International Standard 5541/1

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

Milk and milk products — Enumeration of coliforms — Part 1: Colony count technique at 30 °C

Lait et produits laitiers — Dénombrement des coliformes — Partie 1: Technique par comptage des colonies à 30 °C

First edition — 1986-12-01

UDC 637.1/.3 : 637.075 **Ref. No. ISO 5541/1-1986 (E)**

Descriptors : agricultural products, dairy products, milk, tests, microbiological analysis, determination, coliform bacteria.

Price based on 7 pages

Foreword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

International Standard ISO 5541/1 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*,

NOTE — The method specified in this International Standard has been developed jointly with the International Dairy Federation (IDF) and the Association of Official Analytical Chemists (AOAC) and will also be published by these organizations.

Users should note that all International Standards undergo revision from time to time and that any reference made herein to any other International Standard implies its latest edition, unless otherwise stated.

Printed in Switzerland

Milk and milk products — Enumeration of coliforms — Part 1: Colony count technique at 30 °C

1 Scope and field of application

This part of ISO 5541 specifies a method for the enumeration of coliforms by means of the colony count technique at 30 °C.

The method is applicable to

— milk, and liquid milk products;

— dried milk, dried sweet whey, dried buttermilk, and lactose;

— acid casein, lactic casein and rennet casein;

— caseinate and dried acid whey;

— cheese and processed cheese;

— butter;

— frozen milk products (including edible ices);

— custard, desserts and cream.

This method is to be preferred for samples in which large numbers of coliforms (more than 100 per gram or 10 per millilitre) are suspected.

NOTE — For samples with smaller numbers of coliforms (less than 100 per gram or 10 per millilitre) see ISO 5541/2.

2 Reference

ISO 707, *Milk and milk products — Methods of sampling.*

3 Definition

For the purpose of this part of ISO 5541, the following definition applies.

coliforms: Bacteria which, at 30 °C, form characteristic colonies and which can ferment lactose with the production of gas under the operational conditions described.

4 Principle

4.1 Mixing a defined test portion or a series of decimal dilutions of the sample with the culture medium in Petri dishes, and covering with a layer of the same medium.

4.2 Incubation at 30 °C for 24 h.

4.3 Counting of the characteristic colonies and, if needed, confirmation of colonies by fermentation of lactose, shown by gas production.

4.4 Calculation of the number of coliforms per millilitre or per gram of the original sample.

5 Diluents and media

5.1 Basic materials

In order to improve the reproducibility of the results, it is recommended that, for the preparation of diluents and culture media, dehydrated basic components or complete dehydrated media should be used. The manufacturer's instructions shall be rigorously followed.

The chemical products used shall be of recognized analytical quality.

The water used shall be distilled from glass apparatus or shall be deionized water. It shall be free from substances that might influence the growth of micro-organisms under the test conditions. This shall be periodically checked, particularly in the case of deionized water.

Solutions of sodium hydroxide and hydrochloric acid (approximately 0,1 mol/litre) should be used to adjust the pH of diluents and media.

5.2 Diluents for general use

5.2.1 Peptone/saline solution

NOTE — Peptone/saline solution is the diluent selected by ISO for general use.

Composition

Peptone	1,0 g
Sodium chloride (NaCl)	8,5 g
Water	1 000 ml

Preparation

Dissolve the components in water, heating if necessary. Adjust the pH so that, after sterilization, it is 7,0 ± 0,1 at 25 °C.

5.2.2 Quarter-strength Ringer's solution

Composition

Sodium chloride (NaCl)	2,25 g
Potassium chloride (KCl)	0,105 g
Calcium chloride, anhydrous ($CaCl_2$)	0,06 g
Sodium hydrogencarbonate ($NaHCO_3$)	0,05 g
Water	1 000 ml

Preparation

Dissolve the salts in the water. Adjust the pH so that, after sterilization, it is 6,9 ± 0,1 at 25 °C.

5.2.3 Peptone solution

Composition

Peptone	1,0 g
Water	1 000 ml

Preparation

Dissolve the peptone in the water. Adjust the pH so that, after sterilization, it is 7,0 ± 0,1 at 25 °C.

5.2.4 Phosphate buffer solution

Composition

Potassium dihydrogenphosphate (KH_2PO_4)	42,5 g
Water	1 000 ml

Preparation

Dissolve the salt in 500 ml of water. Adjust the pH so that, after sterilization, it is 7,2 ± 0,1 at 25 °C. Dilute to 1 000 ml. Store this stock solution at 0 to 5 °C.

Add 1,00 ml of this solution to 1 000 ml of water.

5.3 Diluents for special purposes

5.3.1 Sodium citrate solution (for cheese, processed cheese and roller-dried milk).

Composition

Trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	20 g
Water	1 000 ml

Preparation

Dissolve the salt in the water by heating at 45 to 50 °C. Adjust the pH so that, after sterilization, it is 7,5 ± 0,1 at 25 °C.

5.3.2 Dipotassium hydrogenphosphate solution (for cheese, processed cheese, casein, acid casein, dried lactic caseins, rennet casein, caseinates, dried acid whey and roller-dried milk).

Composition

Dipotassium hydrogenphosphate (K_2HPO_4)	20 g
Water	1 000 ml

Preparation

Dissolve the salt in the water by heating at 45 to 50 °C. Adjust the pH. For primary dilution of acid casein and lactic casein, the pH after sterilization should be 8,4 ± 0,1 at 25 °C. For caseinates, cheese, processed cheese, dried acid whey and roller-dried milk, it should be 7,5 ± 0,1 at 25 °C.

5.4 Distribution, sterilization and storage of diluent

Dispense the diluent (5.2 or 5.3) for the primary dilution into flasks or bottles (6.4). Dispense the diluent for further decimal dilutions (5.2) into test tubes or bottles (6.6). The quantities dispensed shall be such that, after sterilization, each flask or bottle (6.4) contains 90 ml of diluent or a multiple of 90 ml, and each test tube or bottle (6.6) contains 9,0 ml of diluent or a multiple of 9,0 ml (or other required quantities). Stopper the test tubes, flasks or bottles.

Sterilize by autoclaving at 121 ± 1°C for 15 min (a longer period may be necessary for larger volumes). If the diluent is not to be used immediately, store it in the dark at 0 to 5 °C, for no longer than one month, in conditions which do not allow any change in its volume or composition.

5.5 Culture media

5.5.1 Violet red bile lactose (VRBL) agar, solid selective medium

Composition

Peptone	7,0 g
Yeast extract	3,0 g
Lactose ($C_{12}H_{22}O_{11} \cdot H_2O$)	10 g
Sodium chloride (NaCl)	5 g
Bile salts	1,5 g
Neutral red	0,03 g

Crystal violet	0,002 g
Agar	12 to 18 g[1)]
Water	1 000 ml

Preparation

Proceed as follows in order to conserve the selective power and specificity of the medium.

Dissolve the components or the dehydrated complete medium in the water and leave to stand for several minutes. Then mix vigorously and adjust the pH so that, after boiling, it is 7,4 ± 0,1 at 25 °C. Bring to the boil, swirling from time to time.

Allow to boil for 2 min. Dispense the medium in 100 to 150 ml quantities into sterile flasks (6.5). Immediately cool the medium to 45 ± 1 °C, in a water bath (6.15).

Avoid overheating the medium or heating it for too long (or reheating it). Consequently, do not sterilize in the autoclave, and check the sterility of the medium at the time of use (see 8.5.1).

Use the medium within 3 h of its preparation.

5.5.2 Lactose bile brilliant green broth, confirmatory medium

Composition

Peptone	10 g
Lactose ($C_{12}H_{22}O_{11}\cdot H_2O$)	10 g
Dehydrated ox bile	20 g
Brilliant green	0,013 3 g
Water	1 000 ml

Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling.

If necessary, adjust the pH so that, after sterilization, it is 7,2 ± 0,1 at 25 °C.

Dispense the medium, in quantities of 10 ml, in test tubes (6.7) containing Durham tubes (6.8).

Sterilize in an autoclave (6.1) at 121 ± 1°C for 15 min.

The Durham tubes shall not contain air bubbles after sterilization.

1) According to the manufacturer's instructions.

6 Apparatus and glassware

NOTE — Disposable apparatus is an acceptabe alternative to re-usable glassware if it has suitable specifications. Re-usable glassware should be capable of undergoing repeated sterilization and should be chemically inert.

Usual microbiological laboratory equipment and in particular:

6.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave) (autoclave operating either separately or as part of an apparatus for preparing and distributing media). Apparatus that will come into contact with the diluent, the test sample, or the dilutions, except for apparatus that is supplied sterile (plastic bags, plastic pipettes, etc.) shall be sterilized by one of the following methods:

a) by being kept at 170 to 175 °C for not less than 1 h in an oven;

b) by being kept at 121 ± 1 °C for not less than 20 min in an autoclave.

6.2 Blending equipment

One of the following shall be used:

a) a rotary blender, operating at a rotational frequency between 8 000 and 45 000 min^{-1}, with glass or metal bowls fitted with lids, resistant to the conditions of sterilization;

b) a peristaltic-type blender (stomacher), with sterile plastic bags;

c) mortar with pestle.

NOTE — The bowls, plastic bags or mortar should have sufficient capacity to allow the sample to be properly mixed with the appropriate amount of diluent. In general, the volume of the container should be equal to about twice the volume of the test sample plus diluent.

6.3 Mixer, capable of mixing 1 or 2 ml of the test sample (in the case of liquid products), or the decimal dilutions, in a tube of adequate dimensions with 9 or 18 ml of diluent, in order to obtain a homogeneous suspension, and working on the principle of eccentric rotation of the contents of the test tube (Vortex mixer).

6.4 Flasks or **bottles,** of sufficient capacity to contain the 90 ml of diluent used for the initial suspension, or multiples of 90 ml, and leave adequate head-space for mixing.

6.5 Flasks, of capacity 150 to 250 ml, to hold the violet red bile lactose agar (5.5.1).

6.6 Test tubes (or **flask** or **bottles**), of sufficient capacity to contain, and leave adequate head-space for mixing, 10 ml (or a multiple of 10 ml, if necessary) of the test sample (if it is liquid) or of the primary dilution (in other cases) or further decimal dilutions.

6.7 Test tubes, of capacity 20 ml, to hold the lactose bile brilliant green broth (5.5.2).

6.8 Durham tubes, of appropriate dimensions for use with the test tubes (6.7).

6.9 Pipettes (plugged with cotton wool), of nominal capacity 1 ml and having an outlet of diameter 2 to 3 mm.

NOTE — Use only pipettes with unbroken tips and, when appropriate, having graduations distinctly marked to contrast sharply with the contents.

6.10 Graduated pipettes (plugged with cotton wool), of large capacity, for example 10 or 20 ml.

NOTE — Use only pipettes with unbroken tips and, when appropriate, having graduations distinctly marked to contrast sharply with the contents.

6.11 Petri dishes, made of glass or plastic, diameter 90 to 100 ml.

6.12 Glass beads, diameter about 6 mm.

6.13 pH-meter, accurate to ± 0,1 pH unit at 25 °C.

6.14 Balance, with sufficient weighing capacity and accurate to within 1 % of the net mass being weighed.

6.15 Water bath, capable of being maintained at a temperature of 45 ± 1 °C.

6.16 Water bath, capable of being maintained at a temperature of 37 ± 1 °C.

6.17 Incubator, designed to be capable of maintaining a temperature of 30 ± 1°C at all points within it.

7 Sampling

See ISO 707.

8 Procedure

NOTES

1 The operations described in 8.1 to 8.5 should not be carried out in direct sunlight.

2 Normal aseptic precautions should be taken whenever necessary.

8.1 Preparation of the test portion and primary dilution

Prepare dilutions so as to obtain plates with colony counts of more than 10, if possible, and fewer than 150.

To avoid damaging the micro-organisms by sudden changes in temperature, the temperature of the diluent during the operations described below shall be approximately the same as that of the test sample, unless prescribed otherwise.

8.1.1 Milk, and liquid milk products

Agitate the test sample thoroughly so that the micro-organisms are distributed as evenly as possible, by rapidly inverting the sample container 25 times. Foaming should be avoided or foam allowed to disperse. The interval between mixing and removing the test portion should not exceed 3 min.

Remove 1 ml of the test sample with a pipette and add to 9 ml of diluent (5.2) (or 10 ml of test sample to 90 ml of diluent or 11 ml of test sample to 99 ml of diluent). Shake this primary dilution (for example, 25 times, with a movement of about 300 mm, in about 10 s). A 10^{-1} dilution is thus obtained.

Prepare further dilutions in accordance with 8.2.

8.1.2 Dried milk, dried sweet whey, dried buttermilk, and lactose

Thoroughly mix the contents of the closed container by repeatedly shaking and inverting. If the test sample is in the original unopened container, too full to permit thorough mixing, transfer to a larger container. Mix. Open the container, remove the test portion required with a spatula and proceed as indicated below. Immediately close the container again.

Warm a bottle containing 90 ml of the appropriate diluent to 45 ± 1 °C in the water bath (6.15).

Weigh 10 g of the test sample into a suitable glass vessel (for example a beaker) and drop the powder into the dilution bottle containing a suitable diluent (5.2 or, if necessary, for roller-dried milk, 5.3.1 or 5.3.2 at pH 7,5 ± 0,1). Alternatively, weigh 10 g of the test sample directly into the bottle with the diluent.

In order to dissolve, swirl slowly to wet the powder and then shake the bottle 25 times, with a movement of about 300 mm, in about 10 s. A peristaltic-type blender [6.2.b)] may be used as an alternative to shaking.

Replace the bottle in the water bath for 5 min, shaking occasionally. A 10^{-1} dilution in thus obtained.

Prepare further dilutions in accordance with 8.2.

NOTE — For better reconstitution, particularly with roller-dried milk, glass beads (6.12) can be helpful. If used they should be added to the bottle (6.4) before sterilization.

8.1.3 Cheese and processed cheese

Weigh 10 g of the cheese or processed cheese in a dish. Transfer to the container of a rotary blender [6.2.a)], or a peristaltic-type blender [6.2.b)] or a mortar [6.2.c)].

When a rotary blender or a peristaltic-type blender is used, add 90 ml of diluent (5.2, 5.3.1, or 5.3.2 at pH 7,5 ± 0,1). Blend until the cheese is thoroughly dispersed (1 to 3 min). Ideally, ensure that the temperature of the dispersion does not exceed 40 °C, and in any case do not allow it to exceed 45 °C. Allow any foam to disperse. With a mortar, add a minimum of diluent and mix with the pestle to obtain a uniform paste free from lumps. Add the remainder of the diluent to a total of 90 ml of diluent. A 10^{-1} dilution is thus obtained.

Prepare further dilutions in accordance with 8.2.

8.1.4 Acid casein, lactic casein and rennet casein

Weigh 10 g of the product in a dish. Transfer to a dilution bottle containing glass beads (6.12) and 90 ml of dipotassium hydrogenphosphate diluent (5.3.2) at pH 8,4 for acid and lactic caseins.

Leave for 15 min and then raise the temperature to 37 ± 1 °C in a water bath (6.16).

Keep the bottle at 37 °C for a further 15 min and shake vigorously at intervals. A 10^{-1} dilution is thus obtained.

NOTE — Avoid using a rotary blender [6.2.a)] or a peristaltic-type blender [6.2.b)] because of the formation of foam that ensues.

Prepare further dilutions in accordance with 8.2.

8.1.5 Caseinate and dried acid whey

Weigh 10 g of the product in a dish. Sprinkle it very slowly on to the surface of 90 ml of dipotassium hydrogenphosphate diluent (5.3.2) at pH 7,5 ± 0,1 in a dilution bottle, shaking the mixture after each addition.

Alternatively, add the dry product to a minimum volume of the diluent and stir with a glass rod to obtain a uniformly wetted paste free from lumps. Add the remainder of the diluent, to a total of 90 ml of diluent.

Leave for 15 min and then raise the temperature to 37 ± 1 °C in a water bath (6.16). Keep the dilution bottle at 37 °C for a further 15 min. Mix thoroughly with a rotary blender [6.2.a)] or peristaltic-type blender [6.2.b)]. Allow foam to subside before proceeding. A 10^{-1} dilution is thus obtained.

Prepare further dilutions in accordance with 8.2.

8.1.6 Butter

Place the test sample in a container in a water bath at 45 ± 1 °C (6.15). Agitate to facilitate melting and leave until the whole test sample has just melted. Shake and, with a pipette warmed to approximately 45 °C, transfer 10 ml into a flask containing 90 ml of diluent (5.2). Shake each time before making further transfers. A peristaltic-type blender [6.2.b)] may be used for mixing. A 10^{-1} dilution is thus obtained.

Alternatively, use only the aqueous phase for dilution, as follows.

Take a test portion of 50 g (containing about 8 ml of water) and add 42 ml of diluent (5.2.3) warmed to 45 °C. Place the container in a water bath at 45 ± 1 °C (6.15) until the butter melts. Shake well and allow to separate for no longer than 15 min. Pipette from the bottom layer; 1 ml is equivalent to 1 g of butter.

Prepare further dilutions in accordance with 8.2.

8.1.7 Frozen milk products (including edible ices)

Proceed as in the case of butter (8.1.6) (first alternative), but using a water bath at no more than 37 °C (6.16). The temperature of the test sample shall not be allowed to exceed 37 °C. A 10^{-1} dilution is thus obtained.

8.1.8 Custard, desserts, fermented milk, and cream

Weigh 10 g of the product into a flask (6.4) containing glass beads (6.12).

For custard, desserts and sweet cream, add 90 ml of diluent (5.2) and shake to disperse. For fermented milk and sour cream, use diluent 5.2 or 5.3.2 at pH 7,5 ± 0,1. A peristaltic-type blender [6.2.b)] may be used. A 10^{-1} dilution is thus obtained.

Prepare further dilutions in accordance with 8.2.

8.2 Further decimal dilutions

Transfer, by means of a fresh pipette, 1 ml of the primary dilution into another tube containing 9 ml of sterile diluent, avoiding contact between the pipette and the diluent. A fresh pipette should be used for each dilution.

Alternatively, transfer 10 ml of the primary dilution to a bottle containing 90 ml of sterile diluent, or 11 ml of the primary dilution to 99 ml of sterile diluent. In a routine procedure, if a 10^{-3} dilution is required, transfer 1 ml of primary dilution to 99 ml of sterile diluent.

Mix carefully, either by aspirating 10 times with a fresh pipette, or in the mechanical mixer (6.3) for 5 to 10 s to obtain a 10^{-2} dilution. The frequency of rotation of the latter shall be chosen so that the liquid, as it swirls, rises to within 20 to 30 mm of the rim of the vessel.

If necessary, repeat these operations using the 10^{-2} and further dilutions to obtain 10^{-3}, 10^{-4}, etc., dilutions until the appropriate number of micro-organisms has been obtained (see 8.1).

When 10 ml plus 90 ml, 11 ml plus 99 ml or 1 ml plus 99 ml have been taken, shake manually (for example 25 times, with a movement of about 300 mm, in about 10 s).

8.3 Duration of the procedure

The time between initial measurement of a test portion or the end of the preparation of the primary dilution and mixing dilutions and medium shall be not more than 15 min, unless prescribed otherwise.

8.4 Inoculation

Prepare two dishes from the liquid product and/or from each dilution chosen (see 8.1).

With a pipette, transfer 1 ml of liquid product or the appropriate dilutions to the centre of each dish. Touch the tip of the pipette onto a dry area in the Petri dish. Use another pipette to inoculate each dilution into the dishes.

8.5 Pouring

8.5.1 Pour about 12 ml of the VRBL agar (5.5.1) at 45 ± 1 °C into each Petri dish. (See the note.)

Mix immediately after pouring, by rotating the Petri dish sufficiently to obtain evenly dispersed colonies after incubation. Allow to solidify on a cool, horizontal surface.

Prepare a control dish, with 12 ml of the medium, to check its sterility.

NOTE — In order to ensure that the temperature is 45 ± 1 °C before pouring, place a thermometer into a portion of 1,5 % (*m*/*m*) agar solution in a separate container identical with that used for the medium. This control portion should be exposed to the same heating and cooling as the medium itself.

8.5.2 After complete solidification, pour about 4 ml of the VRBL agar (5.5.1) at 45 ± 1 °C, onto the surface of the inoculated medium. Allow to solidify.

8.6 Incubation

Incubate the plates in an inverted position. Stack not more than six high. Stacks of plates should be separated from one another and from the walls and top of the incubator.

Incubate at 30 ± 1 °C for 24 ± 2 h.

8.7 Counting

After incubation of the plates, select those with more than 10 and fewer than 150 colonies. Count the dark red coloured colonies having a diameter of at least 0,5 mm, characteristic for coliforms.

Confirm doubtful colonies, which can in particular be expected in the case of milk products containing sugars other than lactose, immediately after the incubation period, according to 8.8.

Calculate the coliform count per gram or millilitre, taking into account the result of the confirmatory test, if carried out, by the formula given in 9.2.

8.8 Confirmatory test

Inoculate five colonies of each type, if available, into tubes of lactose bile brilliant green broth (5.5.2) and incubate at 30 ± 1 °C for 24 ± 2 h. Consider colonies which show gas formation in the Durham tube as coliforms.

Calculate the number of colonies of coliforms per plate from the percentage(s) of confirmed coliform colonies and the number of colonies of each type in the plate.

9 Expression of results

9.1 Use counts from all plates containing more than 10 and fewer than 150 colonies.

9.2 The number of coliforms per millilitre (liquid product) or per gram (other products) is equal to

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

where

ΣC is the sum of coliform colonies counted as in 8.7 or 8.8;

n_1 is the number of plates counted in the first dilution to which 8.7 has been applied;

n_2 is the number of plates counted in the second dilution to which 8.7 has been applied;

d is the dilution factor from which the first counts were obtained.

9.3 In reporting the number of coliforms, round off the number in 9.2 to two significant figures. When the digit to be rounded off is 5, round off so that the figure immediately to the left is even.

NOTE — If there are more than two countable dilutions the formula should be modified to take the further dilution into account.

Thus for three dilutions, the number of coliforms per millilitre or per gram is equal to

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

9.4 If there are only counts less than 10, report the number of coliforms per millilitre or gram as "less than 10 × d", where d is the reciprocal of the lowest dilution.

9.5 If there are only counts exceeding 150, calculate an estimated count from dishes having a count nearest 150 colonies and multiply by the reciprocal of the dilution factor. Report as the "Estimated number of coliforms per millilitre" or "per gram".

9.6 The result may be expressed as a number between 1,0 and 9,9 multiplied by 10^x, where x is the appropriate power of 10.

10 Test report

The test report shall show the method used and the results obtained, indicating clearly the method of expression used. It shall also mention any operating details not specified in this part of ISO 5541, or regarded as optional, together with details of any incidents likely to have influenced the results.

The test report shall include all the information necessary for the complete identification of the sample.

Bibliography

[1] ISO 4832, *Microbiology — General guidance for enumeration of coliforms — Colony count technique at* 30 °C. International Organization for Standardization, 1978.

[2] ISO 8261, *Milk and milk productss — Preparation of test samples and dilutions for microbiological examination.* International Organization for Standardization. (To be published.)