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Milk and milk products — Detection of *Enterobacter sakazakii*

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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.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of normative document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

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An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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/TS 22964|IDF/RM 210 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 worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has 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 technical committees is to prepare International Standards. Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of IDF National Committees casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a Standing Committee may decide to publish another type of normative document which is called by IDF: *Reviewed method*. Such a method represents an agreement between the members of a Standing Committee and is accepted for publication if it is approved by at least 50 % of the committee members casting a vote. A *Reviewed method* is equal to an ISO/PAS or ISO/TS and will, therefore, also be published jointly under ISO conditions.

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/TS 22964|IDF/RM 210 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 the Joint ISO-IDF Action Team on *Harmonization*, of the Standing Committee on *Microbiological methods of analysis*, under the aegis of its project leaders, Mr D.J.C. van den Berg (NL) and Mr H. Joosten (CH).

Milk and milk products — Detection of *Enterobacter sakazakii*

1 Scope

This Technical Specification specifies a method for the detection of *Enterobacter sakazakii* in milk powder and powdered infant formula.

The method is also applicable to environmental samples collected from milk powder or infant formula factories.

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 8261|IDF 122, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

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

3 Terms and definitions

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

3.1
presumptive *Enterobacter sakazakii*
microorganisms which form typical colonies on a chromogenic isolation agar, when tests are carried out in accordance with this Technical Specification

3.2
Enterobacter sakazakii
microorganisms which form typical colonies on a chromogenic isolation agar, form yellow colonies on tryptone soya agar and display biochemical characteristics as described, when tests are carried out in accordance with this Technical Specification

4 Principle (see also annex A)

4.1 Pre-enrichment in non-selective liquid medium

The pre-enrichment medium is inoculated with the test portion and incubated at 37 °C ± 1 °C for 16 h to 20 h.

4.2 Enrichment in selective liquid medium

The selective enrichment medium is inoculated with the culture obtained in 4.1 and incubated at 44 °C ± 0,5 °C for 22 h to 26 h.

4.3 Plating out and identification

A chromogenic agar is inoculated with the enrichment culture obtained in 4.2 and incubated at 44 °C ± 1 °C for 22 h to 26 h.

4.4 Confirmation

Typical colonies are selected from the chromogenic agar, and isolates producing a yellow pigment on tryptone soya agar are biochemically characterized.

5 Culture media and reagents

5.1 General

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity. The water shall be free from substances that might inhibit the growth of microorganisms under the test conditions specified in this Technical Specification. See also ISO 6887-1 and ISO 8261|IDF 122.

In order to improve the reproducibility of the results, it is recommended that, for the preparation of culture media, dehydrated basic components or dehydrated complete media be used. In that case, follow the manufacturer's instructions rigorously. See also ISO 6887-1.

The pH values given refer to a temperature of 25 °C. Adjustments, if necessary, are made by adding either hydrochloric acid [c(HCl) = 1 mol/l] or sodium hydroxide solution [c(NaOH) = 1 mol/l].

If not used immediately, store the prepared culture media and reagents under conditions that do not produce any change in their composition, in the dark at a temperature between 0 °C and 5 °C, for no longer than 1 month, unless otherwise stated.

5.2 Culture media

5.2.1 Buffered peptone water (BPW)

5.2.1.1 Composition

Enzymatic digest of casein	10,0 g
Sodium chloride (NaCl)	5,0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12\ H_2O$)	9,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,5 g
Water	1 000 ml

5.2.1.2 Preparation

Dissolve each of the components in the water, by heating if necessary. Adjust the pH, if necessary, to 7,0 ± 0,2 at 25 °C. Distribute the BPW in flasks or tubes according to the analytical needs. Sterilize at 121 °C for 15 min.

5.2.2 Modified lauryl sulfate tryptose broth (mLST)/vancomycin medium

5.2.2.1 Modified lauryl sulfate tryptose broth (mLST)

5.2.2.1.1 Composition

Sodium chloride (NaCl)	34,0 g
Enzymatic digest of animal and plant tissue	20,0 g
Lactose ($C_{12}H_{22}O_{11}$)	5,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	2,75 g
Dipotassium hydrogen phosphate (K_2HPO_4)	2,75 g
Sodium lauryl sulfate ($C_{12}H_{25}NaO_5S$)	0,1 g
Water	1 000 ml

5.2.2.1.2 Preparation

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

Adjust the pH, if necessary, to 6,8 ± 0,2 at 25 °C. Dispense 10 ml of mLST into tubes of dimensions 18 mm × 160 mm.

Sterilize the tubes at 121 °C for 15 min.

5.2.2.2 Vancomycin solution

5.2.2.2.1 Composition

Vancomycin	10 mg
Water	10 ml

5.2.2.2.2 Preparation

Dissolve the vancomycin in the distilled water. Mix and sterilize by filtration.

The vancomycin solution may be kept at 0 °C to 5 °C for 15 days.

5.2.2.3 mLST/vancomycin medium

Add 0,1 ml of vancomycin solution (5.2.2.2.2) to 10 ml of mLST solution (5.2.2.1.2) so as to obtain a final vancomycin concentration of 10 µg per millilitre of mLST.

The complete mLST/vancomycin medium may be kept at 0 °C to 5 °C for 1 day.

5.2.3 *Enterobacter sakazakii* isolation agar (ESIA™)[1)]

5.2.3.1 Composition

Pancreatic peptone of casein	7,0 g
Yeast extract	3,0 g
Sodium choride (NaCl)	5,0 g
Sodium desoxycholate	0,6 g
5-Bromo-4-chloro-3-indolyl α-D-glucopyranoside ($C_{14}H_{15}BrClNO_6$)	0,15 g
Crystal violet	2 mg
Agar	12,0 g to18,0 g[a]
Water	1 000 ml

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

5.2.3.2 Preparation

Dissolve each of the components in the water by boiling. Adjust the pH, if necessary, to 7,0 ± 0,2 at 25 °C. Sterilize at 121 °C for 15 min.

Cool to between 44 °C and 47 °C. Pour about 15 ml of ESIA™ medium into sterile empty Petri dishes and allow to solidify on a cool even surface.

The medium may be kept at 0 °C to 5 °C for up to 14 days.

5.2.4 Tryptone soya agar (TSA)

5.2.4.1 Composition

Enzymatic digest of casein	15,0 g
Enzymatic digest of soya	5,0 g
Sodium chloride (NaCl)	5,0 g
Agar	9,0 g to 18,0 g[a]
Water	1 000 ml

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

5.2.4.2 Preparation

Dissolve each of the components in the water by boiling. Adjust the pH, if necessary, to 7,3 ± 0,2 at 25 °C. Sterilize at 121 °C for 15 min. Cool to between 44 °C and 47 °C. Pour about 15 ml of TSA into sterile empty Petri dishes and allow to solidify on a cool even surface.

1) ESIA™ is the trade name of a product supplied by AES Laboratoire, Rue Maryse Bastié, Ker Lann, F-35172 Bruz (FR) . This information is given for the convenience of users of this Technical Specification|IDF Reviewed Method and does not constitute an endorsement by either ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.2.5 Media and reagents for biochemical characterization

5.2.5.1 Reagent for detection of oxidase

5.2.5.1.1 Composition

N,N,N′,N′-Tetramethyl-*p*-phenylenediamine dihydrochloride ($C_{10}H_{16}N_2 \cdot 2HCl$)	1,0 g
Water	100 ml

5.2.5.1.2 Preparation

Dissolve the component in the water immediately before use.

5.2.5.2 L-Lysine decarboxylation medium

5.2.5.2.1 Composition

L-Lysine monohydrochloride ($C_6H_{14}N_2O_2 \cdot HCl$)	5,0 g
Yeast extract	3,0 g
Glucose ($C_6H_{12}O_6$)	1,0 g
Bromocresol purple	0,015 g
Water	1 000 ml

5.2.5.2.2 Preparation

Dissolve each of 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 5 ml of L-lysine decarboxylation medium into tubes of dimensions 18 mm × 160 mm.

Sterilize the tubes at 121 °C for 15 min.

5.2.5.3 L-Ornithine decarboxylation medium

5.2.5.3.1 Composition

L-Ornithine monohydrochloride ($C_5H_{12}N_2O_2 \cdot HCl$)	5,0 g
Yeast extract	3,0 g
Glucose ($C_6H_{12}O_6$)	1,0 g
Bromocresol purple	0,015 g
Water	1 000 ml

5.2.5.3.2 Preparation

Dissolve each of 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 5 ml of L-ornithine decarboxylation medium into tubes of dimensions 18 mm × 160 mm. Sterilize the tubes at 121 °C for 15 min.

5.2.5.4 L-Arginine dihydrolation medium

5.2.5.4.1 Composition

L-Arginine monohydrochloride ($C_6H_{14}N_4O_2 \cdot HCl$)	5,0 g
Yeast extract	3,0 g
Glucose ($C_6H_{12}O_6$)	1,0 g
Bromocresol purple	0,015 g
Water	1 000 ml

5.2.5.4.2 Preparation

Dissolve each of 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 5 ml of L-arginine dihydrolation medium into tubes of dimensions 18 mm × 160 mm. Sterilize the tubes at 121 °C for 15 min.

5.2.5.5 Media for fermentation of carbohydrates (peptone water with phenol red, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose and amygdaline)

5.2.5.5.1 Basic medium

5.2.5.5.1.1 Composition

Enzymatic digest of casein	10 g
Sodium chloride (NaCl)	5 g
Phenol red	0,02 g
Water	1 000 ml

5.2.5.5.1.2 Preparation

Dissolve each of the components in the water, by heating if needed. Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Dispense the basic medium into flasks of suitable capacity. Sterilize at 121 °C for 15 min.

5.2.5.5.2 Carbohydrate solutions (D-sorbitol, L-rhamnose, D-sucrose, D-melibiose or amygdaline), 80 mg/ml

5.2.5.5.2.1 Composition

Carbohydrate	8 g
Water	100 ml

5.2.5.5.2.2 Preparation

Dissolve separately each of the four carbohydrate components in the water so as to obtain four carbohydrate solutions. Sterilize all by filtration.

5.2.5.5.3 Complete carbohydrate fermentation mediums

5.2.5.5.3.1 Composition

Basic medium (5.2.5.5.1)	875 ml
Carbohydrate solution (5.2.5.5.2)	125 ml

5.2.5.5.3.2 Preparation

For each carbohydrate, add the prepared carbohydrate solution (5.2.5.5.2) aseptically to basic medium (5.2.5.5.1) and mix. Dispense 10 ml of complete medium of each carbohydrate aseptically into tubes of dimensions 18 mm × 160 mm.

5.2.5.6 Simmons citrate medium

5.2.5.6.1 Composition

Sodium citrate ($Na_3C_6H_5O_7$)	2,0 g
Sodium chloride (NaCl)	5,0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	1,0 g
Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	1,0 g
Magnesium sulfate ($MgSO_4$)	0,2 g
Bromothymol blue	0,08 g
Agar	8,0 g to 18,0 g[a]
Water	1 000 ml

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

5.2.5.6.2 Preparation

Dissolve each of the components or the dehydrated complete medium in the water by boiling. Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Dispense 10 ml of Simmons citrate medium into tubes (6.7) of dimensions 18 mm × 160 mm. Sterilize the tubes at 121 °C for 15 min.

Let the tubes stand in a tilted position so as to obtain a butt 2,5 cm deep.

6 Apparatus and glassware

Disposable glassware is an acceptable alternative to reusable glassware, provided that it has suitable specifications.

Usual microbiological laboratory equipment and, in particular, the following:

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

See ISO 7218.

6.2 Total delivery pipettes, having a nominal capacity of 1 ml.

6.3 **Water bath**, capable of being maintained at 44 °C ± 0,5 °C.

6.4 **Petri dishes**, made of glass or plastic, of diameter 90 mm to 100 mm.

6.5 **Incubators**, capable of operating at 25 °C ± 1 °C, 30 °C ± 1 °C and 44 °C ± 1 °C, respectively.

6.6 **Loop**, made of platinum-iridium or nickel chromium, of diameter approximately 3 mm, or disposable loops.

6.7 **Test tubes**, of diameter 18 mm and length 160 mm (plugged or with screw caps).

6.8 **pH meter**, accurate to 0,1 pH unit at 25 °C ± 1 °C.

7 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this Technical Specification. A recommended sampling method is given in ISO 707|IDF 50.

8 Preparation of test sample

Prepare test samples in accordance with ISO 8261|IDF 122.

9 Procedure (see the scheme in Annex A)

9.1 Test portion

To prepare the primary dilution, add x g of the test sample (Clause 8) to 9 times x ml of pre-enrichment medium (5.2), which is the ratio of test sample to pre-enrichment medium specified in this method.

Allow dry samples to disperse in the liquid without stirring. If a sample has not been dissolved completely after 30 min, than mix it gently with the medium.

9.2 Pre-enrichment

Incubate the inoculated pre-enrichment medium (9.1) at 37 °C ± 1 °C for 18 h ± 2 h.

9.3 Selective enrichment

After incubation of the inoculated pre-enrichment medium, transfer 0,1 ml of the obtained culture (9.2) into 10 ml of mLST/vancomycin medium (5.2.2.3). Incubate at 44 °C ± 0,5 °C for 24 h ± 2 h.

It is recommended to use either a water bath (6.3) or a forced-air incubator to ensure that the maximum temperature (44,5 °C) is not exceeded.

9.4 Isolation of presumptive *Enterobacter sakazakii*

After incubation of the inoculated mLST/vancomycin medium (9.3), streak a loopful (*ca*. 10 µl) onto the surface of the *Enterobacter sakazakii* isolation agar plate (5.2.3.2). Incubate the plate at 44 °C ± 1 °C for 24 h ± 2 h.

After incubation, examine the chromogenic plate for the presence of typical colonies of presumptive *Enterobacter sakazakii*.

NOTE Typical colonies are small to medium sized (1 mm to 3 mm) green to blue-green colonies. Non-typical colonies are often slightly transparent and violet coloured.

9.5 Confirmation

9.5.1 Production of a yellow pigment

9.5.1.1 Selection of colonies

Select one to five of the typical colonies of presumptive *Enterobacter sakazakii* examined on the incubated chromogenic plate (9.4).

9.5.1.2 Incubation

Streak the selected colonies (9.5.1.1) onto the surface of the TSA plate (5.2.4.2) so that after incubation separate colonies can be observed. Incubate the plate at 25 °C ± 1 °C for 44 h to 48 h. After incubation, examine the TSA plates for the presence of yellow-pigmented colonies.

When only one colony is selected (9.5.1.1) and transferred to the TSA plate and after incubation no yellow-pigmented colonies can be seen, select four more typical colonies (9.5.1.1) and proceed according to 9.5.1.2. If there are fewer than five typical colonies, select all of them.

CAUTION — Some exceptional strains of *Enterobacter sakazakii* might not form a yellow pigment under the test conditions specified in this Technical Specification, or the pigment is lost due to sub-culturing. In such cases using this method might, therefore, overlook such strains.

9.5.2 Biochemical confirmation

9.5.2.1 General

Miniaturized biochemical identification kits, currently available commercially and permitting the identification of *Enterobacter sakazakii*, may be used.

9.5.2.2 Selection of colonies

Select one yellow pigmented colony from each tryptone soya agar plate (9.5.1.2) for further biochemical characterization according to 9.5.2.3 to 9.5.2.8.

9.5.2.3 Oxidase

Using a glass rod or disposable inoculation needle, take a portion of each selected characteristic colony (9.5.2.2).

Streak the taken portion on a filter paper moistened with the oxidase reagent (5.2.5.1) or on a commercially available disc. Do not use a nickel/chromium loop or wire.

Consider the test to be negative when the colour of the filter paper has not changed to mauve, violet or deep blue within 10 s.

9.5.2.4 L-Lysine decarboxylase

Using a loop, wire or glass rod, inoculate the L-lysine decarboxylation medium (5.2.5.2) with each of the selected colonies (9.5.2.2) just below the surface of the liquid medium. Incubate the tubes at 30 °C ± 1 °C for 24 h ± 2 h.

A violet colour after incubation indicates a positive reaction. A yellow colour indicates a negative reaction.

9.5.2.5 L-Ornithine decarboxylase

Using a loop, wire or glass rod, inoculate the L-ornithine decarboxylation medium (5.2.5.3) with each of the selected colonies (9.5.2.2) just below the surface of the liquid medium. Incubate the tubes at 30 °C ± 1 °C for 24 h ± 2 h.

A violet colour after incubation indicates a positive reaction. A yellow colour indicates a negative reaction.

9.5.2.6 L-Arginine dihydrolase

Using a loop, wire or glass rod, inoculate the L-arginine dihydrolation medium (5.2.5.4) with each of the selected colonies (9.5.2.2) just below the surface of the liquid medium. Incubate the tubes at 30 °C ± 1 °C for 24 h ± 2 h.

A violet colour after incubation indicates a positive reaction. A yellow colour indicates a negative reaction

9.5.2.7 Fermentation of various sugars

Using a loop, wire or glass rod, inoculate each carbohydrate fermentation medium (5.2.5.5.3) with each of the selected colonies (9.5.2.2) just below the surface of the liquid medium. Incubate the tubes at 30 °C ± 1 °C for 24 h ± 2 h.

A yellow colour after incubation indicates a positive reaction. A red colour indicates a negative reaction.

9.5.2.8 Utilization of citrate

Using a loop, wire or glass rod, streak the selected colonies (9.5.2.2) onto the slant surface of Simmons citrate medium (5.2.5.6). Incubate the tubes at 30 °C ± 1 °C for 24 h ± 2 h.

The reaction is positive if the medium turns blue.

9.6 Interpretation of the results of the confirmation tests

Interpret the results according to Table 1.