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Milk and milk products — Determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB)

Lait et produits laitiers — Détermination de la concentration minimale inhibitrice (CMI) d'antibiotiques applicable aux bifidobacteria et bactéries lactiques non-entérocoques

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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.

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 10932|IDF 223 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

Foreword

IDF (the International Dairy Federation) is a non-profit organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

The main task of Standing Committees is to prepare International Standards. Draft International Standards adopted by the Standing Committees are circulated to the National Committees for endorsement prior to publication as an International Standard. Publication as an International Standard requires approval by at least 50 % of IDF National Committees casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. IDF shall not be held responsible for identifying any or all such patent rights.

ISO 10932 | IDF 223 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by a Joint ISO-IDF Project Group on *Minimal inhibitory concentration (MIC) of antibiotics* of the Standing Committee on *Analytical methods for dairy microorganisms* under the aegis of its project leader, Mr. M. Danielsen (DK).

Introduction

There are several reports on minimal inhibitory concentration (MIC) determination of lactic acid bacteria according to various methods. However, the MIC value obtained depends on the determination used and the strain cultivation technique. For example, MIC determined by different quantitative methods are not always equivalent. Also some media components are antagonistic to certain antibiotics.

Consequently, a standardized MIC determination which employs a suitable growth medium having little or no antagonistic effects towards the antibiotics studied is necessary.

Two EU projects (PROSAFE and ACE-ART) were launched to tackle these issues, and propose appropriate media and method to measure MIC. This International Standard is based on the SOP (standard operating procedure) proposed by ACE-ART.

Milk and milk products — Determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB)

WARNING — Antibiotics are substances that may be hazardous. Necessary precautions should be taken to avoid contact with these substances. In particular, kanamycin may cause harm to the unborn child (risk phrase R61) and chloramphenicol may cause cancer (risk phrase R45).

1 Scope

This International Standard specifies a method for determining the minimal inhibitory concentration (MIC) of a series of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB).

NOTE Unlike the disk diffusion method, which is semi-quantitative, the frequently used broth microdilution method gives quantitative MICs of the test organism in a dilution series of the antibiotics. The lowest concentration of an antibiotic that prevents visible growth of a test organism is considered to be the MIC.

This International Standard recommends the broth microdilution method as the standard method.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6887-5, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

ISO/TS 11133-2, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing of culture media*

3 Terms and definitions

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

3.1
minimal inhibitory concentration
MIC
lowest concentration that, under defined *in vitro* conditions, prevents visible growth of bacteria within a defined period of time

[ISO 20776-1:2006[6], 2.4]

NOTE MIC is expressed in micrograms per millilitre.

4 Principle

Most individual colonies from an agar plate are picked up and suspended in sterile saline. However, *Bifidobacterium* spp. are suspended in pre-reduced LSM-Cys medium.

The bacterial suspension is diluted with recommended medium.

The microdilution plate is prepared with a series of twofold dilutions of antibiotic.

The diluted bacterial suspension is distributed into the wells of the plate and incubated under recommended conditions.

The lowest concentration of an antibiotic that prevents visible growth is considered to be the MIC.

5 Diluents, culture media and reagents

5.1 Basic materials

Use only reagents of recognized analytical grade, unless otherwise specified, and sterile distilled or demineralized water or water of equivalent purity. See ISO 6887-5.

5.2 Diluents

See ISO 6887-5.

5.3 Culture media

5.3.1 MRS agar

5.3.1.1 Composition

Peptone 1 (tryptic digest of casein)	10,0 g
Meat extract	10,0 g
Yeast extract (dried)	5,0 g
Glucose	20,0 g
Polysorbate 80 (polyethoxylated sorbitan mono-oleate)[a]	1,0 ml
Dipotassium hydrogen phosphate (K_2HPO_4)	2,0 g
Sodium acetate trihydrate ($NaCH_3CO_2 \cdot 3H_2O$)	5,0 g
Diammonium citrate [$(NH_4)_2HC_6H_5O_7$]	2,0 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0,2 g
Manganese sulfate tetrahydrate ($MnSO_4 \cdot 4H_2O$)	0,05 g
Agar	10 g to 15 g[b]
Water up to	1 000 ml[c]

[a] Tween 80 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard, and does not constitute an endorsement by ISO or IDF of this product.

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

[c] When using hand-made microdilution plates (8.4.5.1), the MRS medium should be prepared at twice the higher concentration by only adding water up to 500 ml.

5.3.1.2 Preparation

Suspend the ingredients in the water. Heat the suspension to boiling with frequent agitation until complete dissolution. If needed, adjust the pH (6.7) to 6,35 ± 0,2 with dilute hydrochloric acid or dilute sodium hydroxide before autoclaving. After autoclaving, the pH range of the MRS agar medium should be 6,2 ± 0,2 at 25 °C. Distribute the medium in portions of 100 ml ± 1 ml into bottles (6.8) of capacity 150 ml or in portions of 200 ml ± 2 ml into bottles (6.8) of capacity 250 ml.

Sterilize in the autoclave (6.5) maintained at 121 °C for 15 min. If the medium is to be used immediately, cool it in a water bath (6.6) to between 44 °C and 47 °C. If not used immediately, melt the MRS agar (5.3.1.1) in a boiling water bath (6.6) and mix carefully to avoid gas bubbles, then cool it in a water bath (6.6) to between 44 °C and 47 °C.

Pour 15 ml to 20 ml of prepared medium into Petri dishes (6.10). Allow the medium to cool. Solidify by placing the Petri dishes with the lids in place on a cool horizontal surface.

Before use, dry the agar surface in accordance with ISO/TS 11133-1.

The prepared MRS agar plates may be stored in an airtight plastic bag in the dark and held between 2 °C and 8 °C for up to 2 weeks.

Test agar plates for microbial contamination in accordance with ISO/TS 11133-2.

The complete MRS agar is commercially available, but the results obtained may differ significantly from one supplier to another. If used, therefore, check the commercial MRS agar against the same medium prepared in accordance with this International Standard.

5.3.2 MRS-cysteine agar (MRS-Cys agar)

MRS-Cys agar consists of MRS agar (5.3.1) with addition of 0,3 g of L-cysteine per litre of medium.

5.3.2.1 Basic medium — MRS agar

See 5.3.1.

5.3.2.2 L-Cysteine stock solution

5.3.2.2.1 Composition

L-Cysteine hydrochloride	0,3 g
Water up to	10,0 ml

5.3.2.2.2 Preparation

Dissolve the L-cysteine hydrochloride in the water. Sterilize through a 0,2 µm filter (6.12) into a sterile test tube (6.13).

The L-cysteine stock solution may be stored in the dark and held between 2 °C and 8 °C for up to 1 week. Do not expose the solution to direct sunlight.

5.3.2.3 Complete medium

5.3.2.3.1 Composition

Basic medium (5.3.1)	100 ml
L-Cysteine stock solution (5.3.2.2)	1,0 ml

5.3.2.3.2 Preparation

Immediately before use, melt the MRS agar (5.3.1) in a boiling water bath (6.6). Cool it in a water bath (6.6) maintained at a temperature between 44 °C and 47 °C.

Aseptically add 1,0 ml of L-cysteine stock solution (5.3.2.2) to 100 ml of MRS agar (5.3.1). Mix very carefully while avoiding gas bubbles.

Pour 15 ml to 20 ml of prepared medium into Petri dishes (6.10). Allow the medium to cool. Solidify by placing the Petri dishes with the lids in place on a cool horizontal surface. Before use, dry the agar surface in accordance with ISO/TS 11133-1.

The prepared MRS-Cys agar plates may be stored in an airtight plastic bag in the dark and held between 2 °C and 8 °C for up to 1 week.

Test agar plates for microbial contamination in accordance with ISO/TS 11133-2.

The complete MRS-Cys agar is commercially available, but the results obtained may differ significantly from one supplier to another. If used, therefore, check the commercial MRS-Cys agar against the same medium prepared in accordance with this International Standard.

5.3.3 M17-sucrose agar

M17-sucrose agar consists of M17 agar (5.3.3.1) with addition of 5,0 g sucrose per litre of medium.

5.3.3.1 Basic medium — M17 agar

5.3.3.1.1 Composition

Tryptone (pancreatic digest of casein)	5,0 g
Soy peptone	5,0 g
Beef extract	5,0 g
Yeast extract (dried)	2,5 g
Ascorbic acid ($C_6H_8O_6$)	0,5 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0,25 g
Disodium glycerophosphate ($C_3H_7PO_6Na_2 \cdot 5H_2O$)	19,0 g
Agar	10 g to 15 g[a]
Water up to	950 ml
[a] Depending on the gel strength of the agar.	

5.3.3.1.2 Preparation

Suspend the ingredients in the water. Heat to boiling with frequent agitation until complete dissolution. If needed, adjust the pH (6.7) to 7,35 ± 0,2 with dilute hydrochloric acid or dilute sodium hydroxide before autoclaving. After autoclaving, the pH range of the M17 agar medium should be 7,2 ± 0,2 at 25 °C. Distribute the medium in portions of 95 ml ± 1 ml into bottles (6.8) of capacity 150 ml or in portions of 190 ml ± 2 ml into bottles (6.8) of capacity 250 ml.

Sterilize in the autoclave (6.5) maintained at 121 °C for 15 min. If the medium is to be used immediately, cool it in a water bath (6.6) to between 44 °C and 47 °C. If not used immediately, melt the M17 agar (5.3.3.1) in a boiling water bath (6.6) and mix carefully to avoid gas bubbles, then cool it in a water bath (6.6) to between 44 °C and 47 °C.

5.3.3.2 Sucrose stock solution

5.3.3.2.1 Composition

Sucrose	5,0 g
Water up to	50 ml

5.3.3.2.2 Preparation

Dissolve the sucrose in the water. Sterilize through a 0,2 µm filter (6.12) into a sterile test tube (6.13).

5.3.3.3 Complete medium

5.3.3.3.1 Composition

Basic medium (5.3.3.1)	95,0 ml
Sucrose stock solution (5.3.3.2)	5,0 ml

5.3.3.3.2 Preparation

Immediately before use, melt the M17 agar (5.3.3.1) in a boiling water bath (6.6). Cool it in a water bath (6.6) to between 44 °C and 47 °C. Aseptically add 5,0 ml of sucrose stock solution (5.3.3.2) to 95,0 ml of M17 agar (5.3.3.1). Mix very carefully while avoiding gas bubbles.

Pour 15 ml to 20 ml of prepared medium into Petri dishes (6.10). Allow the medium to cool and solidify by placing the Petri dishes with the lids in place on a cool horizontal surface.

Before use, dry the agar surface in accordance with ISO/TS 11133-1.

The prepared M17-sucrose agar plates may be stored in an airtight plastic bag in the dark and held between 2 °C and 8 °C for up to 2 weeks.

Test agar plates for microbial contamination in accordance with ISO/TS 11133-2.

5.3.4 M17-lactose agar

M17-lactose agar consists of M17 agar (5.3.3.1) with addition of 5,0 g lactose per litre of medium.

5.3.4.1 Basic medium — M17 agar

See 5.3.3.1.

5.3.4.2 Lactose stock solution

5.3.4.2.1 Composition

Lactose	5,0 g
Water up to	50 ml

5.3.4.2.2 Preparation

Dissolve the lactose in the water. Sterilize through a 0,2 µm filter (6.12) into a sterile test tube (6.13).

5.3.4.3 Complete medium

5.3.4.3.1 Composition

Basic medium (5.3.3.1)	95,0 ml
Lactose stock solution (5.3.4.2)	5,0 ml

5.3.4.3.2 Preparation

Immediately before use, melt the M17 agar (5.3.3.1) in a boiling water bath (6.6). Cool it in a water bath (6.6) to between 44 °C and 47 °C. Aseptically add 5,0 ml of lactose stock solution (5.3.4.2) to 95,0 ml of M17 agar (5.3.3.1). Mix very carefully while avoiding gas bubbles.

Pour 15 ml to 20 ml of prepared medium into Petri dishes (6.10). Allow the medium to cool. Solidify by placing the Petri dishes with the lids in place on a cool horizontal surface.

Before use, dry the agar surface in accordance with ISO/TS 11133-1.

The prepared M17-lactose agar plates may be stored in an airtight plastic bag in the dark and held between 2 °C and 8 °C for up to 2 weeks.

Test agar plates for microbial contamination in accordance with ISO/TS 11133-2.