisaccharides (55 % ± 2 %), tetrasaccharides (33 % ± 2 %) and penta- & hexasaccharides (12 % ± 2 %).

*; linkage: n = 1-4; (x = β-1,6 > β-1,4, β-1,3); (y = β-1,4 > β-1,3, β-1,6) [15].

5.3.1.3 Preparation

Suspend the ingredients in 950 ml of distilled water while heating carefully (e.g., using a heating plate or a water bath) with frequent agitation until complete solution is obtained.

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.

Autoclave the basic medium at 115 °C ± 3 °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 1-week maximum under conditions not producing any change in its composition.

NOTE TOS-medium being sensitive to heat, excessive heat-treatment may, therefore, negatively influence the properties of the medium. For preparation conditions see also ISO/TS 11133-1.

5.3.2 **MUP Supplement** (MUP-solution; Li-Mupirocin [6])

Use freshly prepared supplement-solution in distilled water and sterilized by membrane filtration (0,22 µm) as described in 5.3.3.

Dissolve the MUP in distilled water. For example, dissolve 50 mg Li-Mupirocin in 50 ml of distilled water, or other useful proportions needed for completion of the final medium.

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-solution 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. Adjust the completed medium in the water bath (6.5) at 48 °C until pouring.

The completed TOS-MUP medium finally contains 50 µg/ml of the antibiotic Li-Mupirocin (MUP).

6 Apparatus and glassware

Sterilization of equipment that will come 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 8261 | IDF 122 as well as ISO/TS 11133-1. The glassware shall be resistant to repeated sterilisation.

Use the usual microbiological laboratory equipment (see ISO 7218) for the preparation of test samples and dilutions, as specified in ISO 8261 | IDF 122 and, in particular, the following.

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

6.1.1 Incubator, capable of operating at 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 approx. of 70 % to 90 % of nitrogen; with a volume fraction approx. of 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 being controlled at 37 °C ± 1 °C, providing an anaerobic atmosphere (see 6.1.2).

6.2 Mechanical stirrer, capable of mixing the contents of the test tubes (test tube agitator, e.g., Vortex mixer).

6.3 Colony counting equipment, as specified in ISO 7218.

6.4 Magnifying lens, magnification 8x to 10x.

6.5 Water baths, capable of operating at 20 °C ± 1 °C, 45 °C ± 1 °C and 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, approx. of height 150 mm and of diameter 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.

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

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

7 Sampling

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

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

8 Procedure

The following procedures are based on the corresponding standards taking into account the recommendations given in [3,11].

8.1 Preparation of the test portion and primary dilution

The operation described in 8.1 to 8.4 shall be carried out 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 to prevent further contamination. Open the container aseptically.

8.1.1 Dried milk products such as infant milk formulae containing bifidobacteria

Proceed as described in the following steps (see also ISO 8261 | IDF 122):

1. Thoroughly mix the content of the closed packing by repeatedly shaking and inverting it

2. Open the packing, draw the test portion required with a sterile spatula and proceed as below indicated. Immediately close the bag again. It is recommend to use an airtight clip and to put the bag inside a tight glass jar to enable storage at 4 °C.

3. Weigh 90 g ± 0,1 g of diluent in each of the 250 ml pre-sterilized bottles (6.7). Close the bottles.

4. Warm the 250 ml bottles containing the 90 g of diluent in the water bath (6.5) at 45 °C.

5. Weigh to the nearest 0,05 g, 10 g ± 0,1 g of test sample. Add the weighed sample 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.

6. To dissolve the test sample, swirl slowly to wet the powder. Then shake the bottle 10 times, with a movement of about 30 cm, for approx. 7 s.

7. Place the bottles in the water bath at 45 °C for 5 min. while shaking occasionally

8. Immediately cool to room temperature while shaking under running tap water for 2 min. Keep all suspensions refrigerated (see ISO 7218) for 30 min. Start with the examination as fast as possible.

To obtain acceptable repeatability of the method, it is important to follow the aforementioned conditions (time limits, mixing intensity). The time period (soaking at 45 °C, rapidly cooling down and refrigerating) shall also be followed strictly.

8.1.2 Probiotic yogurt or yogurt-like products containing bifidobacteria

Proceed as described in the following steps (see also ISO 7889 | IDF 117):

1. Adjust the diluent to room temperature preferably by using a water bath (6.5) at 20 °C.

2. Weigh 90 g ± 0,1 g of the diluent in each of the 250 ml pre-sterilized bottles (6.7). Close the bottles.

3. Thoroughly mix the content of the closed sample-packaging by repeatedly shaking and inverting it (preferably 10 times, with a movement of about 30 cm, for approx. 7 s) – or, if not possible, thoroughly mix the content with a sterile spatula or similar after opening the packaging to get homogenous samples.

4. Open the packaging, draw the test portion required using a sterile spatula or a pipette and proceed as indicated below.

5. Weigh to the nearest 0,05 g, 10 g ± 0,1 g of test sample. Add the weighed sample to the diluent in each bottle.

6. Shake the bottle 10 times, with a movement of about 30 cm, for approx. 7 s.

7. Start with the examination as fast as possible.

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

8.2 Microscopic examination

To carry out a preliminary microscopic examination of several fields of a smear or the liquid or the primary dilution of the test sample (8.1) previously dried and stained with methylene blue, 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.

8.3 Preparation of decimal dilutions

See ISO 6887-1. Take the following into account:

1. The uncertainty of measurement of the volumes shall not exceed ± 2 %.

2. The described operation shall be carried out by gentle mixing and avoiding air inclusion.

3. During the procedure, the temperature of the decimal dilutions of the sample shall not exceed 20 °C.

Prepare the dilutions as follows:

a) Shake the primary dilution (8.1) preferably 10 times, with a manual movement of about 30 cm for approx. 7 s to obtain homogeneity.

b) Transfer, by means of a pipette, 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., 1x during 3 s. For each dilution step use a fresh sterile pipette (6.8).

Do not dip the pipette more than 1 cm below the surface of the liquid. Avoid filling the pipette with air bubbles. When transferring, do not dip the pipette into the new diluent.

When using graduated sterile pipettes (e.g. 1 ml) it is import to dip the pipette inside onto the inner surface of the tube (Petri dish) whilst draining. Take care to drain the pipettes completely, especially at higher sample concentrations.

8.4 Inoculation

Transfer by dripping, 1 ml of the appropriate dilution steps (4 decimal dilutions steps within the countable area are recommended) into each empty Petri dish with two replicate plates per dilution. Pour 12 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 [1], 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 standard.

8.5 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 [3].

8.6 Incubation

Immediately after solidification of the medium, invert all Petri dishes in the anaerobic culture jar or anaerobic incubator (6.1) and incubate in the incubator (6.1.1) at 37 °C for 72 h ± 3 h.

8.7 Counting of the colonies

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

Count all plates of the selected dilutions by considering all colonies on the plate on the day the incubation is completed.

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 pin-point 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).

8.8 Reading of the Petri dishes - Confirmation

Recognize Bifidobacterial colonies by their whitish colour and acetic acid odour. Select typical colonies from the plates used for counting and examine microscopically.

Optionally, a F6PPK-assay can be performed to confirm the results [12, 13].

NOTE Some of the bifidobacterial strains may appear in different colony size as well as colony appearance on the same plate.

9 Calculation and expression of test results

9.1 Calculation

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

Calculate the number, *N*, of presumptive bifidobacteria in the test sample per gram by using the following equation:

$$N = \frac{\sum x_i}{\left(n_1 + 0{,}1 \times n_2 + 0{,}01 \times n_3\right) \times d}\left[\frac{CFU}{g}\right]$$

where

Σx_i is the sum of colonies counted on all dishes retained (8.7);

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 formula respectively as follow:

$$N = \frac{\sum x_i}{\left(n_1 + 0{,}1 \times n_2\right) \times d}\left[\frac{CFU}{g}\right]$$

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

9.2 Expression of test results

Express the results in two significant digits.

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

The result is expressed as the number of bifidobacteria per gram 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.

EXAMPLES 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 in the following examples.

Example 1

Table 1 – Example 1

Dilution	Plate 1	Plate 2
D4	300	298
D5	30	25
D6	2	3

D4, means decimal dilution 10^{-4}; D5, decimal dilution 10^{-5} and D6, decimal dilution 10^{-6}.

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

$$N = \frac{658}{2{,}22 \times 10^{-4}} = 296 \times 10^4 = 3{,}0 \times 10^6 \left[\frac{CFU}{g}\right]$$

Example 2

Table 2 — Example 2

Dilution	Plate 1	Plate 2
D5	311	286
D6	27	21
D7	2	0

D5, means decimal dilution 10^{-5}; D6, decimal dilution 10^{-6} and D7, decimal dilution 10^{-7}.

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

$$N = \frac{647}{2{,}22 \times 10^{-5}} = 291 \times 10^5 = 3{,}0 \times 10^7 \left[\frac{CFU}{g}\right]$$

10 Precision

10.1 Interlaboratory test

Details of the interlaboratory test ("IDF-Bifido" – Ring trial: 2006) on the precision of the method are summarized in Annex A. 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 in Europe and in 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 to the relevant Codex Standard [1].

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; that are *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, $\log_{10}$-transformed) obtained under repeatability conditions and is expected to be with a probability of 95 % [see 8, 9 and 10].

Table 3 shows the repeatability of different milk products obtained in the "IDF-Bifido" – Ring trial 2006, expressed as repeatability limit (r). These results were calculated by applying the robust analyses proposed by Wilrich [10] taking into account all variations (deviations) reflecting usual and practical conditions. Further details are given in Annex A and [11].

Table 3 – Repeatability limits, r robust; [log CFU / g]

Product	Description	Type	r
Yogurt 1	Commercial European probiotic yogurt product containing *B.animalis (BB12®), L.acidophilus (LA5®), S.thermophilus*	Liquid	0,115
Yogurt 2	Commercial European probiotic yogurt product containing *B.animalis (Digestivum essensis Kultur), L.bulgaricus, S.thermophilus*	Firm	0,182
Yogurt 3	Commercial European probiotic yogurt product containing *B.animalis, L.casei (belactiva 3), S.thermophilus*	Liquid	0,123
Yogurt 4	Commercial Asian probiotic yogurt product containing *B.breve (KB41-3213), L.casei, S.thermophilus*	Liquid	0,118
Yogurt 5	Commercial Asian probiotic yogurt product containing *B.longum (SBT2928), L.gasseri, L.delbrueckii* subsp. *bulgaricus, S.thermophilus*	Firm	0,543
Yogurt 6	Commercial Asian probiotic yogurt product containing *B.animalis (BB12®), L.acidophilus, S.thermophilus*	Firm	0,213
Infant milk	Commercial probiotic infant milk product containing *B.animalis (BB12®)*	Powder	0,221

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 (that are *reproducibility conditions*).

The reproducibility limit (R) is the value less than or equal to which the absolute difference between two test results (presumptive bifidobacteria per gram, $\log_{10}$-transformed) obtained under reproducibility conditions and is expected to be with a probability of 95 % [see 8, 9 and 10].

Table 4 shows the reproducibility of different milk products obtained in the "IDF-Bifido"- Ring trial 2006, expressed as reproducibility limit (R). These results were calculated by applying the robust analyses proposed by Wilrich [10] taking into account all variations (deviations) reflecting usual and practical conditions. Further details are given in Annex A and [11].

Table 4 – Reproducibility limits, R robust; [log CFU / g]

Product	Description	Type	R
Yogurt 1	Commercial European probiotic yogurt product containing *B.animalis (BB12®), L.acidophilus (LA5®), S.thermophilus*	Liquid	0,227
Yogurt 2	Commercial European probiotic yogurt product containing *B.animalis (Digestivum essensis Kultur), L.bulgaricus, S.thermophilus*	Firm	0,389
Yogurt 3	Commercial European probiotic yogurt product containing *B.animalis, L.casei (belactiva 3), S.thermophilus*	Liquid	0,538
Yogurt 4	Commercial Asian probiotic yogurt product containing *B.breve (KB41-3213), L.casei, S.thermophilus*	Liquid	0,400
Yogurt 5	Commercial Asian probiotic yogurt product containing *B.longum (SBT2928), L.gasseri, L.delbrueckii* subsp. *bulgaricus, S.thermophilus*	Firm	0,543
Yogurt 6	Commercial Asian probiotic yogurt product containing *B.animalis (BB12®), L.acidophilus, S.thermophilus*	Firm	0,291
Infant milk	Commercial probiotic infant milk product containing *B.animalis (BB12®)*	Powder	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 was defined, thereby distinguishing among three different types of products (see Table 5): The results obtained for yogurt 5 exhibited irregularities possibly due to inhomogeneity and were excluded from the calculation (for more details see [11]).

Depending on the type of product, apply the following precision data (repeatability limits as well as reproducibility limits, Table 5):

a) Liquid yogurts, including all yogurt drinks or similar;

b) Firm and creamy yogurts (including set-style yogurts); and

c) Powder products, including infant milk formulae or similar.

Table 5 – Precision data collectively defined for the product types

Product - Type	s_r collective	s_R collective	**r**	**R**
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**
NOTE All precision data are expressed as decadic logarithms of colony counts per gram sample [log CFU / g].				

Further explanations with reference to practical applications are given in Example 3.

Example 3: Examination of a yogurt product

Table 6 — 1st examination

Dilution	Plate 1	Plate 2
D5	128	145
D6	9	11
D7	0	0

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

$$\log N_1 = \log 1{,}332 \times 10^7 = 7{,}13 \left[\log \frac{CFU}{g} \right]$$

Table 7 — 2nd examination

Dilution	Plate 1	Plate 2
D5	186	171
D6	17	21
D7	1	0

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

$$\log N_2 = \log 1{,}784 \times 10^7 = 7{,}25 \left[\log \frac{CFU}{g} \right]$$

Test of the validity based on Example 3 (see above):

In order to verify whether the repeatability is in agreement with the defined precision data of this standard, proceed as followed:

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

$$\left| \log N_1 - \log N_2 \right| = \left| 7{,}12 - 7{,}25 \right| = 0{,}13 \left[\log \frac{CFU}{g} \right]$$

2. Compare the absolute value calculated with that listed in Table 5. Under repeatability conditions the following requirement should be met:

$$\left|\log N_1 - \log N_2\right| \le r\left[\log \frac{CFU}{g}\right]$$

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

a) In case of liquid yogurt: 0,13 > r_{Tab5} = **0,12** ► **not valid !!**

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

b) In case of firm or creamy yogurt: 0,13 ≤ r_{Tab5} = **0,20** ► **valid !!**

3. Express the test results as follows:

a) In case of valid results (agreement with specified precision data is confirmed): Quote the final result(s) by calculating the arithmetical mean from the test results.

b) If the requirement as shown above is not met, this examination should preferably be repeated. If a repetition is not possible, the individual test results have to be expressed, with an indication “data not validated”.

11 Mastery of the method

To assure the GLP-requirements, it is recommended to prove the mastery of this standard by assessing the analyst performance of each analyst when applying the pour plate technique (see ISO Standard 14461-1).

12 Test report

The test report shall specify:

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` Ringtrial

An international collaborative study involving 23 laboratories located in 11 countries was carried out considering commercially available probiotic milk products (fermented and non-fermented). The whole trial was sub-divided into 3 phases, phase A , the assessment of the analyst performance of all participating partners (international), Phase B, the examination of an infant milk formulae, and phase C, the examination of selected probiotic yogurt products. Phase C was carried out as two-centre study by considering typical commercial yogurt products representative for the European and Asian market.

The "IDF-Bifido"-Ringtrial was organized by the University of Natural Resources and Applied Life Sciences (BOKU), Dept. Food Science & Technology, Vienna in 2005/06. 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 Free University of Berlin, Institute of Biometry (for more details see Zitz et al., 2007 [11])

Table A.1 — Interlaboratory results

PARAMETER	SAMPLES						
	International	Europe			Asia		
	Infant Milk	Yogurt 1	Yogurt 2	Yogurt 3	Yogurt 4	Yogurt 5	Yogurt 6
	K, L, N	A,B	C,D	E,F	A,B	C,D	E,F
Number of Participants	23	17	17	17	6	6	6
Samples size	3	2	2	2	2	2	2
Total Exam. / Participant	12	4	4	4	4	4	4
Mean Value (log10 CFU/g)	7,344	6,337	7,813	6,723	7,262	7,674	7,824
Accepted Datasets	23	17	16	17	6	6	6
Results used to estimate r, R	69	34	32	34	12	12	12
Repeatability SD, $s_{r\ robust}$ [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
Reproducibility SD,$s_{R\ robust}$[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

[a] Based on robust analyses in accordance with the draft version [10], none of the datasets had to be excluded.

Description of test samples used :	
Infant milk formula	Commercial probiotic infant milk product containing *B.animalis (BB12®)*
Yogurt 1	Comm. Europ. probiotic yogurt product cont. *B.animalis (BB12®), L.acid. (LA5®), S.th.*
Yogurt 2	Comm. Europ. probiotic yogurt product cont. *B.animalis (Dig. essensis cultur), L.bulg., S.th.*
Yogurt 3	Comm. Europ. probiotic yogurt product cont. *B.animalis, L.casei (belactiva 3), S.th.*
Yogurt 4	Comm. Asian probiotic yogurt product cont. *B.breve (KB41-3213), L.casei, S.thermophilus*
Yogurt 5	Comm. Asian probiotic yogurt product cont. *B.longum (SBT2928), L.gasseri, L. bulg., S.th.*
Yogurt 6	Comm. Asian probiotic yogurt product cont. *B.animalis (BB12®), L.acidophilus, S.th.*

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