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

Part 2: Enumeration method

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

Partie 2: Méthode de dénombrement

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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 on 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 the following URL: www.iso.org/iso/foreword.html.

This document was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food analysis — Horizontal methods*, in collaboration with ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 11290-2:1998), which has been technically revised. It also incorporates the amendment ISO 11290-2:1998/Amd.1:2004.

The main changes, compared to ISO 11290-2:1998, are the following.

— The enumeration of *Listeria monocytogenes* has been modified as listed below.

— Primary suspension with buffered peptone water, half-Fraser broth with or without supplements, and all appropriate diluents referred to in ISO 6887 (all parts).

— Resuscitation step deleted.

— Microscopic aspect, catalase and CAMP test for confirmation are optional.

— Inclusion of new performance characteristics.

— Moreover, enumeration of *Listeria* spp. has been included in the scope and the title changed accordingly.

A list of parts in the ISO 11290 series can be found on the ISO website.

Introduction

The main changes, listed in the foreword, introduced in this document compared to ISO 11290-2:1998 are considered as major (see ISO 17468[28]). The technical changes were assessed and were considered to have no significant effect on the method performance characteristics or test results.

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products for which it may be necessary to use different or specific methods. Nevertheless, in all cases, this horizontal method is intended to be applied as far as possible and deviations from this only be made for justified technical reasons.

When this document is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from it in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this document so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

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

Part 2: Enumeration method

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *L. monocytogenes* and *Listeria* 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. In particular, it is strongly recommended that tests for detecting *L. monocytogenes* are undertaken in laboratories providing biosafety level 2 conditions. It is strongly recommended that female laboratory staff are made aware of the particular risk to the developing foetus presented by infection of the mother through exposure to *L. monocytogenes* and *Listeria* spp., and that pregnant personnel and persons with recognized underlying conditions or diseases that impair cell-mediated immunity do not manipulate cultures of *L. monocytogenes* and *Listeria* spp.

1 Scope

This document specifies a horizontal method for

— the enumeration of *L. monocytogenes*, and

— the enumeration of *Listeria* spp. (including *L. monocytogenes*).

This document is applicable to

— products intended for human consumption and for the feeding of animals, and

— environmental samples in the area of food production and food handling.

It is possible that certain additionally described *Listeria* species may not be enumerated or confirmed by this method.[3],[6],[9],[11]

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 food and animal feeding stuffs — 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 11290-1, *Microbiology of the food chain — Horizontal method for the detection and enumeration of Listeria monocytogenes* and of *Listeria* spp. — Part 1: Detection method

3 Terms and definitions

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

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

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

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

3.1
Listeria monocytogenes
microorganisms which form typical colonies on solid selective media described and which display the morphological, physiological and biochemical characteristics described when the analysis is carried out in accordance with this document

3.2
enumeration of *Listeria monocytogenes*
determination of the number of colony-forming units (cfu) of *Listeria monocytogenes*, per gram, per millilitre, per square centimetre, or per sampling device when the analysis is carried out in accordance with this document

3.3
***Listeria* spp.**
microorganisms which form typical colonies on solid selective media and which display the morphological, physiological and biochemical characteristics described when tests are carried out in accordance with this document

3.4
enumeration of *Listeria* spp.
determination of the number of colony-forming units (cfu) of *Listeria* spp per gram, per millilitre, per square centimetre, or per sampling device, when the analysis is carried out in accordance with this document

4 Principle

4.1 General

Within the limits of this document, the enumeration of *L. monocytogenes* and of *Listeria* spp. requires five successive steps, as described in the flowchart in Annex A.

4.2 Initial suspension

Preparation of the initial suspension in an appropriate diluent according to the sample type.

4.3 Surface plating

Surface plating on Agar *Listeria* according to Ottaviani and Agosti[13],[14] of a specified quantity of the test sample for liquid products or of the initial suspension for other products and/or decimal dilutions if required.

4.4 Incubation

Incubation of the Petri dishes at 37 °C and examination after 24 h and after a further 24 h.

4.5 Confirmation

Confirmation of presumptive colonies of *L. monocytogenes* and/or of presumptive *Listeria* spp. by means of appropriate morphological and/or biochemical tests.

4.6 Enumeration

From the number of confirmed colonies, calculation of the number of *L. monocytogenes* and/or of *Listeria* spp. per gram, per millilitre, per square centimetre, or per sampling device.

5 Culture media and reagents

For current laboratory practices, refer to ISO 11133.

Composition of culture media and reagents and their preparation are described in Annex B.

6 Equipment and consumables

Usual microbiological laboratory apparatus (as specified in ISO 7218) and, in particular, the following.

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

As specified in ISO 7218.

6.2 Drying cabinet or **incubator**, capable of being maintained between 25 °C and 50 °C.

6.3 Incubators, capable of operating at 37 °C ± 1 °C and 25 °C ± 1 °C (optional).

6.4 Water bath, capable of operating at 47 °C to 50 °C.

6.5 Sterile loops, approximately 3 mm in diameter or 10 µl, and inoculating **needle** or **wire.**

6.6 Glass or **plastic spreaders,** sterile.

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

6.8 Total-delivery graduated pipettes or automatic pipettes, of nominal capacities 1 ml and 10 ml.

6.9 Petri dishes, of diameter 90 mm and/or 140 mm.

6.10 Microscope, preferably with phase-contrast, and with slides and cover slips.

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

7 Sampling

Sampling is not part of the method specified in this document. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject. For food and feed samples, refer to ISO/TS 17728. For environmental samples, use ISO 18593 and see Reference [23].

It is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage (see ISO 7218).

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard dealing with the product concerned [see ISO 6887 (all parts) and ISO 18593]. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilutions

Buffered peptone water, as well as other appropriate diluents referred to in ISO 6887 (all parts) and any specific International Standard appropriate to the product concerned, may be used as diluent for the initial suspension.

Half-Fraser broth (as specified in ISO 11290-1), supplemented with selective agents or not, may be used as a diluent for the initial suspension when both the detection method (as specified in ISO 11290-1) and this enumeration method are carried out on the same test sample. The selective agents should be added (if required) to the suspension preferentially after enumeration, prior to the detection method.

If supplemented half-Fraser is used, inoculate the plates as soon as possible, up to 45 min.

9.2 Inoculation and incubation

9.2.1 Distribute, by means of a sterile pipette (6.8), 0,1 ml of the initial suspension (or sample if liquid) and 0,1 ml of further decimal dilutions each inoculated onto the surface of a small dish (90 mm) of Agar *Listeria* according to Ottaviani and Agosti (see B.2).

When, for certain products, it is desirable to estimate low numbers of *L. monocytogenes* and/or *Listeria* spp., the limits of detection may be raised by a factor of 10 by examining 1 ml of the test sample if the initial product is liquid, or 1 ml of the initial suspension for the other products. Distribute the 1 ml of inoculum either on the surface of a large Petri dish (140 mm) or over the surface of three small dishes (90 mm), dried beforehand if necessary in the incubator (6.2). If only the initial suspension is used, also prepare duplicate plates using an additional three small Petri dishes or one large dish of medium (see ISO 7218).

Repeat the procedure using 0,1 ml of the initial suspension (or sample if liquid) and 0,1 ml of further decimal dilutions if necessary each inoculated onto the surface of a small dish (90 mm) of agar medium.

9.2.2 Carefully spread the inoculum as quickly as possible over the surface of the agar plate without touching the sides of the dish with the spreader (6.6). Use a fresh sterile spreader for each dilution. Leave the plates closed and upright for about 15 min at ambient temperature for the inoculum to be absorbed into the agar.

It is possible to use the same spreader for all the dishes of a given sample, by beginning with the higher dilution.

9.2.3 Incubate the Agar *Listeria* according to Ottaviani and Agosti plates prepared in 9.2.2 inverted at 37 °C (6.3) for 24 h ± 2 h and for an additional incubation at 37°C for 24 ± 2h.

9.3 Enumeration of characteristic colonies

9.3.1 After incubation for 24 h ± 2 h (before excessive development of colonies with large and overlapping opaque halos, which may make reading difficult), and for an additional 24 h ± 2 h (which may allow better development of colonies and of an opaque halo), examine the Petri dishes (9.2.3) for the presence of presumptive colonies of *L. monocytogenes* (see 9.3.2) and/or *Listeria* spp. (see 9.3.3).

9.3.2 Consider as *L. monocytogenes* the blue-green colonies surrounded by an opaque halo (typical colonies). Colonies of *L. ivanovii* are also blue-green and surrounded by an opaque halo.

NOTE 1 Some strains of *L. monocytogenes* exposed to stress conditions, particularly acid stress, may show a very weak halo (or even no halo).

NOTE 2 Some rare *L. monocytogenes* are characterized by a slow PIPLC (phosphatidyl inositol phospholipase C) activity. Such bacteria are detected when the total duration of incubation is more than, for example, 4 days. Some of these strains could be pathogenic.[10] No *L. monocytogenes* strains have been described as PIPLC negative.

9.3.3 Consider as presumptive *Listeria* spp. the blue-green colonies with or without opaque halo.

NOTE Some organisms other than *Listeria* spp. may produce blue colonies on this medium. See Annex C.

9.3.4 Count all the colonies presumed to be *L. monocytogenes* (9.3.2) on each Petri dish containing less than 150 *L. monocytogenes* characteristic colonies, or less than 360 *L. monocytogenes* characteristic colonies if 140 mm Petri dishes are used.

9.3.5 Count all the colonies presumed to be *Listeria* spp. (9.3.3.) on each Petri dish containing less than 150 *Listeria* spp. characteristic colonies, or less than 360 *Listeria* spp. characteristic colonies if 140 mm Petri dishes are used.

In case of mixed cultures of blue-green colonies with or without opaque halo, or in case of blue-green colonies with large and overlapping opaque halos, it is preferable to count the colonies on each Petri dish containing less than 100 *Listeria* spp. characteristic colonies, or less than 240 *Listeria* spp. characteristic colonies if 140 mm Petri dishes are used.

9.4 Confirmation of *L. monocytogenes* or *Listeria* spp.

9.4.1 Selection of colonies for confirmation

9.4.1.1 Consider each group of three 90 mm Petri dishes used for the initial suspension as one dish.

If on one dish there are fewer than five presumptive colonies, take all of them for confirmation.

9.4.1.2 For confirmation of presumptive *L. monocytogenes*, take from each Petri dish, representing each dilution, five colonies in total, representative for each colony type (e.g. with large halo and small halo).

Streak the selected colonies onto the surface of pre-dried plates of a non-selective agar, for example blood agar, nutrient agar, tryptone soya yeast extract agar (TSYEA) (B.1) in a manner which will allow isolated colonies to develop.

Use of blood agar for pure culture enables interpretation of haemolysis, when positive, already at that stage (see 9.4.2.5 and Annex D). If streaking on blood agar does not show haemolysis, then the haemolysis test shall be done by stabbing (9.4.2.5.2) or in liquid medium (9.4.2.5.3).

Place the Petri dishes in the incubator set at 37°C for 18 h to 24 h or until growth is satisfactory.

If the colonies are not isolated, pick a typical *L. monocytogenes* colony onto another non-selective dish.

Carry out the following tests (9.4.2) from colonies of a pure culture on the non-selective agar.

9.4.1.3 For confirmation of presumptive *Listeria* spp. take, from each Petri dish, representing each dilution, five colonies in total, representative for each colony type (e.g. large and small colonies, with or without halo).

For confirmation of *Listeria* spp. use plates of TSYEA.

Streak the selected colonies onto the surface of pre-dried plates of TSYEA (B.1), in a manner which will allow isolated colonies to develop.

Place the Petri dishes in the incubator set at 37°C for 18 h to 24 h or until growth is satisfactory.

Typical colonies on TSYEA of *Listeria* spp. are 1 mm to 2 mm in diameter, convex, colourless and opaque with an entire edge. When the plates are held to the light (artificial or natural) at about 45 degree angle, colonies exhibit a blue-grey colour and a granular surface.

If the colonies are not isolated, pick a typical *Listeria* spp. colony onto another non-selective dish.

Carry out the following tests (9.4.3) from typical colonies of a pure culture on TSYEA.

9.4.2 Confirmation of *L. monocytogenes*

9.4.2.1 General

Carry out the confirmation tests for *L. monocytogenes*. Appropriate positive and negative control strains for each of the confirmation tests shall be used.

Perform at minimum the mandatory tests as listed (in bold) in Table 1.

Table 1 — Confirmation tests for *L. monocytogenes*

Tests	*L. monocytogenes* confirmation tests	Results
Mandatory	**Beta-haemolysis (9.4.2.4)**	**+**
	L-Rhamnose (9.4.2.7)	**+**
	D-Xylose (9.4.2.7)	**-**
Optional	Microscopic aspect (9.4.3.4)	Slim short rods or coccobacilli
	Catalase (9.4.2.2)	+
	Motility at 25 °C (9.4.2.3)	+
	CAMP test (9.4.2.6)	+

Details on results for confirmation tests can be found in Annex D.

NOTE An alternative procedure as mentioned in ISO 7218 can be used to confirm the isolate as *Listeria monocytogenes*, providing the suitability of the relevant procedure is verified.

If shown to be reliable, miniaturized galleries for the biochemical identification of *Listeria monocytogenes* may be used (see ISO 7218).

Rare strains of *L. monocytogenes* do not show beta-haemolysis or a positive reaction to the CAMP test under the conditions described in this document. If typical colonies on Agar *Listeria* according to Ottaviani and Agosti with PIPLC activity even if it is low, are negative for haemolysis, it is recommended to perform additional tests (e.g. Gram stain, catalase, motility, CAMP test, PCR), in order to determine whether this isolate is a non-haemolytic *L. monocytogenes.*

9.4.2.2 Catalase reaction (optional)

Take an isolated colony obtained in 9.4.1 and suspend it in a drop of hydrogen peroxide solution (B.3) on a slide. The immediate formation of gas bubbles indicates a positive reaction.

NOTE A catalase reaction performed from a colony originating from a blood agar can sometimes lead to false-positive results.

9.4.2.3 Motility test (optional)

Take an isolated colony obtained in 9.4.1 and suspend it in a tube containing a non-selective nutrient liquid medium.

Incubate in the incubator (6.3) set at 25 °C for 8 h to 24 h until the medium turns cloudy.

Take a drop of the above culture using a loop (6.5) onto a clean glass microscope slide. Place a cover slip on top and examine it under a microscope (6.10).

Listeria spp. (including *L. monocytogenes*) appear as slim, short rods with tumbling motility.

Cultures grown at a temperature above 25 °C may fail to exhibit this motion. Always compare them to a known culture. Cocci, large rods or rods with rapid swimming motility are not *Listeria* spp.

As an alternative test for motility, using an inoculating needle (6.5), dilute in sterile water (or other appropriate diluent) a fragment of isolated colony obtained on non-selective agar. *Listeria* spp. (including *L. monocytogenes*) appear as slim, short rods with tumbling motility.

As another alternative test for motility, using an inoculating needle (6.5), stab the motility agar (B.4) with a culture taken from a typical colony obtained in 9.4.1. Incubate at 25 °C for 48 h ± 2 h.

Examine for growth around the stab. *Listeria* spp. are motile, giving a typical umbrella-like growth pattern. If growth is not sufficient, incubate for up to an additional five days and observe the stab again.

NOTE Some new *Listeria* species have been recently isolated.[3],[6],[9],[11],[20],[24],[25] Most of them are not motile in the motility agar.

9.4.2.4 Microscopic aspect (optional)

Make a microscopic preparation (e.g. Gram stain, wet microscopy) from an isolated colony obtained in 9.4.1. *Listeria* spp. (including *L. monocytogenes*) appear as Gram-positive (if this stain is performed), slim, short rods or coccobacilli, with tumbling motility when originating from a fresh culture.

For Gram stain microscopic preparation see ISO 7218.

9.4.2.5 Haemolysis tests

9.4.2.5.1 General

Choose one of the haemolysis tests (9.4.2.5.2 or 9.4.2.5.3).

NOTE There exist rare strains of *L. monocytogenes* which do not show *β*-haemolysis or a positive reaction to the CAMP test under the conditions described in this document.

9.4.2.5.2 Haemolysis on blood agar

If the morphological and physiological characteristics are indicative of *Listeria* spp., inoculate blood agar plates (B.5) to determine the haemolytic reaction.

Dry the agar surface well before use. Take an isolated colony obtained in 9.4.1 using a wire (6.5), then stab a section of agar. Repeat for each culture. On the same plate if possible, stab positive (*L. monocytogenes*) and negative (*L. innocua*) control cultures. For example, *L. monocytogenes* 4b WDCM 00021, or *L. monocytogenes* 1/2a WDCM 00109 and *L. innocua* WDCM 00017 may be used.

After incubation at 37 °C (6.3) for 24 h ± 2 h, examine the test strains and controls. *L. monocytogenes* shows narrow, clear, light zones of haemolysis; *L. innocua* shows no clear zone around the stab.

L. seeligeri shows mostly a weak zone of haemolysis. *L. ivanovii* usually shows wide, clearly delineated zones of haemolysis. Examine the plates in bright light to compare test cultures with controls.

NOTE 1 The haemolytic reaction is more readily seen by removing any colony growth on the surface of the agar around the inoculum mark.

NOTE 2 The haemolysis test can be performed by stabbing the blood agar plate used for the CAMP test.

9.4.2.5.3 Haemolysis reaction using red blood corpuscles

The haemolytic reaction may also be carried out using red blood corpuscles as follows.

Disperse the colony in 150 µl of a non-selective liquid nutrient medium, incubate at 37 °C (6.3) for 2 h. Add 150 µl of a suspension of red blood corpuscles (B.7). Incubate at 37 °C (6.3) for 15 min to 60 min, then refrigerate at 5 °C (6.11) for approximately 2 h. Examine for haemolytic activity. If the reaction is not definite, leave at 5 °C (6.11) for up to 24 h ± 2 h. A sedimentation of red blood corpuscles (formation of a red point at the bottom of the tubes) indicates a negative reaction.

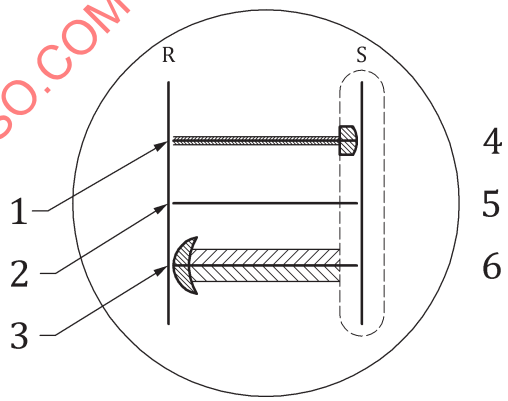
Include positive and negative controls. For control strains see 9.4.2.5.2.

9.4.2.6 CAMP test (optional)

If the result of the haemolysis test is difficult to interpret, the CAMP test is recommended to demonstrate clearly that haemolysis is due to listeriolysin activity.

A *β*-haemolytic strain of *Staphylococcus aureus* (e.g. WDCM 00034) and a strain of *Rhodococcus equi* (e.g. WDCM 0028) are required to undertake the CAMP test. Not all strains of *S. aureus* are suitable for the CAMP test. Streak each of the *S. aureus* and *R. equi* cultures in single lines across the blood agar plate (B.5 or B.8.4) so that the two cultures are parallel and diametrically opposite (see Figure 1). A thin, even inoculum is required. This can be obtained by using an inoculation loop or a wire (6.5) held at right angles to the agar.

Streak a well isolated colony of the strain under test (9.4.1.) in a similar fashion at right angles to these cultures so that the test culture and *S. aureus* and *R. equi* cultures do not touch but at their closest are about 1 mm to 2 mm apart. Several test strains may be streaked on the same plate.



Key

1	narrow band of ß-haemolysis	4	*L. monocytogenes*
2	no haemolysis	5	*L. innocua*
3	wide band of ß-haemolysis	6	*L. ivanovii*

Figure 1 — Inoculation and interpretation of CAMP test plates

The vertical lines in Figure 1 represent streaks of *S. aureus* (S) and *R. equi* (R). Horizontal lines represent streaks of the test cultures. Hatched areas indicate the locations of enhanced haemolysis.

The dotted area indicates the zone of influence of the *S. aureus* culture.

Simultaneously, streak control cultures of *L. monocytogenes, L. innocua* and *L. ivanovii*. For example, *L. monocytogenes* 4b WDCM 00021, *L. monocytogenes* 1/2a WDCM 00109, *L. innocua* WDCM 00017 and *L. ivanovii* WDCM 00018 may be used. For maintaining stock cultures see ISO 11133. If blood agar (B.5) is used, incubate the plates at 37 °C for 18 h to 24 h.

If double-layer plates (B.8.4) are used, incubate at 37 °C for 12 h to 18 h.

The positive reaction with *R. equi* is seen as a wide (5 mm to 10 mm) "arrow-head" of haemolysis. The reaction is considered as negative if a small zone of weak haemolysis extends only about 1 mm at the intersection of the test strain with the diffusion zone of the *R. equi* culture.

A positive reaction with *S. aureus* appears as a small zone of enhanced haemolysis extending only about 2 mm from the test strain and within the weakly haemolytic zone due to growth of the *S. aureus* culture. Large zones of haemolysis do not occur in the area of *S. aureus* and *L. monocytogenes*.

9.4.2.7 Carbohydrate utilization

Using a loop (6.5), inoculate each of the carbohydrate utilization broths (B.9) with the cultures obtained from the non-selective agar (9.4.1). Incubate at 37 °C. Positive reactions (acid formation) are indicated by a yellow colour and occur mostly within 24 h to 48 h for microvolumes tubes, and up to five days for macrovolumes tubes. L-Rhamnose and D-Xylose are used for the confirmation of *L. monocytogenes*, which is L-Rhamnose positive and D-Xylose negative (see Annex D).

NOTE There exist rare strains of *L. monocytogenes* which do not ferment L-Rhamnose.[12][15]

For microvolumes, reactions are more rapid. The level of inoculation compared to the total volume is an important factor. For each chosen protocol, it is important to verify the time taken to obtain a yellow coloration. It is advised to use controls. For example *L. monocytogenes* 4b WDCM 00021, *L. innocua* WDCM 00017 and *L. ivanovii* WDCM 00018 may be used. Maintain stock cultures as specified in ISO 11133.

9.4.3 Confirmation of *Listeria* spp.

9.4.3.1 General

Carry out the confirmation tests for *Listeria* spp. from typical colonies (9.4.1.3). Appropriate positive and negative control strains for each of the confirmation tests shall be used.

Perform at minimum the mandatory tests as listed (in bold) in Table 2.

For further tests, if identification of species of *Listeria* is required, see Annex D.

Table 2 — Confirmation tests for *Listeria* spp.

Tests	*Listeria* spp.	Results
Mandatory	**Microscopic aspect (9.4.2.2)**	**Slim short rods or coccobacilli**
	Catalase (9.4.2.2)	**+**
Optional	VP test (9.4.3.5)	+
	Motility at 25°C (9.4.2.3)	+

NOTE 1 An alternative procedure as mentioned in ISO 7218 can be used to confirm the isolate as *Listeria* spp., providing the suitability of the relevant procedure is verified.

NOTE 2 It is possible that some new *Listeria* species recently isolated may not correspond to this scheme in particular for motility, VP test and growth at 37°C (see, for example, References [3], [6], [9], [11], [20], [24] and [25]).

9.4.3.2 Catalase reaction

Take an isolated colony obtained in 9.4.1 and perform the test as described in 9.4.2.2.

9.4.3.3 Motility test (optional)

Take an isolated colony obtained in 9.4.1 and perform the test as described in 9.4.2.3.

9.4.3.4 Microscopic aspect

Take an isolated colony obtained in 9.4.1 and perform the test as described in 9.4.2.4.

9.4.3.5 Voges – Proskauer (VP) reaction (optional)

Using a loop (6.5), inoculate a tube containing 3 ml of the VP medium (B.10.1). Incubate at 37 °C for 24 h ± 2 h. After incubation add 0,6 ml of 5 % α-naphthol solution (B.10.2) and 0,2 ml of 40 % potassium hydroxide solution (B.10.3). Shake well, slope the tube (to increase the area of the air-liquid interface). Examine after 15 min and 1 h. A positive reaction is indicated by a strong red colour. *Listeria* spp. are VP positive.

NOTE Some new *Listeria* species have been recently isolated.[3],[6],[9],[11],[20],[24],[25] Most of them are VP negative.

9.5 Interpretation of morphological and physiological properties and of the biochemical reactions

All *Listeria* spp. are small, Gram-positive rods or coccobacilli that give a positive reaction in the catalase test.

L. monocytogenes are confirmed according to tests listed in Table 1 and *Listeria* spp. are confirmed according to tests listed in Table 2.

9.6 Additional characterization of isolated strains (optional)

Isolates which are considered to be *L. monocytogenes* (9.5) may be sent for further characterization to a recognized national or regional *Listeria* Reference Laboratory, or (if not available) to the World Health Organization Collaborating Centres for *Listeria*. The dispatch shall be accompanied by all possible information concerning the strain(s).

For further tests, if identification of species of *Listeria* is required, see Annex D.

10 Expression of results

See ISO 7218.

Calculate and report the counts in cfu per gram, per millilitre, per square centimetre, or per sampling device for *L. monocytogenes* and/or *Listeria* spp. depending on the purpose of the assay.

11 Performance characteristics of the method

11.1 Method validation study

Results of the interlaboratory study to determine the precision of the method are summarized in Annex E. Repeatability and reproducibility limits were determined using five types of food contaminated at various levels. The values derived from the interlaboratory study may not be applicable to concentration ranges and food types other than those given in Annex E.

11.2 Repeatability limit

The absolute difference between two independent single ($\log_{10}$-transformed) test results (number of cfu per gram or per millilitre) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in the same laboratory by the same operator using the same apparatus within the shortest feasible time interval, will in no more than 5 % of cases exceed the repeatability limit *r*.

As a general indication of repeatability limit (*r*), the following values (medians of values per matrix and contamination level, see Annex E) may be used when testing food samples in general (except powdered infant formulae):

r = 0,30, range [0,19; 0,40] (expressed as a difference between $\log_{10}$-transformed test results), or

r = 1,99, range [1,55; 2,50] (expressed as a ratio between test results).

EXAMPLE A test result of 100 or $1,0 \times 10^2$ or $\log_{10}$ 2,00 cfu per gram of food product was observed in a given laboratory. Under repeatability conditions, the difference between $\log_{10}$-transformed results should not be greater than ± 0,30 $\log_{10}$ units. So the result from a second test result of the same sample should be between 1,70 (2,00 - 0,30) and 2,30 (2,00 + 0,30) $\log_{10}$ units. For non log-transformed results, the ratio between the first test result and the second test result from the same sample should not be greater than 1,99. So the second test result should be between 50 (= 100/1,99) and 199 (=100 × 1,99) cfu per gram.

11.3 Reproducibility limit

The absolute difference between two single ($\log_{10}$-transformed) test results (number of cfu per gram or per millilitre) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in no more than 5 % of cases exceed the reproducibility limit *R*.

As a general indication of reproducibility limit (*R*), the following values (medians of values per matrix and contamination level, see Annex E) may be used when testing food samples in general (except powdered infant formulae):

R = 0,43, range [0,25; 0,54](expressed as a difference between $\log_{10}$-transformed test results), or

R = 2,68, range [1,79; 3,47] (expressed as a ratio between test results).

EXAMPLE 1 A test result of 100 or $1,0 \times 10^2$ or $\log_{10}$ 2,00 cfu per gram of food product was observed in a first laboratory. Under reproducibility conditions, the difference between $\log_{10}$-transformed results should not be greater than ± 0.43 $\log_{10}$ units. So the results from a second laboratory should be between 1,57 (2,00 - 0,43) and 2,43 (2,00 + 0,43) $\log_{10}$ units. For non log-transformed data the ratio between the test results from this first laboratory and a second laboratory should not be greater than 2,68. So the result from the second laboratory should be between 37 (= 100/2,68) and 268 (=100 × 2,68) cfu per gram.

EXAMPLE 2 A laboratory wants to know the maximum value it may find, which is still in compliance with a pre-set limit (e.g. a limit of 100 or $\log_{10}$ 2). For this, the R value (on the log scale) has to be multiplied by a factor of 0,59[1]. The maximum value is 0,25 (0,43 × 0,59) as a difference between $\log_{10}$-transformed test results or 1,78 ($10^{0,25}$) as a ratio between test results. So results up to $\log_{10}$ 2,25 ($\log_{10}$ 2 + $\log_{10}$ 0,25) or 178 (100 × 1,78) do not indicate non-compliance with the limit.

12 Test report

The test report shall specify the method used and the results obtained. It shall also mention all operating details not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the results (see Clause 10).

The test report shall contain all information necessary for the complete identification of the sample.

1) The factor 0,59 reflects the fact that a test with a one-sided 95 % interval is used to test whether the limit is exceeded; it is obtained from the following formula: $0,59 = \frac{1,64}{1,96 \times \sqrt{2}}$

13 Quality assurance

The laboratory should have a clearly defined quality control system to ensure that the equipment, reagents and techniques are suitable for the test. The use of positive controls, negative controls and blanks are part of the test. Performance testing of culture media is specified in B.5 and described in ISO 11133.

Annex A
(normative)

Diagram of procedure

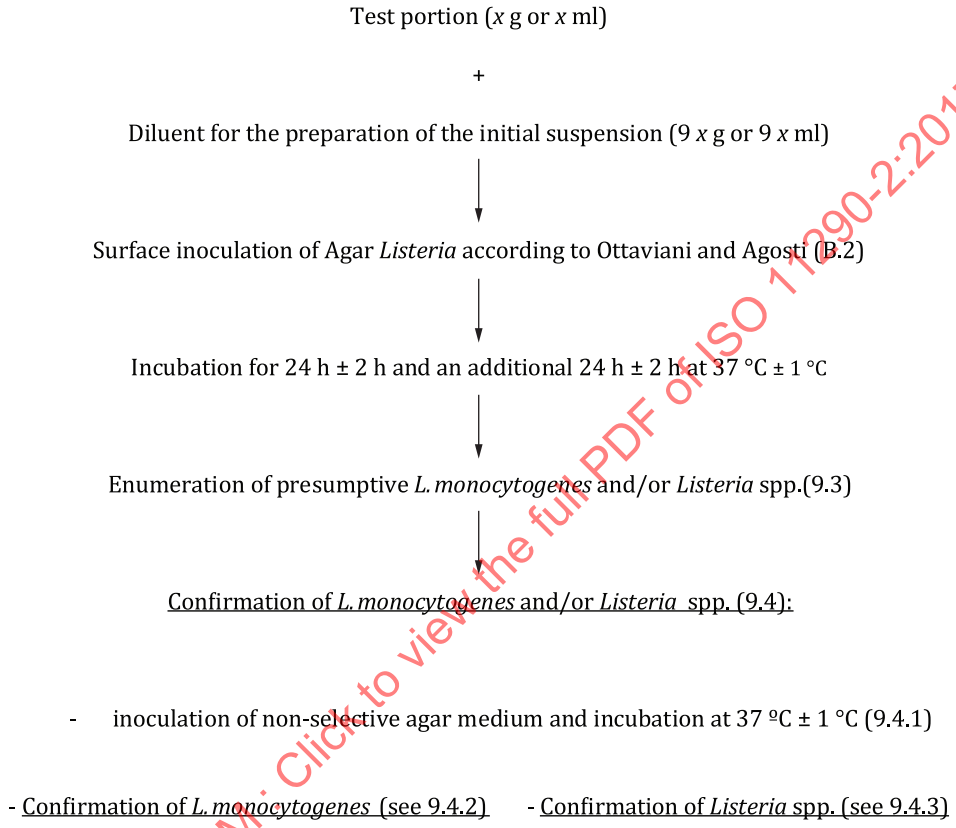


Figure A.1 — Diagram of procedure

Annex B
(normative)

Composition and preparation of media and reagents

B.1 Tryptone soya yeast extract agar (TSYEA)

B.1.1 Composition

Enzymatic digest of casein	17 g
Papaic digest of soyabean meal	3 g
Yeast extract	6 g
Glucose	2,5 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	2,5 g
Agar	12 g to 18 g[a]
Water	1 000 ml

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

B.1.2 Preparation

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

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

Sterilize for 15 min in the autoclave at 121 °C.

B.1.3 Preparation of agar plates

Place in each Petri dish 18 ml to 20 ml of the freshly prepared complete medium, then allow to solidify.

B.2 Agar *Listeria* according to Ottaviani and Agosti[13],[14]

B.2.1 Base medium

B.2.1.1 Composition

Enzymatic digest of animal tissues	18 g
Enzymatic digest of casein	6 g
Yeast extract	10 g

Sodium pyruvate	2 g
Glucose	2 g
Magnesium glycerophosphate	1 g
Magnesium sulfate (anhydrous)	0,5 g
Sodium chloride	5 g
Lithium chloride	10 g
Disodium hydrogen phosphate (anhydrous)	2,5 g
5-Bromo-4-chloro-3-indolyl-ß-D-glucopyranoside	0,05 g
Agar	12 g to 18 g [a]
Water	930 ml [b]

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

[b] 925 ml if Amphotericin B solution is used (see B.2.5.2).

B.2.1.2 Preparation

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

Sterilize for 15 min in the autoclave at 121 °C.

Adjust the pH, if necessary, so that after sterilization it is 7,2 ± 0,2.

B.2.2 Nalidixic acid solution

Nalidixic acid sodium salt	0,02 g
Sodium hydroxide (0,05 mol/l)	5 ml

Dissolve the nalidixic acid sodium salt in 5 ml of sodium hydroxide and sterilize by filtration through a 0,45 µm membrane.

B.2.3 Ceftazidime solution

Ceftazidime	0,02 g
Water	5 ml

Dissolve the ceftazidime in 5 ml of water and sterilize by filtration through a 0,45 µm membrane.

B.2.4 Polymyxin B solution

Polymyxin B sulfate	76 700 IU
Water	5 ml

Dissolve the polymyxin B in 5 ml of water. Sterilize by filtration through a 0,45 µm membrane.

B.2.5 Antibiotic supplement

B.2.5.1 Cycloheximide solution

Cycloheximide	0,05 g
Ethanol	2,5 ml
Water	2,5 ml

Dissolve the cycloheximide in 2,5 ml of ethanol then add 2,5 ml of water. Sterilize by filtration through a 0,45 µm membrane.

B.2.5.2 Amphotericin B solution (as an alternative to cycloheximide solution)

Amphotericin B	0,01 g
HCl (1 mol/l)	2,5 ml
Dimethylformamide (DMF)	7,5 ml

Dissolve the amphotericin in the HCl/DMF solution. Sterilize by filtration through a 0,45 µm membrane. Other techniques of dissolution (e.g. in water or in hot medium base) may be performed according to media suppliers.

WARNING — The HCl/DMF solution is toxic, handle with care.

B.2.6 Supplement

Dissolve 2 g of L-α-phosphatidylinositol[2] in 50 ml of water.

2 g of soy lecithin containing at least 9 % to 15 % unfractionated phosphatidylinositol may be used instead of L-α- phosphatidylinositol.[4],[7],[16]

Stir for about 30 min until a homogeneous suspension is obtained. Autoclave at 121 °C for 15 min and cool to 47 °C to 50 °C.

B.2.7 Complete medium

B.2.7.1 Composition

Base medium (B.2.1)	930 ml [a]
Nalidixic acid solution (B.2.2)	5 ml
Ceftazidime solution (B.2.3)	5 ml
Polymyxin B solution (B.2.4)	5 ml
Cycloheximide solution (B.2.5.1)	5 ml
or Amphotericin B solution (B.2.5.2)	10 ml
Supplement (B.2.6)	50 ml

[a] 925 ml if Amphotericin B solution is used.

2) P 6636® supplied by Sigma is an example of a suitable product available commercially. This information is given for the convenience of the users of this document and does not constitute an endorsement by ISO of this product.

B.2.7.2 Preparation

Add the solutions to the molten base previously cooled in a water bath at 47 °C to 50 °C (6.4), mixing thoroughly between each addition.

The pH of the complete medium shall be 7,2 ± 0,2 at 25 °C.

The medium shall be homogeneously slightly opalescent.

B.2.7.3 Preparation of agar plates

Place in each 90 mm Petri dish 18 ml to 20 ml or in each 140 mm Petri dish 45 ml to 50 ml of the freshly prepared complete medium, then allow to solidify.

B.2.8 Performance testing for the quality assurance of the culture media

Performance testing of the culture media shall be carried out according to ISO 11133, which includes definitions for productivity, selectivity and specificity. Table B.1 gives details of control strains to be used for performance testing of culture media specified in this document.

NOTE For productivity testing, in the case of enumeration of *Listeria* spp., it could be interesting to add another control strain (from another *Listeria* species).

Table B.1 — Performance testing of culture media for *Listeria monocytogenes*

Media[a]	Function	Incubation	Control strain	WDCM number[c]	Reference media	Method of control	Criteria	Characteristic reaction
Agar *Listeria* according to Ottaviani and Agosti	Productivity	(48 ± 4) h/(37 ± 1) °C	*Listeria monocytogenes* 4b	00021[b]	TSA	Quantitative	$P_R \geq 0,5$	Blue green colonies with opaque halo
			Listeria monocytogenes 1/2a	00109				
	Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0)	—
			Enterococcus faecalis[d]	00009 or 00087				
	Specificity		*Listeria innocua*	00017	—	Qualitative	—	Blue green colonies without opaque halo

[a] Full names of media abbreviated terms.

[b] Strains to be used as a minimum.

[c] Refer to the reference strain catalogue available at www.wfcc.info for information on culture collection strain numbers and contact details.

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

B.3 Hydrogen peroxide solution

Use a mass fraction of 3 %, i.e. 10 volume solution.

B.4 Motility agar

B.4.1 Composition

Enzymatic digest of casein	20,0 g
Enzymatic digest of animal tissues	6,1 g
Agar	3,5 g
Water	1 000 ml

B.4.2 Preparation

Dissolve the components in the water by boiling.

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

Dispense the medium into tubes in quantities of about 5 ml.

Sterilize for 15 min in the autoclave at 121 °C.

B.5 Blood agar

B.5.1 Base

B.5.1.1 Composition

Enzymatic digest of animal tissues	15 g
Liver digest	2,5 g
Yeast extract	5 g
Sodium chloride	5 g
Agar	9 g to 18 g [a]
Water	1 000 ml

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

B.5.1.2 Preparation

Dissolve the components in the water by boiling.

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

Dispense the medium into flasks of suitable capacity to obtain portions appropriate for the test.

Sterilize for 15 min in the autoclave at 121 °C.

B.5.2 Defibrinated blood (sheep, calf or bovine)

B.5.3 Complete medium

B.5.3.1 Composition

Base (B.5.1)	100 ml
Defibrinated blood (B.5.2)	5 ml to 7 ml

B.5.3.2 Preparation

Add the blood to the base previously cooled in a water bath at 47 °C to 50 °C (6.4). Mix well.

Dispense the medium into sterile Petri dishes in portions appropriate for the test. Allow to solidify.

B.6 Phosphate-buffered saline (PBS)

B.6.1 Composition

Disodium hydrogen phosphate dihydrate	8,98 g
Sodium dihydrogen phosphate	2,71 g
Sodium chloride	8,5 g
Water	1 000 ml

B.6.2 Preparation

Dissolve the components in the water.

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

Sterilize in the autoclave for 15 min at 121 °C.

B.7 Red blood corpuscle suspension

Maintain the red blood corpuscles at 5 °C ± 2 °C before use.

Before use, examine for signs of haemolysis (reddening) in the top layer of the serum. If no haemolysis has occurred, introduce 2 ml of blood corpuscles from the bottom layer into 98 ml of PBS buffer (B.6).

If haemolysis has occurred, suspend approximately 4 ml of the blood corpuscles layer into 10 ml of PBS buffer and mix gently, then centrifuge. If the supernatant liquid clearly becomes red, due to significant haemolysis, do not use the stock suspension and discard it. Otherwise, decant the supernatant liquid and add 2 ml of this blood corpuscle solution to 98 ml of PBS buffer. Keep the suspension for 5 days at 5 °C ± 2 °C. Discard it if haemolysis occurs.

B.8 CAMP (Christie, Atkins, Munch-Petersen) medium and test strains

B.8.1 General

Blood agar plates (B.5) may be used for this test, but it is preferable to use double-layered agar plates with a very thin blood layer (B.8.4).

B.8.2 Base

See B.5.1.

B.8.3 Blood medium

See B.5.3.

B.8.4 Complete medium

Dispense the base (B.8.2) into sterile Petri dishes in quantities of about 10 ml and allow to solidify. Pour a very thin layer of the blood medium (B.8.3 using blood) using amounts not greater than 3 ml per plate.

Allow to solidify. If the blood medium is added to Petri dishes containing the base which have been prepared in advance, it may be necessary to warm the Petri dishes for 20 min by placing them in an incubator set at 37 °C before pouring the blood medium layer.

B.9 Carbohydrate utilization broth (L-Rhamnose and D-Xylose)

B.9.1 Base

B.9.1.1 Composition

Enzymatic digest of animal tissues	10 g
Meat extract	1 g
Sodium chloride	5 g
Bromocresol purple	0,02 g
Water	1 000 ml

B.9.1.2 Preparation

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

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

Dispense the medium into tubes of suitable capacity to obtain portions appropriate for the test.

Sterilize for 15 min in the autoclave at 121 °C.

B.9.2 Carbohydrate solutions

B.9.2.1 Composition

Carbohydrate [a]	5 g
Water	100 ml

[a] L-Rhamnose or D-Xylose.

B.9.2.2 Preparation

Dissolve the components in the water, and sterilize by filtration through a 0,45 µm membrane.

B.9.3 Complete medium

For each carbohydrate, add aseptically *x* ml of solution B.9.2 to 9*x* ml of the base (B.9.1).

B.10 Reagents for Voges-Proskauer (VP) Reaction

B.10.1 VP medium

B.10.1.1 Composition

Enzymatic digest of animal tissues	7 g
Sodium chloride	5 g
Glucose	5 g
Water	1 000 ml

B.10.1.2 Preparation

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

Adjust the pH, if necessary, so that after sterilization it is 6,9 ± 0,2 at 25 °C.

Dispense the medium into tubes in quantities of 3 ml.

Sterilize for 15 min in the autoclave at 121 °C.

NOTE Commercially available pre-prepared formulations with a pH within 0,5 pH unit of 6,9 are acceptable.

B.10.2 *α*-Naphthol, ethanolic solution

B.10.2.1 Composition

α-Naphthol	5 g
Ethanol, 96 % (volume fraction)	100 ml

B.10.2.2 Preparation

Dissolve the *α*-naphthol in the ethanol.

WARNING — The α-naphthol is toxic, handle with care.

B.10.3 Potassium hydroxide solution

B.10.3.1 Composition

Potassium hydroxide	40 g
Water	100 ml

B.10.3.2 Preparation

Dissolve the potassium hydroxide in the water.

Annex C
(informative)

Distinction of *Listeria* spp. from other genera

Table C.1 — Distinction of *Listeria* spp. from other bacterial genera or species

	Gram appearance	Catalase	Motility (20 °C to 25 °C)	VP test	Growth at 37°C
Listeria spp.	Gpb/Gpcb	+	+	+	+
Bacillus spp. [a]	Large Gpb[b] spore bearer (young culture)	+	V	V	+
Carnobacterium spp. [a]	Gpb	-	-	-	+
Staphylococcus spp.[a]	Gpc	+	-	V	+
Streptococcus spp. [a]	Gpc	-	-	V	+
Lactobacillus spp.	Gpb	- (occasional +)	- (occasional +)	-	+
Brochothrix spp.	Gpb	+	-	+	-
Kurthia spp.	Gpb	+	+	-	+
Erysipelothrix spp.	Gpb	-	-	-	+
Corynebacterium spp.	Gpb	+	-	- (occasional +)	+
Enterococcus spp. [a]	Gpc	-	-	+	+
Cellulosimicrobium funkei [a]	Gpcb	+	+	-	+
Kocuria kristinae [a]	Gpc	+	-	+ (occasional -)	+
Marinilactibacillus psychrotolerans [a,c]	Gpb	+	+	+	+
Rothia terrae [a]	Gpc	+	-	+	+

[a] Known to grow on Agar *Listeria* according to Ottaviani and Agosti and other chromogenic media and sometimes forming blue or bluish colonies.

[b] *Bacillus oleronius* displays a variable reaction to the Gram stain; most cells appear as Gram-negative.[19]

[c] Distinction of *M. psychrotolerans* from *Listeria* spp. can be aided by its different colony characteristics on TSYEA (opaque, pale-yellowish, lenticular colonies, 2 mm to 3 mm in diameter after 24 h of incubation at 37 °C).[19]

Gpb: Gram positive bacillus

Gpcb: Gram positive coccobacillus

Gpc: Gram positive coccus or coccoid

V: variable reaction

NOTE 1 Some new *Listeria* species recently isolated may not correspond to this scheme in particular for motility, VP test and growth at 37°C (see, for example, References [3], [6], [9], [11], [20], [24] and [25]).

NOTE 2 Some rare *Listeria* strains are slow or not catalase positive.