INTERNATIONAL STANDARD

ISO 15213-2

First edition
2023-11

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

Part 2: Enumeration of *Clostridium perfringens* by colony-count technique

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

Partie 2: Dénombrement de Clostridium perfringens *par la technique de comptage des colonies*

Reference number
ISO 15213-2:2023(E)

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Published in Switzerland

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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 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).

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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).

This first edition of ISO 15213-2 cancels and replaces ISO 7937:2004, which has been technically revised.

The main changes are as follows:

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

— 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 *Clostridium (C.) perfringens* present in the sample;

— the selective medium has been re-named from sulfite-cycloserine agar (SC) to tryptose sulfite cycloserine agar (TSC agar) without changes in the formulation;

— the confirmation methods described have been modified according to ISO 14189;

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

— in Annex B, criteria for the performance testing of culture media have been added;

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

— in Annex D (informative), two molecular methods have been added for differentiation between pathogenic and non-pathogenic *C. perfringens* and one molecular method for the differentiation of *C. perfringens* type A strains carrying a chromosomally encoded *cpe* gene or a plasmid encoded *cpe* gene.

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 are 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 enumeration 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. 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 7937:2004, 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 2: Enumeration of *Clostridium perfringens* by colony-count technique

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for enumeration 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 enumeration of *Clostridium (C.) perfringens* by 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
presumptive *C. perfringens*
presumptive *Clostridium perfringens*
spore-forming bacteria forming countable typical colonies in 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 in the specified selective medium under obligate anaerobic conditions and possess the enzyme acid phosphatase

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

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

3.4
enumeration of *C. perfringens*
enumeration of *Clostridium perfringens*
determination of the number of colony-forming units (cfu) of *confirmed C. perfringens* (3.2) found per gram, per millilitre, per square centimetres 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. Additional plates are prepared under the same conditions using decimal dilutions of the test sample. After solidification of the agar 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 is performed before plating. Additionally, a method for molecular differentiation between human pathogenic and non-pathogenic *C. perfringens* strains is described in Annex D.

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 *C. perfringens* requires four successive stages as specified in the normative 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

Petri dishes are inoculated with a specified quantity of the test sample if the initial product is liquid, or a specified quantity of the initial suspension, in the case of other products. Additional Petri dishes are inoculated, under the same conditions, using decimal dilutions of the test sample or of the initial suspension. A selective medium is added (pour-plate technique) and then overlaid with the same medium.

The plates are incubated under anaerobic conditions at 37 °C for 20 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

Confirmatory tests are carried out. The result is calculated as the colony count of confirmed *C. perfringens* per sample volume. Additionally, the method described in Annex D can be used for molecular differentiation between human pathogenic and non-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 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 Freezer, capable of operating at −20 °C ± 2 °C and at −70 °C ± 3 °C.

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

6.5 pH-meter, having a maximum permissible error of calibration of ±0,1 pH unit at 25 °C.

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

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

6.8 Sterile graduated pipettes or **automatic pipettes**, of nominal capacities 10 ml, 1 ml and 0,1 ml.

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

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

6.11 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

The procedure as given in Annex A shall be followed.

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.

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 (6.11) 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 (6.7). 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 that the water bath is equipped 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 till 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, silage).

9.4 Inoculation and incubation

9.4.1 Take two sterile Petri dishes with a diameter of approximately 90 mm (6.10). Transfer to each dish, by means of a sterile pipette (6.8), 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 *C. perfringens*, 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 (6.10).

9.4.2 Take one other sterile Petri dish (6.10). Use another sterile pipette (6.8) 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 (6.8) 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 (6.10) that will give colony counts of between 10 and 150 colonies per plate (on 90 mm Petri dishes) or between 10 and 365 colonies per plate (on 140 mm Petri dishes).

9.4.5 Pour about 12 ml to 15 ml for 90 mm Petri dishes or 30 ml to 35 ml for 140 mm Petri dishes of the tryptose sulfite cycloserine agar (TSC agar) (see B.2), molten and tempered at 44 °C to 47 °C (6.11), into each Petri dish (6.10).

9.4.6 Carefully mix the inoculum with the medium by rotating the Petri dishes 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 TSC agar (see B.2) for 90 mm Petri dishes (6.10) or 12 ml for 140 mm Petri dishes (6.10) 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 (6.4) the plates at 37 °C in an anaerobic atmosphere (6.1).

9.5 Enumeration of typical colonies

9.5.1 After 20 h ± 2 h of incubation, examine the plates (see 9.4.8) for presumptive *C. perfringens*. Longer incubation can result in excess blackening of the plates.

Typical colonies, which show black or grey to yellow-brown staining (even if the colour is faint) on the TSC agar, 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, 6.4).

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 365 colonies (for 140 mm Petri dishes); count these colonies and record the number of presumptive colonies per dish. Then choose, at random, five such colonies from each dish for confirmation (see 9.6). For enumeration of plates with low or high numbers of presumptive colonies, see ISO 7218.

9.6 Confirmation of *C. perfringens*

9.6.1 Selection of colonies for confirmation

9.6.1.1 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.

9.6.1.2 Streak each of the selected colonies with a sterile loop (6.9) onto one non-selective blood agar plates, e.g. Columbia blood agar (see 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. Streaks should obtain well-isolated colonies.

Incubate the plates in an anaerobic atmosphere (6.1) at 37 °C (6.4) 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 or by the sulfite indole motility (SIM) agar test.

NOTE Alternative procedures (see ISO 7218) can be used to confirm the isolate(s) as *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 (6.6) for a maximum of 48 h before reading. For plates which were incubated anaerobically, maintain the anaerobic atmosphere.

9.6.2 Acid phosphatase test

9.6.2.1 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.

9.6.2.2 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.4) 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.

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

9.6.3 Sulfite indole motility (SIM) agar test

Colonies grown anaerobically on blood agar plates or nutrient agar plates are stabbed into SIM tubes (B.5). The tubes are incubated for 22 h ± 2 h at 37 °C (6.4). 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.6) are positive.

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

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

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

9.6.5 Interpretation

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

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 confirmed *C. perfringens* or, if the method of Annex D was also used for differentiation, of confirmed human pathogenic *C. perfringens,* 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 pre-treatment for the selection of spores (9.3) was used, the result is reported as number of confirmed *C. perfringens* spores or, if the method of Annex D was also used for differentiation, of confirmed human pathogenic *C. perfringens* spores in cfu per gram, per millilitre, per square centimetre or per sampling device.

In cases when no typical colonies of *C. perfringens* have been detected, or when no typical colonies are confirmed as *C. perfringens*, 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.

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 the interlaboratory repeatability standard deviation (s_r) is given in Table 1.