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Microbiology of the food chain — Horizontal method for the detection and enumeration of *Clostridium* spp. —

Part 1: Enumeration of sulfite-reducing *Clostridium* spp. by colony-count technique

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche et le dénombrement de Clostridium *spp. —*

Partie 1: Dénombrement des bactéries Clostridium *spp. sulfito-réductrices par la technique de comptage des colonies*

Reference number
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Contents

Page

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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This first edition of ISO 15213-1 cancels and replaces ISO 15213:2003, which has been technically revised.

The main changes are as follows:

— the Scope has been expanded to include samples from the primary production stage;

— the scope of the method has been changed from "sulfite-reducing bacteria" to "sulfite-reducing *Clostridium* spp.": therefore, typical colonies on the iron sulfite agar plates are confirmed;

— the concentration of sulfite in the iron sulfite agar has been reduced from 1,0 g/l to 0,5 g/l;

— the heat treatment of 10 min at 80 °C has been made optional, in the case of high background flora or for the enumeration of only spores of sulfite-reducing *Clostridium* spp. present in the sample;

— the option for using tubes for inoculation has been removed;

— the option for incubating the samples at 50 °C for the enumeration of thermophilic sulfite-reducing bacteria has been removed;

— a description of how the confirmation of typical colonies has to be performed has been added;

— the flow diagram in Annex A giving a short description of the procedure has been revised;

— in Annex C, the performance characteristics have been added;

— Annex D has been added to provide a special protocol for the enumeration of sulfite-reducing *Clostridium* spp. in feed.

A list of all parts in the ISO 15213 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Sulfite-reducing *Clostridium* spp. are obligate anaerobic, Gram-positive, spore-forming, rod-shaped bacteria. The most important species which belong to this group are *Clostridium (C.) perfringens, C. bifermentans, C. sporogenes* and *C. botulinum*. Some species can cause foodborne illness. As ubiquitous bacteria they are predominantly found in nature. The *Clostridium* species inhabit soils and the intestinal tract of animals and humans.

Sulfite-reducing *Clostridium* spp., including *C. perfringens,* are widely used as microbial indicators of clostridial contamination in the manufacturing of foods (e.g. meat production). These have the capacity to produce heat-resistant spores. Outside the dairy industry, the use of sulfite-reducing *Clostridium* spp. as a microbial indicator is limited to a relatively small number of foods. Its current application in non-dairy foods is either an indication of faecal contamination (especially *C. perfringens*, see also ISO 15213-2 and ISO/TS 15213-3) and/or as an indicator of sanitation/process control related to potential growth and survival of anaerobic spore-forming bacteria.

This document describes the horizontal method for the enumeration of sulfite-reducing *Clostridium* spp. in food, feed, environmental samples and samples from the primary production stage. The method for the enumeration of *C. perfringens* is described in ISO 15213-2. The method for the detection of *C. perfringens* is described in ISO/TS 15213-3. These three parts are published as a series of International Standards because the methods are closely linked to each other. These methods are often conducted in association with each other in a laboratory, and the media and their performance characteristics can be similar.

The main technical changes listed in the Foreword, introduced in this document compared with ISO 15213:2003, are considered as major (see ISO 17468).

These changes have a major impact on the performance characteristics of the method.

Microbiology of the food chain — Horizontal method for the detection and enumeration of *Clostridium* spp. —

Part 1: Enumeration of sulfite-reducing *Clostridium* spp. by colony-count technique

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for the enumeration of sulfite-reducing *Clostridium* spp. are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety aspects, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

1 Scope

This document specifies the enumeration of sulfite-reducing *Clostridium* spp. by the colony-count technique.

This document is applicable to:

— products intended for human consumption;

— products for feeding animals;

— environmental samples in the area of food and feed production and handling;

— samples from the primary production stage.

NOTE This method has been validated in an interlaboratory study for the following food categories:

— ready-to-eat, ready-to-reheat meat products;

— eggs and egg products (derivates);

— processed fruits and vegetables;

— infant formula and infant cereals;

— multi-component foods or meal components.

It has also been validated for the following other categories:

— pet food and animal feed;

— environmental samples (food or feed production).

As this method has been validated for at least five food categories, this method is applicable for a broad range of food. For detailed information on the validation, see Clause 11 and Annex C. Since the method is not commonly used for samples in the primary production stage, this category was not included in the interlaboratory study. Therefore, no performance characteristics were obtained for this category.

This horizontal method was originally developed for the examination of all samples belonging to the food chain. Based on the information available at the time of publication of this document, this method is considered to be fully suited to the examination of all samples belonging to the food chain. However, because of the large variety of products in the food chain, it is possible that this horizontal method is not

appropriate in every detail for all products. Nevertheless, it is expected that the required modifications are minimized so that they do not result in a significant deviation from this horizontal method.

This technique is suitable for, but not limited to, the enumeration of microorganisms in test samples with a minimum of 10 colonies counted on a plate. This corresponds to a level of contamination that is expected to be higher than 10 cfu/ml for liquid samples or higher than 100 cfu/g for solid samples.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of the food chain — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

ISO 19036:2019, *Microbiology of the food chain — Estimation of measurement uncertainty for quantitative determinations*

3 Terms and definitions

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

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
sulfite-reducing *Clostridium* spp.
genus of microorganisms of the family of *Clostridiaceae,* usually capable of growth in/on iron sulfite agar (ISA) under anaerobic conditions, forming typical or less typical colonies, and displaying certain characteristics with biochemical confirmation tests

Note 1 to entry: The biochemical confirmation tests are described in 9.6.

3.2
enumeration of sulfite-reducing *Clostridium* spp.
determination of the number of colony-forming units (cfu) of *sulfite-reducing Clostridium spp.* (3.1) bacteria per gram, per millilitre, per square centimetre or per sampling device when a specified test is conducted

Note 1 to entry: Specified tests are given in Clause 9.

4 Principle

4.1 General

A specified quantity of the liquid test sample, or of an initial suspension in the case of other products, is dispensed into an empty Petri dish and mixed well with a specified molten agar culture medium to form a poured plate. Other plates are prepared under the same conditions using decimal dilutions of the test sample. After solidification of the agar culture medium, an overlay is used to prevent the

development of spreading colonies on the surface of the medium. If it is the intention to count only spores, a heat treatment of 10 min at 80 °C needs to be performed before plating.

When the number of cfu is expected to be at or near the limit of determination, the use of duplicate plates is preferable. If duplicate plates are used, the minimum for the sum of colonies on both plates should be 10 colonies. In this case, the level of contamination is expected to be higher than 5 cfu/ml for liquid samples or higher than 50 cfu/g for solid samples.

A pour-plate technique with overlay is especially suited for the enumeration of products expected to contain spreading colonies that can obscure colonies of the target microorganisms.

The enumeration of sulfite-reducing *Clostridium* spp. requires four successive stages as specified in Annex A.

4.2 Preparation of dilutions

For the preparation of decimal dilutions from the test portion, follow the procedure as specified in the ISO 6887 series.

4.3 Enumeration

The plates are incubated under anaerobic conditions at 37 °C for 48 h. After incubation, the number of typical colonies, which show black or grey to yellow-brown staining, are counted. The colour of the colonies and the surrounding zone changes due to the formation of iron(II)sulfide as a result of the reaction between sulfide ions and trivalent iron [Fe(III)] present in the medium.

4.4 Confirmation

Typical colonies are picked for confirmation.

NOTE When no confirmation is performed, the results can be reported as “anaerobic sulfite-reducing bacteria”.

5 Culture media and reagents

Follow current laboratory practices in accordance with ISO 7218. The composition of culture media and reagents and their preparation are specified in Annex B. For performance testing of culture media, follow the procedures in accordance with ISO 11133 and Annex B.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications. The usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following shall be used.

6.1 Appropriate apparatus for achieving an anaerobic atmosphere, a jar that can be hermetically sealed or any other appropriate equipment which enables anaerobic atmosphere conditions to be maintained for the total incubation time of the culture medium. Other systems of equivalent performance, such as anaerobic cabinets, may be used. Follow the manufacturer’s instructions for installation and maintenance.

The composition of the atmosphere required can be achieved by means of the addition of a gas mixture (e.g. from a gas cylinder) after evacuation of air from the jar, by displacement of the atmosphere in a cabinet or by any other appropriate means (such as commercially available gas packs). In general, anaerobic incubation requires an atmosphere of less than 1 % volume fraction oxygen, 9 % volume fraction to 13 % volume fraction carbon dioxide.

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

6.3 Incubator, capable of operating at 37 °C ± 1 °C.

6.4 pH-meter, having an accuracy of calibration of ± 0,1 pH unit at 25 °C.

6.5 Refrigerator, capable of operating at 5 °C ± 3 °C.

6.6 Sterile bottles, flasks or tubes, of appropriate capacity. Bottles, flasks or tubes with non-toxic metallic or plastic screwcaps may be used.

6.7 Sterile graduated pipettes or **automatic pipettes**, of nominal capacities of 10 ml and 1 ml.

6.8 Sterile loops, of approximately 1 µl volume, or inoculation needle or wire.

6.9 Sterile Petri dishes, with a diameter of approximately 90 mm and (optional) large size (diameter approximately 140 mm).

6.10 Thermostatically controlled water bath, capable of operating at 44 °C to 47 °C and 80 °C ± 2 °C.

7 Sampling

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

Recommended sampling techniques are given in the following documents:

— ISO/TS 17728 for food and animal feed;

— ISO 707 for milk and milk products;

— ISO 6887-3 for raw molluscs, tunicates and echinoderms from primary production areas;

— ISO 13307 for primary production stage;

— ISO 17604 for carcasses;

— ISO 18593 for surfaces.

It is important that the laboratory receives a sample that is representative of the product under consideration. The sample should not have been damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned. Follow the procedures as specified in the ISO 6887 series. If there is no specific International Standard available, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 General

A flow diagram of the procedure is given in Annex A.

9.2 Test portion, initial suspension and dilutions

Follow the procedures in accordance with the ISO 6887 series and the specific International Standard dealing with the product concerned.

Prepare a single decimal dilution series from the test portion if the product is liquid, or from the initial suspension in the case of other products.

A special protocol for preparing the initial suspension of feed samples is given in Annex D.

9.3 Heat treatment to select spores

If it is the intention to count only spores, heat the decimal dilution series to 80 °C in a water bath (6.10) for 10 min ± 1 min. Heat treatment shall be given within 15 min after preparation of the initial suspension to avoid germination of spores. If the tube is not placed in the water bath within 15 min, it should be placed immediately in melting ice for a maximum of 2 h.

The temperature during heat treatment should be monitored by placing an appropriate thermometer in a reference bottle of the same size as the sample bottle and containing the same volume of water at the same initial temperature as the sample being treated (6.6). The tubes should not be hermetically sealed during the heat treatment. The time taken to reach 80 °C shall not exceed 5 min and can be minimized by ensuring the water level to be at least 4 cm above the level of the sample and equipping the water bath with a circulating-water pump to maximize heat exchange.

Start the time of heating (10 min) when the temperature of the reference sample has reached 80 °C. After heat treatment, the samples should be cooled immediately until approximately 20 °C.

Heat treatment should also reduce the competitive flora in some matrices containing a high level of background flora (e.g. liquid whey, feed silage).

9.4 Inoculation and incubation

9.4.1 Take two sterile Petri dishes with a diameter of approximately 90 mm (6.9). Transfer to each dish, by means of a sterile pipette (6.7), 1 ml of the test sample if liquid, or 1 ml of the initial suspension (10^{-1} dilution) in the case of other products. If plates from more than one dilution are prepared, this may be reduced to one dish (see ISO 7218).

When, for certain products, it is necessary to estimate low numbers of sulfite-reducing *Clostridium* spp., the limit of enumeration may be lowered by a factor of 10 by examining 10 ml of the initial suspension in three large (140 mm) Petri dishes (6.9).

9.4.2 Take one other sterile Petri dish (6.9). Use another sterile pipette (6.7) to dispense 1 ml of the 10^{-1} dilution (liquid product) or 1 ml of the 10^{-2} dilution (other products).

9.4.3 If necessary, repeat the procedure with further dilutions, using a new sterile pipette (6.7) for each decimal dilution.

9.4.4 If appropriate and possible, select only the critical dilution steps (at least two consecutive decimal dilutions) for the inoculation of the Petri dishes (6.9) that will give colony counts of between 10 and 150 colonies per plate (on 90 mm Petri dishes) or between 10 and 360 colonies per plate (on 140 mm Petri dishes).

9.4.5 Pour about 12 ml to 15 ml for 90 mm Petri dishes or 45 ml to 50 ml for 140 mm Petri dishes of the iron sulfite agar (ISA) medium (Clause B.2), molten and tempered at 44 °C to 47 °C (6.10), into each Petri dish (6.9).

9.4.6 Carefully mix the inoculum with the medium by rotating the Petri dishes (6.9) and allow the mixture to solidify by leaving the Petri dishes standing on a cool horizontal surface.

9.4.7 After complete solidification, pour about 5 ml of the ISA medium (Clause B.2) for 90 mm Petri dishes (6.9) or 10 ml for 140 mm Petri dishes (6.9) as overlay, to prevent the development of spreading colonies on the surface of the medium. Allow to solidify as specified in 9.4.6.

9.4.8 Invert the plates obtained in 9.4.7 and incubate the plates at 37 °C (6.3) in an anaerobic atmosphere (6.1).

9.5 Enumeration of typical colonies

9.5.1 After 48 h ± 2 h of incubation, examine the plates (see 9.4.8) for presumptive sulfite-reducing *Clostridium* spp.

Typical colonies, which show black or grey to yellow-brown staining on the ISA medium, are counted.

Upon removal of the plates from the anaerobic atmosphere, plates shall be counted within 30 min as the colour of the colonies can rapidly fade and disappear upon exposure to oxygen. If anaerobic jars are used, the plates should be checked jar by jar or in small portions if the incubation was performed in an anaerobic incubator (6.1, 6.3).

NOTE Diffuse, unspecific blackening of the medium can occur. The growth of anaerobic bacteria, which produce hydrogen (not H_2S), can also reduce the sulfite present and lead to a general blackening of the medium, which makes enumeration of typical colonies difficult.

9.5.2 Select the plates (see 9.5.1) containing less than 150 presumptive colonies (for 90 mm Petri dishes) or less than 360 colonies (for 140 mm Petri dishes). Count these colonies and record their number as presumptive colonies per dish. Then choose at random five such colonies for subculturing for the confirmation tests (see 9.6).

For enumeration of plates with low or high numbers of presumptive colonies, see ISO 7218.

9.6 Confirmation of sulfite-reducing *Clostridium* spp.

9.6.1 For confirmation, take five presumptive colonies from each dish retained for enumeration (see 9.5.2). If more than one morphology is present among the colonies, select one of each morphology for subculture and confirmation.

9.6.2 Streak each of the selected colonies with a sterile loop (6.8) onto two non-selective blood agar plates, e.g. Columbia blood agar (Clause B.3). If blood is not available, Columbia agar base or another nutrient-rich medium (e.g. tryptone soya agar or brain heart infusion agar) can be used.

Allow the plates to equilibrate at room temperature if they were stored at a lower temperature. If necessary, dry the surface of the plates before use (see ISO 11133).

Several isolates can be streaked onto identified sectors of each of the two non-selective agar plates. Streaks should obtain well-isolated colonies.

From each pair of plates, one is incubated in an aerobic atmosphere and the other in an anaerobic atmosphere (6.1) at 37 °C for 20 h ± 2 h (6.3). After incubation, the plates can be refrigerated at 5 °C (6.5) for a maximum of 48 h before reading. For plates which were incubated anaerobically, maintain the anaerobic atmosphere.

9.6.3 Typical colonies are confirmed as follows:

— If growth from one typical colony occurs on the anaerobically incubated (blood) agar plate and not on the aerobically incubated (blood) agar plate, the colony belongs to the genus *Clostridia*. This colony and other colonies with the same morphology on ISA medium are counted as sulfite-reducing *Clostridium* spp.

— If growth occurs from one typical colony on both the anaerobically and aerobically incubated blood agar plates, the colony does not belong to the genus *Clostridia*. Therefore, this colony and other colonies with the same morphology on ISA medium cannot be counted as sulfite-reducing *Clostridium* spp.

NOTE Alternative procedures (see ISO 7218) can be used to confirm the isolate as sulfite-reducing *Clostridium* spp., provided that the suitability of the alternative procedure has been validated (see ISO 16140-4 or ISO 16140-6).

10 Expression of results

For calculation of the results, follow the procedure(s) in accordance with ISO 7218. Calculate and report the results as the number of sulfite-reducing *Clostridium* spp. in cfu per gram, per millilitre or per square centimetre. When the sampled area is not known, report as per sampling device, such as a cloth, sponge swab or stick.

If heat treatment for the selection of spores (9.3) was used, the result is reported as number of sulfite-reducing *Clostridium* spp. spores in cfu per gram, per millilitre, per square centimetre or per sampling device.

In cases where no typical colonies of the target organism have been detected, follow ISO 7218 for the expression of results for special cases.

11 Validation of the method

11.1 Interlaboratory study

Results of the interlaboratory study (step 6 in ISO 17468) to determine the performance characteristics of the method are described in 11.2.

NOTE In the interlaboratory study, the repeatability and reproducibility were only determined at a low level, as the enumeration of sulfite-reducing *Clostridium* spp. is performed as a hygiene indicator and at low levels (e.g. the maximum acceptable levels for infant formula are between 10 cfu/g and 30 cfu/g).

11.2 Performance characteristics

The performance characteristics of the method (repeatability and reproducibility standard deviations) were determined in an interlaboratory study. It is possible that the values derived from the interlaboratory study are not applicable to concentration ranges and (food) categories other than those used in the study. All data are given in Annex C.

A summary of different values of the interlaboratory repeatability standard deviation (s_r) is given in Table 1.

Table 1 — Summary of s_r values from the interlaboratory study

(Food) category	(Food) item	s_r values of low inoculation level
Ready-to-eat, ready-to-reheat meat products	Corned beef	0,091
Multi-component foods or meal components	Instant soup	0,049
infant formula and infant cereals	Powdered infant formula	0,040
Eggs and egg products (derivates)	Egg powder	0,11
Processed fruits and vegetables	Canned pineapple	0,060
Environmental samples (food or feed production)	Environmental swab	0,054
Pet food and animal feed	Feed silage	0,048

A summary of different values of the interlaboratory reproducibility standard deviation (s_R) is given in Table 2.

Table 2 — Summary of s_R values from the interlaboratory study

(Food) category	(Food) item	s_R values of low inoculation level
Ready-to-eat, ready-to-reheat meat products	Corned beef	0,93
Multi-component foods or meal components	Instant soup	0,41
infant formula and infant cereals	Powdered infant formula	0,12
Eggs and egg products (derivates)	Egg powder	0,61
Processed fruits and vegetables	Canned pineapple	0,35
Environmental samples (food or feed production)	Environmental swab	0,20
Pet food and animal feed	Feed silage	0,26

12 Test report

The test report shall specify at least the following:

— the test method used, with reference to this document, i.e. ISO 15213-1;

— the sampling method used, if known;

— all operating conditions not specified in this document, or regarded as optional or informative (including informative annexes), together with details of any incidents which can have influenced the test result(s);

— any deviations from this document;

— all information necessary for the complete identification of the sample;

— the test result(s) obtained;

— the date of the test;

— when necessary or if requested by the client, an estimate of the measurement uncertainty of quantitative test results, in accordance with ISO 19036:2019, Clause 9.

13 Quality assurance

The laboratory should have a quality control system to ensure that the equipment, reagents and techniques are suitable for the method. The use of positive controls, negative controls and blanks are part of the method. Performance testing of culture media is specified in Annex B and described in ISO 11133.

Annex A
(normative)

Flow diagram of the procedure

Figure A.1 shows the flow diagram of procedure for the enumeration of sulfite-reducing *Clostridium* spp. by colony-count technique in food, animal feed, environmental and primary production stage samples.

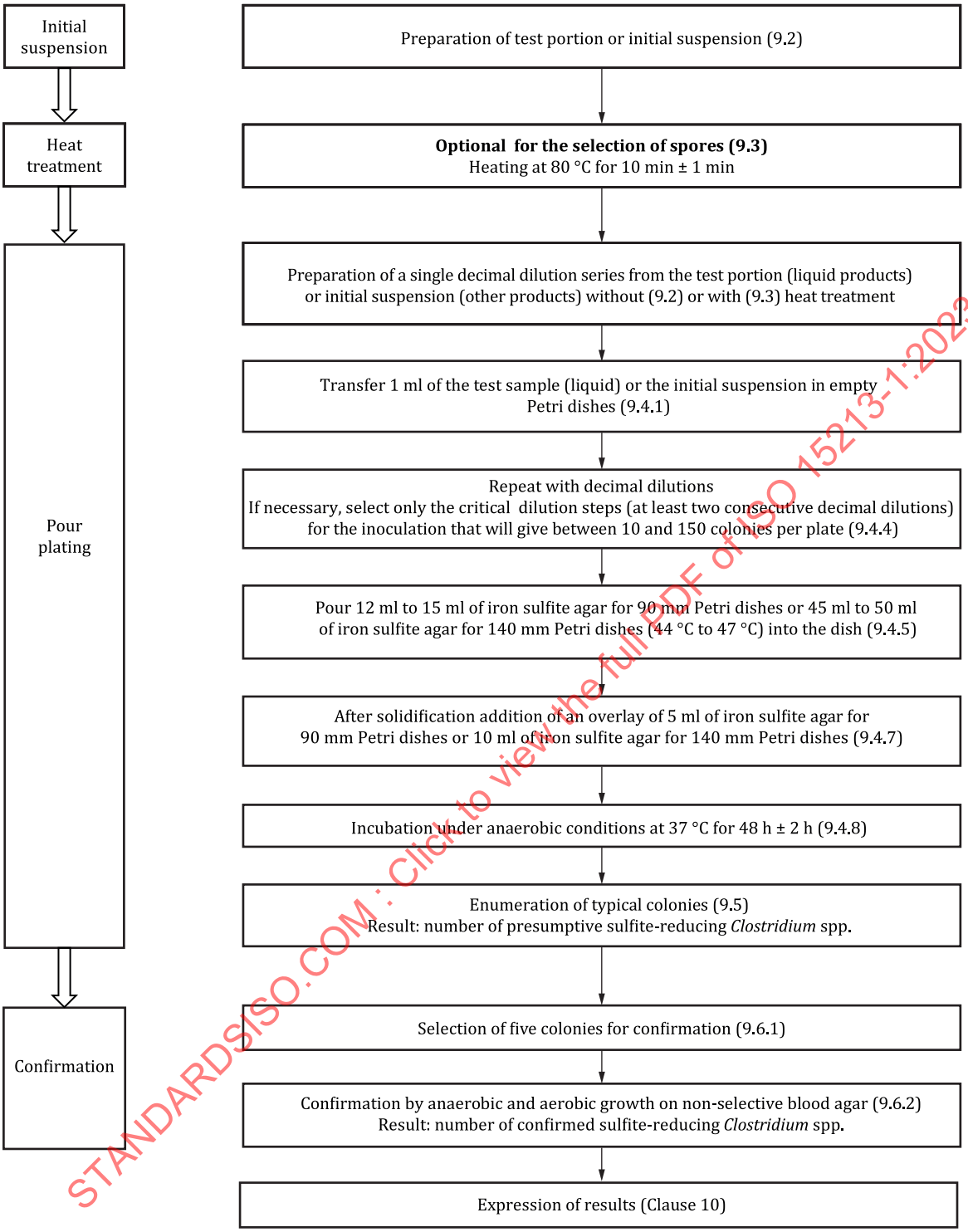

Figure A.1 — Flow diagram of procedure for the enumeration of sulfite-reducing *Clostridium* spp. by colony-count technique

Annex B
(normative)

Culture media and reagents

B.1 General

The general specifications of ISO 11133 are applicable to the preparation and performance testing of the culture media described in this annex. If culture media or reagents are prepared from dehydrated complete media/reagents or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf life of the media indicated in this annex has been determined in some studies. The user shall verify this under its own storage conditions (in accordance with ISO 11133).

Performance testing of culture media is described in Clause B.4.

B.2 Iron sulfite agar (ISA)

B.2.1 Composition

Peptone[a]		15,0 g
Enzymatic digest of soya		5,0 g
Yeast extract		5,0 g
Sodium disulfite (sodium metabisulfite), anhydrous ($Na_2S_2O_5$)	(CAS Registry Number®[d] 7681-57-4)	0,5 g
Iron(III) ammonium citrate ($C_6H_8FeNO_4$)[b]	(CAS No. 1185-57-5)	1,0 g
Agar[c]		9,0 to 18,0 g
Water		1 000 ml

[a] For example, enzymatic digest of casein.

[b] This reagent should contain at least 150 g/kg of iron.

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

[d] Chemical Abstracts Service (CAS) Registry Number® is a trademark of the American Chemical Society (ACS). This information is given in the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

B.2.2 Preparation

Dissolve the ingredients in water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,6 ± 0,2 at 25 °C (6.4).

Sterilize for 15 min in the autoclave (6.2) set at 121 °C.

Store the medium, at 5 °C (6.5) for up to four weeks in closed containers or tubes (6.6). Prior to use, the stored medium is melted completely and cooled down to 44 °C to 47 °C (6.10) before use.

B.3 Columbia blood agar (CBA)

B.3.1 Columbia blood agar base

B.3.1.1 Composition

Enzymatic digest of animal tissue		23,0 g
Starch soluble ($C_{12}H_{22}O_{11}$)	(CAS No. 9005-84-9)	1,0 g
Sodium chloride (NaCl)	(CAS No. 7647-14-5)	5,0 g
Agar[a]		8,0 to 18,0 g
Water		1 000 ml
[a] Depending on the gel strength of the agar.		

B.3.1.2 Preparation

Dissolve the ingredients in water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C (6.4).

Dispense the medium in flasks (6.6) of suitable capacity to obtain portions appropriate for the test.

Sterilize for 15 min in the autoclave (6.2) set at 121 °C. Store the medium, at 5 °C (6.5) for up to four weeks in closed containers or tubes (6.6).

B.3.2 Defibrinated blood (horse or sheep blood)

B.3.3 Complete base

B.3.3.1 Composition

Base (B.3.1)	100 ml
Defibrinated blood (B.3.2)	5 ml

B.3.3.2 Preparation

Add the blood to the base previously cooled to 44 °C to 47 °C (6.10). Mix well.

B.3.3.3 Preparation of sheep blood agar plates

Dispense the medium (B.3.3.1) into sterile Petri dishes (6.9) in portions appropriate for the test. Allow to solidify.

Immediately before use, dry the agar plates following the procedure as given by ISO 11133. Store the poured plates, protected for drying, at 5 °C (6.5) for up to four weeks.

B.4 Performance testing

For the definition of selectivity and productivity refer to ISO 11133. In general, follow the procedures for performance testing described in ISO 11133.

The performance testing for the quality assurance of the culture media is given in Table B.1.

Table B.1 — Performance testing for the quality assurance of the culture media

Medium	Function	Incubation	Control strains	WDCM numbers[a]	Reference medium	Method of control	Criteria[c,e]
ISA	Productivity	(48 ± 2) h/ (37 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b] 00080	A suitable non-selective medium for anaerobes	Quantitative	$P_R \geq 0,5$ ($P_R \geq 0,7$ when compared to ISA batch already validated)
	Specificity		*Escherichia coli*[d]	00013 or 00012	—	Qualitative	Growth (1 to 2) No blackening of colonies
CBA	Confirmation	(20 ± 2) h / (37 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b]	—	Qualitative	Good growth (2) Colonies with beta-haemolysis

[a] Refer to the reference strain catalogue on http://www.wfcc.info for information on culture collection strain numbers and contact details; WDCM: World Data Centre for Microorganisms.

[b] Strain to be used as a minimum.

[c] P_R = productivity ratio.

[d] Strain free of choice; one of the strains has to be used as a minimum.

[e] Growth is categorized as 0: no growth; 1: weak growth (partial inhibition); 2: good growth (see ISO 11133).

Annex C
(informative)

Performance characteristics of the method

An interlaboratory study involving 20 laboratories in 7 countries was carried out. The following (food) items [representing the (food) categories as indicated] were included in the study: canned corned beef, instant soup, powdered infant formula, egg powder, canned pineapple, environmental swabs and feed silage. The (food) samples were each tested at a low level of contamination. The study was organized in 2019 by Merck KGaA, Darmstadt, Germany and FrieslandCampina as part of the development of this document.

The values of the performance characteristics, for each (food) item and category, derived from this interlaboratory study are shown in Tables C.1 to C.7, and were calculated in accordance with ISO 17468.

Table C.1 — Results of data analysis obtained with canned corned beef (category: ready-to-eat, ready-to-reheat meat products)

Parameter	Low level
Number of participating collaborators	20
Number of collaborators retained after evaluation of the data	13
Number of samples	26
Number of sample results retained after evaluation of the data	26
Mean value Σa ($\log_{10}$ cfu/g)	0,61
Interlaboratory repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,091
Interlaboratory reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,931
NOTE 1 Strain used for inoculation: *C. perfringens* (WDCM 00201).	
NOTE 2 The found s_R is high, due to big variances found by the different participants for canned corned beef.	

Table C.2 — Results of data analysis obtained with instant soup (category: multi-component foods or meal components)

Parameter	Low level
Number of participating collaborators	20
Number of collaborators retained after evaluation of the data	13
Number of samples	26
Number of sample results retained after evaluation of the data	26
Mean value Σa ($\log_{10}$ cfu/g)	0,58
Interlaboratory repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,049
Interlaboratory reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,41
NOTE Strain used for inoculation: *C. perfringens* (WDCM 00201).	

Table C.3 — Results of data analysis obtained with powdered infant formula (category: infant formula and infant cereals)

Parameter	Low level
Number of participating collaborators	20
Number of collaborators retained after evaluation of the data	8
Number of samples	16
Number of sample results retained after evaluation of the data	16
Mean value Σa ($\log_{10}$ cfu/g)	0,48
Interlaboratory repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,040
Interlaboratory reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,12
NOTE Strain used for inoculation: *C. sporogenes* (WDCM 00008).	

Table C.4 — Results of data analysis obtained with egg powder (category: eggs and egg products (derivates))

Parameter	Low level
Number of participating collaborators	20
Number of collaborators retained after evaluation of the data	7
Number of samples	14
Number of sample results retained after evaluation of the data	14
Mean value Σa ($\log_{10}$ cfu/g)	0,52
Interlaboratory repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,11
Interlaboratory reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,61
NOTE Strain used for inoculation: *C. sporogenes* (WDCM 00008).	

Table C.5 — Results of data analysis obtained with canned pineapple (category: processed fruits and vegetables)

Parameter	Low level
Number of participating collaborators	20
Number of collaborators retained after evaluation of the data	13
Number of samples	26
Number of sample results retained after evaluation of the data	26
Mean value Σa ($\log_{10}$ cfu/g)	0,60
Interlaboratory repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,060
Interlaboratory reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,35
NOTE Strain used for inoculation: *C. bifermentans* (WDCM 00079).	

Table C.6 — Results of data analysis obtained with environmental swab (category: environmental samples (food or feed production))

Parameter	Low level
Number of participating collaborators	20
Number of collaborators retained after evaluation of the data	14
Number of samples	28
Number of sample results retained after evaluation of the data	28
Mean value Σa ($\log_{10}$ cfu/g)	0,60
Interlaboratory repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,054
Interlaboratory reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,20
NOTE Strain used for inoculation: *C. bifermentans* (WDCM 00079).	

Table C.7 — Results of data analysis obtained with feed silage (category: pet food and animal feed)

Parameter	Low level
Number of participating collaborators	20
Number of collaborators retained after evaluation of the data	13
Number of samples	26
Number of sample results retained after evaluation of the data	26
Mean value Σa ($\log_{10}$ cfu/g)	0,61
Interlaboratory repeatability standard deviation, s_r ($\log_{10}$ cfu/g)	0,048
Interlaboratory reproducibility standard deviation, s_R ($\log_{10}$ cfu/g)	0,26
NOTE Strain used for inoculation: *C. perfringens* (WDCM 00201).	