National Food Safety Standard of the People's Republic of China

GB4789.35-2010

National Food Safety Standard
Food microbiological examination:
Lactic acid bacteria

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Preface

The Standard substitutes the GB/T 4789.35-2003 “Microbiological Examination for Food Hygiene : Examination of Lactic Acid Bacteria in Beverages Containing Lactic Acid Bacteria”.

Compared with the GB/T4789.35-2008, main changes are following:

- The count methods of lactic acid bacteria, lactobacillus, bifidobacteria and streptococcus thermophilus are revised.

The appendix A of the standard is the normative appendix.

The replaced former editions are:

- GB 4789.35-1996, GB/T 4789.35-2003, GB/T4789.35-2008

National Food Safety Standard

Food microbiological examination: Lactic Acid Bacteria

1. Scope

The Standard defines the examination method of lactic acid bacteria in foods containing lactic acid bacteria.

The Standard is applicable to the examination of lactic acid bacteria in foods containing live lactic acid bacteria.

2. Normative References

The documents referred in the standard are requisite for the application of the standard.

For dated references, only the version dated is applicable to the standard.

For undated references, the latest version including all modification notices are applicable to the standard.

3. Terms and Definitions

3.1 Lactic acid bacteria

The common name of a group of bacteria capable of fermenting saccharides to produce large amount of lactic acid.

The lactic acid bacteria in the standard are mainly Lactobacillus, Bifidobacterium and Streptococcus genus.

4. Equipment and Materials

In addition to conventional sterilization and cultivation equipment used in microbiological laboratory, other equipment and materials needed are as follows:

4.1 Thermostatic Cultivator: 36℃±1℃.

4.2 Refrigerator: 2℃~5℃

4.3 Homogenizer and sterile homogenizing bag, homogenizing cup or sterile pestle.

4.4 Balance: accuracy of 0.1g.

4.5 Sterile tube: 18mmX180mm, 15mmX100mm

4.6 Sterile pipette: 1ml (with 0.01ml of scale), 10ml(with 0.1ml of scale) or micropipettor and tips.

4.7 Sterile conical beaker: 500ml, 250ml

5. Culture Mediums and Reagents

5.1 MRS (man rogosa sharpe) Culture Medium and improved MRS with Li-Mupirocin Culture Medium : refer to Appendix A.1.

5.2 MC (modified chalmers) Culture Medium: refer to Appendix A.2.

5.3 0.5% Sucrose Fermentation Tube: refer to Appendix A.3.

5.4 0.5% Cellobiose Fermentation Tube: refer to Appendix A.3.

5.5 0.5% Maltose Fermentation Tube: refer to Appendix A.3.

5.6 0.5% Mannite Fermentation Tube: refer to Appendix A.3.

5.7 0.5% Salicin Fermentation Tube: refer to Appendix A.3.

5.8 0.5% Sorbitol Fermentation Tube: refer to Appendix A.3.
5.9 0.5% Lactose Fermentation Tube: refer to Appendix A.3.
5.10 Aesculin Fermentation Tube: refer to Appendix A.4.
5.11 Gram's Staining Solution: refer to Appendix A.5.
5.12 Li-Mupirocin, chemically pure.

6. Examination Procedures

For the examination procedures of lactic acid bacteria, please refer to Fig. 1.

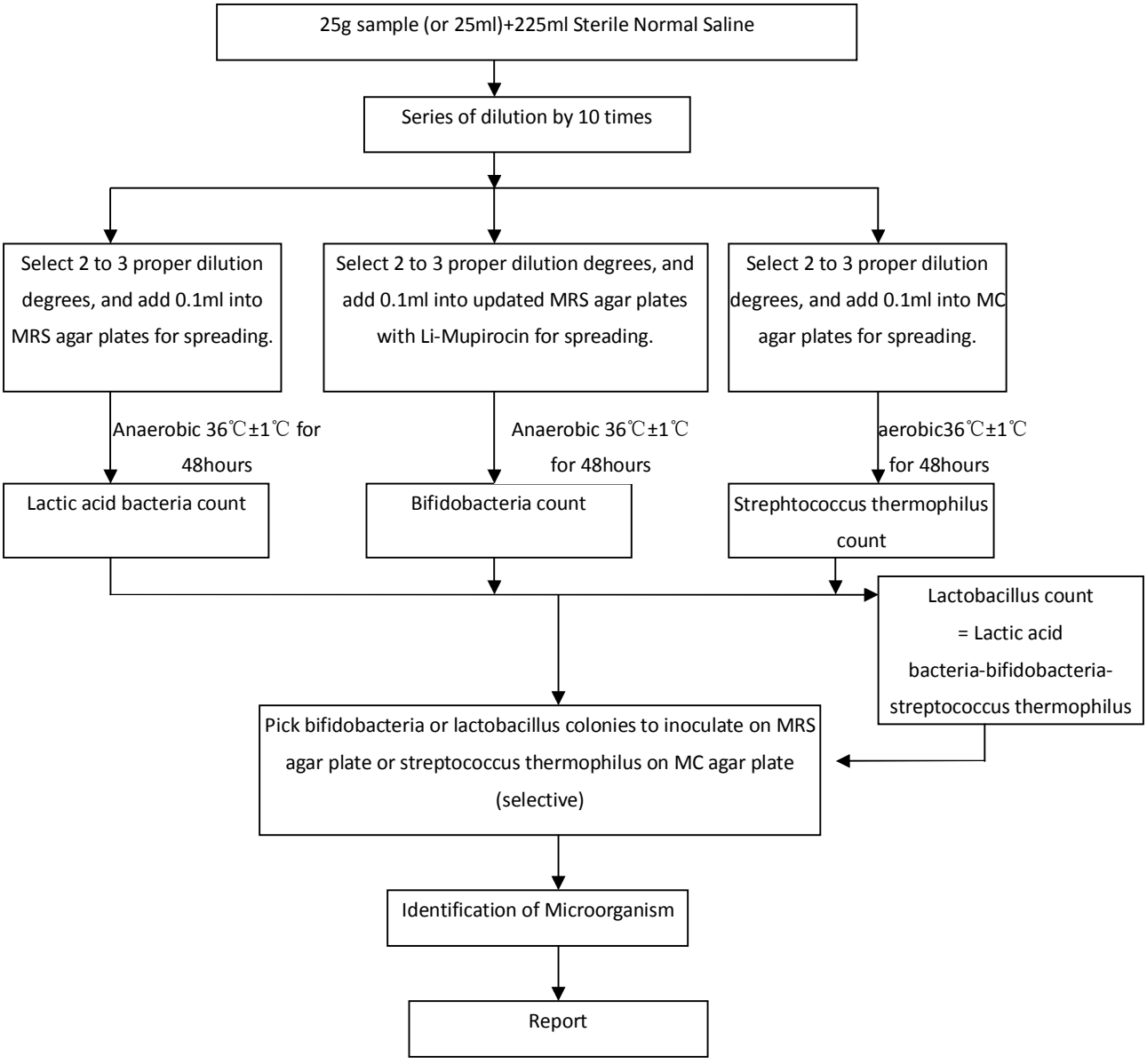

Figure 1. Examination Procedures of Lactic Acid Bacteria

7. Operation Procedures

7.1 Sample preparation

7.1.1 The whole process of sample preparation shall observe aseptic operation procedures.

7.1.2 Freezing samples shall be thawed at 2℃~5℃ for no more than 18h, and it could also be carried out at a temperature lower than 45℃ for no more than 15 min.

7.1.3 Solid and semi-solid food: measure 25g sample with aseptic operation into sterile

homogenizing cup containing 225ml normal saline, homogenize at 8000-10000r/m for 1-2 min, and then formulate into 1:10 sample solution; or place it into sterile homogenizing bag containing 225ml normal saline, beat with slaping type homogenizer for 1-2 min, and then formulate into 1:10 sample solution.

7.1.4 Liquid sample: First, sufficiently shake the liquid samples, absorb 25ml sample solution with sterile pipette into a sterile conical beaker containing 225ml normal saline (proper amount of sterile beads are placed into the beaker in advance), and then shake up to formulate into 1:10 sample solution.

7.2 Procedures

7.2.1 Absorb 1ml 1:10 sample solution with 1ml sterile pipette or micropipettor, and place into a sterile test tube containing 9ml normal saline along the wall of the tube (it is noted that the tip of the pipette shall not touch the diluent), shake up the test tube or blow it repeatedly with another piece of sterile pipette to homogeneity, and then formulate into 1:100 sample solution.

7.2.2 Get another 1ml sterile pipette or micropipettor tip, follow the above operation procedures to conduct escalating dilutions on the sample solutions by 10 times. For each time of dilution, one 1ml sterile pipette or tip shall be replaced.

7.2.3 Lactic acid bacteria count

7.2.3.1 Lactic acid bacteria count

According to the estimation of live bacteria count to be tested, select 2 to 3 proper continuous dilution degrees, for each dilution degree, absorb 0.1ml of dilution sample and place it onto 2 MRS agar plates, and then spread the surface with L-shape bar. Count all colonies on the plate after anaerobic incubation at 36’C+/-1’C for 48hr+/-2hr.

The whole experiment process shall be completed within 15minutes.

7.2.3.2 Bifidobacteria count

According to the estimation of live bacteria count to be tested, select 2 to 3 proper continuous dilution degrees, for each dilution degree, absorb 0.1ml of dilution sample and place it onto 2 updated MRS agar plates with Li-Mupirocin, and then spread the surface with L-shape bar. Count all colonies on the plate after anaerobic incubation at 36’C+/-1’C for 48hr+/-2hr.

The whole experiment process shall be completed within 15minutes.

7.2.3.3 Streptococcus thermophilus count

According to the estimation of live bacteria count to be tested, select 2 to 3 proper continuous dilution degrees, for each dilution degree, absorb 0.1ml of dilution sample and place it onto 2 MC agar plates, and then spread the surface with L-shape bar. Do colony count after aerobic incubation at 36’C+/-1’C for 48hr+/-2hr. The colony morphology of streptococcus thermophilus on the MC agar plate: the colony is medium in size and less than normal. The colony is round and red, the diameter is 2mm+/-1mm, and the back of the colony is pink.

The whole experiment process shall be completed within 15minutes.

7.2.3.4 Lactobacillus count

The lactobacillus count equals lactic acid bacteria minus bifidobacteria 7.2.3.2, and minus strephtococcus thermophilus count 7.2.3.3.

7.3 Colonies count

It could be observed with naked eyes, apply magnifying glass or bacteria colony counter

when necessary, and record the dilution times and corresponding plate count. Plate count number is represented by colony-forming units (CFU).

7.3.1 Select the plates for the number of colonies between 30CFU and 300CFU, and without spreading growth on the plate. For plate with colony count under 30CFU, the number of colony is recorded, while for plate count over 300, it shall be recorded as uncountable. For each dilution degree, the average number of two plates shall be applied.

7.3.2 For those plates with large piece of colony growing, they shall not be applied. If the piece of colony covers less than one half of the plate area, and the colonies on the remaining half of the plate area scatter evenly, it shall be counted of this half of the plate and then multiply by 2, to represent the entire plate count.

7.3.3 When there occurs chain like growth on the plate without evident border line between colonies on the plate, each chain shall be calculated as one colony.

7.4 Formulation of results

7.4.1 If there is only one dilution degree whose plate count fall in the proper counting scope, the average plate count of both plate shall be calculated, and then multiply the average value by corresponding dilution times, to serve as the total plate count in one gram (or ml) of sample.

7.4.2 If there are two continuous dilution degrees whose plate count falls in the proper counting scope, they shall be calculated as in Formula (1):

$$N = \sum C/(n_1 + 0.1n_2)d \qquad \cdots\cdots(1)$$

Where,

N – Colony count in sample;

$\sum C$ - The total number of colonies on the plates (including the plates within the range of proper plate count;

N_1 - The number of colonies on the plates of the first proper dilution degree;

N_2 - The number of colonies on the plates of the second proper dilution degree;

d - Dilution Factor (the first dilution degree).

7.4.3 If the colony numbers on the plates of all dilution degrees are all over 300CFU, count the plates with the maximum dilution degree. For other plates, they shall be recorded as uncountable, and the results shall be obtained by multiplying the average colony number by the maximum dilution times.

7.4.4 If the colony numbers on the plates of all dilution degree are all less than 30CFU, it shall be calculated by multiplication of average colony number on the minimum dilution degree plates by the dilution times.

7.4.5 If, for plates of all dilution degrees (including the original liquid samples), there is no colony growth, then it shall be calculated as multiplying the minimum dilution degree by a factor smaller than 1.

7.4.6 If, for plates of all dilution degrees, the colony number falls outside the range between 30CFU and 300CFU, part of which are less than 30CFU or more than 300CFU, then it shall be calculated for the plates whose colony number is closest to 30CFU or 300CFU, as the average colony number multiply by dilution times.

7.5 Colony count report

7.5.1 When the plate count falls within 100CFU, it shall be rounded up and reported as integer.

7.5.2 When the plate count is larger than or equal to 100CFU, the third digit shall be rounded up, and take the first two digits, while the following digits are replaced by 0; it could also be indicated as exponential of 10CFU, round-up and then take the two significant digits.

7.5.3 For sampling by weight, CFU/g is applied as the report unit, while for sampling by volume, CFU/ml is applied as the report unit.

8. Results and reports

To issue the report cccording to the colony count, the unit of report is CGU/g(ml)

9. Lactic acid bacteria identification (Optional)

9.1 Pure incubation

Pick 3 or more separate colonies, streptococcus thermophilus shall be inoculated on MC agar plates, while lactobacillus shall be inoculated on MRS agar plates. Then, incubate under facultative anaerobic conditions at 36℃±1℃ for 48 hours.

9.2 Identification

9.2.1 The identification of bifidobacteria shall be subject to GB/T 4789.34.

9.2.2 Smear slide microscopy: lactobacillus is multiple in morphology, in elongated shape, bending rod shape or short rod shape. It has no spore, and is positive in Gram's Staining. Streptococcus thermophilus is in ball shape or clavate shape, with a diameter between 0.5um and 2.0um, distributed in pairs or chain, without spore, and it is positive in Gram's Staining.

9.2.3 For the main biochemical reaction of lactic acid bacteria, pls refer to Table 1 and Table 2.

Table 1. Biochemical reactions within common lactobacilius genus

Genus	Aesculin	Cellobiose	Maltose	Mannite	Salicin	Sorbitol	Sucrose
L.casei subsp. casei	+	+	+	+	+	+	+
L.delbrueckii subsp. bulgaricus	_	_	_	_	_	_	_
L.acidophilus	+	+	+	_	+	_	+
L.reuteri	ND	_	+	_	_	_	+
L.rhamnosus	+	+	+	+	+	+	+
L.plantarum	+	+	+	+	+	+	+
Note: +indicates over 90% of the strains mentioned above are positive; -indicates over 90% of the strains mentioned above are negative; while ND indicates not detectable.							

Table 2. The main biochemical reactions in streptococcus thermophilus

Genus	Synanthrin	Lactose	Mannite	Salicin	Sorbitol	Hippuric Acid	Aesculin
S.thermophilus	_	+	_	_	_	_	_
Note: + indicates over 90% of the strains mentioned above are positive; - indicates over 90% of the strains mentioned above are negative.							

Appendix A
(Normative Appendix)
Culture Media and Reagents

A.1 MRS Culture Medium

A.1.1 Ingredients

Peptone	10.0g
Beaf powder	5.0g
Yeast powder	4.0g
Glucose	20.0 g
Tween 80	1.0ml
$K_2HPO_4.7H_2O$	2.0g
$CH_3COONa.3H_2O$	5.0g
Triammonium citrate	2.0g
$MgSO_4.7H_2O$	0.2g
$MnSO_4.4H_2O$	0.05g
Agar powder	15.0g
pH 6.2	

A.1.2 Preparation method

Put the ingredients mentioned above into 1000ml distilled water, heat for dissolving. Adjust the pH value, autoclave at 121℃ for 15-20min after distribution.

A.1.3 updated MRS with Li-Mupirocin

A.1.3.1 preparation of Li-Mupirocin reserve solution: weigh 50mg Li-Mupirocin into 50ml distilled water, sterilization by filter of 0.22um micro-hole filterable membrane.

A.1.3.2 Preparation method

Put the A.1.1 ingredients into950ml distilled water, heat for dissoclving. Ajesut the pH value, autoclave at 121℃ for 15-20min after distribution.

Cool down to 48℃ in water bath, then use injector with 0.22um micro-hole filterable membrane to inject Li-Mupirocin reserve solution into melting agar. The concentration of Li-Mupirocin should be 50ug/ml.

A.2 MC Culture Medium

A.2.1 Ingredients

Soya peptone	5.0g
Beef powder	3.0g
Yeast powder	3.0g
Glucose	20.0g
Lactose	20.0g
Calcium carbonate	10.0g
Agar	15.0g
Distilled water	1000ml
1% Neutral red solution	5.0ml
pH 6.0	

A.2.2 Preparation method

Put the former 7 ingredients into distilled water, heat for dissolving. Adjust the pH value, and

then add into the neutral red solution. Autoclave at 121℃ for 15-20min after distribution.

A.3 Lactobacillus Saccharides Fermentation Tube

A.3.1 Ingredients

Beaf extract	5.0g
Peptone	5.0g
Yeast extract	5.0g
Tween 80	0.5ml
Agar	1.5g
1.6% Bromocresol purple ethanol solution	1.4ml
Distilled Water	1000ml.

A.3.2 Preparation method

Add saccharides needed per 0.5%, and then distribute into small test tubes. Autoclave at 121℃ for 15-20min.

A.4 Aesculin Fermentation Tube

A.4.1 Ingredients

Peptone	5.0g
K_2HPO_4	1.0g
Aesculin	3.0g
Ferric citrate	0.5g
1.6% Bromocresol purple ethanol solution	1.4ml
Distilled water	100ml

A.4.2 Preparation method

Dissolve the ingredients mentioned above into distilled water, and then autoclave at 121℃ for 15-20min.

A.5 Gram's Staining Solution

A.5.1 Crystal Violet Staining Solution

A.5.1.1 Ingredients

Crystal violet	1.0g
95% Ethanol	20ml
1% Ammonium oxalate water solution	80ml

A.5.1.2 Preparation method

Dissolve the crystal violet completely into ethanol, and then mix up with ammonium oxalate solution.

A.5.2 Gram's Iodine Solution

A.5.2.1 Ingredients

Iodine	1.0g
Potassium iodide	2.0g
Distilled water	300ml

A.5.2.2 Preparation method

First mix iodine and potassium iodide together, and then add into small amount of distilled water, and shake up sufficiently, after complete dissolution, add distilled water to it to 300ml.

A.5.3 Safranin Restaining Solution

A.5.3.1 Ingredients

Safranin	0.25g
95% Ethanol	10ml
Distilled water	90ml

A.5.3.2 Preparation method

Dissolve the safarin into ethanol, and then dilute with distilled water.

A.5.4 Staining Method

A.5.4.1 Fix the smear slide on the flame of alcohol burner, drip crystal violet staining solution, dye for 1 min, and then rinse with water;

A.5.4.2 Drip the Gram's Iodine Solution， react for 1 min, and then rinse with water;

A.5.4.3 Drip 95% of ethanol to decolor for about 15~30s, until the staining solution is washed off, and it shall not be overdone, and then rinse with water;

A.5.4.4 Drip the restaining solution, restain for 1min. Rinse with water, and then dry it for microscopy.