



BSI Standards Publication

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

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National foreword

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The UK participation in its preparation was entrusted to Technical Committee AW/5, Chemical analysis of milk and milk products.

A list of organizations represented on this committee can be obtained on request to its secretary.

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223**

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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 bifidobactéries 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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

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) <sup>a</sup>	1,0 ml
Dipotassium hydrogen phosphate (K <sub>2</sub> HPO <sub>4</sub> )	2,0 g
Sodium acetate trihydrate (NaCH <sub>3</sub> CO <sub>2</sub> ·3H <sub>2</sub> O)	5,0 g
Diammonium citrate [(NH <sub>4</sub> ) <sub>2</sub> HC <sub>6</sub> H <sub>5</sub> O <sub>7</sub> ]	2,0 g
Magnesium sulfate heptahydrate (MgSO <sub>4</sub> ·7H <sub>2</sub> O)	0,2 g
Manganese sulfate tetrahydrate (MnSO <sub>4</sub> ·4H <sub>2</sub> O)	0,05 g
Agar	10 g to 15 g <sup>b</sup>
Water up to	1 000 ml <sup>c</sup>
<p>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.</p> <p>b Depending on the gel strength of the agar.</p> <p>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.</p>	

#### 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  $6,35 \pm 0,2$  with dilute hydrochloric acid or dilute sodium hydroxide before autoclaving. After autoclaving, the pH range of the MRS agar medium should be  $6,2 \pm 0,2$  at 25 °C. Distribute the medium in portions of  $100 \text{ ml} \pm 1 \text{ ml}$  into bottles (6.8) of capacity 150 ml or in portions of  $200 \text{ ml} \pm 2 \text{ ml}$  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 <sub>6</sub> H <sub>8</sub> O <sub>6</sub> )	0,5 g
Magnesium sulfate heptahydrate (MgSO <sub>4</sub> ·7H <sub>2</sub> O)	0,25 g
Disodium glycerophosphate (C <sub>3</sub> H <sub>7</sub> PO <sub>6</sub> Na <sub>2</sub> ·5H <sub>2</sub> O)	19,0 g
Agar	10 g to 15 g <sup>a</sup>
Water up to	950 ml
<sup>a</sup> 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  $7,35 \pm 0,2$  with dilute hydrochloric acid or dilute sodium hydroxide before autoclaving. After autoclaving, the pH range of the M17 agar medium should be  $7,2 \pm 0,2$  at 25 °C. Distribute the medium in portions of  $95 \text{ ml} \pm 1 \text{ ml}$  into bottles (6.8) of capacity 150 ml or in portions of  $190 \text{ ml} \pm 2 \text{ ml}$  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.



### 5.3.5 Elliker agar

#### 5.3.5.1 Composition

Pancreatic digest of casein	20,0 g
Yeast extract (dried)	5,0 g
Gelatin	2,5 g
Dextrose	5,0 g
Lactose	5,0 g
Sucrose	5,0 g
Sodium chloride (NaCl)	4,0 g
Sodium acetate trihydrate (Na CH <sub>3</sub> CO <sub>2</sub> ·3H <sub>2</sub> O)	1,5 g
Ascorbic acid (C <sub>6</sub> H <sub>8</sub> O <sub>6</sub> )	0,5 g
Agar	10 g to 15 g <sup>a</sup>
Water up to	1 000 ml
<sup>a</sup> Depending on the gel strength of the agar.	

#### 5.3.5.2 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  $6,95 \pm 0,2$  with dilute hydrochloric acid or dilute sodium hydroxide before autoclaving. After autoclaving, the pH range of the MRS agar medium should be  $6,8 \pm 0,2$  at 25 °C. Distribute the medium in portions of  $100 \text{ ml} \pm 1 \text{ ml}$  into bottles (6.8) of 150 ml capacity or in portions of  $200 \text{ ml} \pm 2 \text{ ml}$  into bottles (6.8) of 250 ml capacity.

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 Elliker agar (5.3.5.1) in a boiling water bath (6.6) while mixing 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 Elliker 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.

### 5.3.6 IST medium

#### 5.3.6.1 Composition

Hydrolysed casein	11,0 g
Peptone	3,0 g
Glucose	2,0 g
Sodium chloride	3,0 g
Soluble starch	1,0 g
Disodium hydrogenphosphate	2,0 g
Sodium acetate	1,0 g
Magnesium glycerophosphate	0,2 g
Calcium gluconate	0,1 g
Cobalt(II) sulfate	0,001 g
Copper(II) sulfate	0,001 g
Zinc sulfate	0,001 g
Iron(II) sulfate	0,001 g
Manganese(II) chloride	0,002 g
Menadione	0,001 g
Cyanocobalamin	0,001 g
L-Cysteine hydrochloride	0,02 g
L-Tryptophan	0,02 g
Pyrodoxine	0,003 g
Pantothenate	0,003 g
Nicotinamide	0,003 g
Biotin	0,000 3 g
Thiamine	0,000 04 g
Adenine	0,01 g
Guanine	0,01 g
Xanthine	0,01 g
Uracil	0,01 g
Water up to	1 000 ml <sup>a</sup>
a When using hand-made microdilution plates (8.4.5.1), the IST medium should be prepared at twice the concentration by only adding water up to 500 ml.	

#### 5.3.6.2 Preparation

Suspend the ingredients in the water. Mix the suspension well until complete dissolution. Distribute the medium in portions of 100 ml ± 1 ml into bottles (6.8) of 150 ml capacity or in portions of 200 ml ± 2 ml into bottles (6.8) of 250 ml capacity.

Sterilize in the autoclave (6.5) at 121 °C for 15 min. If needed, adjust the pH (6.7) so that, after sterilization, it is 7,4 ± 0,2. If the medium is to be used immediately, cool it in a water bath (6.6) to 32 °C.



The prepared IST medium (5.3.6.1) may be stored in the dark and held between 2 °C and 8 °C for up to 2 weeks with the cap tightened.

Test the medium for microbial contamination in accordance with ISO/TS 11133-2.

### 5.3.7 IST lactose medium

IST lactose medium consists of IST medium and 10,0 g lactose per litre of medium.

#### 5.3.7.1 Composition

IST medium (5.3.6)	90,0 ml
Lactose stock solution (5.3.4.2)	10,0 ml

#### 5.3.7.2 Preparation

Prepare the IST medium (5.3.6) and the lactose stock solution (5.3.4.2). Aseptically mix the prepared medium and stock solution in the proportions specified in 5.3.7.1 in a sterilized bottle (6.8) of 150 ml capacity. If using hand-made microdilution plates (8.4.5.1), prepare the IST with the mass fraction of 1 % lactose medium at an IST concentration twice higher (see footnote to the composition table in 5.3.6.1).

The prepared IST lactose medium may be stored with the cap tightened in the dark and held between 2 °C and 8 °C for up to 2 weeks. Test the medium for microbial contamination in accordance with ISO/TS 11133-2.

### 5.3.8 LSM medium

LSM medium consists of 90 % IST medium and 10 % MRS medium.

#### 5.3.8.1 Composition

IST medium (5.3.6)	90,0 ml
MRS medium (5.3.1) without agar	10,0 ml

#### 5.3.8.2 Preparation

Separately prepare the IST medium (5.3.6) and MRS medium (5.3.1) without agar. Mix both before autoclaving in the proportions specified in 5.3.8.1. Adjust the pH (6.7) to  $6,85 \pm 0,1$  with dilute hydrochloric acid or dilute sodium hydroxide before autoclaving. After autoclaving, the pH range of the LSM medium should be  $6,7 \pm 0,1$ .

If using hand-made microdilution plates (8.4.5.1), prepare the LSM medium by using a twice higher concentration of IST medium (see footnote to the composition table in 5.3.6.1) and MRS medium (see footnote to the composition table in 5.3.1.1).

Distribute the pH-adjusted medium (5.3.8.1) into bottles (6.8) of 150 ml capacity. 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 28 °C or 37 °C.

The prepared LSM medium may be stored with the cap tightened in the dark and held between 2 °C and 8 °C for up to 2 weeks.

Test the medium for microbial contamination in accordance with ISO/TS 11133-2.



### 5.3.9 LSM-cysteine medium (LSM-Cys medium)

LSM-Cys medium consists of LSM medium (5.3.8) with addition of 0,3 g L-cysteine per litre of medium.

#### 5.3.9.1 Composition

LSM medium (5.3.8)	100,0 ml
L-Cysteine hydrochloride	0,03 g

#### 5.3.9.2 Preparation

Add 0,03 g of L-cysteine hydrochloride into 100 ml of LSM medium that has not been autoclaved (5.3.8). Mix well until complete dissolution. Adjust the pH (6.7) to  $6,85 \pm 0,1$  with dilute hydrochloric acid or dilute sodium hydroxide before autoclaving. After autoclaving, the pH of the LSM-Cys medium should be  $6,7 \pm 0,1$ .

If using hand-made microdilution plates (8.4.5.1), prepare the LSM-Cys medium at a concentration twice higher (see 5.3.8.2).

Distribute the pH-adjusted medium (5.3.9) into bottles (6.8) of 150 ml capacity.

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 37 °C.

The prepared LSM-Cys medium may be stored with the cap tightened in the dark and held between 2 °C and 8 °C for up to 1 week.

Test the medium for microbial contamination in accordance with ISO/TS 11133-2.

### 5.3.10 Saline

Saline consists of a 0,85 % mass fraction of sodium chloride in water.

#### 5.3.10.1 Composition

Sodium chloride	0,85 g
Water up to	100 ml

#### 5.3.10.2 Preparation

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

## 6 Apparatus and glassware

**6.1 General.** Sterilize as specified in ISO 7218 all equipment that comes into contact with the test sample, the diluent, the dilutions or the culture medium. The glassware shall be resistant to repeated sterilization.

Usual microbiological laboratory equipment required for the preparation of test samples and dilutions as specified in ISO 7218 and in particular the following.

**6.2 Incubators,** capable of maintaining temperatures of  $28\text{ °C} \pm 1\text{ °C}$ ,  $32\text{ °C} \pm 1\text{ °C}$ , and  $37\text{ °C} \pm 1\text{ °C}$ , respectively.



**6.3 Anaerobic incubators**, capable of maintaining temperatures of  $28\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ ,  $32\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ , and  $37\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ , respectively or **anaerobic culture jars**, providing an atmosphere of an approximate volume fraction of 9 % to 13 % of carbon dioxide.

**6.4 Colony-counting equipment**, see ISO 7218.

**6.5 Autoclave**, capable of maintaining a temperature of  $121\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ .

**6.6 Water baths**, capable of maintaining all temperatures between  $28\text{ }^{\circ}\text{C}$  and  $55\text{ }^{\circ}\text{C}$  and at boiling point.

**6.7 pH meter**, temperature compensated, accurate to  $\pm 0,1$  pH unit at  $25\text{ }^{\circ}\text{C}$ .

**6.8 Bottles or flasks**, of capacity 150 ml or 250 ml and with suitable caps or stoppers (to hold the culture medium).

**6.9 Pipettes**, sterile, calibrated for bacteriological use, capable of delivering  $0,05\text{ ml} \pm 0,002\text{ ml}$ ,  $0,1\text{ ml} \pm 0,02\text{ ml}$ , ISO 7550[5],  $1,0\text{ ml} \pm 0,02\text{ ml}$  and  $10\text{ ml} \pm 0,2\text{ ml}$ , ISO 648[1] class A.

**6.10 Petri dishes**, sterile, made of clear colourless glass or plastic, of diameter 90 mm, of minimal internal depth 10 mm. The bottoms shall have no irregularities that may interfere with counting colonies.

**6.11 Spatula**, sterile, made of glass or metal.

**6.12 Filtration equipment**, sterile, with cellulose acetate membrane filters of nominal size of openings  $0,2\text{ }\mu\text{m}$ .

**6.13 Test tubes**, sterile, of capacity 5 ml, 20 ml or 50 ml with suitable sealing caps.

**6.14 Spreader**, sterile, made of glass, metal or plastic.

**6.15 Drying cabinet**, oven or incubator (e.g. a laminar airflow cabinet), ventilated (for drying the surface of agar plates).

**6.16 Cryotubes**, sterile, of capacity 2 ml.

**6.17 Spectrophotometer**, capable of measuring optical density at 625 nm.

**6.18 Microdilution plates**, 96 well standard plates.

## 7 Sampling

A representative sample should be sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 | IDF 50[2].

## 8 Procedure

### 8.1 Propagation

**8.1.1** Prior to the susceptibility assay, propagate a strain to be tested on the agar medium under the recommended incubation conditions (see Table 1). If a strain does not grow well in the recommended medium or conditions, another carbon source may be added or the growth condition changed to facilitate growth.



**Table 1 — Growth conditions for propagation**

Species	Medium (agar)	Temperature °C	Atmosphere	Incubation time h
<i>Bifidobacterium</i> spp.	MRS-Cys	37	Anaerobic	24 to 48
<i>Lactobacillus brevis</i>	MRS	28	Anaerobic or ambient	16 to 24
<i>Lactobacillus plantarum/pentosus</i>	MRS	28	Anaerobic or ambient	16 to 24
<i>Lactobacillus sakei</i>	MRS	28	Anaerobic or ambient	16 to 24
<i>Lactobacillus delbrueckii</i> group	MRS	37	Anaerobic	16 to 24
Other lactobacilli	MRS	37	Anaerobic or ambient	16 to 24
<i>Lactococcus lactis</i>	M17-lactose or Elliker	32	Anaerobic or ambient	16 to 24
<i>Streptococcus thermophilus</i>	M17-sucrose or Elliker	37	Anaerobic or ambient	16 to 24

**8.1.2** A strain stored as frozen can first be recovered in the liquid medium of the growth condition in Table 1 prior to its propagation on the agar medium.

## 8.2 Quality control strains and testing

- Lactobacillus plantarum* ATCC® 14917<sup>TM1)</sup> (at 28 °C).
- Lactobacillus paracasei* ATCC® 334<sup>TM1)</sup> (at 37 °C).
- Lactococcus lactis* ATCC® 19435<sup>TM1)</sup> (at 32 °C).
- Streptococcus thermophilus* LMG 18311<sup>1)</sup> (at 37 °C).
- Bifidobacterium longum* ATCC® 15707<sup>TM1)</sup> (at 37 °C).

The following quality control testing procedures are possibilities for testing strains.

When testing a strain of interest, examine a quality control strain which belongs to the same species of the test strain each time:

- for lactobacilli to be cultivated at 28 °C, use *Lactobacillus plantarum* ATCC® 14917<sup>TM1)</sup>;
- for lactobacilli to be cultivated at 37 °C, use *Lactobacillus paracasei* ATCC® 334<sup>TM1)</sup>;
- for *Bifidobacterium* spp., use *Bifidobacterium longum* ATCC® 15707<sup>TM1)</sup>.

## 8.3 Growth conditions for antibiotic susceptibility test

See Table 2.

1) 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.



**Table 2 — Growth conditions for antibiotic susceptibility test**

Organism	Medium	Temperature °C	Atmosphere	Incubation time h
<i>Bifidobacterium</i> spp.	LSM-Cys	37	Anaerobic	48
<i>Lactobacillus brevis</i>	LSM	28	Anaerobic	48
<i>Lactobacillus plantarum/pentosus</i>	LSM	28	Anaerobic	48
<i>Lactobacillus sakei</i>	LSM	28	Anaerobic	48
Other lactobacilli	LSM	37	Anaerobic	48
<i>Lactococcus lactis</i>	IST	32	Anaerobic	48
<i>Streptococcus thermophilus</i>	IST-lactose	37	Anaerobic	48

For testing *Bifidobacterium* spp., it is recommended to pre-reduce the medium under anaerobic conditions one day prior to testing.

## 8.4 Preparation of microdilution plate

### 8.4.1 Concentration range of antibiotics

The concentration range of antibiotics is listed in Table 3.

**Table 3 — Concentration range of antibiotics**

Antibiotic	Class	Concentration range µg/ml
Gentamicin	Aminoglycoside	0,5 to 256
Kanamycin	Aminoglycoside	2 to 1 024
Streptomycin	Aminoglycoside	0,5 to 256
Neomycin	Aminoglycoside	0,5 to 256
Tetracycline	Tetracycline	0,125 to 64
Erythromycin	Macrolide	0,016 to 8
Clindamycin	Lincosamide	0,032 to 16
Chloramphenicol	Chloramphenicol	0,125 to 64
Ampicillin	β-Lactam	0,032 to 16
Vancomycin	Glycopeptide	0,25 to 128
Quinupristin and dalfopristin combination	Streptogramin	0,016 to 8
Linezolid	Oxazolidinone	0,032 to 16
Trimethoprim	Dihydrofolate reductase inhibitor	0,125 to 64
Ciprofloxacin	Fluoroquinolone	0,25 to 128
Rifampicin	Rifamycin	0,125 to 64

## 8.4.2 Layout of microdilution plate

### 8.4.2.1 General

The layout of the microdilution plate is shown in Table 4 and Table 5.

**Table 4 — Layout of microdilution plate — Panel 1**

Antibiotic	1 <sup>a</sup>	2	3	4	5	6	7	8	9	10	11	12 <sup>b</sup>
Gentamicin	P	0,5	1	2	4	8	16	32	64	128	256	N
Kanamycin	P	2	4	8	16	32	64	128	256	512	1024	N
Streptomycin	P	0,5	1	2	4	8	16	32	64	128	256	N
Tetracycline	P	0,125	0,25	0,5	1	2	4	8	16	32	64	N
Erythromycin	P	0,016	0,032	0,063	0,125	0,25	0,5	1	2	4	8	N
Clindamycin	P	0,032	0,063	0,125	0,25	0,5	1	2	4	8	16	N
Chloramphenicol	P	0,125	0,25	0,5	1	2	4	8	16	32	64	N
Ampicillin	P	0,032	0,063	0,125	0,25	0,5	1	2	4	8	16	N
<p>a Positive control well without the antibiotic, but with the test strain and the medium containing solvent which is used to dissolve the antibiotic at the highest concentration.</p> <p>b Negative control well without the test strain and the antibiotic, but with the medium.</p>												

**Table 5 — Layout of microdilution plate — Panel 2**

Antibiotic	1 <sup>b</sup>	2	3	4	5	6	7	8	9	10	11	12 <sup>c</sup>
Neomycin	P	0,5	1	2	4	8	16	32	64	128	256	N
Vancomycin	P	0,25	0,5	1	2	4	8	16	32	64	128	N
Quinupristin and dalfopristin combination or virginiamycin <sup>a</sup>	P	0,016	0,032	0,064	0,125	0,25	0,5	1	2	4	8	N
Linezolid	P	0,032	0,064	0,125	0,25	0,5	1	2	4	8	16	N
Trimethoprim	P	0,125	0,25	0,5	1	2	4	8	16	32	64	N
Ciprofloxacin	P	0,25	0,5	1	2	4	8	16	32	64	128	N
Rifampicin	P	0,125	0,25	0,5	1	2	4	8	16	32	64	N
<p>a The microdilution plate combined with a quinupristin and dalfopristin combination is difficult to acquire. Instead, pre-made microdilution plates with virginiamycin can be used, which is a similar antibiotic used, for example, in veterinary practice. Virginiamycin was also used in the interlaboratory trial (Annex A).</p> <p>b Positive control well without the antibiotic, but with the test strain and the medium containing solvent which is used to dissolve the antibiotic at the highest concentration.</p> <p>c Negative control well without the test strain and the antibiotic, but with the medium.</p>												



#### 8.4.2.2 Dilution of antibiotic

For the microdilution plate assay, first prepare a stock solution at the recommended concentration of an antibiotic. When preparing the stock solution, adjust the mass of the antibiotic for the potency.

Calculate the potency,  $w_p$ , by using the equation:

$$w_p = \frac{w_{as} \left( \frac{w_{ac}}{1 - w_{H_2O}} \right)}{w_{ac}}$$

where

$w_{as}$  is the assay purity;

$w_{ac}$  is the active fraction, which is the free acid or base and not the salt — use a minimum amount of solvent to solubilize the antibiotic powder;

$w_{H_2O}$  is the water content due to any hydrate present.

Use one of the following equations to calculate the amount, as mass,  $m$ , or volume,  $V$ , of powder or diluent needed for a stock solution:

$$m = \frac{V \rho}{w_p}$$

$$V = \frac{mw_p}{\rho}$$

An example using gentamicin is shown in Table 6.

Table 6 — Stepwise preparation of antibiotic working solution					
Step	Solution source	Concentration	Source volume	Water (5.1)	Double strength solution
		µg/ml	ml	ml	µg/ml
1	Stock solution	5 120	1	9	512
2	Step 1	512	1	1	256
3	Step 1	512	1	3	128
4	Step 1	512	1	7	64
5	Step 4	64	1	1	32
6	Step 4	64	1	3	16
7	Step 4	64	1	7	8
8	Step 7	8	1	1	4
9	Step 7	8	1	3	2
10	Step 7	8	1	7	1





#### 8.4.2.3 Preparation of final solution

Dilute 1 ml of each solution source mentioned in Table 6, steps 1 to 10 with water (5.1).

#### 8.4.2.4 Distribution of the final solution

Dispense, for step 1 to step 10 (8.4.2.3), 50 µl of the double strength solution obtained into a well of the microdilution plates (6.18) listed in Table 4 or Table 5.

#### 8.4.2.5 Storage of microdilution plates

If the microdilution plates are not used immediately, they may be sealed and stored below –20 °C until needed.

The antibiotic in frozen microdilution plates usually remains stable for several months. Check the performance of the microdilution plates with a control strain.

#### 8.4.3 Preparation of microdilution plates

VetMIC<sup>TM2)</sup> antibiotic pre-coated microdilution plates can be used. Applying a 100 µl solution (8.4.5.2) gives the defined antibiotic concentration indicated in the instruction.

The plates can be kept for 2 years at room temperature.

#### 8.4.4 Preparation of inoculum

**8.4.4.1** Pick up individual colonies from an agar plate (8.1). Suspend the colonies obtained in a sterile glass or plastic culture tube containing 2 ml to 5 ml of sterile saline.

In the case of *Bifidobacterium* spp., suspend the obtained colonies in pre-reduced LSM-Cys medium.

**8.4.4.2** Suspend colonies until the solution turbidity reaches McFarland standard 1 or an optical density at 625 nm of 0,16 to 0,2 by spectrophotometer (6.17). The suspended solution corresponds to approximately  $3 \times 10^8$  cfu/ml.

For *S. thermophilus* and *Lactob. delbrueckii*, the McFarland 1 suspension of some strains might be lower than  $3 \times 10^8$  cfu/ml. In this case, a McFarland turbidity corresponding to approximately  $3 \times 10^8$  cfu/ml should be used.

#### 8.4.5 Dilution of the bacterial suspension with recommended medium

##### 8.4.5.1 Hand-made microdilution plates

Dilute the bacterial suspension 500 times in the recommended medium (Table 2). The medium is diluted twice with the antibiotic solution and, therefore, should have twice the concentration of bacteria compared to 8.4.5.2 (see 5.3.8.2).

Distribute the diluted bacterial suspension after its preparation within 30 min.

##### 8.4.5.2 Pre-coated microdilution plates

Dilute the bacterial suspension 1 000 times in the recommended medium.

Distribute the diluted bacterial suspension after its preparation within 30 min.

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2) VetMIC<sup>TM</sup> is the trade name of a product supplied by the National Veterinary Institute, Uppsala, Sweden. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.



## 8.4.6 Distribution of bacterial suspension into the well of the microdilution plate

### 8.4.6.1 Hand-made microdilution plates

When using frozen-stored plates, thaw the frozen antibiotic solution under anaerobic conditions (6.3) immediately before use.

Distribute 50 µl of diluted suspension (8.4.5.1) into each well (approximately  $3 \times 10^4$  cfu/well) of the microdilution plate (8.4.2).

Incubate the plates under the conditions mentioned in Table 2. When using anaerobic jars, pile the plates with a lid between every plate to generate a homogeneous environment in the jar.

### 8.4.6.2 Pre-coated microdilution plates

When using pre-coated plates (8.4.3), distribute 100 µl of the diluted suspension (8.4.5.2) into each well (approximately  $3 \times 10^4$  cfu/well) of the microdilution plate (8.4.3).

Incubate the plates under the conditions mentioned in Table 2. When using anaerobic jars, pile the plates with a lid between every plate for generating a homogeneous environment in the jar.

## 8.4.7 Reading of MIC

Read the MICs visually after 48 h incubation.

Following incubation, check the negative control wells for presence of visible growth. If contamination is noted, reject all data generated from the strain concerned.

**NOTE** The absence of growth in positive control wells implies that the tested strain is sensitive to the solvent which is used to dissolve the antibiotic (e.g. citric acid included in the buffer used for ampicillin). In such cases, reading of the MIC for this particular antibiotic is not relevant.

If negative and positive controls are checked and approved, growth is determined visually for each antibiotic by comparing with the positive control.

Preferably place the panel on top of a viewing device, in the form of a rack with an enlarging mirror. A bench lamp giving indirect light facilitates reading.

Bacterial growth is easily detected in the mirror as a pellet at the bottom of the well. Discard any series of wells where discontinuity in growth is observed (e.g. growth at 16 µg/ml and 64 µg/ml, but not at 32 µg/ml).

The end point is defined as the lowest antibiotic concentration at which there is no visual growth. Report this concentration as the MIC of that antibiotic for that particular strain.

Trimethoprim antagonists in the medium might allow some slight growth. Therefore, read the end point at the concentration in which there is 80 % or greater reduction in growth as compared to the control.

If a strain does not grow well in the recommended medium or condition, another carbon source might be added or the growth condition changed to facilitate growth.

However, it is necessary to prove that the addition of another carbon source or changing the growth condition does not drastically (more or less than twofold) affect the evaluation of MICs by examining the control strain and other strains of the same species.

## 8.4.8 Determination of epidemiological cut-off value

In the ACE-ART project, MICs served to determine the epidemiological cut-off value rather than the clinical breakpoint of LAB species.

Following the EUCAST definition, an epidemiological cut-off value serves to distinguish between wild type organisms (free of acquired and mutational resistance mechanisms to the tested agent) and non-wild type organisms (harbouring an acquired or mutational resistance mechanism to the tested agent).



## 9 Expression of results

Express minimum inhibitory concentrations in micrograms per millilitre.

## 10 Precision

### 10.1 Interlaboratory test

The values for repeatability and reproducibility limit were derived from the results of interlaboratory tests carried out in accordance with CLSI M23-A2[17]. Details of the interlaboratory test on the precision of the method are summarized in Annex A.

The values are expressed for the 95 % probability level and may not be applicable to concentration ranges and matrices other than those given.

### 10.2 Repeatability

The absolute difference between two individual single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than the values shown in Annex A for each strain.

### 10.3 Reproducibility

The absolute difference between two individual single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than the values shown in Annex A for each strain.

## 11 Test report

The test report shall contain at least the following information:

- a) all information necessary for the complete identification of the strain;
- b) the sampling method used, if known;
- c) the test method used, with reference to this International Standard (ISO 10932□IDF 223);
- d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incident which may have influenced the test result(s);
- e) the test result(s) obtained and if the repeatability has been checked, the final quoted result obtained.

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## Annex A (informative)

### Interlaboratory trial

#### A.1 General

An international collaborative trial involved eight laboratories in seven countries. The study was split into three parts and a maximum of seven laboratories participated in each part.

The trial was organized in accordance with CLSI M23-A2<sup>[17]</sup>, which was developed specifically for *in vitro* susceptibility testing. It is stated in 6.2.2, “To monitor the performance of *in vitro* dilution tests, it is necessary to establish the limits of acceptable variability in MICs using appropriate QC strains”. Furthermore, “To establish quality control (QC) limits for dilution tests, results from at least seven laboratories from seven separate and distinct institutions should be analyzed” and “For anaerobic bacteria and other special organisms, three lots of agar or broth (in accordance with the applicable standards) should be from different manufacturers if possible.”

For interpretation of the results, it is stated: “Ideally, at least 95 % of the values should be included in the proposed range and will include mode  $\pm 1$  log. A three-dilution range is preferred; however a four-dilution range may sometimes be needed.”

Specifically for the performance of the tests, it is stated “Each of the seven laboratories should test ten replicates of each QC strain on each media lot. Each replicate should use individually prepared inoculum suspensions. The study should be conducted over a minimum of three days with a maximum of four replicates per day resulting in 70 data points for each individual media lot and 210 data points in total”.

The interlaboratory study included the five control strains specified in 8.2. The LSM medium consists of a mixture of two broths (ISO-sensitest and MRS).

ISO-sensitest is a well-defined medium for susceptibility testing, whereas MRS is made to various recipes by different manufacturers. In order to limit the total number of tests, only three different brands of MRS were tested (Oxoid, BD, AES)<sup>3)</sup> whereas only ISO-sensitest from Oxoid was used. Additionally, 25 strains were tested in duplicate in seven laboratories to verify that the method is appropriate for susceptibility testing of bifidobacteria and non-enterococcal lactic acid bacteria.

See Table A.1.

#### A.2 Results

##### A.2.1 General

One laboratory consistently reported higher linezolid MICs than the other laboratories.

In Tables A.2 to A.12, the emboldened entries indicate data within the QC range and the grey fields indicate either below range (no growth) or above range (growth in all wells).

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3) This information is given for the convenience of users of this International Standard, and does not constitute an endorsement by ISO or IDF of these products.









Table A.3 — Results for five *Lactobacillus paracasei* strains

Antibiotic	Microdilution plate														Sum	No growth or contaminated	At mode	Within QC range %
	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128	256				
Gentamicin					1	19	23	16	7	4					70	0	33	83
Kanamycin									1	11	29	16	11	2	70	0	41	80
Streptomycin								1	8	38	21	2			70	0	54	96
Tetracycline						9	60	1							70	0	86	100
Erythromycin		2	48	18	2										70	0	59	100
Clindamycin		10	42	18											70	0	60	100
Chloramphenicol								40	30						70	0	57	100
Ampicillin						19	31	20							70	0	44	71
Vancomycin														70	70	0		— <sup>a</sup>
Neomycin						2	19	19	11	11	8				70	0	27	70
Virginiamycin					11	56	3								70	0	80	96
Ciprofloxacin					1	25	37	7							70	0	53	99
Linezolid						2	39	19		6	4				70	0	36	86
Rifampicin			7	36	23	4									70	0	51	100
Trimethoprim			4	18	25	16	3				2		2		70	0	336	— <sup>a</sup>
a Not applicable.																		

Table A.4 — Results for three *Lactobacillus rhamnosus* strains

Antibiotic	Microdilution plate														Sum	No growth or contaminated	At mode	Within QC range %
	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128	256				
Gentamicin	0	0	0	0	1	0	12	20	9	0	0	0	0	0	42	0	40	80
Kanamycin	0	0	0	0	0	0	0	0	0	0	14	26	2	0	42	0	62	90
Streptomycin	0	0	0	0	0	0	0	0	13	26	3	0	0	0	42	0	62	94
Tetracycline	0	0	0	0	0	17	22	3	0	0	0	0	0	0	42	0	52	60
Erythromycin	0	1	16	22	3	0	0	0	0	0	0	0	0	0	42	0	52	100
Clindamycin	0	0	0	10	22	10	0	0	0	0	0	0	0	0	42	0	52	33
Chloramphenicol	0	0	0	0	0	0	0	11	28	3	0	0	0	0	42	0	67	93
Ampicillin	0	0	0	0	0	6	23	13	0	0	0	0	0	0	42	0	55	76
Vancomycin	0	0	0	0	0	0	0	0	0	0	0	0	0	42	42	0		— <sup>a</sup>
Neomycin	0	0	0	0	0	0	2	13	19	8	0	0	0	0	42	0	45	77
Virginiamycin	0	0	0	0	0	16	26	0	0	0	0	0	0	0	42	0	62	61
Ciprofloxacin	0	0	0	0	0	17	22	3	0	0	0	0	0	0	42	0	52	60
Linezolid	0	0	0	0	0	0	25	11	0	4	2	0	0	0	42	0	60	83
Rifampicin	0	0	1	26	12	3	0	0	0	0	0	0	0	0	42	0	62	89
Trimethoprim	0	0	0	0	0	0	1	1	3	8	1	5	23	0	42	0		— <sup>a</sup>
a Not applicable.																		

Table A.5 — Results for two *Lactobacillus acidophilus* strains

Antibiotic	Microdilution plate														Sum	No growth or contaminated	At mode	Within QC range %
	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128	256				
Gentamicin					2	7	11	7	1						28	0	39	89
Kanamycin									1	6	14	7			28	0	50	96
Streptomycin						2	7	14	4	1					28	0	50	18
Tetracycline				1	4	16	7								28	0	57	82
Erythromycin	2	3	13	9	1										28	0	46	93
Clindamycin			2	2	11	12	1								28	0	43	14
Chloramphenicol							2	23	3						28	0	82	100
Ampicillin				2	4	16	6								28	0	57	93
Vancomycin					21	7									28	0	75	— <sup>a</sup>
Neomycin					2	2	11	5	7	1					28	0	39	82
Virginiamycin					6	22									28	0	79	100
Ciprofloxacin											2	16	10		28	0	57	0
Linezolid							4	20			4				28	0	71	86
Rifampicin				4	11	6	7								28	0	39	75
Trimethoprim												1	27		28	0	96	— <sup>a</sup>
a Not applicable.																		

### A.2.3 *Bifidobacterium longum* ATCC® 15707<sup>TM</sup><sub>1)</sub>

Six laboratories participated in the testing of this strain, which is fewer than the requirement of CLSI M23-A2[17]. However, for several of the antibiotics, the results suggest that quality control ranges can be established.

*B. longum* ATCC® 15707<sup>TM</sup><sub>1)</sub> was more difficult to test than the other strains. For several antibiotics, a laboratory did not produce results similar to other laboratories. One laboratory had MICs approximately twofold lower for aminoglycosides than other laboratories, and those MIC data have been excluded from the aminoglycoside data, leaving 150 datapoints for these four antibiotics (gentamicin, kanamycin, neomycin, and streptomycin).

The MICs for ampicillin were too low for one laboratory and these results were excluded.

Finally, the results for erythromycin from one laboratory were excluded due to MICs which were too low. The MICs of trimethoprim covered a wide range and no quality control parameters could be suggested, which indicates that the method is not applicable for this antibiotic.

The concentrations of clindamycin and rifampicin on the pre-made plates are at the low range of the MICs, which means that in some experiments there is no growth even at the lowest concentration.



Table A.6 — Result for *Bifidobacterium longum* ATCC® 15707<sup>TM1)</sup>

Antibiotic	Microdilution plate																Sum	At mode
	0,02	0,03	0,06	0,12	0,3	0,5	1	2	4	8	16	32	64	128	256	512		
Gentamicin <sup>a</sup>									15	31	98	6					150	65
Kanamycin <sup>a</sup>													29	39	65	17	150	43
Streptomycin <sup>a</sup>										12	64	73	1				150	49
Tetracycline						22	152	6									180	84
Erythromycin <sup>a,b</sup>		25	49	70	5												149	47
Clindamycin		128	46	6													180	71
Chloramphenicol						9	53	118									180	66
Ampicillin <sup>a</sup>				1	54	80	15										150	53
Vancomycin						47	131	2									180	73
Neomycin <sup>a</sup>										12	34	57	46	1			150	38
Virginiamycin			14	108	57		1										180	60
Ciprofloxacin									1	166	13						180	92
Linezolid					3	82	65	1	6	23							180	46
Rifampicin				72	81	27											180	45
Trimethoprim									9	27	55	77	12				180	43
a One dataset missing from a laboratory (not the same laboratory for all antibiotics).																		
b One dataset missing for erythromycin.																		

A further five *B. longum* strains were tested. Most results were within the QC range, but there were several problems reported with contamination and no growth. For most antibiotics, a few results were outside the normal range indicating that the method should be used with caution when testing bifidobacteria.

Table A.7 — Result for five *Bifidobacterium longum* isolates

Antibiotic	Microdilution plate																Sum	No growth or contaminated	At mode	Within QC range
	0,02	0,03	0,06	0,12	0,3	0,5	1	2	4	8	16	32	64	128	256	512				%
Gentamicin							1	2	4	8	19	22	5	1			62	8	31	76
Kanamycin										2	1	5	12	19	12	11	62	8	27	77
Streptomycin								2		6	11	19	4	6		14	62	8	27	57
Tetracycline					7	7	32	8	6		2						62	8	46	67
Erythromycin	11	15	14	19	1	2											62	8	27	70
Clindamycin		29	23	6	3	1											62	8	41	83
Chloramphenicol				1	1	10	38	10	2								62	8	54	86
Ampicillin		1	2	3	20	22	13	1									62	8	31	79
Vancomycin						48	14										62	8	69	89
Neomycin								1	2	4	19	17	4	10	5		62	8	27	63
Virginiamycin				35	27												62	8	50	89
Ciprofloxacin									6	29	7	13	7				62	8	41	60
Linezolid				3	7	32	10	2	8								62	8	46	70
Rifampicin				26	30	3	3										62	8	43	84
Trimethoprim										8	24	7	7	16			62	8	34	

#### A.2.4 *Lactococcus lactis* ATCC® 19435<sup>TM1)</sup>

For all antibiotics except trimethoprim, quality control ranges can be proposed although they are based on 70 datapoints. The MICs for trimethoprim are above range.

One laboratory had contamination in wells with vancomycin and ampicillin, and these results were excluded. The concentrations of gentamicin, kanamycin, neomycin, and vancomycin on the pre-made plates are at the low range of the MICs, which means that in some experiments there is no growth even at the lowest concentration.



**Table A.8 — Result for *Lactococcus lactis* ATCC® 19435<sup>TM1)</sup>**

Antibiotic	Microdilution plate													Sum	At mode
	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128		
Gentamicin					42	17	10	1						70	60
Kanamycin							51	15	3	0	1			70	73
Streptomycin							3	15	48	4				70	69
Tetracycline					29	37	4							70	53
Erythromycin			16	50	4									70	71
Clindamycin				19	51									70	73
Chloramphenicol								37	30	3				70	53
Ampicillin				25	16	20								61	41
Vancomycin				5	54	2								61	89
Neomycin					18	30	10	12						70	43
Virginiamycin					1	7	61	1						70	87
Ciprofloxacin							1	14	44	11				70	63
Linezolid							42	18		10				70	60
Rifampicin							2	8	24	36				70	51

Five additional *Lactoc. lactis* strains were tested and all results shown in Table A.9 were acceptable.

**Table A.9 — Result for five *Lactococcus lactis* isolates**

Antibiotic	Microdilution plate													Sum	No growth or contaminated	At mode	Within QC range
	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128				%
Gentamicin					17	24	21	8						70	0	34	89
Kanamycin							21	24	15	10				70	0	34	86
Streptomycin						1	11	14	8	22	12	2		70	0	31	79
Tetracycline			1	19	40	9	1							70	0	57	71
Erythromycin	2	3	36	29										70	0	51	93
Clindamycin		11	26	30	3									70	0	43	47
Chloramphenicol							27	36	7					70	0	51	100
Ampicillin				15	37	16	2							70	0	53	97
Vancomycin				5	61	4								70	0	87	100
Neomycin					10	8	19	18	11	4				70	0	27	79
Virginiamycin					7	10	39	14						70	0	56	90
Ciprofloxacin						1	6	43	17	3				70	0	61	90
Linezolid					2	13	34	11	2	6	2			70	0	49	83
Rifampicin							3	8	14	24	21			70	0	34	96

## A.2.5 *Streptococcus thermophilus* LMG 18311<sup>1)</sup>

For all antibiotics except trimethoprim, quality control ranges can be proposed although they are based on 70 datapoints. The MICs for trimethoprim are above range.

One laboratory did not report streptomycin MICs.

The concentrations of clindamycin, ampicillin, neomycin, vancomycin, and rifampicin on the pre-made plates are at the low range of the MICs, which means that in some experiments there is no growth even at the lowest concentration.

**Table A.10 — Result for *Streptococcus thermophilus* LMG 18311<sup>1)</sup>**

Antibiotic	Microdilution plate														Sum	At mode
	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128	256		
Gentamicin						35	33	2							70	50
Kanamycin									2	48	20				70	69
Streptomycin <sup>a</sup>							2	29	28	1					60	48
Tetracycline				1	19	35	15								70	50
Erythromycin	2	18	32	17	1										70	46
Clindamycin	11	30	23	6											70	43
Chloramphenicol							21	49							70	70
Ampicillin	21	19	25	5											70	36
Vancomycin				11	59										70	84
Neomycin					3	29	30	8							70	41
Virginiamycin		5	54	11											70	77
Ciprofloxacin						3	50	13	4						70	71
Linezolid <sup>b</sup>						33	26		1	9					69	48
Rifampicin			41	25	4										70	59
<sup>a</sup> One dataset was not reported. <sup>b</sup> One datapoint was not reported.																



Five additional strains of *S. thermophilus* were tested and almost all results were acceptable as shown in Table A.11.

One laboratory reported higher kanamycin and neomycin MICs than the other laboratories.

**Table A.11 — Result for five *Streptococcus thermophilus* isolates**

Antibiotic	Microdilution plate														Sum	No growth or contaminated	At mode	Within QC range %
	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128	256				
Gentamicin					1	12	29	16	5	2					65	5	41	83
Kanamycin								1	11	6	26	15	5	1	65	5	37	61
Streptomycin							14	16	25	10					65	5	36	93
Tetracycline			1	7	21	29	7								65	5	41	91
Erythromycin	9	40	16												65	5	57	80
Clindamycin	42	23													65	5	60	93
Chloramphenicol						3	43	19							65	5	61	89
Ampicillin	13	13	20	12	7										65	5	29	83
Vancomycin				17	44	4									65	5	63	93
Neomycin						4	11	20	16	6	4	3	1		65	5	29	50
Virginiamycin			18	45	2										65	5	64	90
Ciprofloxacin					1	11	35	16	2						65	5	50	91
Linezolid				1	6	45	5		6	2					65	5	64	80
Rifampicin			45	18	2										65	5	64	93

## A.2.6 *Lactobacillus plantarum* ATCC® 14917<sup>TM 1)</sup>

The strain has intrinsic resistance to vancomycin. Quality control ranges can neither be proposed for the aminoglycosides (gentamicin, kanamycin, neomycin, and streptomycin) nor for trimethoprim, as the MIC ranges were too broad for these antibiotics. No differences were observed between the three brands of MRS (data not shown).

**Table A.12 — Result for *Lactobacillus plantarum* ATCC® 14917<sup>TM1)</sup>**

Antibiotic	Microdilution plate															Sum	At mode
	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128	256	512		
Gentamicin						12	23	41	84	20						180	NA
Kanamycin											22	34	85	36	3	180	— <sup>a</sup>
Streptomycin										18	27	94	38	3		180	NA
Tetracycline									28	152						180	84
Erythromycin				44	51	82	3									180	46
Clindamycin					3	84	88	4		1						180	49
Chloramphenicol							1	70	109							180	61
Ampicillin				39	81	58	2									180	45
Vancomycin													180			180	NA
Neomycin						2	19	40	71	34	13	1				180	NA
Virginiamycin						3	174	3								180	97
Ciprofloxacin									1		100	79				180	56
Linezolid						1	32	117		11	19					180	65
Rifampicin						31	114	31	4							180	63
Trimethoprim							17	12	9	7	14	5	116			180	— <sup>a</sup>
Penicillin					7	58	91	24								180	51
<sup>a</sup> Not applicable.																	

### A.3 Quality control parameters

The quality control parameters were set following the recommendations in CLSI M23-A2[17]: “Ideally, at least 95 % of the values should be included in the proposed range and will include mode  $\pm 1$  log. A three-dilution range is preferred; however a four-dilution range may sometimes be needed.”



Table A.13 — Quality control parameters

Antibiotic	<i>Lactobacillus paracasei</i> ATCC® 334 <sup>TM</sup> <sup>a</sup> µg/ml	<i>Lactobacillus plantarum</i> ATCC® 14917 <sup>TM</sup> <sup>a</sup> µg/ml	<i>Bifidobacterium longum</i> ATCC® 15707 <sup>TM</sup> <sup>a</sup> µg/ml	<i>Lactococcus lactis</i> ATCC® 19435 <sup>TM</sup> <sup>a</sup> µg/ml	<i>Streptococcus thermophilus</i> LMG 18311 <sup>a</sup> µg/ml
Gentamicin	1 to 4	— <sup>b</sup>	4 to 32	0,5 to 2	0,5 to 4
Kanamycin	16 to 64	— <sup>b</sup>	64 to 512	2 to 8	8 to 32
Streptomycin	8 to 32	— <sup>b</sup>	8 to 64	2 to 16	2 to 16
Tetracycline	1 to 4	8 to 32	0,5 to 2	0,5 to 2	0,25 to 2
Erythromycin	0,06 to 0,5	0,25 to 2	0,03 to 0,25	0,12 to 0,5	0,06 to 0,25
Clindamycin	0,06 to 0,25	0,5 to 4	0,03 to 0,12	0,25 to 1	0,03 to 0,25
Chloramphenicol	2 to 8	4 to 16	0,5 to 4	2 to 16	2 to 8
Ampicillin	0,5 to 2	0,25 to 2	0,25 to 1	0,12 to 1	0,03 to 0,25
Vancomycin	— <sup>b</sup>	— <sup>b</sup>	0,5 to 2	0,25 to 1	0,25 to 1
Neomycin	2 to 8	<sup>b</sup>	8 to 64	0,5 to 4	0,5 to 4
Virginiamycin	0,25 to 1	1 to 4	0,06 to 0,25	0,5 to 4	0,06 to 0,25
Ciprofloxacin	1 to 4	16 to 64	4 to 16	4 to 16	1 to 8
Linezolid	1 to 4	2 to 8	0,25 to 1	1 to 4	0,5 to 2
Rifampicin	0,12 to 1	1 to 4	0,12 to 0,5	4 to 32	0,12 to 0,5
Trimethoprim	— <sup>b</sup>	— <sup>b</sup>	— <sup>b</sup>	— <sup>b</sup>	— <sup>b</sup>
a 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.					
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