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# Yogurt — Enumeration of characteristic microorganisms — Colony-count technique at 37 °C

*Yaourt — Dénombrement des micro-organismes caractéristiques — Technique de comptage des colonies à 37 °C*

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# Foreword

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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

ISO 7889 | IDF 117 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

# 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 and AOAC International 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 the National Committees casting a vote.

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All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Lactic acid bacteria and starters,* of the Standing Committee on *Microbiological methods of analysis*, under the aegis of its project leader, Prof. B. Bianchi Salvadori (IT).

This edition cancels and replaces the first edition of IDF 117A:1988, which has been technically revised.

# Yogurt — Enumeration of characteristic microorganisms — Colony-count technique at 37 °C

## 1 Scope

This International Standard specifies a method for the enumeration of characteristic microorganisms in yogurt by means of the colony-count technique at 37 °C.

The method is applicable to yogurts in which both characteristic microorganisms (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) are present and viable.

## 2 Normative references

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

ISO 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 feeding stuffs — General rules for microbiological examinations*

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

## 3 Terms and definitions

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

**3.1**
**characteristic microorganisms in yogurt**
*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* as detected under the conditions specified in this International Standard

**3.2**
***Lactobacillus delbrueckii* subsp. *bulgaricus***
thermophilic microorganism which forms lenticular, often sharp-shaped, colonies of diameter 1 mm to 3 mm on acidified MRS medium under the conditions specified in this International Standard

NOTE Under a microscope, these microorganisms appear as rods, generally short, but sometimes in longer forms. They are non-spore forming, Gram-positive, non-motile and catalase-negative.

**3.3**
***Streptococcus thermophilus***
thermophilic microorganism which forms lenticular colonies of diameter 1 mm to 2 mm on M17 medium under the conditions specified in this International Standard

NOTE Under a microscope, these microorganisms appear as spherical or ovoid cells (of diameter 0,7 µm to 0,9 µm) in pairs or in long chains. They are Gram-positive and catalase-negative.

## 4 Principle

**4.1** Decimal dilutions of the test sample are inoculated into:

a) acidified MRS medium, followed by anaerobic incubation at 37 °C ± 1 °C for 72 h, for the count of *Lactobacillus delbrueckii* subsp. *bulgaricus*;

b) complete medium (M17), followed by aerobic incubation at 37 °C ± 1 °C for 48 h, for the count of *Streptococcus thermophilus*.

**4.2** The colonies are counted and confirmed by means of appropriate tests.

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

## 5 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. See also ISO 6887-1 and ISO 8261 | IDF 122.

### 5.1 Diluent

See ISO 6887-1 and ISO 8261 | IDF 122.

### 5.2 Culture media

Use freshly prepared culture media (MRS and M17) which shall not be exposed to direct sunlight.

If the prepared culture media are not used immediately, they shall, unless otherwise specified, be cooled and stored at between 2 °C and 4 °C for no longer than 1 week and under conditions which do not produce any change in their composition.

As for reagents, see storage conditions specified in ISO 7218.

#### 5.2.1 **Acidified MRS medium** (see reference [7])

##### 5.2.1.1 Composition

| | |
|---|---|
| Peptone 1 (tryptic digest of casein) | 10,0 g |
| Meat extract | 10,0 g |
| Yeast extract (dried) | 5,0 g |
| Glucose ($C_6H_{12}O_6$) | 20,0 g |
| Tween 80 (sorbitan mono-oleate) | 1,0 ml |
| Dipotassium hydrogen orthophosphate ($K_2HPO_4$) | 2,0 g |
| Sodium acetate trihydrate ($CH_3CO_2Na{\cdot}3H_2O$) | 5,0 g |
| Diammonium citrate [$C_6H_6O_7(NH_4)_2$] | 2,0 g |
| Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) | 0,2 g |
| Manganese sulfate tetrahydrate ($MnSO_4{\cdot}4H_2O$) | 0,05 g |
| Agar | 9,0 g to 18,0 g[1)] |
| Water up to | 1 000 ml |

1) Depending on the gel strength of the agar or according to manufacturer's instructions.

#### 5.2.1.2 Preparation

Separately dissolve each component in a water bath (6.7) set at boiling. Cool in another water bath (6.7) to 50 °C. Adjust the pH so that after sterilization it is 5,4 ± 0,1 at 25 °C ± 1 °C by adding acetic acid (5.3.3) and checking with the pH-meter (6.8). Transfer the medium in 100 ml portions into 150 ml bottles (6.10) or in 200 ml portions into 250 ml bottles (6.10). Sterilize for 15 min in an autoclave at 121 °C ± 1 °C.

NOTE 1 MRS medium is highly sensitive to heat treatment which may cause differences according to the autoclave used.

NOTE 2 Comparative tests have shown that commercially available MRS media may give counts that are lower than those given by the MRS medium prepared in accordance with this International Standard. Therefore, if used, the former should be checked against the medium prepared according to this International Standard. This may cause problems for yogurt producers and the authorities investigating the requested minimum cell count in yogurt products.

Before beginning the bacteriological examination, completely melt the required amount of medium in a water bath (6.7) set at boiling, or by steaming in a partially closed container. Then cool it in another water bath (6.7).

### 5.2.2 M17 medium (see reference [8])

#### 5.2.2.1 Basic medium

#### 5.2.2.1.1 Composition

| | |
|---|---|
| Peptone 1 (tryptic digest of casein) | 2,5 g |
| Peptone 2 (peptic digest of meat) | 2,5 g |
| Peptone 3 (papain digest of soya) | 5,0 g |
| Yeast extract (dried) | 2,5 g |
| Meat extract | 5,0 g |
| β-Glycerophosphate (disodium salt) ($C_3H_7O_6PNa_2$) | 19,0 g |
| Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) | 0,25 g |
| Ascorbic acid ($C_6H_8O_6$) | 0,50 g |
| Agar | 9,0 g to 18,0 g[1)] |
| Water up to | 950 ml |

#### 5.2.2.1.2 Preparation

Separately, dissolve each component in a water bath (6.7) set at boiling. Cool in another water bath (6.7) to 50 °C. Adjust the pH so that, after sterilization, it is 6,8 ± 0,1 at 25 °C ± 1 °C by using a reagent (5.3) and checking with the pH-meter (6.8). Transfer the medium in 95 ml portions into 150 ml bottles (6.10). Sterilize for 15 min in an autoclave at 121 °C ± 1 °C.

#### 5.2.2.2 Lactose solution

#### 5.2.2.2.1 Composition

| | |
|---|---|
| Lactose ($C_{12}H_{22}O_{11}$) | 10,0 g |
| Water up to | 100 ml |

#### 5.2.2.2.2 Preparation

Dissolve the lactose in the water. Dilute with water to 100 ml. Sterilize for 15 min in an autoclave at 121 °C ± 1 °C.

#### 5.2.2.3 Complete medium (M17)

##### 5.2.2.3.1 Composition

| | |
|---|---|
| Basic medium (5.2.2.1) | 95,0 ml |
| Lactose solution (5.2.2.2) | 5,0 ml |

##### 5.2.2.3.2 Preparation

Immediately before use, melt the basic medium in a water bath (6.7) set at boiling. Cool in another water bath (6.7) to 50 °C. Preheat the lactose solution in a water bath (6.7) set at 50 °C. Add the lactose solution to the basic medium and mix by swirling. Cool the medium in the water bath to between 44 °C and 47 °C.

NOTE Complete M17 media are commercially available but, as in the case of commercially available MRS media, the results obtained may differ significantly from one supplier to the other. Therefore they should be checked against M17 medium prepared according to this International Standard. This may cause problems for yogurt producers and the authorities investigating the requested minimum bacterial count in yogurt products.

### 5.3 Reagents for pH adjustment

**5.3.1 Sodium hydroxide solution**, $c$(NaOH) = 0,1 mol/l approximately.

**5.3.2 Dilute hydrochloric acid**, $c$(HCl) = 0,1 mol/l approximately.

**5.3.3 Acetic acid** ($CH_3COOH$), 100 % (glacial).

**5.4 Reagent for staining**, ethanolic solution of methylene blue, 6 g/l.

**5.5 Reagent for cleaning the container surface**, ethanol 70 % (volume fraction).

## 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. The glassware shall be resistant to repeated sterilization.

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 Incubator**, capable of operating at 37 °C ± 1 °C.

**6.2 Anaerobic incubation cabinet** or **anaerobic jars**, capable of being maintained at 37 °C ± 1 °C, providing an atmosphere of 90 % nitrogen and 10 % carbon dioxide.

**6.3 Blender**, either a peristaltic-type blender (stomacher) with sterile plastic bags, or a rotary blender, capable of operating at a minimum rotational frequency of 20 000 $min^{-1}$, with sterile 200 ml round-bottom centrifuge tubes made of strengthened glass, or metal containers of appropriate capacity.

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

**6.5 Colony-counting equipment**, as specified in ISO 7218.

**6.6 Magnifying lens**, magnification × 8 to × 10.

**6.7 Water baths**, capable of operating between 44 °C and 47 °C, at 45 °C ± 1 °C, at 50 °C ± 1 °C, and capable of boiling.

**6.8 pH-meter**, with temperature compensation, accurate to ± 0,1 pH unit at 25 °C ± 1 °C (see also ISO 7218).

**6.9 Dilution bottles**, of capacity 50 ml to 250 ml, or **test tubes** of diameter and length 18 mm by 180 mm, with suitable seal cap or stopper made of rubber or synthetic material.

**6.10 Flasks** or **bottles**, with rubber stoppers or caps of capacity 150 ml to 250 ml.

**6.11 Test tubes**, with rubber stoppers or caps, of capacity about 20 ml, to hold the culture medium.

**6.12 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).

**6.13 Petri dishes**, made of clear uncoloured glass or plastics, of diameter 90 mm and 140 mm, internal depth of 10 mm minimum. The bottom shall have no irregularities that could interfere with colony counting.

**6.14 Spatula**, of glass or metal, sterilized.

## 7 Sampling

It is important that the laboratory receive a sample which is truly representative and has not 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.

## 8 Preparation of test sample

Prepare the test sample in accordance with ISO 8261.

Take the following precautions before opening the yogurt container. Clean the external surface immediately surrounding the area from which the sample is to be taken, in order to remove any material that might contaminate the sample. The area may be swabbed with 70 % ethanol (5.5) to prevent further contamination. Open the container aseptically.

## 9 Procedure

### 9.1 Preparation of test portion

#### 9.1.1 General

For general requirements, see ISO 8261 | IDF 122.

#### 9.1.2 Non-fruit yogurts

Carefully mix the contents of the sample container using a sterile spatula (6.14). Weigh 10 g ± 0,1 g of test sample in a suitable container [e.g. a 200 ml round-bottom centrifuge tube made of strengthened glass, or the bowl of the rotary blender, or the plastic container of the peristaltic blender (6.3)].

#### 9.1.3 Fruit yogurts

Blend the complete contents of the sample container for 1 min, using the blender (6.3). Weigh 10 g ± 0,1 g of the sample in a suitable container as described in 9.1.2.

## 9.2 Microscopic examination

Carry out a preliminary microscopic examination of several fields of a smear of the test sample (Clause 8), previously dyed with methylene blue (5.4), to estimate the density of the two bacterial types, cocci and rods, and to select the proper range of dilutions to be used for the count of each type.

## 9.3 Preparation of primary dilution

See ISO 8261|IDF 122.

The operations described in 9.3 to 9.6.4 shall not be carried out in direct sunlight.

Add the diluent (5.1) to the test portion (9.1.1 or 9.1.2) until the mass of test portion and diluent is 50 g. Blend for 1 min with the blender (6.3). Dilute to 100 g with the diluent to obtain a $10^{-1}$ dilution.

## 9.4 Preparation of decimal dilutions

See ISO 8261|IDF 122.

## 9.5 Duration of the procedure

See ISO 8261|IDF 122.

## 9.6 Inoculation and incubation

**9.6.1** Proceeding in duplicate pairs (for both *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*), transfer with a sterile pipette (6.12) 1 ml of each dilution into Petri dishes (6.13).

**9.6.2** For *L. delbrueckii* subsp. *bulgaricus*, pour 15 ml of acidified MRS medium (5.2.1) maintained on a water bath (6.7) at 45 °C into each Petri dish (6.13).

**9.6.3** For *S. thermophilus*, pour 15 ml of M17 medium (5.2.2), heated on a water bath (6.7) to between 44 °C and 47 °C, into each Petri dish (6.13).

**9.6.4** Immediately after pouring, carefully mix the inoculum with the medium by rotating the Petri dishes (9.6.2 or 9.6.3). Allow the mixture to solidify by leaving the Petri dishes to stand on a cool horizontal surface.

**9.6.5** Incubate the prepared dishes in an inverted position. Stack not more than six high. Stacks of dishes should be separated from one another and from the walls and top of the incubator (6.1).

**9.6.6** Incubate the plates to be used for the enumeration of *L. delbrueckii* subsp. *bulgaricus* in the anaerobic incubation cabinet or anaerobic jar (6.2) in the incubator (6.1) set at 37 °C for 72 h.

**9.6.7** Incubate the plates to be used for the enumeration of *S. thermophilus* in the incubator (6.1) set at 37 °C for 48 h.

## 9.7 Counting of colonies

**9.7.1** After the specified period of incubation (9.6.6 and 9.6.7), count the colonies showing the features of each characteristic microorganism [*Lactobacillus delbrueckii* subsp. *bulgaricus* (3.2) and *Streptococcus thermophilus* (3.3)] on plates having between 15 and 300 colonies (see Annex A).

NOTE If problems occur with counting *L. delbrueckii* subsp. *bulgaricus* when using MRS medium, LBA medium, developed at NIZO[2)], may be used; see also Annex A.

2) NIZO Food Research, Kernhemseweg 2, Postbus 20 6710 BA, Ede, The Netherlands.

**9.7.2** Examine the plates under subdued light. To facilitate counting, suitable colony-counting equipment (6.5) may be used. Take care not to mistake particles of undissolved sample or precipitated matter for pinpoint colonies. Examine doubtful objects carefully, using a lens (6.6) of higher magnification where required, to distinguish colonies from foreign matter.

### 9.8 Confirmation

Select colonies from the plates used for counting such that the number taken is equal to the square root of the total colony count. Stain these colonies using the Gram method and confirm that they are non-spore forming, Gram-positive, catalase-negative rods (in the case of those grown on the MRS medium), and Gram-positive, catalase-negative chains of cocci or diplococci (in the case of those grown on the M17 medium) (see ISO 7218).

The identity of doubtful strains may be checked according to ISO 9232 | IDF 146.

## 10 Calculation and expression of results

### 10.1 Calculation

**10.1.1** Use counts from plates containing between 15 and 300 colonies as obtained in 9.7.1 or 9.7.2.

**10.1.2** Calculate the number of each characteristic microorganism in the test sample using the following equation:

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

where

$N$ is the numerical value of the number of characteristic microorganisms per gram of test sample;

$\Sigma C$ is the numerical value of the sum of colonies on all plates counted (10.1.1);

$n_1$ is the numerical value of the number of plates counted using the first dilution;

$n_2$ is the numerical value of the number of plates counted using the second dilution;

$d$ is the numerical value of the mass, in grams, of undiluted test sample present in the plate with the first dilution.

EXAMPLE A dilution factor of $10^{-2}$ means that $10^{-2}$ g or $10^{-2}$ ml of the undiluted test sample (in the diluted state) has been put into the dish.

NOTE The first dilution is the dilution with the higher content of test sample.

In case of three dilutions, calculate the number of each characteristic microorganism in the test sample using the following equation.

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

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

### 10.2 Expression of results

**10.2.1** Round the result obtained in 10.1.2 to two significant figures. For a three-figure number, round the third figure to the nearest zero. If the third figure is 5, round to the figure below if the second figure is even, and to the figure above if the second figure is odd.

EXAMPLE

Round: 234 to 230;
235 to 240;
225 to 220;
245 to 240.

**10.2.2** If there are only counts less than 10, report the number of microorganisms per gram as

"less than $10 \times 1/d$" ($d$ being the value corresponding to the lowest dilution).

**10.2.3** If there are only counts exceeding 300, calculate an estimated count from dishes having a count nearest to 300 colonies and multiply by the reciprocal of the value corresponding to the highest dilution. Report as

"estimated minimum number of microorganisms per gram".

**10.2.4** The result shall be expressed as a number from 1,0 to 9,9 multiplied by the appropriate power of 10.

**10.2.5** The total number of characteristic microorganisms, $N$, per gram of sample is equal to:

$$N = N_L + N_S$$

where

$N_L$ is the numerical value of the number of *L. delbrueckii* subsp. *bulgaricus* per gram, calculated in 10.1.2;

$N_S$ is the numerical value of the number of *S. thermophilus* per gram, calculated in 10.1.2.

## 10.3 Examples of calculation

### 10.3.1 *L. delbrueckii* subsp. *bulgaricus*

Assume that a *L. delbrueckii* subsp. *bulgaricus* count gave the following results (two Petri dishes per dilution were incubated):

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

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

then

$$N = \frac{\Sigma C}{(n_1 + 0{,}1\, n_2)\, d} = \frac{295 + 245 + 33 + 40}{(2 + 0{,}1 \times 2)\, 10^{-5}} = \frac{613}{2{,}2 \times 10^{-5}} = 278{,}6 \times 10^5$$

In accordance with 10.2.1, this is equal to $280 \times 10^5$ per gram. The estimated number of *L. delbrueckii* subsp. *bulgaricus*, expressed in accordance with 10.2.4, is therefore $2{,}8 \times 10^7$ per gram.

### 10.3.2 *S. thermophilus*

Similarly, for *S. thermophilus*, an estimated number of $4{,}9 \times 10^8$ per gram of yogurt was obtained.

Thus the total number of characteristic microorganisms is equal to:

$$N = (2{,}8 \times 10^7) + (4{,}9 \times 10^8) = 5{,}18 \times 10^8 \text{ per gram}$$

which, when rounded in accordance with 10.2.4 gives:

$N = 5{,}2 \times 10^8$ per gram of sample.

# 11 Precision

## 11.1 General

Given a Poisson distribution of microorganisms in the substrate, the confidence limits of this method vary according to the count of colonies examined from ± 16 % to ± 52 %. In practice, even greater variation can be found. In various collaborative studies in accordance with IDF 135, the standard deviation of the repeatability ($s_r$) appeared to be 0,20 log units, and the standard deviation of the reproducibility ($s_R$) appeared to be 0,35 log units according to ISO 5725-1 and ISO 5725-2.

More information about the confidence limits for the estimation of small numbers of microorganisms is given in ISO 7218.

NOTE IDF 135 is based on ISO 5725.

## 11.2 Repeatability

Experience indicates that if the higher of two independent tests on the same sample frequently exceeds the lower by 30 %, the procedures should be re-examined to determine sources of error.

# 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 test result(s) obtained or, if the repeatability has been checked, the final quoted results obtained.

# Annex A
(informative)

# Notes on procedure

Neither of the two recommended culture media (acidified MRS and M17) is completely selective.

Most *S. thermophilus* strains do not form visible colonies on the acidified MRS medium in dilutions normally used for the count of *L. delbrueckii* subsp. *bulgaricus*. However, when the number of lactobacilli in the sample of yogurt is considerably lower than the number of streptococci, low dilutions have to be used for the count of *L. delbrueckii* subsp. *bulgaricus*.

Under these conditions, some *S. thermophilus* may form very small or pinpoint colonies on the acidified MRS plate. These colonies can easily be differentiated with the naked eye from the *L. delbrueckii* subsp. *bulgaricus* colonies (the latter being of larger size) and can be checked additionally microscopically. Furthermore, some *L. delbrueckii* subsp. *bulgaricus* strains show poor or no growth on MRS and sometimes it is difficult to differentiate them from *S. thermophilus*.

When strains of *S. thermophilus* grow very slowly or are not able to grow, some specific conditions are recommended such as:

— higher incubation temperature (39 °C to 42 °C and 45 °C for 24 h aerobically);

— decrease in β-glycerophosphate content;

— modification of pH;

— great care when agitating.

When strains of *L. delbrueckii* subsp. *bulgaricus* grow very slowly or are not able to grow, some specific conditions are recommended such as:

— incubation for more than 3 days, up to 5 or 6 days;

— higher incubation temperature (40 °C to 42 °C and 45 °C for 48 h anaerobically);

— modification of pH;

— great care when agitating;

— gas environment ($CO_2$ enrichment, $CO_2$ alone, etc.).

*L. delbrueckii* subsp. *bulgaricus,* when grown anaerobically in yogurt, cannot be enumerated in MRS. This problem does not occur when using LBA medium (see Note in 9.7.1) under anaerobic conditions at 50 °C ± 0,5 °C for 72 h.

On the other hand, most *L. delbrueckii* subsp. *bulgaricus* strains do not form visible colonies on M17 plates with dilutions normally used for counting *S. thermophilus*. This confirms earlier findings (see reference [9]).

Some strains of *L. delbrueckii* subsp. *bulgaricus* may, however, form small pinpoint colonies on the M17 medium, especially with samples of yogurt presenting a much higher number of lactobacilli compared to the number of streptococci. These small rough colonies usually have a woolly or fleecy appearance and they can easily be distinguished with the naked eye (and still better with a magnifying lens) and checked additionally microscopically, from the smooth lenticular colonies of *S. thermophilus* which are of larger size.

The media selection, sample preparation, incubation procedures and counting are the more critical parts of the assay. Once the technicians become familiar with the methods, accuracy improves significantly.

Furthermore, no appropriate test strains can be used to test selectivity of strains, since strains used in yogurt are numerous and different from those used in tests so that some strains of a certain species (e.g. *S. thermophilus*) can grow even in media selective for another species (e.g. for *L. delbrueckii* subsp. *bulgaricus*).

# Bibliography

[1] ISO 707, *Milk and milk products — Guidance on sampling*

[2] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[3] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[4] ISO 9232 | IDF 146, Y*ogurt — Identification of characteristic microorganisms (*Lactobacillus delbrueckii *subsp.* bulgaricus *and* Streptococcus thermophilus)

[5] 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*

[6] IDF 135, *Milk and milk products — Precision characteristics of analytical methods — Outline of collaborative study procedure*

[7] De MAN, J.K., ROGOSA, M. and SHARPE, M.E. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol*., **23**, 1960, pp. 130-135

[8] TERZAGHI, B.E. and SANDINE, W.E. Improved medium for lactic streptococci and their bacteriophages. *J. Appl. Microbiol*., **29**, 1975, pp. 807-813

[9] SHANKAR, P.A. and DAVIES, F.L. Recent developments in yoghurt starters. II. A note on the suppression of *Lactobacillus bulgaricus* in media containing β-glycerophosphate and application of such media to selective isolation of *Streptococcus thermophilus* from yoghurt. *J. Soc. Dairy Technol*., **30**(1),1977, pp. 28-30 (Annex B)

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