International Standard

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IDF 220

Milk products — Enumeration of bifidobacteria — Colony-count technique

Produits laitiers — Dénombrement des bifidobacteria présumés — Technique par comptage des colonies

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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 document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

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

This second edition cancels and replaces the first edition (ISO 29981 | IDF 220:2010), which has been technically revised.

The main changes are as follows:

— diluents which can be used have been added;

— preparation of the test portion and primary dilution in cases of dried milk products has been added;

— a new culture medium, TOS agar, has been introduced;

— storage of incubated plates has been included;

— expression of results has been changed to be in accordance with ISO 7218;

— performance testing of the culture media has been introduced;

— performance characteristics, with the results of an interlaboratory study, which are based on the method of this second edition, have been included as Annex C.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

IDF (the International Dairy Federation) is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/ food control authorities.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

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This document was prepared by IDF *Standing Committee on Methods for Dairy Microbiology* and ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by ISO and IDF.

The work was carried out by the IDF/ISO Action Team (D09) of the *Standing Committee on Methods for Dairy Microbiology* under the aegis of its project leader Masamichi Muto (JP).

Introduction

Bifidobacteria are non-acid-fast, non-spore-forming, Gram-positive, non-motile and catalase-negative chemoorganotrophs bacilli, which produce acetic acid, lactic acid and formic acid. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate shunt in which fructose-6-phosphate phosphoketolase (F6PPK, EC 4.1.2.22) cleaves fructose-6-phosphate into acetyl phosphate and erythrose-4-phosphate.

Many reports show that bifidobacteria have various physiological functions and bifidobacteria are widely applied to foods in milk products such as yoghurt, infant formula and milk powders, and also in non-milk products such as starter and probiotic cultures. Many bifidobacteria-containing products describe the bacterial cell counts on the product label which is an important indicator of the functionality. An accurate bifidobacteria enumeration method, such as the one given in this document, is important to guarantee the bacterial cell counts.

The main technical changes listed in the Foreword, introduced in this document compared to ISO 29981 | IDF 220:2010, are considered as major (see ISO 17468). These technical changes have a major impact on the performance characteristics of the method.

Milk products — Enumeration of bifidobacteria — Colony-count technique

1 Scope

This document specifies a method for the selective enumeration of bifidobacteria in milk products by using a colony-count technique at 37 °C under anaerobic conditions.

The method is applicable to milk products, such as fermented (e.g. yoghurts) and non-fermented milks (e.g. pasteurized milks, skim milks, whey protein concentrates), milk powders and formulae (e.g. infant formulae, follow-up formulae for older infants, products for young children) where these microorganisms are present and viable, in combination with other lactic acid bacteria or alone. The method is also applicable to starter and probiotic cultures. For proposed quality criteria of dairy products, see, for example, CXS 243-2003[6].

Bifidobacteria used in milk products usually belong to the following species (e.g. References [7] and [10]):

— *Bifidobacterium adolescentis*;

— *B. animalis* subsp. *animalis*;

— *B. animalis* subsp. *lactis*;

— *B. bifidum*;

— *B. breve*;

— *B. longum* subsp. *infantis*;

— *B. longum* subsp. *longum*.

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 6887-1, *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

ISO 6887-5, *Microbiology of the food chain — 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 the food chain — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

ISO 19036:2019, *Microbiology of the food chain — Estimation of measurement uncertainty for quantitative determinations*

3 Terms and definitions

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

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

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

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

3.1
bifidobacteria
anaerobic microorganisms of the family *Bifidobacteriaceae*, usually capable of growth in/on TOS-MUP agar or TOS agar by forming typical colonies and displaying certain characteristics in microscopic examination

Note 1 to entry: The morphology of typical colonies of bifidobacteria in/on TOS-MUP agar and TOS agar is described in 9.6. The microscopic examination and other confirmation test are described in 9.7.

4 Principle

4.1 General

The enumeration of bifidobacteria requires three successive stages as specified in Annex A.

TOS-MUP agar contains the antibiotic mupirocin lithium salt (MUP), which inhibits the growth of most lactic acid bacteria commonly used in products, such as fermented and non-fermented milks (e.g. pasteurized milks, skim milk, whey protein concentrate), milk powders and infant formulae, as well as starter and probiotic cultures (see Reference [8]).

Owing to the proven selectivity of the MUP antibiotic when added to the base medium, usually there is no growth of typical yoghurt bacteria (*Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus*), mesophilic cultures (e.g. *Lactococcus lactis*), *Lactobacillus acidophilus, Lacticaseibacillus casei* and *Lacticaseibacillus rhamnosus* on the medium specified. This property has been tested with a representative number of reference strains and isolates.

For the enumeration of bifidobacteria from samples containing only bifidobacteria, TOS agar with or without the antibiotic MUP can be used.

4.2 Preparation of initial suspension and decimal dilutions

An initial dilution and decimal dilutions are prepared from the test sample.

4.3 Isolation and selection for confirmation

TOS-MUP agar or TOS agar is inoculated with a specified quantity of the test sample if the product is liquid, or of the initial suspension in the case of other products. Other plates are prepared under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

The dishes are incubated anaerobically at 37 °C for 72 h. Alternatively, if the colony size is large enough to count accurately, the dishes can be examined after 48 h incubation.

4.4 Confirmation

Colonies of presumptive bifidobacteria can be confirmed by microscopic examination and/or appropriate tests (e.g. F6PPK-assay, see References [11] and [14]).

Confirmation of presumptive bifidobacteria by microscopic examination is required, but is optional in the case of test samples containing only bifidobacteria.

4.5 Calculation

The number of bifidobacteria per millilitre or gram of the test sample is calculated from the number of confirmed typical colonies per dish.

5 Culture media and reagents

Follow current laboratory practices in accordance with ISO 7218. The composition of culture media and reagents and their preparation are specified in Annex B. For performance testing of culture media, follow the procedures in accordance with Clause B.5 and ISO 11133.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications. The usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following shall be used.

6.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave), as specified in ISO 7218.

6.2 Autoclave, capable of operating at a temperature of 115 °C ± 3 °C and equipped with short heating and cooling cycles.

6.3 Equipment for culture in an anaerobic atmosphere, as specified in ISO 7218, capable of operating at a temperature of 37 °C ± 1 °C, providing an anaerobic atmosphere of volume fraction 5 % to 20 % of carbon dioxide; a volume fraction of approximately 70 % to 90 % of nitrogen; with a volume fraction of approximately 10 % of hydrogen (not obligatory). The gas mixture should not contain more than a volume fraction of 1 % of oxygen.

6.4 Refrigerator (optional), capable of operating at 5 °C ± 3 °C.

6.5 Water baths, one capable of being maintained at 37 °C ± 1 °C and another capable of being maintained between 44 °C and 47 °C.

6.6 Sterile test tubes or flasks, of appropriate capacity. Bottles or flasks with non-toxic metallic or plastic screwcaps may be used.

6.7 pH meter, accuracy to within ±0,1 pH unit at 25 °C.

6.8 Sterile graduated pipettes or automatic pipettes, of nominal capacities 25 ml, 10 ml, 1 ml and 0,1 ml.

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

6.10 Peristaltic blender (stomacher), with sterile bags, possibly with a device for adjusting speed and time, as specified in ISO 7218.

6.11 Microscope (optional), preferably with phase-contrast, and with slides and cover slips, as specified by ISO 7218.

6.12 Colony-counting equipment with a magnifying lens (optional), e.g. 8 times to 10 times, as specified in ISO 7218.

7 Sampling

Sampling is not part of the method specified in this document. Follow the specific International Standard dealing with the product concerned. If there is no specific International Standard dealing with the sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

Recommended sampling techniques are given in ISO 707 | IDF 50 for milk and milk products.

It is important that the laboratory receives a sample that is representative of the product under consideration. The sample should not have been damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned: follow the procedures specified in ISO 6887-1 and ISO 6887-5. If there is no specific International Standard available, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 General

Take all necessary precautions to ensure sample preparation and examination in the laboratory are conducted under aseptic conditions (see ISO 7218).

Perform the procedures specified in 9.2 to 9.5 by gentle mixing, to limit exposure to aerobic conditions such as air bubbles.

9.2 Preparation of the test portion and primary dilution

9.2.1 Dried milk products (e.g. infant milk formulae) including dehydrated starter and probiotic cultures

Follow the procedures specified in ISO 6887-5 and the mentioned steps below.

Warm the bottles of diluent in the water bath (6.5) at 37 °C.

Massage the bag (by hand) to dissolve the test portion. Then mix in a peristaltic blender (6.10) for 2 min.

9.2.2 Non-dried fermented (e.g. yoghurt) and non-fermented milk-based products (e.g. pasteurized milks)

Follow the procedures specified in ISO 6887-5 and the mentioned steps below.

Thoroughly mix the contents of the closed sample container by repeatedly shaking and inverting it (preferably 10 times, with a movement of about 300 mm, for about approximately 7 s). If not possible, thoroughly mix the content with a sterile spatula or similar after opening the packaging to obtain homogeneous samples.

Take the test portion required using a sterile spatula or a pipette.

Prepare the test portion and initial suspension (primary dilution) as described by ISO 6887-1, using a diluent listed in ISO 6887-5 which is adjusted to laboratory ambient temperature.

Mix the content as described above or use peristaltic blender (6.10).