Technical Specification

ISO/TS 15213-3

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

Part 3: Detection of *Clostridium perfringens*

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

Partie 3: Recherche de Clostridium perfringens

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Foreword

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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 document 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).

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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).

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

Clostridium (C.) perfringens is a gram-positive, anaerobic, spore-forming bacterium. As a ubiquitous bacterium, *C. perfringens* is predominantly found in soil, but also in the intestinal tract of humans and animals. Therefore, the presence of *C. perfringens* in high numbers can be an indication of inadequate preparation or handling of food.

High numbers of *C. perfringens* in ready-to-eat-food can cause human illness, mainly diarrhoea. The strains are classified into toxin types, depending on the ability to produce different so called “major” and “minor” toxins. Food poisonings related to *C. perfringens* are mostly caused by *C. perfringens* isolates with the ability to produce *C. perfringens* enterotoxin (CPE).

A characteristic feature is the heat resistance of the spores; they have the ability to germinate and multiply in ready-to-eat food after the cooking process. Ingestion of contaminated food is followed by gastrointestinal disease, when enzyme-resistant *C. perfringens* enterotoxins are set free during sporulation in the small intestine. The strains are classified into different types.

This document describes the horizontal method for the detection of *C. perfringens* in food, feed, environmental samples and samples from the primary production stage. The method for the enumeration of sulfite-reducing *Clostridium* spp. is described in ISO 15213-1 and ISO 15213-2 describes the method for the enumeration of *C. perfringens*. 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.

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

Part 3: Detection of *Clostridium perfringens*

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for the detection of *Clostridium perfringens* 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 detection of *Clostridium* (*C.*) *perfringens*.

This document is applicable to:

— products intended for human consumption;

— products intended for animal feeding;

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

— samples from the primary production stage.

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.

NOTE Interlaboratory studies with a small number of participating laboratories (<10) were conducted for the following food categories:

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

— eggs and egg products (derivates);

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

— processed fruits and vegetables;

— infant formula and infant cereals (with probiotics);

— multi-component foods or meal components.

It has also been validated with a small number of participating laboratories for the following other category:

— environmental samples (food or feed production).

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. The method has not been validated for the category 'pet food and animal feed', as the test samples used for the interlaboratory study were already naturally contaminated with *C. perfringens*. Given the limited number of participating laboratories in the interlaboratory studies, the calculated performance characteristics can be used as indicative values of the method performance. For detailed information on the validation, see Clause 11 and Annexes C to F.

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*

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
presumptive *C. perfringens*
presumptive *Clostridium perfringens*
spore-forming bacteria forming typical colonies on a specific selective medium under obligate anaerobic conditions

Note 1 to entry: Presumptive *C. perfringens* are spore-forming bacteria that are able to produce typical colonies under the conditions specified in this document.

3.2
confirmed *C. perfringens*
confirmed *Clostridium perfringens*
bacteria that produce characteristic colonies on the specified selective medium under obligate anaerobic conditions and either possess the enzyme acid phosphatase, or are able to produce sulfite, are not able to produce indole and are not motile (SIM agar)

3.3
human pathogenic *C. perfringens*
human pathogenic *Clostridium perfringens*
confirmed *C. perfringens* (3.2) strains which possess the ability to produce *C. perfringens* enteroxin (CPE), encoded by the *cpe* gene

Note 1 to entry: The *cpe* gene can be located either chromosomally or on large plasmids. These isolates are able to produce CPE in the small intestine on sporulation and cause human illness.

3.4
detection of *C. perfringens*
detection of *Clostridium perfringens*
determination of confirmed *C. perfringens* (3.2) in a particular mass, volume of product, on a surface area or object, when a specified test is conducted

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

4 Principle

4.1 General

The detection of *C. perfringens* requires three successive stages as specified in Annex A.

4.2 Enrichment in selective liquid medium

A selective culture medium (at ambient temperature) is inoculated with a specified quantity of the test sample if the initial product is liquid, or a specified quantity of an initial suspension in the case of other products. The inoculated selective medium is incubated at 46 °C for 18 h.

4.3 Isolation on selective solid medium

From the cultures obtained in 4.2, two selective plating media are inoculated. The plates are incubated at 37 °C and at 46 °C respectively for 24 h anaerobically.

4.4 Confirmation

Confirmatory tests are carried out. The result is expressed as *C. perfringens* detected or not detected per sample volume. Additionally, the method mentioned in Annex G can be used for molecular differentiation between non-pathogenic and human pathogenic *C. perfringens* strains.

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 apparatus (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 Drying cabinet or **oven**, ventilated, capable of operating between 25 °C and 50 °C.

6.4 Freezers, capable of operating at -20 °C ± 2 °C and below -70 °C.

6.5 Incubator(s), capable of operating at 37 °C ± 1 °C, 46 °C ± 1 °C.

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

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

6.8 Sterile bottles, **flasks** or **tubes**, of appropriate capacity. Bottles, flasks or tubes with non-toxic metallic of plastic screw-caps may be used.

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

6.10 Sterile loops, of approximate diameter of 3 mm (10 µl volume) and of 1 µl volume, or inoculation needle or wire.

6.11 Sterile Petri dishes, with a diameter of approximately 90 mm.

6.12 Thermostatically controlled water bath, capable of operating at 44 °C to 47 °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

The procedure as given in Annex A shall be followed.

9.2 Test portion and initial suspension

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

Prepare the initial suspension in the case the product of concern is not liquid. Add 1 ml (6.9) of the liquid sample or 1 ml (6.9) of the initial suspension (0,1 g product) to 9 ml of rapid perfringens medium (RPM, B.2). Alternatively, 10 ml (6.9) of the liquid sample or of the initial suspension (1 g product) is added to 90 ml of RPM (B.12).

It is possible to composite or pool samples of the same type, to reduce workload when a large number of samples are required to be examined. This can be necessary to reflect microbiological quality of a large batch of product of environmental samples or required by regional legislation.

Similarly, a number of test portions may be pooled and examined together in larger quantities of media, or the (pre)enrichment cultures from the test portions may be pooled and carried out as a single test. These pooling procedures are described in ISO 6887-1. Whether it is possible to pool samples of a certain type shall be verified according to the protocol described in ISO 6887-1.

NOTE Validation of this method can be conducted according to the appropriate documents in ISO 16140 (all parts).

9.3 Selective enrichment

Incubate the selective enrichment broth RPM in closed tubes or bottles (9.2) at 46 °C (6.5) for 18 h ± 2 h.

9.4 Isolation

Allow the selective plating media (B.3 and B.4) to equilibrate at room temperature if they were stored at a lower temperature. If necessary, dry the surfaces of the plates (see ISO 11133) in a drying cabinet or oven (6.3) before use.

From the selective enrichment obtained at 9.3, inoculate by means of a 10 µl loop (6.10) the surface of a Petri dish (6.11) containing the selective medium tryptose sulfite cycloserine agar (TSC agar, B.3) and a Petri dish (6.11) containing the selective medium Lactose egg-yolk neomycin agar (LENA, B.4).

Incubate the TSC agar plates anaerobically (6.1) in an incubator (6.5) at 37 °C for 24 h ± 2 h. Incubate the LENA plates anaerobically (6.1) in an incubator (6.5) at 46 °C for 24 h ± 2 h.

NOTE After inoculation of the TSC agar plates an overlay of TSC agar can be used to prevent the development of spreading colonies on the surface of the medium. Pour about 5 ml of the TSC medium (see B.3) as overlay and allow to solidify by leaving the Petri dishes standing on a cool horizontal surface.

9.5 Confirmation of *C. perfringens*

9.5.1 Selection of colonies for confirmation

9.5.1.1 Typical colonies on TSC agar are black or grey to yellow-brown staining, even if the colour is faint.

Typical colonies on LENA show yellow colour (acid fermentation from lactose) and precipitation (lecithinase reaction).

Upon removal of the TSC agar plates from the anaerobic atmosphere, plates shall be read 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, 6.5).

For confirmation, take five presumptive *C. perfringens* colonies from each dish containing typical colonies (see 9.4). If more than one morphology is present among the colonies, select one of each morphology for subculture and confirmation.

9.5.1.2 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).

Streak each of the selected colonies with a sterile loop (6.10) onto one non-selective blood agar plate, e.g. Columbia blood agar (B.5). 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 with or without blood. Several isolates can be streaked onto identified sectors of a non-selective agar plates. Streaks should obtain well-isolated colonies.

Incubate the plates in an anaerobic atmosphere (6.1) at 37 °C (6.5) for 20 h ± 2 h. Right after incubation, select well-isolated freshly grown colonies for confirmation. Confirmation may be done either by the acid phosphatase test (9.5.2) or by the sulfite indole motility (SIM) agar test (9.5.3).

NOTE Alternative procedures (see ISO 7218) can be used to confirm whether the typical colonies are *C. perfringens,* provided that the suitability of the alternative procedure has been validated (see ISO 16140-4 or ISO 16140-6).

After incubation, these plates can be refrigerated at 5 °C (6.7) for a maximum of 48 h before reading. For plates which were incubated anaerobically, maintain the anaerobic atmosphere.

9.5.2 Acid phosphatase test

9.5.2.1 It is known that, beside *C. perfringens*, some other *Clostridium* strains (e.g. some strains of *C. baratii*) can produce acid phosphatase, but this ability is very limited. Therefore, only a very low percentage of false positives is expected.

9.5.2.2 Colonies grown anaerobically on blood or nutrient agar plates are spread on filter paper and 2 to 3 drops of the acid phosphatase reagent (B.6) are placed onto the colonies. If a commercially available test kit is used, follow the manufacturer's instructions.

NOTE It is possible to drip acid phosphatase reagent on colonies, if no further investigation of the colonies is needed.

9.5.2.3 A purplish colour developed within 3 min to 4 min is considered as a positive reaction.

9.5.3 Sulfite indole motility (SIM) agar test

Colonies grown anaerobically on blood or nutrient agar plates are stabbed into SIM agar tubes (B.7). The tubes are incubated for 22 h ± 2 h at 37 °C (6.5), in an anaerobic atmosphere (6.1) with the caps of the SIM agar tubes loosened. After incubation the tubes are read for:

— Sulfite production: tubes showing blackening are positive

— Motility: tubes showing growth outside the inoculation stab are positive

— Indole production: tubes giving a red coloured ring directly after adding Kovacs reagent (B.8) are positive

C. perfringens is positive for sulfite production and negative for indole production and motility.

9.5.4 Differentiation between human pathogenic and non-pathogenic *C. perfringens* strains (optional)

Additionally, the method described in Annex G can be used for molecular differentiation between human pathogenic and non-pathogenic *C. perfringens* strains.

9.5.5 Interpretation

C. perfringens produces black or grey to yellow-brown staining on TSC agar, even if the colour is faint, and possesses acid phosphatase, or are positive for sulfite production, negative for indole production and mobility on SIM agar.

10 Expression of results

In accordance with the interpretation of the results, indicate *C. perfringens* detected or not detected in a test portion of *x* g or *x* ml of product, or on the surface area swabbed or per sampling device.

11 Indicative performance characteristics of the method

11.1 Validation based on principles of ISO 17468

Using the standardized reference method, an interlaboratory study with a small number of participating laboratories (<10) was conducted based on the principles of ISO 17468.

The indicative performance characteristics of the method as derived from the interlaboratory study are described in 11.2.

NOTE In this document, the words “category”, “item”, “matrix” and ”type” are combined with “food” to improve the clarity of this document. However, the word ”food” is interchangeable with “feed” and the other areas of the food chain as mentioned in Clause 1 of this document.

11.2 Indicative performance characteristics

The indicative performance characteristics of the method (specificity, sensitivity, LOD_{50}) were determined in interlaboratory studies. All data are given in Annex C to F. It is possible that the values derived from the interlaboratory studies are not applicable to (food) categories other than those used in the study.

A summary of the indicative LOD_{50} values is given in Tables 1 till 4.

Table 1 — Summary of the indicative LOD_{50} values from the interlaboratory study for TSC isolation agar and acid phosphatase as confirmation method

(Food) category	(Food) item	LOD_{50} in cfu/test portion	Test portion size	Strain used in the interlaboratory study
Ready-to-eat, ready-to-reheat meat products	Canned meat	2,9 (1,7 – 5,1)	1 gram	*Clostridium perfringens* CECT[a] 4110
Eggs and egg products	Egg powder	0,9 (0,4 – 2,3)	1 gram	*Clostridium perfringens* NCTC 13170 (WDCM 00201)
Ready-to-eat, ready-to-reheat fishery products	Canned fish	3,2 (1,3 – 7,6)	1 gram	*Clostridium perfringens* CECT 4110
Processed fruits and vegetables	Canned pineapple	1,0 (0,6 – 1,5)	1 gram	*Clostridium perfringens* NCTC 13170 (WDCM 00201)
Infant formula and infant cereals	Infant formula with probiotics	4,9 (3,1 – 7,6)	1 gram	*Clostridium perfringens* CECT 4110
Multi-component foods or meal components	Instant soup	0,5 (0,2 – 1,4)	1 gram	*Clostridium perfringens* NCTC 13170 (WDCM 00201)
Environmental samples (food or feed production)	Swabs	1,7 (0,7 – 4,4)	Cloth	*Clostridium perfringens* CECT 4110

[a] Colección Española de Cultivos Tipo (CECT)

Table 2 — Summary of the indicative LOD_{50} values from the interlaboratory study for TSC isolation agar and SIM agar test as confirmation method

(Food) category	(Food) item	LOD_{50} in cfu/test portion	Test portion size	Strain used in the interlaboratory study
Ready-to-eat, ready-to-reheat meat products	Canned meat	2,9 (1,7 – 5,1)	1 gram	*Clostridium perfringens* CECT 4110
Eggs and egg products	Egg powder	2,0 (0,9 – 5,1)	1 gram	*Clostridium perfringens* NCTC 13170 (WDCM 00201)
Ready-to-eat, ready-to-reheat fishery products	Canned fish	3,0 (1,2 – 7,2)	1 gram	*Clostridium perfringens* CECT 4110
Processed fruits and vegetables	Canned pineapple	1,1 (0,8 – 1,7)	1 gram	*Clostridium perfringens* NCTC 13170 (WDCM 00201)
Infant formula and infant cereals	Infant formula with probiotics	2,9 (1,9 – 4,5)	1 gram	*Clostridium perfringens* CECT 4110
Multi-component foods or meal components	Instant soup	0,5 (0,2 – 1,4)	1 gram	*Clostridium perfringens* NCTC 13170 (WDCM 00201)
Environmental samples (food or feed production)	Swabs	2,5 (1,4 – 4,5)	Cloth	*Clostridium perfringens* CECT 4110

Table 3 — Summary of the indicative LOD_{50} values from the interlaboratory study for LENA isolation agar and acid phosphatase as confirmation method

(Food) category	(Food) item	LOD_{50} in cfu/test portion	Test portion size	Strain used in the interlaboratory study
Ready-to-eat, ready-to-reheat meat products	Canned meat	3,0 (1,9 – 4,7)	1 gram	*Clostridium perfringens* CECT 4110
Eggs and egg products	Egg powder	0,7 (0,3 – 1,9)	1 gram	*Clostridium perfringens* NCTC 13170 (WDCM 00201)
Ready-to-eat, ready-to-reheat fishery products	Canned fish	3,5 (2,0 – 6,1)	1 gram	*Clostridium perfringens* CECT 4110
Processed fruits and vegetables	Canned pineapple	1,0 (0,6 – 1,6)	1 gram	*Clostridium perfringens* NCTC 13170 (WDCM 00201)
Infant formula and infant cereals	Infant formula with probiotics	3,6 (2,5 – 5,2)	1 gram	*Clostridium perfringens* CECT 4110
Multi-component foods or meal components	Instant soup	0,8 (0,3 – 2,0)	1 gram	*Clostridium perfringens* NCTC 13170 (WDCM 00201)
Environmental samples (food or feed production)	Swabs	2,1 (1,2 – 3,8)	Cloth	*Clostridium perfringens* CECT 4110

Table 4 — Summary of the indicative LOD_{50} values from the interlaboratory study for LENA isolation agar and SIM agar test as confirmation method

(Food) category	(Food) item	LOD_{50} in cfu/test portion	Test portion size	Strain used in the interlaboratory study
Ready-to-eat, ready-to-reheat meat products	Canned meat	3,0 (1,9 – 4,7)	1 gram	*Clostridium perfringens* CECT 4110
Eggs and egg products	Egg powder	1,9 (0,8 – 4,5)	1 gram	*Clostridium perfringens* NCTC 13170 (WDCM 00201)
Ready-to-eat, ready-to-reheat fishery products	Canned fish	5,3 (3,0 – 9,3)	1 gram	*Clostridium perfringens* CECT 4110
Processed fruits and vegetables	Canned pineapple	1,3 (0,9 – 1,9)	1 gram	*Clostridium perfringens* NCTC 13170 (WDCM 00201)
Infant formula and infant cereals	Infant formula with probiotics	3,5 (2,5 – 4,8)	1 gram	*Clostridium perfringens* CECT 4110
Multi-component foods or meal components	Instant soup	1,3 (0,8 – 2,0)	1 gram	*Clostridium perfringens* NCTC 13170 (WDCM 00201)
Environmental samples (food or feed production)	Swabs	2,0 (1,1 – 3,7)	Cloth	*Clostridium perfringens* CECT 4110

12 Test report

The test report shall specify at least the following:

— the test method used, with reference to this document, i.e. ISO/TS 15213-3:2024;

— the sampling method used, if known;

— the size of the test portion and/or the nature of the subject examined;

— 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.

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 diagram of the procedure for the detection of *C. perfringens* in food, animal feed, environmental and primary production stage samples.

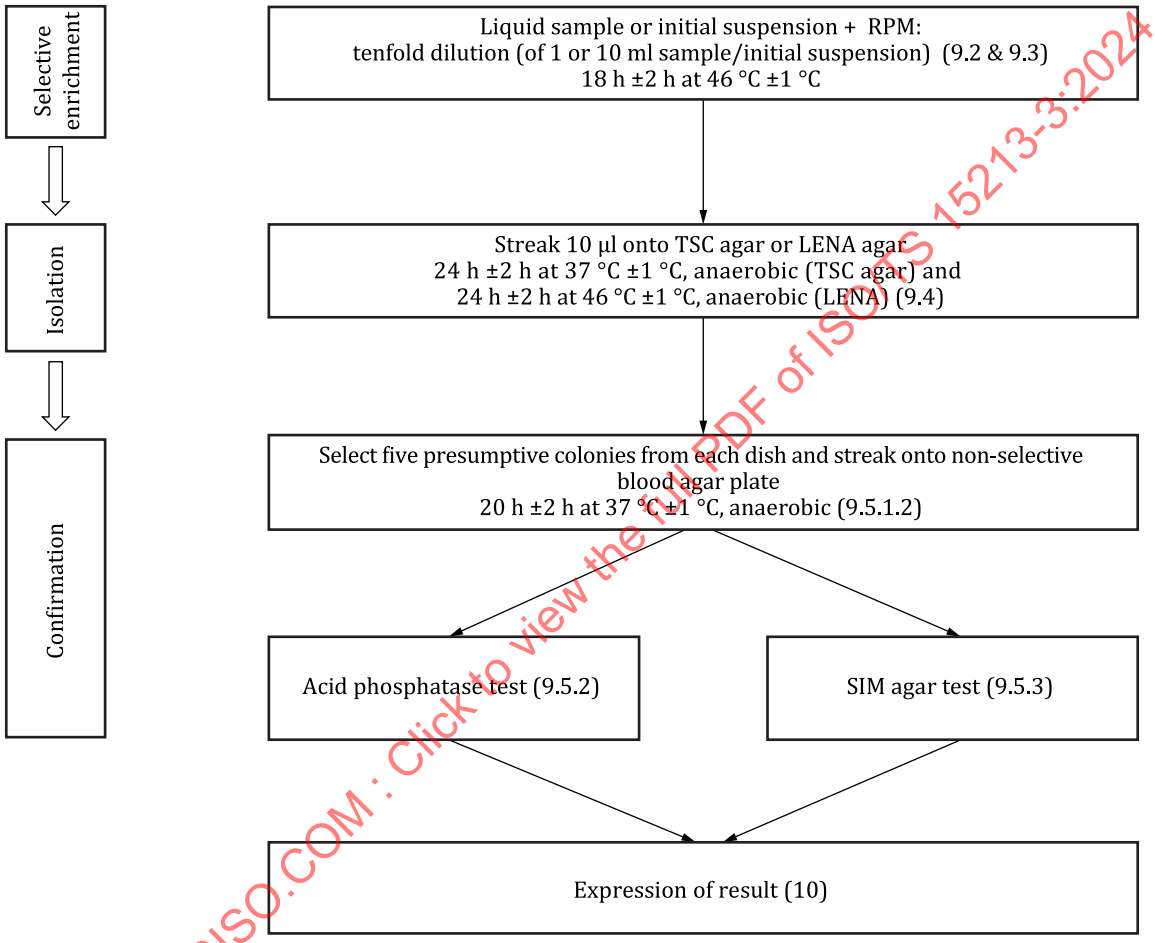

Figure A.1 — Flow diagram of the procedure for the detection of *C. perfringens*

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 these under their own storage conditions (in accordance with ISO 11133).

Performance testing of culture media is described in B.9 and Table B.1.

B.2 Rapid perfringens medium (RPM)

B.2.1 Rapid perfringens base medium

B.2.1.1 Composition

Yeast extract		11,0 g
Enzymatic digest of casein		15,0 g
Peptone [a]		10,0 g
Glucose ($C_6H_{12}O_6$)	(CAS[c] No. 50-99-7)	10,5 g
Sodium thioglycollate ($C_2H_3NaO_2S$)	(CAS No. 367-51-1)	0,5 g
Sodium chloride (NaCl)	(CAS No. 7647-14-5)	5,5 g
L-cystine ($C_6H_{12}N_2O_4S_2$)	(CAS No. 56-89-3)	0,5 g
Resazurin sodium salt ($C_{12}H_6NNaO_4$)	(CAS No. 62758-13-8)	0,001 g
Dipotassium hydrogenphosphate (HK_2O_4P)	(CAS No. 7758-11-4)	10,0 g
Iron(II) sulfate heptahydrate ($H_{14}FeO_{11}S$)	(CAS No. 7782-63-0)	1,0 g
Gelatin ($AlH_3KO_8S_2$)	(CAS No. 9000-70-8)	120,0 g
Agar [b]		1,0 to 1,5 g
Water		1 000 ml

[a] For example enzymatic digest of animal tissue.

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

[c] CAS Registry Number ® is a trademark of CAS corporation. 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.2.1.2 Preparation

Dissolve the ingredients in the water, by heating if necessary.

Adjust the pH (6.6), if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Sterilise for 5 min in the autoclave set at 121 °C (6.2).

Store the medium, at 5 °C (6.7) for up to 4 weeks in closed containers or tubes (6.8). Prior to use, the stored medium is melted completely and cooled down to 44 °C to 47 °C (6.12) before use.

B.2.2 Neomycin-polymyxin solution

B.2.2.1 Composition

Neomycin sulfate ($C_{23}H_{52}N_6O_{25}S_3$)	(CAS No. 1405-10-3)	0,75 g
Polymyxin-B sulfate ($C_{55}H_{96}N_{16}O_{13}{\cdot}H_2SO_4$) (7 500 IU/mg)	(CAS No. 1405-20-5)	0,125 g
Water		100 ml

B.2.2.2 Preparation

Dissolve the ingredients and filter sterilise (0,2 µm).

Store the solution, at 5 °C (6.7) for up to 4 weeks in closed containers or tubes (6.8).

B.2.3 Milk powder solution

B.2.3.1 Composition

Instant whole milk powder	100 g
Water	1 000 ml

B.2.3.2 Preparation

Dissolve the ingredients in the water, by heating if necessary.

Sterilise for 5 min in the autoclave set at 121 °C (6.2).

Store the solution, at 5 °C (6.7) for up to 4 weeks in closed containers or tubes (6.8). Prior to use, the stored medium is pre-warmed to 44 °C to 47 °C (6.12).

B.2.4 Complete rapid perfringens medium (RPM)

B.2.4.1 Composition

Rapid perfringens medium base [melted and cooled-down at 47 °C to 50 °C (B.2.1)]	300 ml
Neomycin-polymyxin solution (B.2.2)	6 ml
Milk powder solution [pre-warmed at 44 °C to 47 °C (B.2.3)]	300 ml

B.2.4.2 Preparation

Add to 300 ml pre-warmed rapid perfringens medium base, 6 ml neomycin-polymyxin solution and 300 ml pre-warmed Lithmus milk. Mix and fill 10 ml into sterile tubes (6.8) or 90 ml into sterile bottles (6.8).

B.3 Tryptose sulfite cycloserine agar (TSC agar)

NOTE See reference [12].

B.3.1 Base medium

B.3.1.1 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 No. 7681-57-4)	1,0 g
Iron(III) ammonium citrate [b] ($C_6H_8FeNO_4$)	(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.

B.3.1.2 Preparation

Suspend the ingredients in the water and dissolve by heating and stirring.

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

Allow to cool to 44 °C to 47 °C (6.12).

The base medium may be stored at 5 °C (6.7) for up to 4 weeks in closed containers or tubes. Prior to the preparation of the complete medium, the stored medium is melted completely and cooled down to 44 °C to 47 °C (6.12) before adding the cycloserine solution (see B.3.2).

B.3.2 D-cycloserine solution

B.3.2.1 Composition

D-cycloserine ($C_3H_6N_2O_2$)	(CAS No. 68-41-7)	4,0 g
Water		100 ml

B.3.2.2 Preparation

Dissolve the D-cycloserine in the water and filter through a membrane of 0,2 µm pore size.

Dispense aseptically into suitable volumes, store at -20 °C (6.4) and use within 4 weeks of preparation.

Alternatively, the dispended volumes of cycloserine can be stored at -70 °C (6.4) for a maximum of 12 months.

B.3.3 Complete medium

B.3.3.1 Composition

Base medium (B.3.1)	100 ml
D-cycloserine solution (B.3.2)	1 ml

B.3.3.2 Preparation

Add to each 100 ml of sterile molten base (B.3.1) cooled to 44 °C to 47 °C (6.12), 1 ml of D-cycloserine solution (B.3.2) to obtain a final cycloserine concentration of 0,4 g per litre TSC agar.

The final pH of the medium should correspond to 7,6 ± 0,2 at 25 °C (6.6). Mix well and dispense into Petri dishes (6.11).

Store the TSC agar plates, at 5 °C (6.7) for up to 4 weeks.

B.4 Lactose egg-yolk neomycin agar (LENA)

B.4.1 Lactose agar base

B.4.1.1 Composition

Peptone [a]		10,0 g
Meat extract		10,0 g
Sodium chloride (NaCl)	(CAS No. 7647-14-5)	5,0 g
Lactose ($C_{12}H_{24}O_{12}$)	(CAS No. 10039-26-6)	10,0 g
Phenol red ($C_{19}H_{14}O_5S$)	(CAS No. 143-74-8)	0,1 g
Agar [b]		9,0 to 18,0 g
Water		1 000 ml

[a] For example enzymatic digest of casein.

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

B.4.1.2 Preparation

Suspend the ingredients in the water, and dissolve by heating and stirring.

Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 (6.6) at 25 °C.

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

Store the medium, at 5 °C (6.7) for up to 4 weeks in closed containers or tubes (6.8). Prior to use, the stored medium is melted completely and cooled down to 44 °C to 47 °C (6.12) before use.

B.4.2 Neomycin solution

B.4.2.1 Composition

Neomycin sulfate ($C_{23}H_{52}N_6O_{25}S_3$)	(CAS No. 1405-10-3)	250 mg
Water		10 ml

B.4.2.2 Preparation

Dissolve the ingredients and filter sterilise (0,2 µm).

Store the solution, at 5 °C (6.7) for up to 4 weeks in closed containers or tubes (6.8).

B.4.3 Egg-yolk emulsion (concentration approximately 20 % (e.g. 200 ml/l) or according to the manufacturer's instructions)

B.4.3.1 General

If a commercial preparation is available, it may be used.

B.4.3.2 Preparation

Use fresh hen eggs with intact shells. Clean the eggs with a brush using a liquid detergent. Rinse them under running water, then disinfect the shells either by immersing them in ethanol (70 % volume fraction, e.g. 700 ml/l) for 30 s and allowing them to dry in the air, or by spraying them with alcohol followed by flame sterilization.

Proceeding using aseptic conditions, break each egg and separate the yolk from its white by repeated transfer of the yolk from one half of the shell to the other. Place the yolks in a sterile flask (6.8) and add four times their volume of sterile water. Mix thoroughly. Heat the mixture in the water bath (6.12) set at 44 °C to 47 °C for 2 h and leave for 18 h to 24 h at 5 °C (6.7) to allow a precipitate to form. Aseptically collect the supernatant liquid into a fresh sterile flask for use.

The emulsion may be stored at 5 °C (6.7) for up to 72 h.

B.4.4 Complete Lactose egg-yolk neomycin agar

B.4.4.1 Composition

Lactose agar base (melted and cooled down at 44 °C to 47 °C) (B.4.1)	100 ml
Neomycin solution (B.4.2)	1,0 ml
Egg-yolk emulsion (B.4.3)	5,0 ml

B.4.4.2 Preparation

Add the neomycin solution (B.4.2) and the egg-yolk emulsion to the molten and cooled down to 44 °C to 47 °C (6.12) Lactose agar base medium (B.4.1). Mix well and dispense into Petri dishes (6.11).

Store the LENA medium plates, at 5 °C (6.7) for up to 4 weeks.

B.5 Columbia blood agar (CBA)

B.5.1 Columbia blood agar base

B.5.1.1 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.5.1.2 Preparation

Dissolve the ingredients 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 (6.6).

Dispense the medium in flasks (6.8) of suitable capacity to obtain portions appropriate for the test.

Sterilise for 15 min in the autoclave (6.2) set at 121 °C. Store the medium, at 5 °C (6.7) for up to four weeks in closed containers or tubes (6.8)

B.5.2 Defibrinated blood (horse or sheep blood)

B.5.3 Complete base

B.5.3.1 Composition

Base (B.5.1)	100 ml
Defibrinated blood (B.5.2)	5 ml

B.5.3.2 Preparation

Add the blood (B.5.2) to the base (B.5.1) previously cooled to 44 °C to 47 °C (6.12). Mix well.

B.5.3.3 Preparation of blood agar plates

Dispense the medium (B.5.3.1) into sterile Petri dishes (6.11) in portions appropriate for the test. Allow to solidify.

Immediately before use, dry the agar plates following the procedures as given in ISO 11133. Store the poured plates, protected for drying, at 5 °C (6.7) for up to four weeks.

B.6 Acid phosphatase reagent

NOTE Acid phosphatase reagent composition and preparation is the same as in ISO 14189, but different from the original description in the literature.

B.6.1 Composition

NOTE Acid phosphatase reagent composition and preparation is the same as in ISO 14189, but different from the original description in the literature.

1-naphthylphosphate disodium salt [a] ($C_{10}H_7Na_2O_4P$)	(CAS No. 2183-17-7)	0,4 g
Fast Blue B Salt (o-Dianisidine bis(diazotized) zinc double salt) ($C_{14}H_{12}Cl_4N_4O_2Zn$)	(CAS No. 14263-94-6)	0,8 g
Acetate buffer (pH 4,6 ± 0,2)		20 ml

[a] Instead of 1-naphthylphosphate disodium salt, 1-naphthylphosphate monosodium salt (CAS No. 81012-89-7) can be used.

B.6.2 Preparation

WARNING — Fast Blue B Salt is toxic and can cause cancer. Appropriate precautions shall be taken when weighing out, preparing and discarding the reagent.

Prepare the acetate buffer by dissolving 0,3 ml glacial acetic acid (CAS No. 64-19-7) and 0,4 g sodium acetate (CAS No. 127-09-3) in deionised water and make up to 1 000 ml. Alternatively, use a commercially available product.

Dissolve the ingredients in the acetate buffer and allow to stand for 60 min ± 5 min at 5 °C ± 3 °C (6.7) to allow precipitation. Then filter the solution through a fluted filter to remove the precipitate. Store the prepared solution at 5 °C (6.7) for no longer than two weeks. If precipitation occurs again filter again once more before use.

B.7 Sulfite indole motility agar (SIM agar)

B.7.1 Composition

Peptone [a]		6,0 g
Enzymatic digest of soya		20 g
Ferrous ammonium sulfate (anhydrous) ($FeH_8N_2O_8S_2$)	(CAS No. 10045-89-3)	0,2 g
Sodium thiosulfate ($Na_2O_3S_2$)	(CAS No. 7772-98-7)	0,2 g
Agar		2,5 to 4,5 g
Water		1 000 ml

[a] For example enzymatic digest of casein.

B.7.2 Preparation

Dissolve the ingredients in the water, by heating if necessary. Fill 10 ml into tubes (6.8) and sterilise for 15 min in the autoclave set at 121 °C (6.2).

Allow to cool in upright position.

B.8 Kovacs reagent

B.8.1 Composition

4-(methylamino)benzaldehyde (C_8H_9NO)	(CAS No. 556-21-8)	5,0 g
Hydrochloric acid, ρ – 1,18 g/ml to 1,19 g/ml		25 ml
2-Methyl-2-butanol ($C_5H_{12}O$)	(CAS No. 75-85-4)	75 ml

B.8.2 Preparation

Mix components and store reagent in closed flask in the dark at 5 °C (6.7) for up to 6 months.

B.9 Performance testing

Follow the procedures for performance testing described in ISO 11133. Selectivity and productivity are defined in ISO 11133.

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

Medium/ reagent	Function	Incubation	Control strains	WDCM numbers [a]	Reference medium	Method of control	Criteria [c]	Characteristic reactions
RPM	Productivity	(18 ± 2) h / (46 ± 1) °C	*Clostridium perfringens*	00007 [b] 00080	-	Qualitative	> 10 colonies on TSC agar or on LENA	Characteristic colonies according to each medium
TSC agar [e]	Productivity	(24 ± 2) h / (37 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	0n 0007 [b] 00080 00174	A suitable non-selective medium for anaerobes	Qualitative	Good growth (2)	Black colonies
	Selectivity		*Bacillus subtilis* subsp. *spizizenii*	00003	-	Qualitative	Total inhibition (0)	-
LENA	Productivity	(24 ± 2) h / (46 ± 1) °C anaerobic conditions	*Clostridium perfringens*	00007 [b] 00080	-	Qualitative	Good growth (2)	Yellow colonies precipitation
	Selectivity		*Escherichia coli* [d]	00012 or 00013	-	Qualitative	Total inhibition (0)	-
	Specificity		*Bacillus subtilis subsp. spizizenii*	00003	-	Qualitative	-	Yellow colonies without precipitation halo
CBA	Productivity	(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] Growth is categorised as 0: no growth; 1: weak growth (partial inhibition); 2: good growth (see ISO 11133).

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

[e] In case of both quantitative and qualitative use for the medium, only results of the quantitative tests are required.

Table B.1 *(continued)*

Medium/ reagent	Function	Incubation	Control strains	WDCM numbers [a]	Reference medium	Method of control	Criteria [c]	Characteristic reactions
Acid phosphatase	Confirmation	In 3 to 4 min reaction time	*Clostridium perfringens* [d]	00007 [b] 00080 00174	-	Qualitative		Positive reaction: Purplish colour
		In 3 to 4 min reaction time	*Bacillus subtilis* subsp. *spizizenii* [d]	00003	-	Qualitative		Negative reaction: No colour change
SIM-agar in combination with Kovacs reagent	Confirmation	(22 ± 2) h / (37 ± 1) °C anaerobic atmosphere	*Clostridium perfringens* [d]	00007 [b] 00080 00174		Qualitative		Positive reaction: Good growth (2), blackening of the tube, no growth outside the inoculation stab and no red coloured ring after adding Kovacs reagent
			Escherichia coli [d]	00012 or 00013 [b]		Qualitative		Negative reaction: Good growth (2), no blackening of the tube, possible growth outside the inoculation stab and red coloured ring after adding Kovacs reagent

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

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

[e] In case of both quantitative and qualitative use for the medium, only results of the quantitative tests are required.

Annex C
(informative)

Indicative performance characteristics of the method using TSC isolation agar and acid phosphatase confirmation test

An interlaboratory study with a limited number of participating laboratories (9 laboratories in 7 countries) was carried out. The following (food) items, were included in the study: ready-to-eat, ready-to-re-heat meat products [item: canned meat], eggs and egg products (derivates) [item: egg powder], ready-to-eat, ready-to-re-heat fishery products [item: canned fish], processed fruits and vegetables [item: canned pineapple], infant formula and infant cereals (with probiotics) [item: probiotic formula], multi-component foods or meal components [item: instant soup], and environmental samples (food or feed production) [item: cloths]. The (food) items were each tested at three different levels of contamination, plus an uninoculated sample (negative control). The study was organized in 2022 by FrieslandCampina, Leeuwarden, The Netherlands and Merck KGaA, Darmstadt, Germany as part of ISO/TC 34/SC 9/WG 23 "sulfite-reducing *Clostridium* spp. and *Clostridium perfringens*".

The method submitted to the interlaboratory study was that of this document.

Data obtained by some collaborators have been excluded from the calculations only on basis of clearly identified technical reasons (e.g. positive results in the negative controls, no increase in number of positives at increasing inoculation level, all negative results after confirmation).

The values of the indicative 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 based on the principles of ISO 17468. The study was performed in 4 different levels (L0, L1, L2, L3) in 6-fold due to the fact that laboratories had to inoculate samples using the provided reference materials and therefore guarantee that different levels are obtained in this study.

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

Parameter	**Blank**	**Low contamination level** *1,7 cfu/test portion*	**Medium contamination level** *5,1 cfu/test portion*	**High contamination level** *15,3 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	4	4	4	4
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %	-	50,0	70,8	91,7
LOD_{50} (95 % confidence interval), in cfu/test portion	2,9 (1,7 – 5,1)			
NOTE Strain used for inoculation: *Clostridium perfringens* CECT 4110.				

Table C.2 — Results of data analysis obtained with egg powder (category: eggs and egg products)

Parameter	Blank	Low contamination level *0,8 cfu/test portion*	Medium contamination level *2,5 cfu/test portion*	High contamination level *7,4 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	3	3	3	3
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %	-	55,6	77,8	100
LOD_{50} (95 % confidence interval), in cfu/test portion	0,9 (0,4 – 2,3)			
NOTE Strain used for inoculation: *Clostridium perfringens* NCTC 13170 (WDCM 00201).				

Table C.3 — Results of data analysis obtained with canned fish (category: ready-to-eat, ready-to-reheat fishery products)

Parameter	Blank	Low contamination level *1,7 cfu/test portion*	Medium contamination level *5,1 cfu/test portion*	High contamination level *15,3 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	3	3	3	3
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %	-	38,9	66,7	94,4
LOD_{50} (95 % confidence interval), in cfu/test portion	3,2 (1,3 – 7,6)			
NOTE Strain used for inoculation: *Clostridium perfringens* CECT 4110.				

Table C.4 — Results of data analysis obtained with canned pineapple (category: processed fruits and vegetables)

Parameter	Blank	Low contamination level *0,7 cfu/test portion*	Medium contamination level *2,2 cfu/test portion*	High contamination level *6,6 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	5	5	5	5
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %	-	46,7	80,0	100
LOD_{50} (95 % confidence interval), in cfu/test portion	0,8 (0,5 – 1,3)			
NOTE Strain used for inoculation: *Clostridium perfringens* NCTC 13170.				

Table C.5 — Results of data analysis obtained with probiotic formula (category: infant formula and infant cereals)

Parameter	Blank	Low contamination level *1,7 cfu/test portion*	Medium contamination level *5,1 cfu/test portion*	High contamination level *15,3 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	5	5	5	5
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %	-	33,3	63,3	76,7
LOD_{50} (95 % confidence interval), in cfu/test portion	4,9 (3,1 – 7,6)			
NOTE Strain used for inoculation: *Clostridium perfringens* CECT 4110.				

Table C.6 — Results of data analysis obtained with instant soup (category: multi-component foods or meal components)

Parameter	Blank	Low contamination level *0,7 cfu/test portion*	Medium contamination level *2,2 cfu/test portion*	High contamination level *6,6 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	3	3	3	3
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %	-	72,2	94,4	100
LOD_{50} (95 % confidence interval), in cfu/test portion	0,4 (0,2 – 1,2)			
NOTE Strain used for inoculation: *Clostridium perfringens* NCTC 13170 (WDCM 00201).				

Table C.7 — Results of data analysis obtained with cloth samples (category: environmental samples (food or feed production))

Parameter	Blank	Low contamination level *1,7 cfu/test portion*	Medium contamination level *5,1 cfu/test portion*	High contamination level *15,3 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	3	3	3	3
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %	-	44,4	88,9	100
LOD_{50} (95 % confidence interval), in cfu/test portion	1,7 (0,7 – 4,4)			
NOTE Strain used for inoculation: *Clostridium perfringens* CECT 4110.				

Annex D
(informative)

Indicative performance characteristics of the method using TSC isolation agar and SIM agar test

An interlaboratory study with a limited number of participating laboratories (9 laboratories in 7 countries) was carried out. The following (food) items, were included in the study: ready-to-eat, ready-to-re-heat meat products [item: canned meat], eggs and egg products (derivates) [item: egg powder], ready-to-eat, ready-to-re-heat fishery products [item: canned fish], processed fruits and vegetables [item: canned pineapple], infant formula and infant cereals (with probiotics) [item: probiotic formula], multi-component foods or meal components [item: instant soup], and environmental samples (food or feed production) [item: cloths]. The (food) items were each tested at three different levels of contamination, plus an uninoculated sample (negative control). The study was organized in 2022 by FrieslandCampina, Leeuwarden, The Netherlands and Merck KGaA, Darmstadt, Germany as part of ISO/TC 34/SC 9/WG 23 "sulfite-reducing *Clostridium* spp. and *Clostridium perfringens*".

The method submitted to the interlaboratory study was that of this document.

Data obtained by some collaborators have been excluded from the calculations only on basis of clearly identified technical reasons (e.g. positive results in the negative controls, no increase in number of positives at increasing inoculation level, all negative results after confirmation).

The values of the indicative performance characteristics, for each (food) item and category, derived from this interlaboratory study are shown in Tables D.1 to D.7, and were calculated based on the principles of ISO 17468. The study was performed in 4 different levels (L0, L1, L2, L3) in 6-fold due to the fact that laboratories had to inoculate samples using the provided reference materials and therefore guarantee that different levels are obtained in this study.

Table D.1 — Results of data analysis obtained with canned meat (category: ready-to-eat, ready-to-re-heat meat products)

Parameter	**Blank**	**Low contamination level** *1,7 cfu/test portion*	**Medium contamination level** *5,1 cfu/test portion*	**High contamination level** *15,3 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	4	4	4	4
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %	-	50,0	70,8	91,7
LOD_{50} (95 % confidence interval), in cfu/test portion	2,9 (1,7 – 5,1)			
NOTE Strain used for inoculation: *Clostridium perfringens* CECT 4110.				

Table D.2 — Results of data analysis obtained with egg powder (category: eggs and egg products)

Parameter	**Blank**	**Low contamination level** *0,8 cfu/test portion*	**Medium contamination level** *2,5 cfu/test portion*	**High contamination level** *7,3 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	3	3	3	3
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %	-	38,9	66,7	77,8
LOD_{50} (95 % confidence interval), in cfu/test portion	2,0 (0,9 – 5,1)			
NOTE Strain used for inoculation: *Clostridium perfringens* NCTC 13170 (WDCM 00201).				

Table D.3 — Results of data analysis obtained with canned fish (category: ready-to-eat, ready-to-reheat fishery products)

Parameter	**Blank**	**Low contamination level** *1,7 cfu/test portion*	**Medium contamination level** *5,1 cfu/test portion*	**High contamination level** *15,3 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	3	3	3	3
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %		44,4	66,7	94,4
LOD_{50} (95 % confidence interval), in cfu/test portion	3,0 (1,2 – 7,2)			
NOTE Strain used for inoculation: *Clostridium perfringens* CECT 4110.				

Table D.4 — Results of data analysis obtained with canned pineapple (category: processed fruits and vegetables)

Parameter	**Blank**	**Low contamination level** *0,8 cfu/test portion*	**Medium contamination level** *2,5 cfu/test portion*	**High contamination level** *7,3 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	6	6	6	6
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %	-	46,7	80,0	100
LOD_{50} (95 % confidence interval), in cfu/test portion	1,0 (0,6 – 1,5)			
NOTE Strain used for inoculation: *Clostridium perfringens* NCTC 13170 (WDCM 00201).				

Table D.5 — Results of data analysis obtained with probiotic formula (category: infant formula and infant cereals)

Parameter	Blank	Low contamination level *1,7 cfu/test portion*	Medium contamination level *5,1 cfu/test portion*	High contamination level *15,3 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	5	5	5	5
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %		46,7	76,7	90,0
LOD_{50} (95 % confidence interval), in cfu/test portion	2,9 (1,9 – 4,5)			
NOTE Strain used for inoculation: *Clostridium perfringens* CECT 4110.				

Table D.6 — Results of data analysis obtained with instant soup (category: multi-component foods or meal components)

Parameter	Blank	Low contamination level *0,7 cfu/test portion*	Medium contamination level *2,2 cfu/test portion*	High contamination level *6,6 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	3	3	3	3
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %		72,2	94,4	100
LOD_{50} (95 % confidence interval), in cfu/test portion	0,4 (0,2 – 1,2)			
NOTE Strain used for inoculation: *Clostridium perfringens* NCTC 13170 (WDCM 00201).				

Table D.7 — Results of data analysis obtained with cloth samples (category: environmental samples (food or feed production))

Parameter	Blank	Low contamination level *1,7 cfu/test portion*	Medium contamination level *5,1 cfu/test portion*	High contamination level *15,3 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	4	4	4	4
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %		33,3	75,0	100
LOD_{50} (95 % confidence interval), in cfu/test portion	2,5 (1,4 – 4,5)			
NOTE Strain used for inoculation: *Clostridium perfringens* CECT 4110.				

Annex E
(informative)

Indicative performance characteristics of the method using LENA isolation agar and acid phosphatase confirmation test

An interlaboratory study with a limited number of participating laboratories (9 laboratories in 7 countries) was carried out. The following (food) items, were included in the study: ready-to-eat, ready-to-re-heat meat products [item: canned meat], eggs and egg products (derivates) [item: egg powder], ready-to-eat, ready-to-re-heat fishery products [item: canned fish], processed fruits and vegetables [item: canned pineapple], infant formula and infant cereals (with probiotics) [item: probiotic formula], multi-component foods or meal components [item: instant soup], and environmental samples (food or feed production) [item: cloths]. The (food) items were each tested at three different levels of contamination, plus an uninoculated sample (negative control). The study was organized in 2022 by FrieslandCampina, Leeuwarden, The Netherlands and Merck KGaA, Darmstadt, Germany as part of ISO/TC 34/SC 9/WG 23 "sulfite-reducing *Clostridium* spp. and *Clostridium perfringens*".

The method submitted to the interlaboratory study was that of this document.

Data obtained by some collaborators have been excluded from the calculations only on basis of clearly identified technical reasons (e.g. positive results in the negative controls, no increase in number of positives at increasing inoculation level, all negative results after confirmation).

The values of the indicative performance characteristics, for each (food) item and category, derived from this interlaboratory study are shown in Tables E.1 to E.7, and were calculated based on the principles of ISO 17468. The study was performed in 4 different levels (L0, L1, L2, L3) in 6-fold due to the fact that laboratories had to inoculate samples using the provided reference materials and therefore guarantee that different levels are obtained in this study.

Table E.1 — Results of data analysis obtained with canned meat (category: ready-to-eat, ready-to-re-heat meat products)

Parameter	**Blank**	**Low contamination level** *1,7 cfu/test portion*	**Medium contamination level** *5,1 cfu/test portion*	**High contamination level** *15,3 cfu/test portion*
Number of participating collaborators	9	9	9	9
Number of samples per collaborator	6	6	6	6
Number of collaborators retained after evaluation of the data	5	5	5	5
Test portion size, in gram	1	1	1	1
Specificity, in %	100	-	-	-
Sensitivity, in %	-	42,4	66,7	93,3
LOD_{50} (95 % confidence interval), in cfu/test portion	3,0 (1,9 – 4,7)			
NOTE Strain used for inoculation: *Clostridium perfringens* CECT 4110.				