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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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## Contents

Page

|                                                |    |
|------------------------------------------------|----|
| Foreword.....                                  | iv |
| Foreword.....                                  | v  |
| 1 Scope.....                                   | 1  |
| 2 Normative references .....                   | 1  |
| 3 Terms and definitions.....                   | 1  |
| 4 Principle .....                              | 2  |
| 5 Culture media, diluents and reagents.....    | 2  |
| 6 Apparatus and glassware.....                 | 4  |
| 7 Sampling .....                               | 5  |
| 8 Preparation of test sample .....             | 5  |
| 9 Procedure.....                               | 5  |
| 9.1 Preparation of test portion .....          | 5  |
| 9.2 Microscopic examination .....              | 6  |
| 9.3 Preparation of primary dilution .....      | 6  |
| 9.4 Preparation of decimal dilutions .....     | 6  |
| 9.5 Duration of the procedure .....            | 6  |
| 9.6 Inoculation and incubation .....           | 6  |
| 9.7 Counting of colonies .....                 | 6  |
| 9.8 Confirmation .....                         | 7  |
| 10 Calculation and expression of results ..... | 7  |
| 10.1 Calculation .....                         | 7  |
| 10.2 Expression of results.....                | 7  |
| 10.3 Examples of calculation .....             | 8  |
| 11 Precision .....                             | 9  |
| 11.1 General .....                             | 9  |
| 11.2 Repeatability.....                        | 9  |
| 12 Test report.....                            | 9  |
| Annex A (informative) Notes on procedure ..... | 10 |
| Bibliography.....                              | 11 |

## 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 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.

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## Foreword

**IDF (the International Dairy Federation)** is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO 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  $37\text{ °C} \pm 1\text{ °C}$  for 72 h, for the count of *Lactobacillus delbrueckii* subsp. *bulgaricus*;
- b) complete medium (M17), followed by aerobic incubation at  $37\text{ °C} \pm 1\text{ °C}$  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 <sub>6</sub> H <sub>12</sub> O <sub>6</sub> )                                           | 20,0 g                        |
| Tween 80 (sorbitan mono-oleate)                                                                    | 1,0 ml                        |
| Dipotassium hydrogen orthophosphate (K <sub>2</sub> HPO <sub>4</sub> )                             | 2,0 g                         |
| Sodium acetate trihydrate (CH <sub>3</sub> CO <sub>2</sub> Na·3H <sub>2</sub> O)                   | 5,0 g                         |
| Diammonium citrate [C <sub>6</sub> H <sub>6</sub> O <sub>7</sub> (NH <sub>4</sub> ) <sub>2</sub> ] | 2,0 g                         |
| Magnesium sulfate heptahydrate (MgSO <sub>4</sub> ·7H <sub>2</sub> O)                              | 0,2 g                         |
| Manganese sulfate tetrahydrate (MnSO <sub>4</sub> ·4H <sub>2</sub> O)                              | 0,05 g                        |
| Agar                                                                                               | 9,0 g to 18,0 g <sup>1)</sup> |
| 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  $5,4 \pm 0,1$  at  $25 \text{ °C} \pm 1 \text{ °C}$  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  $121 \text{ °C} \pm 1 \text{ °C}$ .

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                         |
| $\beta$ -Glycerophosphate (disodium salt) ( $\text{C}_3\text{H}_7\text{O}_6\text{PNa}_2$ ) | 19,0 g                        |
| Magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ )               | 0,25 g                        |
| Ascorbic acid ( $\text{C}_6\text{H}_8\text{O}_6$ )                                         | 0,50 g                        |
| Agar                                                                                       | 9,0 g to 18,0 g <sup>1)</sup> |
| 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  $6,8 \pm 0,1$  at  $25 \text{ °C} \pm 1 \text{ °C}$  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  $121 \text{ °C} \pm 1 \text{ °C}$ .

#### 5.2.2.2 Lactose solution

##### 5.2.2.2.1 Composition

|                                                       |        |
|-------------------------------------------------------|--------|
| Lactose ( $\text{C}_{12}\text{H}_{22}\text{O}_{11}$ ) | 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  $121 \text{ °C} \pm 1 \text{ °C}$ .

### 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**,  $c(\text{NaOH}) = 0,1 \text{ mol/l}$  approximately.

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

**5.3.3 Acetic acid** ( $\text{CH}_3\text{COOH}$ ), 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  $37 \text{ °C} \pm 1 \text{ °C}$ .

**6.2 Anaerobic incubation cabinet or anaerobic jars**, capable of being maintained at  $37 \text{ °C} \pm 1 \text{ °C}$ , 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  $20\,000 \text{ min}^{-1}$ , 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  $\times 8$  to  $\times 10$ .

**6.7 Water baths**, capable of operating between 44 °C and 47 °C, at  $45 \text{ °C} \pm 1 \text{ °C}$ , at  $50 \text{ °C} \pm 1 \text{ °C}$ , and capable of boiling.

**6.8 pH-meter**, with temperature compensation, accurate to  $\pm 0,1$  pH unit at  $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$  (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  $1\text{ ml} \pm 0,02\text{ ml}$  and  $10\text{ ml} \pm 0,2\text{ ml}$  (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  $10\text{ g} \pm 0,1\text{ g}$  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  $10\text{ g} \pm 0,1\text{ g}$  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<sup>2)</sup>, may be used; see also Annex A.

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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.

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

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

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

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:

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