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Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of coliforms — Most probable number technique

Microbiologie des aliments — Méthode horizontale pour la recherche et le dénombrement des coliformes — Technique du nombre le plus probable

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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 4831 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

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This third edition of ISO 4831 cancels and replaces ISO 4831:1991 and ISO 5541-2:1986. Clauses 4, 9 and 10 of ISO 4831:1991 have been technically revised. The main changes are as follows:

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— the alternative procedure of incubation at 35 °C has been deleted;

— detection and enumeration of coliforms are covered (Clauses 4 and 9);

— description of the MPN and the CCT have been omitted (Clause 10) and reference is given to ISO 7218.

Considering the nature of the changes to the previous edition of this International Standard, it is considered that the validation of alternative methods based on ISO 4831:1991 is not affected by this revision.

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Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods, which are specific to these products, may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method in the case of particular products.

The harmonization of test methods cannot be immediate and, for certain groups of products, International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this International Standard so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

The technique described in this International Standard is less precise than that described in ISO 4832 [1], but allows a microbiological examination to be carried out on a larger test portion, thus permitting a lower number of coliforms per gram or per millilitre of product to be detected. Moreover, since the definition of "coliforms" adopted in the two documents is different, the microorganisms enumerated are not necessarily the same.

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For any particular product, the method to be chosen will be specified in the International Standard dealing with that product.

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For the purposes of a practicable test method, the definition of "coliforms" given in Clause 3 and used as the basis for the procedure is not necessarily identical to corresponding definitions given in other published texts. A proportion of strains of the microorganisms described in other published texts as "coliforms" (including *Escherichia coli*) fail to produce enough gas to be detectable by use of a Durham tube (i.e. "anaerogenic strains"). Therefore the method described in this International Standard will not detect all strains of the microorganisms referred to in other publications as "(presumptive) coliforms" (e.g. certain strains of *Citrobacter*, *Enterobacter*, *Klebsiella*) (see Reference [2]).

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Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of coliforms — Most probable number technique

1 Scope

This International Standard gives general guidelines for the detection and the enumeration of coliforms. It is applicable to

— products intended for human consumption and for the feeding of animals, and

— environmental samples in the area of food production and food handling.

Enumeration is carried out by calculation of the most probable number (MPN) after incubation in a liquid medium at 30 °C or 37 °C.

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NOTE The temperature is subject to agreement between the parties concerned. In the case of milk and milk products, the temperature of incubation is 30 °C.

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This enumeration method is applicable when the number sought is expected to be in the range 1 to 100 per millilitre or per gram of test sample.

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A limitation on the applicability of this International Standard is imposed by the susceptibility of the method to a large degree of variability. The information given in Clause 11 provides guidance on the applicability of the method and on the interpretation of the results.

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 (all parts), *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

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

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

ISO/TS 11133-2:2003, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing of culture media*

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3 Terms and definitions

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

3.1
coliforms
bacteria which, at the specified temperature (i.e. 30 °C or 37 °C, as agreed) cause fermentation of lactose with the production of gas under the test conditions specified in this International Standard

3.2
detection of coliforms
determination of the presence or absence of these bacteria, in a particular quantity of product, when tests are carried out in accordance with the method specified in this International Standard

3.3
enumeration of coliforms
most probable number of coliforms found per millilitre or per gram of the test sample, when the test is carried out in accordance with the method specified in this International Standard

4 Principle

4.1 Detection of coliforms

4.1.1 A tube of selective enrichment broth is inoculated with the test portion and incubated at 30 °C or 37 °C (as agreed) for 24 h or 48 h.

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4.1.2 A tube of confirmation medium is inoculated from the tube obtained in 4.1.1 when opacity and/or gas formation has been noted, and incubated at 30 °C or 37 °C (as agreed) for 24 h or 48 h.

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4.1.3 The presence of coliforms is confirmed in the case that opacity and gas formation have been noted after examination of the tube obtained in 4.1.2.

4.2 Enumeration by the MPN technique

4.2.1 Three tubes of double-strength liquid selective enrichment medium are inoculated with a specified quantity of the test sample if the initial product is liquid, or with a specified quantity of an initial suspension in the case of other products.

4.2.2 Three tubes of single-strength liquid selective enrichment medium are inoculated with a specified quantity of the test sample if the initial product is liquid, or with a specified quantity of an initial suspension in the case of other products. Then, under the same conditions, further tubes of single-strength medium are inoculated with decimal dilutions of the test sample or of the initial suspension.

4.2.3 The tubes containing double-strength selective enrichment medium are incubated at 30 °C or 37 °C (as agreed) for 24 h, and the tubes containing single-strength medium are incubated for 24 h or 48 h, after which period these tubes are examined for gas formation or opacity preventing the detection of gas formation.

4.2.4 A series of tubes of the confirmation medium are inoculated with the cultures from the tubes of double-strength selective enrichment medium, and with the cultures from the tubes of single-strength selective enrichment medium in which gas formation or opacity preventing the detection of gas formation has been noted.

4.2.5 The tubes from 4.2.4 are incubated at 30 °C or 37 °C (as agreed) for 24 h or 48 h and the tubes are examined for gas formation.

4.2.6 The most probable number of coliforms per millilitre or per gram of sample (i.e. the MPN) is calculated from the number of tubes in the new series (4.2.5) showing gas formation. A table for determination of most probable numbers is used.

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5 Culture media and diluents

5.1 General

See ISO 7218, ISO/TS 11133-1 and ISO/TS 11133-2 for the preparation, production and performance testing of culture media.

5.2 Diluents

See ISO 6887 (relevant part), ISO 8261 or the specific International Standard dealing with the product under examination.

5.3 Selective enrichment medium: Lauryl sulfate tryptose broth

5.3.1 Composition

	a) Double-strength medium	b) Single-strength medium
Enzymatic digest of milk and animal proteins	40 g	20 g
Lactose ($C_{12}H_{22}O_{11}·H_2O$)	10 g	5 g
Dipotassium hydrogen phosphate (K_2HPO_4)	5,5 g	2,75 g
Potassium dihydrogen phosphate (KH_2PO_4)	5,5 g	2,75 g
Sodium chloride	10 g	5 g
Sodium lauryl sulfate	0,2 g	0,1 g
Water	1 000 ml	1 000 ml

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5.3.2 Preparation

Dissolve the different components or the dehydrated complete medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Dispense the media in quantities of 10 ml into tubes of dimensions of approximately 16 mm × 160 mm (6.4) containing Durham tubes (6.5) in the case of single-strength medium, and into test tubes of dimensions of approximately 20 mm × 200 mm (6.4) [not containing Durham tubes (6.5)] in the case of the double-strength medium.

Sterilize in an autoclave set at 121 °C for 15 min. The Durham tubes shall not contain air bubbles after sterilization.

5.3.3 Performance testing for the quality assurance of the culture medium

For the definitions of selectivity and productivity, refer to ISO/TS 11133-1. Performance testing relating to lauryl sulfate tryptose broth is given in ISO/TS 11133-2:2003, Table B.1.

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5.4 Confirmation medium: Brilliant green lactose bile broth

5.4.1 Composition

Enzymatic digest of casein	10 g
Lactose ($C_{12}H_{22}O_{11}·H_2O$)	10 g
Dehydrated ox bile	20 g
Brilliant green	0,013 3 g
Water	1 000 ml

5.4.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,2 ± 0,2 at 25 °C.

Dispense the medium in quantities of 10 ml in test tubes of approximately 16 mm × 160 mm (6.4) containing Durham tubes (6.5).

Sterilize in an autoclave set at 121 °C for 15 min. The Durham tubes shall not contain air bubbles after sterilization.

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5.4.3 Performance testing for the quality assurance of the culture medium

For the definitions of selectivity and productivity, refer to ISO/TS 11133-1. Performance testing relating to lactose bile brilliant green broth is given in ISO/TS 11133-2:2003, Table B.1.

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6 Apparatus and glassware

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) or **wet sterilization** (autoclave).

See ISO 7218.

6.2 Incubator, capable of operating at 30 °C ± 1 °C or 37 °C ± 1 °C.

6.3 Loop, made of platinum-iridium, or nickel-chromium, approximately 3 mm in diameter, or disposable loops.

6.4 Test tubes, of dimensions approximately 16 mm × 160 mm and 20 mm × 200 mm.

6.5 Durham tubes, of a size suitable for use in the test tubes of dimensions 16 mm × 160 mm (6.4).

6.6 Total-delivery pipettes, having nominal capacities of 1 ml and 10 ml.

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

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7 Sampling

Sampling should have been carried out in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

8 Preparation of the test sample

Prepare the test sample in accordance with ISO 6887 (relevant part), ISO 8261 or the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure (see Annex A)

9.1 Detection method (see Figure A.1)

9.1.1 Test portion and initial suspension

See ISO 6887 (relevant part), ISO 8261 or the specific International Standard appropriate to the product concerned.

9.1.2 Inoculation and incubation

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9.1.2.1 Depending on the limit of detection that is required, x ml of the test sample if liquid, or x ml of the initial suspension in the case of other products, is transferred to a tube containing 10 ml of double-strength selective enrichment medium [5.3.1a)] when 1 ml $< x <$ 10 ml, or to a tube containing 10 ml of single-strength selective enrichment medium [5.3.1b)] when $x \leqslant$ 1 ml.

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9.1.2.2 Leave the tube of double-strength medium (9.1.2.1) in the incubator (6.2) set at 30 °C or 37 °C (as agreed) for 24 h ± 2 h.

9.1.2.3 Leave the tube of single-strength medium (9.1.2.1) in the incubator (6.2) at 30 °C or 37 °C (as agreed) for 24 h ± 2 h or, if neither gas formation nor opacity preventing the detection of gas formation is observed at this stage, continue incubation for another 24 h ± 2 h.

9.1.3 Confirmation (see Figure A.3)

9.1.3.1 From the incubated tube from 9.1.2.2, inoculate with a loop (6.3) a tube of confirmation medium (5.4). Incubate in the incubator (6.2) set at 30 °C or 37 °C (as agreed) for 24 h ± 2 h or, if gas formation is not observed at this stage, for 48 h ± 2 h.

9.1.3.2 Carry out the same procedure as described in 9.1.3.1 for the incubated tubes from 9.1.2.3 showing gas formation, or opacity preventing the detection of gas formation, when either of these features is first observed (i.e. after 24 h ± 2 h or after 48 h ± 2 h).

9.1.4 Interpretation (see Figure A.1)

A tube from 9.1.3.1 or 9.1.3.2 in which gas formation is observed after 24 h ± 2 h or 48 h ± 2 h is considered as a positive tube.

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