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EAS 68-2-2 (2007) (English): Milk and milk products — Methods of microbiological examination Part 2-2: Enumeration of coliforms Most probable number technique at 30 °C

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EAST AFRICAN STANDARD

Milk and milk products — Methods of microbiological examination Part 2-2: Enumeration of coliforms — Most probable number technique at 30 °C

EAST AFRICAN COMMUNITY

First Edition 2006

Foreword

Development of the East African Standards has been necessitated by the need for harmonizing requirements governing quality of products and services in East Africa. It is envisaged that through harmonized standardization, trade barriers which are encountered when goods and services are exchanged within the Community will be removed.

In order to achieve this objective, the Partner States in the Community through their National Bureaux of Standards, have established an East African Standards Committee.

The Committee is composed of representatives of the National Standards Bodies in Partner States, together with the representatives from the private sectors and consumer organizations. Draft East African Standards are circulated to stakeholders through the National Standards Bodies in the Partner States. The comments received are discussed and incorporated before finalization of standards, in accordance with the procedures of the Community.

East African Standards are subject to review, to keep pace with technological advances. Users of the East African Standards are therefore expected to ensure that they always have the latest versions of the standards they are implementing.

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International Standard 5541/2

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

Milk and milk products — Enumeration of coliforms — Part 2: Most probable number technique at 30 °C

Lait et produits laitiers — Dénombrement des coliformes — Partie 2: Technique du nombre le plus probable après incubation à 30 °C

First edition — 1986-12-01

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

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.

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 5541/2 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 2: Most probable number 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 culture technique involving a liquid medium, and calculation of the most probable number (MPN) after incubation 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 comparatively low numbers of coliforms (less than 100 per gram or 10 per millilitre) are suspected.

NOTE — For samples with larger numbers of coliforms (more than 100 per gram or 10 per millilitre) see ISO 5541/1.

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, cause fermentation of lactose with the production of gas and form characteristic growth under the operational conditions described.

4 Principle

4.1 Inoculation of a test portion and/or a series of decimal dilutions of the sample in triplicate into the selective liquid medium prescribed in test tubes containing Durham tubes.

4.2 Incubation of the tubes at 30 °C for 48 h.

4.3 From presumed positive tubes, i.e. those tubes showing gas production in the Durham tube in lactose bile brilliant green broth, inoculation on eosin-methylene blue agar.

4.4 Incubation at 30 °C for 24 h.

4.5 From confirmed positive tubes, i.e. those tubes showing gas production in lactose bile brilliant green broth and characteristic growth on eosin-methylene blue agar, calculation of the number of coliforms per millilitre or per gram of sample (i.e. the MPN) using a table.

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 1,0 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 Lactose bile brilliant green broth, liquid selective 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.

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.

5.5.2 Double-strength broth

Proceed as described in 5.5.1, but use half the quantity of water and distribute in 20 × 200 mm test tubes without Durham tubes.

5.5.3 Eosin-methylene blue agar, confirmatory medium.

Composition

Peptone	10 g
Lactose ($C_{12}H_{22}O_{11}·H_2O$)	10 g
Dipotassium hydrogenphosphate (K_2HPO_4)	2 g
Eosin Y	0,4 g
Methylene blue	0,065 g
Agar	12 to 18 g[1)]
Water	1 000 ml

Preparation

Suspend the solid components in the water. Adjust the pH, if necessary, so that, after sterilization, it is 6,8 ± 0,2 at 25 °C. Bring to the boil to dissolve completely. Dispense the medium in 100 to 150 ml quantities into flasks (6.5). Sterilize in an autoclave at 121 ± 1 °C for 15 min. Cool to 60 °C and shake the medium in order to oxidize the methylene blue (i.e. restore its blue colour) and to suspend the precipitate, which is an essential part of the medium.

Prepare plates containing 10 to 15 ml of medium for use in 8.6.

6 Apparatus and glassware

NOTE — Disposable apparatus is an acceptable 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 eosin-methylene blue agar (5.5.3).

6.6 Test tubes or **flasks** 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 (5.5.1).

1) According to the manufacturer's instructions.

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.

6.18 Loop, of platinium-iridium or nickel-chromium, diameter approximately 3 mm.

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

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 [6.2b)] 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.

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 300 mm, in about 10 s).

Make a sufficient number of dilutions to ensure that all tubes corresponding to the final dilution will yield a negative result.

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

8.4.1 Take three tubes of double-strength broth (5.5.2) and transfer to each of these tubes, using a pipette, 10 ml of the liquid test sample or 10 ml of the primary dilution.

8.4.2 Take three tubes of single-strength broth (5.5.1), and transfer to each of these tubes, using a pipette, 1 ml of the liquid test sample, or 1 ml of the primary dilution.

8.4.3 For each of the following dilutions (from 10^{-1} or 10^{-2}, according to the circumstances) take three tubes of single-strength broth (5.5.1). Transfer 1 ml of the respective dilution into each of these tubes.

Change the pipette for each dilution. Carefully mix the inoculum and the medium.

8.5 Incubation

8.5.1 Incubate the tubes of double-strength broth (8.4.1) at 30 ± 1 °C for 24 ± 2 h.

8.5.2 Incubate the tubes of single-strength broth (8.4.2 and 8.4.3) at 30 ± 1 °C for 48 ± 2 h.

8.6 Inoculation from incubated tubes of double-strength broth

From each of the incubated tubes of double-strength broth (8.4.1) inoculate with a loop (6.18) a tube of single-strength broth (5.5.1). Incubate at 30 ± 1 °C for 48 ± 2 h.

8.7 Confirmatory test

8.7.1 From each of the incubated tubes (8.5.2 and 8.6) showing production of gas in the Durham tubes, streak a loopful on eosin-methylene blue agar (5.5.3). Incubate at 30 ± 1 °C for 24 ± 2 h.

8.7.2 Consider as characteristic growth those colonies that are metallic, red/pink and mucoid in appearance.

If further evidence is needed, inoculate colonies in tubes containing single-strength broth (5.5.1) and check for gas production after incubation (8.5.2).

8.7.3 Record for each dilution the number of confirmed positive tubes.

9 Expression of results

9.1 Selection of dilutions

For each sample examined, select three consecutive dilutions in accordance with one of the three following rules, whichever is appropriate:

a) *When at least one dilution yielding three confirmed positive tubes exists*

Select the highest dilution (i.e. that having the lowest sample concentration) yielding three confirmed positive tubes, together with the next two higher dilutions (i.e. those having sample concentrations of 10^{-1} and 10^{-2} of that of the first dilution selected).

See also rule c).

If insufficient further dilutions were made beyond the highest dilution yielding three confirmed positive tubes, select instead the three highest dilutions in the series (i.e. those having the lowest sample concentration).

b) *When no dilution yielding three confirmed positive tubes exists*

If rule a) cannot be applied, select the three highest dilutions in the series (i.e. those having the lowest sample concentration).

See also rule c).

c) *Special case*

In all cases where more than one of the three dilutions selected in accordance with rules a) and b) does not yield confirmed positive tubes, select from these dilutions the lowest one not yielding confirmed positive tubes (i.e. that having the highest sample concentration) and the two next lower dilutions in the series (i.e. those having sample concentrations of 10 times and 100 times that of the first dilution selected) except when confirmed positive tubes are only found at the level of the first dilution prepared from the sample. In this last case, it is necessary to select the first three dilutions for calculation of the MPN even though this series includes two dilutions yielding no confirmed positive tube.

See examples in table 1.

Table 1 — Examples of selection of dilutions

Example	Number of confirmed positive tubes at each sample concentration[1]				MPN
	10 g or 10 ml	1 g or 1 ml	0,1 g or 0,1 ml	0,01 g or 0,01 ml	
A	3	1	1	0	7 per 10 g or 10 ml
B	2	1	0	0	1,5 per 10 g or 10 ml or 15 per 100 g or 100 ml
C		3	2	0	9 per 1 g or 1 ml
D		3	2	2	21 per 1 g or 1 ml
E		3	0	1	4 per 1 g or 1 ml

1) Selected dilutions are underlined.

9.2 Determination of the MPN index

Determine the MPN index of coliforms from the number of confirmed positive tubes for each dilution selected, in accordance with table 2.

9.3 Calculation of most probable number (MPN)

Obtain the number of coliforms per millilitre or per gram by multiplying the MPN index (see 9.2) by the reciprocal of the lowest dilution selected (i.e. that having the highest sample concentration).

When the lowest dilution selected corresponds to the tubes prepared with double-strength medium (inoculation with 10 ml), first divide the MPN index by 10.

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.

9.4 Precision

It is recognized that wide variations in results may occur with the MPN technique. Results obtained from this method should therefore be used with caution.

Confidence limits are given in table 2.

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 4831, *Microbiology — General guidance for the enumeration of coliforms — Most probable number technique at* 30 °C. International Organization for Standardization, 1978.

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

Table 2 — MPN index and confidence limits

Number of confirmed positive tubes for the three dilutions selected[1]			MPN index	Confidence limits			
First	Second	Third		> 95 %	> 95 %	> 99 %	> 99 %
0	0	0	< 0,30	0,00	0,94	0,00	1,40
0	0	1	0,30	0,01	0,95	0,00	1,40
0	1	0	0,30	0,01	1,00	0,00	1,60
0	1	1	0,61	0,12	1,70	0,05	2,50
0	2	0	0,62	0,12	1,70	0,05	2,50
0	3	0	0,94	0,35	3,50	0,18	4,60
1	0	0	0,36	0,02	1,70	0,01	2,50
1	0	1	0,72	0,12	1,70	0,05	2,50
1	0	2	1,1	0,4	3,5	0,2	4,6
1	1	0	0,74	0,13	2,00	0,06	2,70
1	1	1	1,1	0,4	3,5	0,2	4,6
1	2	0	1,1	0,4	3,5	0,2	4,6
1	2	1	1,5	0,5	3,8	0,2	5,2
1	3	0	1,6	0,5	3,8	0,2	5,2
2	0	0	0,92	0,15	3,50	0,07	4,60
2	0	1	1,4	0,4	3,5	0,2	4,6
2	0	2	2,0	0,5	3,8	0,2	5,2
2	1	0	1,5	0,4	3,8	0,2	5,2
2	1	1	2,0	0,5	3,8	0,2	5,2
2	1	2	2,7	0,9	9,4	0,5	14,2
2	2	0	2,1	0,5	4,0	0,2	5,6
2	2	1	2,8	0,9	9,4	0,5	14,2
2	2	2	3,5	0,9	9,4	0,5	14,2
2	3	0	2,9	0,9	9,4	0,5	14,2
2	3	1	3,6	0,9	9,4	0,5	14,2
3	0	0	2,3	0,5	9,4	0,3	14,2
3	0	1	3,8	0,9	10,4	0,5	15,7
3	0	2	6,4	1,6	18,1	1,0	25,0
3	1	0	4,3	0,9	18,1	0,5	25,0
3	1	1	7,5	1,7	19,9	1,1	27,0
3	1	2	12	3	36	2	44
3	1	3	16	3	38	2	52
3	2	0	9,3	1,8	36,0	1,2	43,0
3	2	1	15	3	38	2	52
3	2	2	21	3	40	2	56
3	2	3	29	9	99	5	152
3	3	0	24	4	99	3	152
3	3	1	46	9	198	5	283
3	3	2	110	20	400	10	570
3	3	3	> 110				

1) See de MAN, J.C. MPN tables, corrected. *Eur. J. Appl. Biotechnol.* **17**, 1983: 301-305.

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