International Standard 3811

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Meat and meat products — Detection and enumeration of presumptive coliform bacteria and presumptive *Escherichia coli* (Reference method)

Viandes et produits à base de viande — Recherche et dénombrement des bactéries présumées coliformes et présumées Escherichia coli *(Méthode de référence)*

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Foreword

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International Standard ISO 3811 was developed by Technical Committee ISO/TC 34, *Agricultural food products*, and was circulated to the member bodies in May 1975.

It has been approved by the member bodies of the following countries :

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Chile	India	Spain
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Australia
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Meat and meat products — Detection and enumeration of presumptive coliform bacteria and presumptive *Escherichia coli* (Reference method)

1 Scope and field of application

This International Standard specifies a reference method for the detection and enumeration of presumptive coliform bacteria and of presumptive *Escherichia coli* (*E. coli*) in meat and meat products.

2 References

ISO 3100, *Meat and meat products — Sampling.*

ISO 3565, *Meat and meat products — Detection of* salmonellae *(Reference method).*

3 Definitions

3.1 presumptive coliform bacteria : Micro-organisms that ferment lactose with the production of gas at 30 °C when the test is carried out according to the method specified.

3.2 presumptive *Escherichia coli* : Presumptive coliform bacteria that ferment lactose with the production of gas at 44 °C and produce indole from tryptophane at 44 °C when the test is carried out according to the method specified.

3.3 count of presumptive coliform bacteria and presumptive *Escherichia coli* : The number of presumptive coliform bacteria and presumptive *E. coli* found per gram of meat or meat product when the test is carried out according to the method specified.

4 Principle

Mincing of a test sample and then maceration of a test portion with a sterile diluent in a mechanical blender. Preparation, from the macerate, of decimal dilutions, which are inoculated in triplicate into a liquid selective medium. From the number of tubes showing gas production after incubation at 30 °C, determination of the most probable number of presumptive coliform bacteria per gram by using the MPN table (see the annex).

For the enumeration of presumptive *E. coli*, inoculation of tubes containing the liquid selective medium and tubes containing tryptone water, from the positive coliform tubes, i.e. those tubes that show gas production, and incubation at 44 °C. From the number of incubated tubes showing gas production in the selective medium and indole production in the tryptone water, determination of the most probable number of presumptive *E. coli* by using the MPN table (see the annex).

5 Culture media, dilution fluid and reagent

5.1 Basic materials

In order to improve the precision of the results, it is recommended that either dehydrated culture medium components of uniform quality and analytical grade chemicals, or dehydrated complete media, be used. The water used shall be distilled water or water of at least equivalent purity.

5.2 Culture media

5.2.1 Lactose bile brilliant green broth (selective medium)

Composition

	a) Double-strength medium	b) Single-strength medium
peptone	20,0 g	10,0 g
lactose	20,0 g	10,0 g
ox bile (dehydrated)	40,0 g	20,0 g
brilliant green, corresponding to the specifications in the annex of ISO 3565	0,026 6 g	0,013 3 g
water	1 000 ml	1 000 ml

NOTE — Because the complete medium may not always produce the expected result, its performance should be checked before use. (A method for this purpose will form the subject of a future International Standard.)

Preparation

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

Adjust the pH with sodium hydroxide solution or hydrochloric acid solution of an appropriate strength so that after sterilization it is 7,2 ± 0,1 at 25 °C.

Transfer 10 ml portions to culture tubes (6.2.1) (16 mm × 160 mm, containing Durham tubes, for single-strength medium, or 20 mm × 200 mm for double-strength medium), or screw-cap bottles of similar capacity.

Sterilize the medium at 121 ± 1 °C for 15 min.

At least 1 cm of the Durham tubes shall be visible above the liquid.

If bubbles are present, remove them if possible by tilting and tapping the tubes. Do not use tubes which contain gas bubbles.

5.2.2 Tryptone water

Composition

tryptone	10,0 g
sodium chloride	5,0 g
water	1 000 ml

Preparation

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

Adjust the pH with sodium hydroxide solution or hydrochloric acid solution of an appropriate strength so that after sterilization it is 7,3 ± 0,2 at 25 °C.

Transfer the medium, in 5 to 10 ml portions, to culture tubes (6.2.1) or to screw-cap bottles of similar capacity.

Sterilize the medium at 121 ± 1 °C for 15 min.

5.3 Dilution fluid

Composition

peptone	1,0 g
sodium chloride	8,5 g
water	1 000 ml

Preparation

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

Adjust the pH with sodium hydroxide solution or hydrochloric acid solution of an appropriate strength so that after sterilization it is 7,0 ± 0,1 at 25 °C.

Transfer part of the dilution fluid, in quantities of 100 to 300 ml, to flasks or screw-cap bottles whose capacity is approximately twice the volume dispensed. Transfer the remainder of the fluid to tubes, or to small flasks or small screw cap bottles, so that after sterilization each contains 9,0 ml.

Sterilize the dilution fluid at 121 ± 1 °C for 15 min.

5.4 Indole reagent (Kovacs reagent)

Composition

p-dimethylaminobenzaldehyde	5,0 g
amyl alcohol	75,0 ml
hydrochloric acid (ϱ_{20} 1,18 to 1,19 g/ml)	25,0 ml

Preparation

Dissolve the aldehyde in the alcohol by gently warming in a water bath (about 50 to 55 °C).

Cool and add the acid.

Protect from light and store at about 4 °C.

The reagent shall be light yellow to light brown.

6 Apparatus and glassware

6.1 Apparatus

6.1.1 Mechanical meat mincer, laboratory size, sterile, fitted with a plate with holes of diameter not exceeding 4 mm.

6.1.2 Mechanical blender, operating at a rotational frequency of not less than 8 000 min^{-1} and not more than 45 000 min^{-1}, with glass or metal blending jars of an appropriate capacity and resistant to the conditions of sterilization.

6.1.3 Apparatus for sterilization of glassware, blender jars, culture media, etc.

6.1.4 Incubator for maintaining the inoculated tubes at 30 ± 1 °C.

6.1.5 Water bath for maintaining the inoculated tubes at 44 ± 0,1 °C.

6.2 Glassware

The glassware shall be resistant to repeated sterilization.

6.2.1 Flasks and culture tubes (16 mm × 160 mm, and 20 mm × 200 mm). Alternatively, screw-cap bottles of similar capacity may be used.

6.2.2 Graduated pipettes, calibrated for bacteriological use only, with a nominal capacity of 1,0 ml and of 10,0 ml, subdivided in 0,1 ml and 1 ml respectively, and with an outflow opening of diameter 2 to 3 mm.

6.3 Sterilization of glassware, etc.

Sterilize the glassware by one of the following methods :

— wet sterilization at 121 ± 1 °C for not less than 20 min;

— dry sterilization at not less than 170 °C for not less than 1 h.

7 Sampling

Proceed from a representative sample of at least 200 g. See ISO 3100.

The representative sample may be stored in the laboratory at a temperature of 0 to 5 °C, but not for longer than 1 h.

8 Procedure

8.1 Pre-treatment of the sample

Grind and mix the sample twice in the meat mincer (6.1.1). Start the examination of the pre-treated sample as soon as possible; it may be stored, if necessary, at a temperature between 0 and 5 °C, but not for longer than 1 h.

8.2 Test portion

Weigh, to the nearest 0,1 g, approximately 10 g of the pre-treated sample (8.1) into a sterile blender jar (6.1.2).

8.3 Maceration and dilution

8.3.1 Add to the test portion nine times the quantity, by mass, of dilution fluid (5.3). Operate the blender (6.1.2), according to its rotational frequency, for sufficient time to give a total number of 15 000 to 20 000 revolutions. Thus, even with the slowest blender, this time will not exceed 2,5 min.

8.3.2 Carry out the following operations (8.3.3 to 8.3.5) simultaneously on two separate portions of the macerate (8.3.1).

8.3.3 Take, directly after maceration, a portion of 1 ml of the macerate (8.3.1) with a sterile 1 ml pipette and add the portion to a tube containing 9 ml of sterile dilution fluid (5.3), avoiding contact between the pipette and the dilution fluid.

8.3.4 Mix the liquids carefully by aspirating with a fresh sterile pipette, filling and emptying the pipette ten times. Transfer, with the same pipette, 1 ml of this dilution (10^{-2}) to another tube containing 9 ml of sterile dilution fluid (5.3), avoiding contact between the pipette and the dilution fluid.

8.3.5 Mix the liquids carefully with a fresh sterile pipette. Repeat the operations until the required number of dilutions up to 10^{-6} has been made.

8.4 Presumptive coliform bacteria

8.4.1 Inoculation

Transfer, with a fresh sterile pipette, six 1 ml portions of the macerate (8.3.1) (dividing them into two triplicate sets) and triplicate 1 ml portions of each of the five following dilutions (8.3.3, 8.3.4 and 8.3.5) of both dilution series to tubes containing 10 ml of single-strength selective broth [5.2.1 b)]. Start with the highest dilution and proceed to the lowest (the macerate), filling and emptying the pipette three times before transferring the 1 ml portions to the tubes. When the number of presumptive coliform bacteria is expected to be very small, transfer also six 10 ml portions of the macerate, in two triplicate sets, to 10 ml portions of the double-strength selective broth [5.2.1 a)], using a sterile 10 ml pipette.

8.4.2 Incubation

Keep the tubes, prepared according to 8.4.1, in the incubator (6.1.4) at 30 ± 1 °C for 48 ± 2 h.

8.4.3 Interpretation

After incubation, examine the tubes and record as positive those in which growth and gas production amounting to at least one-tenth of the volume of the Durham tube are observed.

8.5 Presumptive *Escherichia coli*

8.5.1 Inoculation

Using a sterile 1 ml pipette for each tube, inoculate 1 drop from the presumptive coliform positive tubes (8.4.3) into 10 ml of the single-strength broth [5.2.1 b)] and into 10 ml of the tryptone water (5.2.2), both pre-warmed to 44 °C.

8.5.2 Incubation

Keep the tubes, prepared according to 8.5.1, in the water bath (6.1.5) at 44 ± 0,1 °C for 48 h.

8.5.3 Interpretation

8.5.3.1 Gas production

Examine the tubes containing the inoculated broth after a maximum incubation time of 48 h.

Record as positive those tubes in which growth and gas production amounting to at least one-tenth of the volume of the Durham tube are observed.

8.5.3.2 Indole production

Add to the tubes containing the inoculated tryptone water, after incubation for 48 h, 0,5 ml of the indole reagent (5.4), shake well and examine after 1 min.

A red colour in the alcohol layer indicates the presence of indole.

Record as positive the tubes in which indole has been produced.

9 Expression of results

9.1 Presumptive coliform bacteria

9.1.1 Calculate from the number of presumptive coliform

positive tubes (8.4.3) in the different dilutions the most probable number of these micro-organisms per gram of meat or meat product, as indicated in 9.1.2 to 9.1.4.

9.1.2 For calculation of the MPN, select three consecutive dilutions in accordance with one of the three following rules, whichever is appropriate :

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

Select the highest dilution (i.e. that having the lowest sample concentration) yielding three positive tubes, together with the next two higher dilutions (i.e. those having sample concentrations of 1/10 and 1/100 of that of the first dilution selected).

See also rule c).

If insufficient further dilutions were made beyond the highest dilution yielding three 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 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 positive tubes, select from these dilutions the lowest one not yielding 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 ten times and one hundred times that of the first dilution selected), except when 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 positive tube.

9.1.3 Multiply the MPN index obtained from the table (see the annex) by the reciprocal of the lowest dilution selected (i.e. that having the highest sample concentration) to obtain the most probable number of presumptive coliform bacteria per gram of meat or meat product.

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.

9.1.4 Calculate the average of the results obtained from each of the duplicate dilution series.

9.2 Presumptive *Escherichia coli*

9.2.1 Calculate from the number of presumptive coliform positive tubes that resulted in production of gas (8.5.3.1) and in production of indole (8.5.3.2) in the different dilutions the most probable number of these micro-organisms per gram of meat or meat product, as indicated in 9.2.2 to 9.2.4.

9.2.2 See 9.1.2.

9.2.3 See 9.1.3. (Substitute "presumptive *Escherichia coli*" for "presumptive coliform bacteria".)

9.2.4 See 9.1.4.

9.3 Reporting of result

Report the result as the most probable number of presumptive coliform bacteria (or presumptive *Escherichia coli*, as appropriate) per gram of meat or meat product.

In the event of finding an MPN count of more than 100 of these micro-organisms per gram, express the result as a number between 1,0 and 9,9 multiplied by the appropriate power of 10 (for example an average count of 15 000 would be reported as $1{,}5 \times 10^4$ per gram). In the event of finding an MPN count between 0,3 and 100, express the result as calculated.

In the event of an MPN count of < 0,3 of these micro-organisms per gram when the procedure appropriate to a low count of coliforms has been used (8.4.1), the result may be expressed in the form : "presumptive coliform bacteria (or presumptive *Escherichia coli*) were not detected in 1 g of the meat or meat product".

10 Test report

The test report shall show the method used and the result obtained; it shall also mention all operating conditions not specified in this International Standard, or regarded as optional, as well as any circumstances that may have influenced the result.

The report shall include all details for complete identification of the sample.

Annex

Table for the determination of MPN index when the examination is carried out on a single sample per batch

Number of positive tubes for the three dilutions selected			MPN index	Category*	Confidence limits			
1st	2nd	3rd			95 %		99 %	
0	0	0	<0,3					
0	0	1		0				
0	1	0	0,3	2	<0,1	1,7	<0,1	2,3
0	2	0		0				
1	0	0	0,4	1	0,1	2,1	<0,1	2,8
1	0	1	0,7	2	0,2	2,7	0,1	3,5
1	1	0	0,7	1	0,2	2,8	<0,1	3,6
1	1	1		0				
1	2	0	1,1	2	0,4	3,5	0,2	4,4
1	2	1		0				
1	3	0		0				
2	0	0	0,9	1	0,2	3,8	<0,1	5,0
2	0	1	1,4	2	0,5	4,8	0,2	6,2
2	1	0	1,5	1	0,5	5,0	0,2	6,4
2	1	1	2,0	2	0,7	6,0	0,4	7,6
2	2	0	2,1	1	0,8	6,2	0,5	7,9
2	2	1		0				
2	3	0		0				
3	0	0	2	1	<1	13	<1	18
3	0	1	4	1	1	18	<1	23
3	0	2		0				
3	1	0	4	1	1	21	<1	28
3	1	1	7	1	2	28	2	36
3	1	2		0				
3	2	0	9	1	3	38	1	51
3	2	1	15	1	5	50	3	66
3	2	2	21	2	8	64	5	82
3	2	3		0				
3	3	0	20	1	<10	140	<10	190
3	3	1	50	1	10	240	<10	320
3	3	2	110	1	30	480	20	640
3	3	3	>110					

* **Category 0** : Unacceptable tube combinations, having, under normal conditions, the least chance of being obtained. Combinations not mentioned in the table also belong to this category.

When such a combination is obtained, it is probable that a mistake has been made, faulty technique has been used, or a bacteriostatic substance is present in the product.

Category 1 : Most likely tube combinations. One of these should be obtained in 95 % of cases.

Category 2 : Less likely tube combinations than those of category 1. One of these should be obtained in only 4 % of cases.