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English Version

Animal feeding stuffs - Isolation and enumeration of Lactobacillus spp.

Aliments des animaux - Isolement et dénombrement du
Lactobacillus spp.

Futtermittel - Keimzählung von Lactobacillus spp.

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Foreword

This document (EN 15787:2009) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs”, the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by March 2010, and conflicting national standards shall be withdrawn at the latest by March 2010.

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Introduction

This methodology has been developed to enumerate probiotic lactobacilli to enable the European Commission to control proper labelling of animal feeding products (EU project SMT4-CT98-2235 – “Methods for the official control of probiotics (microorganisms) used as feed additives”) [1]. The described methodology was validated in an interlaboratory study [2].

1 Scope

This European Standard defines general rules for the enumeration of probiotic lactobacilli in feed samples (additives, premixtures and feeding stuffs) that contain lactobacilli as a single bacterial component or in a mixture with other microorganisms. This standard is not applicable to mineral feeds, which are defined as complementary feeding stuffs composed mainly of minerals and containing at least 40% crude ash (Council Directive 79/373/EEC [3]).

There are different categories of feed samples:

- a) Additives containing about 10^{10} colony forming units (CFU)/g;
- b) Premixtures containing about 10^8 CFU/g;
- c) Feeds, meal or pellets, which contain about 10^6 CFU/g and include complete feeding stuffs and milk replacers.

The detection limit is as defined in EN ISO 7218.

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.

EN ISO 6887-1, *Microbiology of food and animal feeding stuffs - 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 6887-1:1999)*

EN ISO 7218, *Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations (ISO 7218:2007)*

ISO 6498, *Animal feeding stuffs – Preparation of test samples*

3 Terms and definitions

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

3.1

lactobacilli (described by their characteristics as used for this standard)

lactobacilli are bacteria which form colonies fitting the description of these species on the specified selective medium after incubation of 48 h to 72 h at a temperature of 37 °C under anaerobic conditions [4]:

Morphology of colonies:

- a) circular;
- b) regular or irregular (starry) surrounding;
- c) convex or conical;
- d) dull or glistening surface;

- e) translucent, white, pale green, dark green.

Colony size varies between 0,5 mm and 3 mm in diameter.

Phase contrast microscopic examination of selected colonies shows that cells are varying from long and slender sometimes bent rods, to short, often coryneform coccobacilli and chain formation is common.

NOTE For a detailed account of morphology see [4].

4 Principle

- a) Preparation of sterile and dry poured agar plates.
- b) Drawing a representative test sample under sterile conditions.
- c) Preparation of the initial suspension to obtain a homogeneous distribution of bacterial cells from the test portion.
- d) Preparation of further decimal dilutions of the initial suspension in order to reduce the number of microorganisms per unit volume to allow, after incubation, the counting of colonies.
- e) Inoculation of the prepared plates with an aliquot of the optimum dilutions and dispersion of the inoculum by using a sterile spreader.
- f) Incubation of inverted plates for 48 h to 72 h at $37\text{ °C} \pm 1\text{ °C}$, under anaerobic conditions.
- g) Counting of typical colonies, considering the specific properties of lactobacilli.
- h) Morphological verification of isolates within the *Lactobacillus* genus through the use of microscope analysis.
- i) Calculation of the colony count per gram or kilogram of feed sample.

5 Diluent, selective media and phenotypic characterisation

5.1 Diluents

5.1.1 Diluent for initial suspension of premixtures, additives and feeding stuffs

This diluent is used to decimally dilute the sample to prepare an initial decimally sample suspension (10^{-1}) in appropriate containers (e.g. universals, bottles or flasks).

Phosphate buffered saline (PBS):

Dissolve 8 g sodium chloride, 0,2 g potassium chloride, 1,15 g disodium hydrogen phosphate, 0,2 g potassium dihydrogen phosphate, pH $7,3 \pm 0,2$ in 1 l of distilled water. Aliquote this saline into appropriate containers (e.g. universals, bottles or flasks). Autoclave all capped containers with the initial diluent at $121\text{ °C} \pm 1\text{ °C}$ for 10 min. To avoid loss during autoclaving, screw cap bottles are recommended.

Bring the diluent to room temperature before use.

Measure the pH of the diluent to ensure the suitable buffer capacity.

5.1.2 Diluent for serial dilutions

This diluent is used to decimally dilute the initial sample suspension and subsequent dilutions.

Peptone salt solution:

A peptone salt solution is made complying with EN ISO 6887-1.

Compose the solution of enzymatic digest of 1 g casein such as pancreatic peptone of casein (or peptone of same quality) and 8,5 g sodium chloride) per liter (l) distilled water. Dissolve the ingredients in water. Adjust the pH to $7,0 \pm 0,2$ at $25 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$. For decimal dilutions, prepare test tubes containing $9,0 \text{ ml} \pm 0,1 \text{ ml}$ after sterilisation or use screw cap bottles to avoid weight loss during autoclaving.

Sterilise in the autoclave for 15 min at $121 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$. Bring the diluent to room temperature before use.

5.2 Media

5.2.1 General

Four different media are proposed:

- a) MRS medium;
- b) MRS supplemented with Triphenyl Tetrazolium Chloride (TTC);
- c) AMRSA: Acidified MRS agar;
- d) LAMVAB: Lactobacillus Anaerobic MRS with vancomycin and bromocresol green.

For routine enumeration of lactobacilli the use of MRS agar will be sufficient assuming that the probiotic strain is present in far higher numbers than any other microorganism. The medium is designed to encourage the growth of the 'lactic acid bacteria' such as lactobacilli, enterococci and pediococci. Selection can be made by pH adjustment, as lactobacilli will tolerate a lower pH than enterococci (pH 5,0 to pH 6,5), with pediococci growing best in this range. When enterococci are expected to be present in similar concentrations as lactobacilli, acidified MRS agar (AMRSA) should be used. When lactobacilli in combination with pediococci are expected, MRS agar supplemented with TTC allows differentiation of colonies by different coloration after anaerobic incubation. LAMVAB is a selective medium for lactobacilli.

5.2.2 Composition

5.2.2.1 MRS agar

The composition of the agar per l of distilled water is as follows [5]:

20,0 g dextrose, 10,0 g polypeptone, 10,0 g meat extract, 5,0 g yeast extract, 5,0 g sodium acetate $3\text{xH}_2\text{O}$, 2,0 g sodium phosphate, 2,0 g tri-ammonium citrate, 1,0 g Tween 80, 0,2 g magnesium sulphate $7\text{xH}_2\text{O}$, 0,05 g manganese sulphate $4\text{xH}_2\text{O}$, agar 15,0 g, pH $6,2 \pm 0,2$.

5.2.2.2 MRS agar supplemented with TTC

Sterilise MRS agar (5.2.2.1) by autoclaving at $121 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ for 15 min. Supplement with 1 ml of a filter sterilised 1 g/100 ml water solution of Triphenyl Tetrazolium Chloride (TTC) per 100 ml MRS agar.

5.2.2.3 AMRSA

Acidified MRS agar can be obtained by adjusting the pH of MRS agar (see 5.2.2.1) to $5,4 \pm 0,1$ with HCl prior to autoclaving.

5.2.2.4 LAMVAB

The medium consists of three different components:

- a) Solution A: MRS broth 104,4 g/l with cysteine hydrochloride (0,5 g/l) and bromocresol green (0,05 g/l).
- b) Solution B: Agar 40 g/l.
- c) Solution C: 2 mg/ml of vancomycin hydrochloride (potency about 1 000 µg/mg) in distilled water.

5.2.3 Preparation

5.2.3.1 MRS agar

Suspend all ingredients in distilled water and sterilise by autoclaving at $121\text{ °C} \pm 1\text{ °C}$ for 15 min.

5.2.3.2 MRS agar supplemented with TTC

Prepare 1 g Triphenyl Tetrazolium Chloride (TTC) in 100 ml water and filter sterilise. Add 1 ml per 100 ml MRS agar medium (see 5.2.3.1) which is tempered at $48\text{ °C} \pm 1\text{ °C}$ after autoclaving.

NOTE TTC is destroyed by autoclaving.

5.2.3.3 AMRSA

Adjust the pH of MRS agar with HCl to $5,4 \pm 0,1$ prior to autoclaving. Sterilise at $121\text{ °C} \pm 1\text{ °C}$ for 15 min.

5.2.3.4 LAMVAB

Adjust the pH of solution A with HCl to $5,0 \pm 0,1$.

Sterilise solutions A and B at $121\text{ °C} \pm 1\text{ °C}$ for 15 min. Sterilise solution C by filtration using a 0,2 µm filter. Solution C is stable for at least three months in a fridge.

Preparation of the medium involves sterilisation of equal volumes of solutions A and B. Cool solution B down to 50 °C in an incubator or water bath. Cool solution A down to room temperature. Add to 500 ml of this solution A, 10 ml of solution C aseptically. Finally, add solution B to the MRS-vancomycin (A+C) mixture. Pour plates immediately after mixing. This procedure results in a final vancomycin concentration of 20 mg/l.

5.3 Phenotypic characterisation

Microscopical observation of the Gram stained microorganism and catalase test identifies the bacteria. A test for catalase is done with a drop of hydrogen peroxide (H₂O₂) on a colony. The formation of bubbles is a positive reaction. Only bacteria that are regular, nonsporing Gram-positive rods, catalase negative, and non-obligatory aerobe are considered.

6 Apparatus and glassware

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

6.1 Equipment for dry sterilisation (oven) and wet sterilisation (autoclave)

According to EN ISO 7218.

6.2 Incubator

Capable of maintaining a temperature of $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

6.3 Blending equipment

Two-speed or a variable adjustable blender (18 000 rpm and 22 000 rpm), with a one litre bowl which is sterilised in an oven for 1 h at 170°C to 180°C .

6.4 Mechanical stirrer

A mechanical stirrer e.g. Vortex Mixer (see EN ISO 7218), or equivalent

6.5 Balance

Balance capable of weighing to the nearest 0,01 g.

6.6 Flasks or screw-cap bottles of appropriate capacities**6.7 Test tubes of appropriate capacities****6.8 Pipettor and sterile tips to dispense 100 μl and 1 ml**

Wide bore tips to pipette homogenised feed stuff for dilution.

6.9 pH meter**6.10 Sterile Petri dishes, 90 mm in diameter****6.11 Equipment for anaerobic incubation**

Appropriate anaerobic jars or chambers

6.12 Laminar flow cabinet**6.13 Water bath**

Capable of maintaining temperatures of $48^{\circ}\text{C} \pm 1^{\circ}\text{C}$ resp. $50^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

6.14 Microscope

Capable of phase-contrast microscopy at 600x to 1 000x magnification.

6.15 Bacterial Cell spreaders

Sterile L- or triangular-shaped spreaders from glass or metal or sterile disposable plastic spreaders.

7 Sampling

Carry out the sampling procedure in accordance with the specific standard appropriate to the product concerned. If such a specific standard is not available, it is recommended that agreement be reached on this subject among the parties concerned. Apply community rules [1] for official control sampling of animal feeds.

NOTE Sampling can be done according to ISO 6497 [6]. Although ISO 6497 is not applicable for microorganisms, due to the lack of other reference, it seems it is the most suitable protocol to be taken into account

WARNING — Take precautions to avoid potential cross-contamination of samples with microorganisms. Particularly after sampling additives and premixtures supplemented with microorganisms. If needed, clean and disinfect sampling equipment between each sample, particularly after sampling additives and premixtures containing microorganisms. Put the sample in a sterile container.

8 Preparation of test sample

The test sample preparation shall be done in accordance with ISO 6498 and the congruent product standard. If such a specific standard is not available, it is recommended that agreement be reached on this subject among the parties concerned. ISO 6498 gives general guidelines on test sample preparation.

9 Procedure

9.1 Feeds with a single or multi-component probiotic microflora

If lactobacilli are the only bacterial component in the feed use MRS agar or AMRSA, or if they are dominant with additional microflora, enumeration can proceed on AMRSA or MRS + TTC or LAMVAB. If lactobacilli are not dominant, use LAMVAB.

9.2 Preparation of poured agar plates

Prepare MRS agar according to the manufacturer's directions.

Carry out acidification of MRS agar for preparation of AMRS agar is carried out before autoclaving.

For MRS + TTC agar, add the filter-sterilised TTC, after autoclaving the MRS agar for 15 min at $121\text{ °C} \pm 1\text{ °C}$, when the medium has cooled down in a water bath to a temperature of $48\text{ °C} \pm 1\text{ °C}$.

For preparation of LAMVAB, add vancomycin from sterilised stock solutions to the medium immediately before pouring.

Pour approximately 15 ml portions of medium are poured into each Petri dish (6.10) under sterile conditions and spread to give a homogeneous layer.

When the medium has solidified, pile quantities of four plates reversed on each other and dry at room temperature or in an incubator at $37\text{ °C} \pm 1\text{ °C}$ for approximately 12 h or over night. Alternatively spread the plates out in a lamina flow cabinet and dry the agar surface with the lids partially removed for about 30 min.

Check the dried plates for sterility.

Dried plates, if correctly protected against dehydration, may be stored for 2 weeks in a fridge. In this case bring the plates to room temperature about 30 min before use.

The initial suspension must be always homogenous (consider the warning in section 9.3.)

9.3 Preparation of the initial suspension and decimal dilutions

Weigh $20 \text{ g} \pm 0,1 \text{ g}$ of plain additive or premixture or $2 \text{ g} \pm 0,01 \text{ g}$ of a microgranul (encapsulated) additive and $50 \text{ g} \pm 0,5 \text{ g}$ of feed sample into a sterile blender's bowl. Add 180 ml or 450 ml of buffered saline at room temperature. Depending on the size of capsules an adequate amount of sample material has to be used in the initial suspension.

NOTE 1 It is recommended to check the pH of the initial suspension and to correct it to a range of pH 7,3 – 8,1.

The numbers of capsules should be more than 25, to obtain a SD (Standard Deviation) of less than 20% for a good repeat of analysis.

Blend additives and premixtures for 3 min at high speed (see 6.3) and dilute immediately.

Blend feeding stuffs for 1 min at high speed (see 6.3); start to blend at low speed to avoid splashing, then turn the switch to high speed. Let the sample stand for 30 min. It is important for pelleted feeds to absorb the liquid. Blend for 2 min at high speed (see 6.3) and immediately dilute using a wide bore pipette.

NOTE 2 Intense foaming in initial suspensions during blending may be avoided by using a feasible antifoaming agent (e.g. silicon antifoaming agent or equivalent) in adequate concentrations.

In the case of encapsulated products the application of adequate additives has to be used (e.g. Polyoxyethylensorbitanmonooleat or equivalent) with adequate temperatures (e.g. $40 \text{ }^\circ\text{C}$).

The weight of the withdrawn pipette volume has to be calibrated in order to receive a correction factor for the calculation of the counts of *Lactobacillus* species.

WARNING — Suspensions containing probiotics tend to settle quickly after blending. This shall be avoided by rapid pipetting subsamples of only properly homogenised samples for further dilutions.

Prepare a series of appropriate dilutions (serial dilutions) using a sterile pipette such as a microdispenser set at 1 ml. Transfer 1 ml of the initial suspension into a tube containing 9 ml of sterile peptone saline brought to room temperature, and mix with a mechanical stirrer. Repeat this procedure using the 10^{-2} dilution and all further dilutions until the appropriate estimate for the number of cells is obtained. After this dilution procedure, inoculate the plates immediately as described in section 9.4.

NOTE 3 It is recommended to examine the content of the copper in the initial suspension by a pretest. Copper contents exceeding 200 mg/kg need the application of chelating agents e.g. iminodiacetic acid in a feasible concentration with regard to the pH-value.

9.4 Inoculation and incubation of plates

Use from each of the chosen dilutions 0,1 ml to inoculate two separate agar plates. Spread it uniformly as quickly as possible on the surface of the medium using a sterile spreader.

WARNING — Selected appropriate dilutions must be homogeneous prior to transferring a subsample onto plates.

Reverse plates, stack them and incubate them anaerobically at $37 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ for 48 h to 72 h.

9.5 Counting of colonies

After the incubation under the above specified conditions, all plates containing more than 30 and not more than 300 colonies are used for the enumeration of colony-forming units (CFU).

Count presumptive lactobacilli positive colonies, and calculate the number of colony-forming units per gram feed according to the weighted arithmetical mean value in accordance with EN ISO 7218.

9.6 Cell morphology – confirmation

Depending on the number of different colony morphologies appearing after incubation, 2 to 5 colonies of each morphology type are selected at random. Half of the colony is Gram stained and microscopically observed. The other half is tested for catalase.

Bacteria which are catalase negative, regular, and nonsporing Gram positive rods are considered to be lactobacilli.

If colonies are detected which do not conform to this, repeat the analysis with AMRSA agar and/or LAMVAB agar. In case of the presence of yeasts, add nystatin to the agars.

10 Expression of results

The number of colony forming units (CFU) per gram of feed is calculated according to EN ISO 7218.

The number of lactobacilli (N) per g or per ml is equal to:

$$N = \frac{\sum C}{v(n_1 + 0,1 n_2)d} \quad (1)$$

$\sum C$ is the sum of the colonies counted on all the plates;

V is the volume of inoculum applied to each dish, in ml;

n_1 is the number of plates counted at the first dilution;

n_2 is the number of plates counted at the second dilution;

d is the dilution from which the first counts were obtained.

Round off the result obtained to two significant figures. For a three-figure number, round off the third to the nearest 0. If the third is 5, round off to the figure below if the first two figures are even numbers, and to the figure above if the first two figures are odd numbers.

The result is the number of micro organisms per gram product, expressed as a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

11 Precision

11.1 General

The precision of the method using different media in terms of repeatability and reproducibility was determined in an interlaboratory study.

11.2 Interlaboratory study

Details of the interlaboratory study on the precision of the method are published [1], [2] and are summarized in Annex B. Repeatability and reproducibility limits were determined using three types of feed samples contaminated at two levels of contamination. The values derived from the interlaboratory study may not be applicable to concentration ranges and matrices other than those given.

11.3 Repeatability

The absolute difference between two independent single (\log_{10} -transformed) test results (number of lactobacilli per g or per ml) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in the same laboratory by the same operator using the same apparatus within the shortest feasible time interval, will in not more than 5% of the cases exceed the repeatability limit r .

11.4 Reproducibility

The absolute difference between two single (\log_{10} -transformed) test results (number of lactobacilli per g or per ml) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5% of cases exceed the reproducibility limit R .

12 Test report

The test report shall specify:

- a) information necessary for the complete identification of the sample;
- b) the sampling method used, if known;
- c) the test method used, with reference to this European Standard;
- d) the temperature of incubation;
- e) operating details not specified in this European Standard, or regarded as optional, together with
- f) details of any incidents which may have influenced the test result(s);
- g) the test result(s) obtained, or, if the repeatability has been checked, the final quoted result obtained.

Annex A (informative)

Notes on procedure

MRS agar allows growth of all lactobacilli but also of other micro-organism species. The addition of TTC to MRS agar provides a useful tool to distinguish e.g. pediococci by different coloration after anaerobic incubation.

On Acidified MRS agar (AMRSA) microorganisms other than lactobacilli used as feed additives such as enterococci or bacilli do not form colonies. Only some yeast, moulds or pediococci may be able to produce colonies under the specified conditions. Colonies of non-lactobacilli can be easily distinguished by different colony morphology or microscopical examination.

All authorised probiotic lactobacilli used in animal feeding products grow on LAMVAB which is a selective medium for lactobacilli. However among the 88 species of lactobacilli there are some species which do not grow very well or not at all on LAMVAB (e.g. *Lactobacillus delbrueckii*, *Lactobacillus helveticus* and some strains of *Lactobacillus acidophilus*). On the LAMVAB Agar most of the other microorganisms used as feed additives are unable to form colonies and only some yeasts, moulds or pediococci may grow under the specified conditions. These colonies can easily be differentiated by microscopical observation and are not used for colony counting.

An antifungal agent such as nystatin (50 U/ml) can be added to MRS agar, AMRSA or LAMVAB to inhibit moulds and yeasts but this may reduce the colony size of lactobacilli.

Annex B (informative)

Results of the interlaboratory study

An interlaboratory study involving 20 laboratories in 12 European Countries was carried out at two different levels of contamination. The study was organised in 2002 and coordinated by Central Science Laboratory [1], [2]. For the interlaboratory study homogeneous test samples were prepared using feeding stuffs which contained lactobacilli in combination with pediococci, enterococci and yeast. Samples with concentrations of around 2×10^7 CFU/g (low level) and 1×10^8 CFU/g (high level) were analysed. The precision data derived from the study are presented in Table 1.

Table B.1 — Precision data obtained from the collaborative study [1], [2].

parameters	Sample			
	MRSA*		MRSA + TTC*	
	low	high	low	High
Number of samples	2	2	2	2
Number of laboratories retained after eliminating outliers	11	11	9	11
Mean value, in \log_{10} cfu/g	7,51	8,00	7,50	7,91
Repeatability standard deviation, s_r , in \log_{10} CFU/g	0,24	0,24	0,26	0,12
Coefficient of variation of Repeatability, CV_r , in %	3,14	2,96	3,42	1,46
Repeatability limit r ($=2,8 \times s_r$)	0,66	0,66	0,72	0,32
Reproducibility standard deviation, s_R , in \log_{10} CFU /g	0,38	0,29	0,39	0,32
Coefficient of variation of reproducibility, CV_R , in %	5,00	3,63	5,21	4,02
Reproducibility limit, R	1,05	0,81	1,10	0,89
* media reference see clause 5				

parameters	Sample			
	AMRSA*		LAMVAB*	
	Low	high	low	high
Number of samples	2	2	2	2
Number of laboratories retained after eliminating outliers	10	9	7	7
Mean value, in log ₁₀ cfu/g	7,40	8,03	7,02	7,68
Repeatability standard deviation, s _r , in log ₁₀ CFU/g	0,24	0,10	0,24	0,24
Coefficient of variation of Repeatability, CV _r , in %	3,21	1,24	3,39	3,13
Repeatability limit <i>r</i> (=2,8 x s _r)	0,67	0,28	0,67	0,67
Reproducibility standard deviation, s _R , in log ₁₀ CFU /g	0,35	0,18	0,34	0,24
Coefficient of variation of reproducibility, CV _R , in %	4,75	2,24	4,82	3,13
Reproducibility limit, R	0,99	0,50	0,95	0,67
* media reference see clause 5				

Bibliography

- [1] European Community project SMT4-CT98-2235. 'Methods for the official control of probiotics used as feed additives (vol. 1-3). 2002. Report EUR 20873/1-3. Office for Official Publications of the European Communities. ISBN 92-894-6249-3 (set)'
- [2] Leuschner R.G.K., J. Bew, V. Coeuret, J.-P. Vernoux, M. Gueguen. 2003. A collaborative study of a method for the enumeration of probiotic lactobacilli in animal feed. *Food Microbiol.* 80, 131-143
- [3] Council Directive (79/373/EEC) of 2 April 1979 on the marketing of compound feeding stuffs (OJ No L 86, 6.4.1979, p.30)
- [4] Bergey's Manual of Systematic Bacteriology, Volume 2 (ISBN 0-683-07893-3) by O.Kandler and N.Weiss, chapter "Genus Lactobacilli"
- [5] Man J.C., de Rogosa, M. and E.M. Sharpe. 1960. *Appl. Bact.* 23, 130-135
- [6] ISO 6497, *Animal feeding stuffs – Sampling*