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Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of *Clostridium perfringens* — Colony-count technique

Microbiologie des aliments — Méthode horizontale pour le dénombrement de Clostridium perfringens *— Technique par comptage des colonies*

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Foreword

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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 7937 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This third edition cancels and replaces the second edition (ISO 7937:1997, EN 13401:1999), which has been technically revised.

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 that 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.

Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of *Clostridium perfringens* — Colony-count technique

1 Scope

This International Standard describes a horizontal method for the enumeration of viable *Clostridium perfringens.* It 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.

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 11133-2:2003, *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*

3 Terms and definitions

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

3.1
Clostridium perfringens
C. perfringens
bacteria that form characteristic colonies (black precipitate, caused by the reduction of sulfite to sulfide, which colours the colonies black) in the specified selective medium, and which give positive confirmatory reactions when the test is carried out by either of two techniques specified in this International Standard

3.2
enumeration of *C. perfringens*
determination of the number of culturable and confirmed *Clostridium perfringens* bacteria per millilitre or per gram of sample when the test is carried out by the method specified in this International Standard

4 Principle

4.1 Petri dishes are inoculated with a specified quantity of the test sample if the initial product is liquid, or a specified quantity of the initial suspension in the case of other products.

Further Petri dishes are inoculated, under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

A selective medium is added (poured-plate technique) and then an overlay of the same medium.

4.2 The plates are incubated anaerobically at 37 °C for 20 h ± 2 h.

4.3 The characteristic colonies are enumerated.

4.4 The numbers of characteristic colonies are confirmed and the number of *C. perfringens* per millilitre or per gram of sample is calculated.

5 Diluent, culture media and reagents

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

5.1 Diluent

See the relevant part of ISO 6887 or ISO 8261.

5.2 Sulfite-cycloserine agar (SC)

NOTE This was originally designated “egg-yolk-free TSC” (see [1]).

5.2.1 Base

5.2.1.1 Composition

Enzymatic digest of protein	15,0 g
Enzymatic digest of soya	5,0 g
Yeast extract	5,0 g
Disodium disulfite ($Na_2S_2O_5$), anhydrous	1,0 g
Ammonium iron(III) citrate[a]	1,0 g
Agar	9,0 g to 18,0 g[b]
Water	1 000 ml

[a] This reagent should contain at least 15 % (mass fraction) of iron.
[b] Depending on the gel strength of the agar.

5.2.1.2 Preparation

Dissolve the components in the water by boiling. Adjust the pH so that after sterilization it will be 7,6 ± 0,2 at 25 °C. Dispense the base into flasks or bottles of appropriate capacity. Sterilize for 15 min at 121 °C. Store in a refrigerator at 5 °C ± 3 °C. Discard unused medium 2 weeks after preparation.

In some cases (see 9.4.3.1), it may be necessary to prepare dishes of SC agar base medium for confirmation with the nitrate motility medium (5.5) and the lactose-gelatin medium (5.8). For this purpose, transfer portions of about 15 ml of the base [melted and cooled to approximately 44 °C to 47 °C using a water bath (6.10)] into Petri dishes and allow to solidify. Immediately before use, dry the plates (see ISO 7218).

5.2.2 D-Cycloserine solution

5.2.2.1 Composition

D-Cycloserine[a]	4,0 g
Water	100 ml

[a] Use white crystalline powder only.

5.2.2.2 Preparation

Dissolve the D-cycloserine in the water and sterilize the solution by filtration.

Store in a refrigerator at 3 °C ± 2 °C.

Discard unused solution 4 weeks after preparation.

5.2.3 Complete medium

Immediately before use in the pour-plate method (see 9.2), to each 100 ml of sterile molten base (5.2.1) cooled to 44 °C to 47 °C, add 1 ml of D-cycloserine solution (5.2.2).

5.2.4 Performance testing for the quality assurance of SC medium

For the definition of selectivity and productivity, refer to ISO/TS 11133-1. To check the performance, refer to ISO/TS 11133-2:2003, Table B.1 [see TS(C)].

5.3 Fluid thioglycollate medium

5.3.1 Composition

Enzymatic digest of casein	15,0 g
L-Cystine	0,5 g
D-Glucose	5,5 g
Yeast extract	5,0 g
Sodium chloride	2,5 g
Sodium thioglycollate (mercaptoacetate)	0,5 g
Agar	0,5 g to 2,0 g[a]
Resazurin	0,001 g
Water	1 000 ml

[a] Depending on the gel strength of the agar.

5.3.2 Preparation

Dissolve the components in the water by boiling. Adjust the pH so that after sterilization it will be 7,1 ± 0,2 at 25 °C.

Dispense 10 ml portions into tubes and sterilize at 121 °C for 15 min.

Before use, this medium shall be de-aerated.

5.3.3 Performance testing for the quality assurance of thioglycollate broth

For the definition of selectivity and productivity, refer to ISO/TS 11133-1. To check the performance, refer to ISO/TS 11133-2:2003, Table B.4.

5.4 Lactose sulfite medium (LS) (optional)

5.4.1 Base medium

5.4.1.1 Composition

Enzymatic digest of casein	5,0 g
Yeast extract	2,5 g
Sodium chloride	2,5 g
Lactose	10 g
L-Cysteine hydrochloride	0,3 g
Water	1 000 ml

5.4.1.2 Preparation

Dissolve the components in the water by boiling (if necessary). Adjust the pH so that after sterilization it will be 7,1 ± 0,2 at 25 °C.

Dispense 8 ml portions into test tubes with inverted Durham tubes (6.7) and sterilize at 121 °C for 15 min.

The medium may be stored at 3 °C ± 2 °C for up to 4 weeks.

5.4.2 Disodium disulfite solution

5.4.2.1 Composition

Disodium disulfite ($Na_2S_2O_5$), anhydrous	1,2 g
Water	100 ml

5.4.2.2 Preparation

Dissolve the disodium disulfite in the water and sterilize the solution by filtration.

Use the solution within a day.

5.4.3 Ammonium iron(III) citrate solution

5.4.3.1 Composition

Ammonium iron(III) citrate	1 g
Water	100 ml

5.4.3.2 Preparation

Dissolve the ammonium iron(III) citrate in the water and sterilize the solution by filtration.

Use the solution within a day.

5.4.4 Complete medium

If the medium is not used on the day of preparation, just prior to completion de-aerate the medium by heating and then cool rapidly. If the medium is in screw-cap bottles, loosen the caps before heating and tighten them before cooling.

Then add 0,5 ml of the disodium disulfite solution (5.4.2) and 0,5 ml of the ammonium iron(III) citrate solution (5.4.3) to each 8 ml of base (5.4.1).

Use the complete medium within a day.

5.5 Nitrate motility medium (optional)

5.5.1 Composition

Enzymatic digest of casein	5,0 g
Meat extract	3,0 g
Galactose	5,0 g
Glycerol	5,0 g
Potassium nitrate (KNO_3)	1,0 g
Disodium hydrogen orthophosphate (Na_2HPO_4)	2,5 g
Agar	1,0 g to 5,0 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

5.5.2 Preparation

Dissolve the components in the water by boiling. Adjust the pH so that it will be 7,3 ± 0,2 at 25 °C after sterilization.

Transfer the medium to culture tubes in 10 ml quantities and sterilize at 121 °C for 15 min. If not used the same day, store in a refrigerator at 5 °C ± 3 °C. Just prior to use, heat in boiling water or steam for 15 min and then cool rapidly to the incubation temperature.

Discard unused medium 4 weeks after preparation.

5.6 **Nitrite detection reagent** (optional)

5.6.1 5-Amino-2-naphthalenesulfonic acid (5-2-ANSA) solution

Dissolve 0,1 g of 5-2-ANSA in 100 ml of 15 % (volume fraction) acetic acid solution. Filter through a filter paper.

Store in a well-stoppered brown bottle (preferably with a bulb type dropper) at 5 °C ± 3 °C.

5.6.2 Sulfanilic acid solution

Dissolve 0,4 g of sulfanilic acid in 100 ml of 15 % (volume fraction) acetic acid solution. Filter through a filter paper.

Store in a well-stoppered brown bottle (preferably with a bulb type dropper) at 5 °C ± 3 °C.

5.6.3 Preparation of complete reagent

Mix equal amounts of the two solutions (5.6.1 and 5.6.2) just before use. Discard unused reagent immediately.

5.7 **Zinc dust** (optional)

5.8 **Lactose-gelatin medium** (optional)

5.8.1 Composition

Enzymatic digest of casein	15,0 g
Yeast extract	10,0 g
Lactose	10,0 g
Gelatin	120,0 g
Phenol red	0,05 g
Water	1 000 ml

5.8.2 Preparation

Dissolve the components, except the lactose and phenol red, in the water. Adjust the pH so that after sterilization it will be 7,5 ± 0,2 at 25 °C.

Add the lactose and phenol red, dispense 10 ml portions into test tubes and sterilize at 121 °C for 15 min. If not used the same day, store in a refrigerator at 5 °C ± 3 °C.

Just prior to use, heat in boiling water or flowing steam for 15 min, then cool rapidly to the incubation temperature.

Discard unused medium 3 weeks after preparation.

6 Apparatus and glassware

Usual microbiological 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 being maintained at 37 °C ± 1 °C.

6.3 Modified atmosphere jars, or any other apparatus appropriate for anaerobic culture.

6.4 pH-meter, capable of being read to the nearest ± 0,01 pH unit at 25 °C, enabling measurements to be made which are accurate to 0,1 pH unit.

6.5 Loops, of platinium-iridium or nickel-chromium, of diameter approximately 3 mm, and **stab-inoculation needles** of the same material, or equivalent **sterile disposable loops** and **inoculating needles**.

6.6 Filtration apparatus, for sterilization of solutions.

6.7 Test tubes, **bottles** or **flasks**, of appropriate capacity, in particular 16 mm × 160 mm test tubes with inverted Durham tubes, for example of length 35 mm and of diameter 7 mm.

6.8 Total-delivery graduated pipettes or **micropipettes**, of nominal capacities 1 ml and 10 ml, graduated in 0,1 ml and 0,5 ml intervals respectively.

6.9 Petri dishes, made of glass or plastics material, of diameter 90 mm to 100 mm.

6.10 Water baths, or similar apparatus, capable of being maintained at 44 °C to 47 °C, and at 46 °C ± 0,5 °C.

6.11 Rubber bulbs, for use with the graduated pipettes when distributing the components of the nitrite-detection reagent (if necessary).

7 Sampling

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.

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample in accordance with ISO 6887-2, ISO 6887-3, ISO 6887-4 or ISO 8261.

9 Procedure

9.1 Test portion, initial suspension and dilutions

See the relevant part of ISO 6887 or ISO 8261.

9.2 Inoculation and incubation (poured-plate technique)

Transfer, by means of a sterile pipette (6.8), 1 ml of the initial suspension, or of the test sample if the initial product is liquid, in duplicate, to the centres of empty Petri dishes (6.9).

Pour 10 ml to 15 ml of the SC agar (5.2.3), maintained at 44 °C to 47 °C in the water bath (6.10), into the dish and mix well with the inoculum by gently rotating each dish. When the medium has solidified, add an overlayer of 10 ml of the same SC agar.

Allow to solidify. Place the plates in modified atmosphere jars or other suitable containers (6.3) and incubate under anaerobic conditions at 37 °C for 20 h ± 2 h. Longer incubation may result in excess blackening of the plates.

Follow the same procedure with the decimal dilutions prepared (see 9.1).

9.3 Counting and selection of colonies

After the specified period of incubation (9.2), select all plates containing less than 150 colonies. From these, select, if possible, plates representing successive dilutions.

Count on each plate the characteristic colonies of presumptive *C. perfringens*.

Select five characteristic colonies and confirm them using one of the techniques described in 9.4.2 and 9.4.3.

9.4 Biochemical confirmation

9.4.1 General

Choose one of the two confirmation techniques described in 9.4.2 and 9.4.3.

Commercially available biochemical galleries may be used if in accordance with ISO 7218.

9.4.2 Confirmation technique using the LS medium

NOTE The reaction obtained in lactose sulfite medium (5.4) when incubated at 46 °C is very specific for *Clostridium perfringens* and *Clostridium absonum*. It is therefore not necessary to ensure that the black colonies picked from the agar are pure before inoculation into the thioglycollate broth and subsequently into the lactose sulfite medium.

9.4.2.1 Inoculation and incubation

Inoculate each selected colony (see 9.3) into fluid thioglycollate medium (5.3). Incubate under anaerobic conditions at 37 °C for 18 h to 24 h.

After incubation, transfer with no delay 5 drops of the thioglycollate culture to the LS medium by means of a sterile pipette. Incubate aerobically at 46 °C for 18 h to 24 h in the water bath (6.10).

9.4.2.2 Interpretation

Examine the tubes of LS medium for the production of gas and the presence of a black colour (iron sulfite precipitate). Durham tubes more than one-quarter full of gas and tubes having a black precipitate are considered positive.

In case of doubt, when the Durham tube in a blackened medium is less than one-quarter full of gas, transfer with no delay, using a sterile pipette, 5 drops of the previous growth in LS medium (9.4.2.1) to another tube of LS medium. Incubate in the water bath (6.10) at 46 °C for 18 h to 24 h. Examine this tube as described above.

Bacteria which form characteristic colonies in the SC medium and which give a positive confirmation with the LS medium are considered as being *C. perfringens*. In all other cases, the tubes should be considered as negative.

9.4.3 Confirmation technique using the nitrate motility medium and the lactose-gelatin medium

9.4.3.1 General

This confirmation technique necessitates well-isolated characteristic colonies. If this is not the case (i.e. the surface area of the plates is overgrown and it is not possible to select well-isolated characteristic colonies), inoculate five characteristic colonies into pre-deaerated fluid thioglycollate medium (5.3).

Incubate under anaerobic conditions at 37 °C for 18 h to 24 h. Streak the colonies on SC base agar plates (see 5.2.1.2), and add an overlay of 10 ml of the SC base agar.

Allow to solidify and incubate anaerobically at 37 °C for 18 h to 24 h. Select from each plate at least one characteristic and well-separated colony. If necessary, repeat the streaking and inoculation on SC base agar plates until well-isolated, characteristic black colonies are obtained.

Confirm this colony as described in 9.4.3.2, 9.4.3.3 and 9.4.3.4.

9.4.3.2 Inoculation and reading of nitrate motility medium

Stab-inoculate each selected colony (see 9.3) into the freshly deaerated nitrate motility medium (5.5).

Incubate under anaerobic conditions at 37 °C for 24 h. Examine the tube of the nitrate motility medium for the type of growth along the stab line. Motility is evident from diffuse growth out into the medium away from the stab line.

Test for the presence of nitrite by adding, with the graduated pipette (6.8) and the rubber bulb (6.11), 0,2 ml to 0,5 ml of the nitrite detection reagent (5.6) to each tube of nitrate motility medium.

WARNING — For health reasons, carry out this test under a fume hood.

The formation of a red colour confirms the reduction of nitrate to nitrite. If no red colour is formed within 15 min, add a small amount of zinc dust (5.7) and allow to stand for 10 min. If a red colour is formed after the addition of zinc dust, no reduction of nitrate has taken place.

9.4.3.3 Inoculation and reading of lactose-gelatin medium

Inoculate each selected colony (see 9.3) into the freshly deaerated lactose-gelatin medium (5.8). Incubate under anaerobic conditions at 37 °C for 24 h.

Examine the tubes of the lactose-gelatin medium for the presence of gas and a yellow colour (due to acid formation) indicating fermentation of lactose. Chill the tubes for 1 h at 5 °C and check for gelatin liquefaction. If the medium has solidified, re-incubate for an additional 24 h and again check for gelatin liquefaction.

9.4.3.4 Interpretation

Bacteria that produce black colonies in SC medium, are non-motile, usually reduce nitrate to nitrite, produce acid and gas from lactose, and liquefy gelatin in 48 h are considered to be *C. perfringens*. Cultures that show a faint reaction for nitrite (i.e. a pink colour) shall be eliminated, since *C. perfringens* consistently gives an intense and immediate reaction.

10 Expression of results

10.1 Method of calculation

See ISO 7218.

10.2 Precision

10.2.1 Interlaboratory test

Precision data for the method described in this International Standard are based on the results of an interlaboratory test (see [2]). Details of this interlaboratory test are summarized in Annex A. Repeatability and reproducibility limit values were determined using three types of food contaminated at various levels and reference materials.

The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.2.2 Repeatability

The absolute difference between two single ($\log_{10}$-transformed) test results (number of *C. perfringens* per gram or per millilitre), or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material by the same operator using the same apparatus within the shortest feasible time interval, will in not more than 5 % of cases exceed the repeatability limit (r).

As a general indication of repeatability (r), the following values may be used when testing food samples in general. These values of r are general means for all matrices considered in the interlaboratory test:

— r = 0,21 for LS confirmation or 0,25 for MN/LG confirmation (expressed as a difference between $\log_{10}$-transformed test results); or

— r = 1,67 for LS confirmation or 1,8 for MN/LG confirmation (expressed as a ratio of the higher to the lower of the two test results).

For reference materials (see Table A.4), the following values may be used:

— r = 0,13 for LS confirmation or 0,12 for MN/LG confirmation (expressed as a difference between $\log_{10}$-transformed test results); or

— r = 1,3 for LS confirmation and for MN/LG confirmation (expressed as a ratio of the higher to the lower of the two test results).

EXAMPLE A first test result of 10 000 or $1{,}0 \times 10^4$ presumptive *C. perfringens* per gram of food was observed. Under repeatability conditions, the ratio of the higher to the lower test result should not be greater than 1,9. So the second result should be between 5 263 (= 10 000/1,9) and 19 000 (10 000 × 1,9) presumptive *C. perfringens* per gram.

10.2.3 Reproducibility

The absolute difference between two single ($\log_{10}$-transformed) test results (number of *C. perfringens* per gram or millilitre), or the absolute ratio between two test results on the normal scale, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases exceed the reproducibility limit (R).

As an indication of reproducibility limit (R), the values of Table 1 may be used for the different food types and reference materials tested. These values are means of the values obtained in the interlaboratory trial for the different levels[1)].

1) In the case of this interlaboratory trial, the values for the reproducibility varied too much between samples to be expressed as a general value applicable to all food samples.

Table 1 — Examples of values for R

Sample type	LS confirmation		MN and LG confirmation	
	R (log)[a]	R [b]	R (log)[a]	R [b]
Cheese	0,26	1,8	0,31	2,1
Meat	0,55	3,5	0,52	3,3
Dried animal feed	0,65	4,5	0,72	5,3
Reference material	0,27	1,9	0,29	1,9

[a] R (log) is the reproducibility limit expressed as a difference between $\log_{10}$-transformed test results.

[b] R is the reproducibility limit expressed as a ratio between test results.

EXAMPLE 1 The first laboratory found a test result of 10 000 or $1,0 \times 10^4$ *C. perfringens* per gram of cheese. Under reproducibility conditions, the ratio of the higher to the lower test result should not be greater than 2,1. So the result of the second laboratory should be between 4 761 (= 10 000/2,1) and 21 000 (10 000 × 2,1) presumptive *C. perfringens* per gram.

EXAMPLE 2 Secondly, a laboratory wants to know the maximum level it may find that is still in compliance with a pre-set limit (for example a limit of 100 000 or $\log_{10}5$). For this, the R value (0,31 on the log scale for cheese) has to be multiplied by a factor of 0,59. This value is 0,18 (0,31 × 0,59) as a difference between $\log_{10}$-transformed test results or 1,52 ($10^{0,18}$) as a ratio between test results. So results up to $\log_{10}5,18$ ($\log_{10}5 + \log_{10}0,18$) or 152 000 (100 000 × 1,52) do not indicate non-compliance with the limit. The factor 0,59 reflects the fact that a test with a one-sided 95 % interval is used to test whether the limit is exceeded. The factor 0,59 is obtained from the following formula:

$$0,59 = \frac{1,64}{1,96 \times \sqrt{2}}$$

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 result(s);

e) the test result(s) obtained or, if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Results of interlaboratory test

An international collaborative test (see [2]) involving 17 laboratories in 15 countries was carried out on cheese, meat, dried animal feed and a reference material. The food/feed samples were each tested at three different levels of contamination with *Clostridium perfringens*.

In accordance with ISO 16140, the following parameters were identified in interlaboratory tests. The test was organized by the Dutch National Institute of Public Health (RIVM) in January 2000 and gave the precision data shown in Tables A.1 to A.4.

Table A.1 — Results of data analysis obtained with cheese samples

Sample	Cheese (low level)	Cheese (medium level)	Cheese (high level)
Number of laboratories with valid results	13	13	13
Number of samples	2	2	2
Number of laboratories retained after eliminating outliers	13	13	13
Number of outliers	0	0	0
Number of accepted samples	26	26	26
Mean value $\overline{x}$ ($\log_{10}$ cfu/g)	2,5/2,5[a]	3,5/3,5[a]	4,5/4,5[a]
Repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,11/0,11[a]	0,06/0,07[a]	0,08/0,10[a]
Repeatability relative standard deviation (%)	4,37/4,59[a]	1,63/1,97[a]	1,85/2,31[a]
Repeatability limit, r			
— as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,30/0,32[a]	0,16/0,19[a]	0,23/0,29[a]
— as ratio on normal scale (cfu/g)	2,0/2,1[a]	1,5/1,6[a]	1,7/1,9[a]
Reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,13/0,13[a]	0,08/0,15[a]	0,11/0,14[a]
Reproducibility relative standard deviation (%)	5,21/5,11[a]	2,32/4,38[a]	2,50/3,11[a]
Reproducibility limit, R			
— as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,36/0,35[a]	0,23/0,43[a]	0,31/0,39[a]
— as ratio on normal scale (cfu/g)	2,3/2,2[a]	1,7/2,7[a]	2,1/2,4[a]

[a] The first result was obtained using lactose-sulfite medium and the second result using nitrate motility with lactose-gelatin medium.

Table A.2 — Results of data analysis obtained with minced meat samples

Sample	Minced meat (low level)	Minced meat (medium level)	Minced meat (high level)
Number of laboratories with valid results	13	13	13
Number of samples	2	2	2
Number of laboratories retained after eliminating outliers	13	13	13
Number of outliers	0	0	0
Number of accepted samples	26	26	26
Mean value $\bar{x}$ ($\log_{10}$ cfu/g)	2,7/2,7[a]	3,6/3,6[a]	4,5/4,5[a]
Repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,06/0,11[a]	0,06/0,10[a]	0,11/0,09[a]
Repeatability relative standard deviation (%)	2,32/4,22[a]	1,67/2,70[a]	2,33/2,01[a]
Repeatability limit, r			
— as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,18/0,32[a]	0,17/0,27[a]	0,29/0,25[a]
— as ratio on normal scale (cfu/g)	1,5/2,1[a]	1,5/1,9[a]	2,0/1,8[a]
Reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,14/0,18[a]	0,18/0,18[a]	0,18/0,22[a]
Reproducibility relative standard deviation (%)	5,01/6,54[a]	5,07/5,05[a]	3,90/4,76[a]
Reproducibility limit, R			
— as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,38/0,49[a]	0,51/0,50[a]	0,49/0,60[a]
— as ratio on normal scale (cfu/g)	2,4/3,1[a]	3,2/3,2[a]	3,1/4,0[a]

[a] The first result was obtained using lactose-sulfite medium and the second result using nitrate motility with lactose-gelatin medium.

Table A.3 — Results of data analysis obtained with dried animal feed

Sample	Feed (low level)	Feed (medium level)	Feed (high level)
Number of laboratories with valid results	13	13	13
Number of samples	2	2	2
Number of laboratories retained after eliminating outliers	13	13	13
Number of outliers	0	0	0
Number of accepted samples	25	26	26
Mean value $\overline{x}$ ($\log_{10}$ cfu/g)	2,6/2,6[a]	3,8/3,9[a]	4,8/4,9[a]
Repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,07/0,10[a]	0,08/0,08[a]	0,06/0,04[a]
Repeatability relative standard deviation (%)	2,85/3,79[a]	2,09/1,93[a]	1,22/0,75[a]
Repeatability limit, r			
— as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,21/0,28[a]	0,22/0,21[a]	0,16/0,10[a]
— as ratio on normal scale (cfu/g)	1,6/1,9[a]	1,7/1,6[a]	1,5/1,3[a]
Reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,32/0,32[a]	0,25/0,24[a]	0,17/0,17[a]
Reproducibility relative standard deviation (%)	12,21/12,03[a]	6,53/6,18[a]	3,50/3,49[a]
Reproducibility limit, R			
— as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,88/0,88[a]	0,69/0,67[a]	0,47/0,47[a]
— as ratio on normal scale (cfu/g)	7,6/7,6[a]	4,9/4,7[a]	3,0/3,0[a]

[a] The first result was obtained using lactose-sulfite medium and the second result using nitrate motility with lactose-gelatin medium.

Table A.4 — Results of data analysis obtained with reference materials

Sample	Reference material
Number of laboratories with valid results	13
Number of samples	2
Number of laboratories retained after eliminating outliers	13
Number of outliers	0
Number of accepted samples	26
Mean value $\bar{x}$ ($\log_{10}$ cfu/capsule)	3,7/3,7[a]
Repeatability standard deviation, s_r ($\log_{10}$ cfu/capsule)	0,05/0,05[a]
Repeatability relative standard deviation (%)	1,24/1,21[a]
Repeatability limit, r	
— as difference on $\log_{10}$ scale ($\log_{10}$ cfu/capsule)	0,13/0,12[a]
— as ratio on normal scale (cfu/capsule)	1,3/1,3[a]
Reproducibility standard deviation, s_R ($\log_{10}$ cfu/capsule)	0,09/0,09[a]
Reproducibility relative standard deviation (%)	2,51/2,39[a]
Reproducibility limit, R	
— as difference on $\log_{10}$ scale ($\log_{10}$ cfu/capsule)	0,26/0,25[a]
— as ratio on normal scale (cfu/capsule)	1,8/1,8[a]

[a] The first result was obtained using lactose-sulfite medium and the second result using nitrate motility with lactose-gelatin medium.

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