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Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of presumptive *Escherichia coli* — Most probable number technique

*Microbiologie des aliments — Méthode horizontale pour la recherche et le dénombrement d'*Escherichia coli *présumés — 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 7251 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This third edition cancels and replaces the second edition (ISO 7251:1993), and also ISO 11866-1:1997 and IDF 170-1:1999.

Clauses 4, 9 and 10 of ISO 7251:1993 have been technically revised. The main changes are as follows:

— the temperature of the second incubation is now 44 °C ± 1 °C (see 4.2.5);

— detection (9.1) and enumeration (9.2) of presumptive *E. coli* are covered;

— the use of five tubes per dilution is allowed for some specific products (see 9.2.2.1);

— some products (dairy products) may hinder the collection of gas (see 9.1.2 and 9.2.2.5);

— the MPN calculation refers to ISO 7218 (see Clause 10).

Introduction

Because of the large variety of products within this field of application, these guidelines may not be appropriate in every detail for certain products, and for some other products it may be necessary to use different methods. Nevertheless, it is hoped that in all cases every attempt will be made to apply the provided guidelines as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons for deviations from them 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 these guidelines. In cases where International Standards already exist for the product to be tested, they should be followed, but 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 these guidelines will be those necessary for well-established technical reasons.

Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of presumptive *Escherichia coli* — Most probable number technique

1 Scope

This International Standard gives general guidelines for the detection and enumeration of presumptive *Escherichia coli* by means of the liquid-medium culture technique and calculation of the most probable number (MPN) after incubation at 37 °C, then at 44 °C. This International Standard is applicable to

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

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

WARNING — Some pathogenic strains of *Escherichia coli* do not grow at 44 °C.

A limitation of the applicability of this International Standard is imposed by the susceptibility of the method to a large degree of variability (see Clause 10).

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 6887-2, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products*

ISO 6887-3, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products*

ISO 6887-4, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products*

ISO 7218, *Microbiology of food and animal feeding stuffs — General rules 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, *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*

3 Terms and definitions

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

3.1
presumptive *Escherichia coli*
bacteria which at 44 °C ferment lactose with the production of gas, and which at 44 °C produce indole from tryptophan, when the test is carried out in accordance with the method specified in this International Standard

3.2
enumeration of presumptive *Escherichia coli*
most probable number of *E. coli* per millilitre or per gram of the test sample when the test is carried out according to the method specified in this International Standard

4 Principle

4.1 Detection method

4.1.1 A liquid selective enrichment medium is inoculated with a specified quantity of the initial suspension of the test sample.

4.1.2 The tube is incubated at 37 °C for up to 48 h. The tube is examined for gas production after 24 h and 48 h.

4.1.3 If the tube has given rise to opacity, cloudiness or gaseous emission, it is subcultured to a tube containing a liquid selective medium (EC broth).

4.1.4 The tube obtained in 4.1.3 is incubated at 44 °C for up to 48 h. The tube is examined for gas production after 24 h and 48 h.

4.1.5 If the tube of medium obtained in 4.1.4 has given rise to gaseous emission, it is subcultured to a tube containing indole-free peptone water.

4.1.6 The tube obtained in 4.1.5 is incubated at 44 °C for 48 h. The tube is examined for indole production resulting from the degradation of tryptophan in the peptone constituent.

4.1.7 Tubes showing opacity, cloudiness or gas production in the liquid selective enrichment medium (4.1.1) and whose subcultures have produced gas in the EC broth and indole in the peptone water at 44 °C are considered to contain presumptive *Escherichia coli*. The results are given as the "presence" or "absence" of presumptive *Escherichia coli* in x g or x ml of product.

4.2 Enumeration method

4.2.1 Three tubes of double-strength liquid selective enrichment medium are inoculated with a specified quantity of the initial suspension.

4.2.2 Three tubes of single-strength liquid enrichment medium are inoculated with a specified quantity of the initial suspension.

Then, under the same conditions, another three tubes of the single-strength medium are inoculated with a specified quantity of decimal dilutions of the initial suspension.

4.2.3 The tubes of double- and single-strength medium are incubated at 37 °C for up to 48 h. The tubes are examined for gas production after 24 h and 48 h.

4.2.4 Each tube of double-strength medium that has given rise to opacity, cloudiness or gaseous emission, and each tube of single-strength medium that has given rise to gaseous emission, is subcultured to a tube containing a liquid selective medium (EC broth).

4.2.5 The tubes obtained in 4.2.4 are incubated at 44 °C for up to 48 h. The tubes are examined for gas production after 24 h and 48 h.

4.2.6 Each tube of medium obtained in 4.2.5 that has given rise to gaseous emission is subcultured to a tube containing indole-free peptone water.

4.2.7 The tubes obtained in 4.2.6 are incubated at 44 °C for 48 h. The tubes are examined for indole production resulting from the degradation of tryptophan in the peptone constituent.

4.2.8 The most probable number of presumptive *Escherichia coli* is determined by means of the MPN table (see Annex A), according to the number of tubes of single- and double-strength medium whose subcultures have produced gas in the EC broth and indole in the peptone water at 44 °C.

5 Diluent, culture media and reagent

For current laboratory practice, see ISO 7218.

5.1 Diluent

In general, see ISO 6887-1, but for milk products see ISO 8261.

5.2 **Selective enrichment medium** (lauryl sulfate broth)

5.2.1 Composition

	a) **Double-strength medium**	b) **Single-strength medium**
Enzymatic digest of plant and animal tissues	40,0 g	20,0 g
Lactose	10,0 g	5,0 g
Dipotassium monohydrogen phosphate (K_2HPO_4)	5,5 g	2,75 g
Potassium dihydrogen phosphate (KH_2PO_4)	5,5 g	2,75 g
Sodium chloride	10,0 g	5,0 g
Sodium lauryl sulfate [$CH_3(CH_2)_{11}OSO_3Na$]	0,2 g	0,1 g
Water	1 000 ml	1 000 ml

5.2.2 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 6,8 ± 0,2 at 25 °C.

Dispense the media in quantities of 9 ml into tubes of dimensions 16 mm × 160 mm (6.4) containing Durham tubes (6.6) in the case of single-strength medium, and 10 ml into test tubes of dimensions 18 mm × 180 mm or 20 mm × 200 mm (6.4) containing Durham tubes (6.6) in the case of the double-strength medium.

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

The Durham tubes shall not contain air bubbles after sterilization.

5.3 **EC broth** (selective medium)

5.3.1 Composition

Enzymatic digest of casein	20,0 g
Lactose	5,0 g
Bile salts No. 3[a]	1,5 g
Potassium monohydrogen phosphate (K_2HPO_4)	4,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,5 g
Sodium chloride	5,0 g
Water	1 000 ml

[a] See Reference [1].

5.3.2 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 6,8 ± 0,2 at 25 °C.

Dispense the medium in quantities of 10 ml into tubes of dimensions 16 mm × 160 mm (6.4) containing Durham tubes (6.6).

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

The Durham tubes shall not contain air bubbles after sterilization.

5.3.3 Performance testing and quality assurance of the culture medium

To test the performance of the medium, see ISO/TS 11133-1 and ISO/TS 11133-2.

5.4 Peptone water, indole free

5.4.1 Composition

Enzymatic digest of casein	10,0 g
Sodium chloride	5,0 g
Water	1 000 ml

5.4.2 Preparation

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

Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C.

Dispense the medium in quantities of 5 ml to 10 ml into tubes of dimensions 16 mm × 160 mm (6.4).

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

5.5 Indole reagent (Kovacs' reagent)

5.5.1 Composition

4-Dimethylaminobenzaldehyde	5,0 g
2-Methylbutan-1-ol or pentan-1-ol	75,0 ml
Hydrochloric acid (ρ_{20} 1,18 g/ml to 1,19 g/ml)	25,0 ml

5.5.2 Preparation

Dissolve the 4-dimethylaminobenzaldehyde in the alcohol by heating gently by means of a water bath maintained at between 50 °C and 55 °C.

Cool and add the acid.

Protect from light and store at approximately 4 °C (see ISO 7218).

The reagent shall be light yellow to light brown.

NOTE A ready-to-use commercially available preparation may also be used.

6 Apparatus and glassware

NOTE Disposable apparatus is an acceptable alternative to reusable glassware if it has similar specifications.

Usual microbiological laboratory equipment 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 37 °C ± 1 °C and 44 °C ± 1 °C.

6.3 Water bath, capable of being maintained at 44 °C ± 1 °C.

6.4 Test tubes, of dimensions approximately 16 mm × 160 mm and 18 mm × 180 mm or 20 mm × 200 mm.

6.5 Sampling loops, made of platinum/iridium or nickel/chromium, approximately 3 mm in diameter, or approximately 10 µl **sterile disposable sampling loops**.

6.6 Durham tubes, of a size suitable for use in the test tubes (6.4).

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

6.8 pH-meter, having a resolution of 0,01 pH units and accurate to within ± 0,1 pH units at 25 °C.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

8 Preparation of test sample

Prepare the test sample 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.

9 Procedure

9.1 Detection method

9.1.1 Test portion and initial suspension

See the appropriate part of ISO 6887 depending on the product concerned, or ISO 8261.

Add 1 ml of the initial suspension to 9 ml of single-strength lauryl sulfate broth [5.2.1 b)] (i.e. 0,1 g or 0,1 ml of the sample) or 10 ml of the initial suspension to 10 ml of double-strength lauryl sulfate broth [(5.2.1 a)] (i.e. 1 g or 1 ml of the sample). For larger volumes of test portions, prepare the initial suspension by adding x ml or x g to $9x$ ml of the diluent (see ISO 6887 or ISO 8261), then add the entire initial suspension to $90x$ ml of single-strength lauryl sulfate broth [5.2.1 b)]. For example, add 5 ml or 5 g of the sample to 45 ml of the diluent, and add this entire initial suspension to 450 ml of single-strength lauryl sulfate broth [5.2.1 b)], or add the test portion to an equal volume of double-strength lauryl sulfate broth [5.2.1 a)].

9.1.2 Incubation of the selective enrichment medium (lauryl sulfate broth)

Incubate single-strength or double-strength lauryl sulfate broth (5.2) in the incubator (6.2) set at 37 °C for 24 ± 2 h. If, at this stage, neither gas production nor opacity preventing the observation of gas production is observed, incubate for up to 48 h ± 2h.

NOTE For live shellfish, an incubation time of 48 h ± 2 h may be used.

For some milk products (e.g. casein), the Durham tube may stick to the bottom of the tubes of selective enrichment medium. If, after 48 h incubation period, opacity is observed but no gas production, inoculate the EC broth with this broth and proceed with the method as described in 9.1.3.

9.1.3 Inoculation and incubation of the selective medium (EC broth)

After incubation of double-strength medium [(5.2.1 a)] according to 9.1.2, if opacity, cloudiness or any visible gas is observed, or after incubation of the single-strength medium [(5.2.1 b)] according to 9.1.2 if visible gas is observed, inoculate a tube of EC broth (5.3) with a sampling loop (6.5) and incubate in a water bath (6.3) or an incubator (6.2) set at 44 °C for 24 h ± 2 h. If, at this stage, there is no visible gas in the EC broth (5.3), extend the incubation time up to a total of 48 h ± 2 h.

NOTE For live shellfish, a total incubation time of not more than 24 h ± 2 h may be used.

9.1.4 Inoculation and incubation of the peptone water

After incubation according to 9.1.3, if visible gas is observed, inoculate a tube of peptone water (5.4), preheated to 44 °C, using a sampling loop (6.5). Incubate for 48 h ± 2 h at 44 °C.

9.1.5 Examination for indole production

Add 0,5 ml of indole reagent (5.5) to the tubes of peptone water (5.4) incubated according to 9.1.4.

Mix well and examine after 1 min. A red colour in the alcoholic phase indicates the presence of indole.

9.1.6 Interpretation

Consider as positive the selective enrichment medium incubated according to 9.1.2 that has given rise (after subculturing and incubation according to 9.1.3 and 9.1.4) to visible gas in the tube of EC broth and to indole production in the tube of peptone water.

9.2 Enumeration method

9.2.1 Test portion, initial suspension and dilutions

See the appropriate part of ISO 6887 depending on the product concerned, or ISO 8261.

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

9.2.2 Inoculation and incubation of the selective enrichment medium (lauryl sulfate broth)

9.2.2.1 A series of three tubes for each dilution is scheduled. It is necessary to incubate a series of five tubes (see Annex A) for live shellfish or other special products, and/or when greater accuracy of the results is required.

9.2.2.2 Take three tubes of double-strength selective enrichment medium [5.2.1 a)]. Using a sterile pipette (6.7), transfer to each of these tubes 10 ml of the initial suspension. These test portions correspond to 1 g of sample per tube.

9.2.2.3 Then take three tubes of single-strength selective enrichment medium [5.2.1 b)]. Using a fresh sterile pipette (6.7), transfer to each of these tubes 1 ml of the initial suspension. These test portions correspond to 0,1 g of sample per tube.

9.2.2.4 For each of the further dilutions (equal to 0,01 g, 0,001 g, etc. of sample per tube), proceed as in 9.2.2.3. Use a new sterile pipette for each dilution. Carefully mix the inoculum and the medium.

9.2.2.5 Incubate the tubes of double-strength selective enrichment medium inoculated in 9.2.2.2 and the tubes of single-strength selective enrichment medium inoculated in 9.2.2.3 and 9.2.2.4 in the incubator (6.2) set at 37 °C for 24 h ± 2 h. If, at this stage, neither gas production nor opacity preventing the observation of gas production is observed, incubate for up to 48 h ± 2 h.

For live shellfish, the incubation time shall be 48 h ± 2 h.

For some milk products (e.g. casein), the Durham tube may stick to the bottom of the tubes of selective enrichment medium. If after the 48 h incubation period, opacity is observed but no gas production, inoculate the EC broth (5.3) with this broth and proceed with the method as described in 9.2.3.

9.2.3 Subculturing and incubation of the selective medium (EC broth)

9.2.3.1 For each tube of double-strength medium incubated according to 9.2.2.5 showing opacity, cloudiness or any visible gas, and for each tube of single-strength medium incubated according to 9.2.2.5 showing visible gas subculture to a tube containing EC broth (5.3) with a sampling loop (6.5).

9.2.3.2 Incubate the tubes inoculated as in 9.2.3.1 in a water bath (6.3) or an incubator (6.2) set at 44 °C for 24 h ± 2 h. If, at this stage, there is no visible gas in the EC broth (5.3), extend the incubation up to a total of 48 h ± 2 h.

For live shellfish, the total incubation time shall be limited to 24 h ± 2 h.

9.2.4 Inoculation and incubation of the peptone water

For each tube incubated according to 9.2.3.2 and showing any visible gas, inoculate a tube of peptone water (5.4), preheated to 44 °C, using a sampling loop (6.5). Incubate for 48 h ± 2 h at 44 °C.

9.2.5 Examination for indole production

Add 0,5 ml of indole reagent (5.5) to the tubes of peptone water (5.4) incubated according to 9.2.4.

Mix well and examine after 1 min. A red colour in the alcoholic phase indicates the presence of indole.

9.2.6 Interpretation

Consider as positive each tube of double-strength [5.2.1 a)] or single-strength [5.2.1 b)] selective enrichment medium incubated according to 9.2.2 that has given rise (after subculturing and incubation according to 9.2.3 and 9.2.4) to any visible gas in the tube of EC broth and to indole production in the tube of peptone water.

For each dilution, count the number of positive result tubes of double-strength [5.2.1 a)] and single-strength [5.2.1 b)] medium.

10 Expression of results

10.1 Detection method

In accordance with the interpretation of the results (9.1.6), report the presence or absence of presumptive *Escherichia coli* in the test portion, specifying the mass in grams, or the volume in millilitres, of the test sample.

10.2 Enumeration method

See Annex A.

EXAMPLE Using a solid sample and three tubes, in 95 % of cases, the confidence limits vary from 13 to 200 presumptive *Escherichia coli* per gram for an MPN of 7,4 × 10 presumptive *Escherichia coli* per gram, and from 4 to 99 presumptive *Escherichia coli* per gram for an MPN of 2,4 × 10 presumptive *Escherichia coli* per gram.

11 Test report

The test report shall specify:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this International Standard;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test results;

e) the test results obtained.

Annex A
(normative)

MPN table

A.1 MPN calculations using three tubes

See ISO 7218.

A.2 MPN calculations using five tubes

See Table A.1.

Table A.1 — MPN table for 5 × 1 g (ml), 5 × 0,1 g (ml) and 5 × 0,01 g (ml)

Number of positive results			MPN index	Category[a] when the number of samples (per batch) tested is					Confidence limits			
				1	2	3	5	10	⩾ 95 %	⩾ 95 %	⩾ 99 %	⩾ 99 %
0	0	0	< 0,18						0,00	0,65	0,00	0,93
0	0	1	0,18	2	2	2	1	1	0,00	0,65	0,00	0,93
0	1	0	0,18	2	2	2	1	1	0,01	0,65	0,00	0,93
0	1	1	0,36	3	3	3	2	2	0,07	0,99	0,02	1,40
0	2	0	0,37	3	2	2	2	1	0,07	0,99	0,02	1,40
0	2	1	0,55	0	0	0	3	3	0,17	1,40	0,09	2,10
0	3	0	0,56	0	3	3	3	3	0,17	1,40	0,09	2,10
1	0	0	0,20	1	1	1	1	1	0,02	0,99	0,01	1,40
1	0	1	0,40	2	1	1	1	1	0,07	1,00	0,02	1,40
1	0	2	0,60	0	0	3	3	3	0,17	1,40	0,09	2,10
1	1	0	0,40	1	1	1	1	1	0,07	1,10	0,03	1,40
1	1	1	0,61	3	2	2	2	1	0,17	1,40	0,09	2,10
1	1	2	0,81	0	0	0	0	3	0,33	2,20	0,20	2,80
1	2	0	0,61	2	1	1	1	1	0,18	1,40	0,09	2,10
1	2	1	0,82	3	3	3	3	2	0,33	2,20	0,20	2,80
1	3	0	0,83	3	3	3	3	2	0,33	2,20	0,20	2,80
1	3	1	1,0	0	0	0	0	3	0,3	2,2	0,2	2,8
1	4	0	1,1	0	0	0	0	3	0,3	2,2	0,2	2,8
2	0	0	0,45	1	1	1	1	1	0,08	1,4	0,04	2,10
2	0	1	0,68	2	1	1	1	1	0,18	1,50	0,09	2,10
2	0	2	0,91	0	3	3	3	3	0,33	2,20	0,20	2,80
2	1	0	0,68	1	1	1	1	1	0,19	1,70	0,10	2,30
2	1	1	0,92	2	2	1	1	1	0,33	2,20	0,20	2,80