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Selective Enumeration of Bifidobacteria in Dairy Products: Development of a Standard Method

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Selective Enumeration of Bifidobacteria in Dairy Products: Development of a Standard Method

Foreword

This issue of the Bulletin presents the Development of a Standard Method for the Selective Enumeration of Bifidobacteria in Milk and Milk Products, produced by a project group set up for the purpose within the IDF/ISO Joint Action Team (JAT) on lactic acid bacteria and starters.

IDF is most grateful to the members of the project group (Dorthea Ellekaer, Wolfgang Kneifel and Ulrike Zitz, Yasuhisa Shimakawa and Koichiro Sonoike, Vojtech Rada, Cristina Alvarez Fernandez, Paul Simpson, Sally Miller, Karlheinz Friedrich and all the co-workers) for their relentless effort in producing this report, as well as to the JAT and all laboratories and institutions supporting the work.

As bifidobacteria play an important role as probiotic microorganisms in fermented dairy products, this Bulletin will no doubt be of major interest to all concerned with the microbiology of such products.

Christian Robert
Director General
March 2007

Preface

After a series of trials a Draft standard for the methodology of bifidobacteria enumeration in dairy products has been developed. This work constituted a considerable and comprehensive task over the past three years, and was possible thanks to the good cooperation of the project team "bifidobacterial enumeration method" and the support of the IDF/ISO Joint Action Team on Lactic Acid Bacteria in general.

The team also took some effort with regard to the trial planning and the statistical evaluation. Prof. Weiss and Prof. Wilrich from Germany contributed a lot to this. Overall the development of the standard was a very instructive experience for all parties involved. Hence we came up with the idea to report this useful experience in a paper, which would fit into the scope of the IDF Bulletin and which describes all the important details of such an «enterprise» in the form of a roadmap. We also think that the information given in the paper can be helpful for future methodological standardisation procedures in dairy microbiology.

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Selective Enumeration of Bifidobacteria in Dairy Products: Development of a Standard Method

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Abstract

Bifidobacteria play an important role as probiotic microorganisms in fermented dairy products. Depending on the individual properties of the strains as well as on their density in the product and the survival conditions in the human intestinal tract, several beneficial effects have been demonstrated for certain bifidobacteria in numerous studies. In this context, selective viable count and bacterial viability have been identified as key criteria for determining the quality of probiotic products. Hence, defined viable count threshold levels for probiotic products are under discussion for implementation. Usually, culture methods are used for routine examination of these products. As far as bifidobacterial enumeration is considered, several methodologies have been proposed during the last two decades, however, with limited applicability due to laborious procedures and/or lacking selectivity. According to experience, the accompanying microflora often negatively influences the discrimination among the different groups of bacteria contained in a product. On this account, a project team within the IDF "Lactic acid bacteria" group of experts has addressed this task and elaborated a procedure for bifidobacterial enumeration, which is easy to perform and of high selectivity. In cooperation with experts in statistical planning and evaluation of analytical methods, this project has led to a Draft standard method, which has undergone several phases of testing, evaluation and modification. This report describes in detail, how the method was developed step by step and assessed for its performance based on selectivity, reproducibility and repeatability. It can be exemplarily used as a roadmap for developing a microbiological standard method.

1 Survey of literature and methodologies for the enumeration of bifidobacteria

During the last three decades considerable activities were focused on research topics and product development in the probiotic area. There is some growing evidence on the beneficial role of probiotics in the human gut and several effects have been demonstrated in vitro and in vivo [Holzapfel et al., 1998; Holzapfel and Schillinger, 2002]. This development has stimulated the emergence of a growing number of probiotic dairy products, which have gained increasing popularity among the consumers. Due to their strain-specific properties and physiological nature, bifidobacteria have been widely used in fermented milk products, also in combination with other lactic acid bacteria such as Lactobacillus delbrueckii subsp. bulgaricus, L. casei, L. acidophilus and Streptococcus thermophilus. Besides Bifidobacterium animalis, also selected strains of B. bifidum, B. breve, B. infantis, and B. longum are candidates applied as probiotics [IDF-"Bifido"-Guideline, 1999]. Although there has been some uncertainty regarding the taxonomy of the different bifidobacterial species, recent studies have demonstrated that representatives of B. lactis and B. animalis obviously constitute two clearly separated sub-groups [Mayer et al., 2003; Masco et al., 2004]. Today, Bifidobacterium animalis spp. lactis can be detected in at least 58 probiotic products worldwide [Masco et al., 2005].

To be able to assure a defined quality of probiotic products with certain health benefits, the demand for an official regulation on the basis of the Codex Standard for Fermented Milks (2003) is highly desirable. At the time of consumption, a targeted population of at least 10^6 CFU/g product probiotic microorgranisms is under discussion [CODEX STAN 243-2003].

In order to be able to assess such products, reliable methods for the selective detection and enumeration of bifidobacteria are needed. On the other hand, the producer guarantees a certain microbial density in the yogurt during its entire shelf-life period. Depending on their universal use as well as on their easy applicability, plate count methods are still preferred for quality control of dairy products [Hartemink et al., 1996]. Various bifidobacteria enumeration media of varying selectivity have been proposed during the last years. It has also been shown that the accompanying microflora

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is a criterion of crucial relevance in the applicability of the method.

The aim of a group of experts within IDF was to identify and validate a suitable medium, which allows the selective detection and enumeration of the different species of bifidobacteria used in dairy products. In this context, products containing high quantities of other lactic acid bacteria should also be covered. It is evident from the literature that larger differences in the magnitude of the product flora may lead to the inability to count bifidobacterial colonies. Moreover, some strains of bifidobacteria are partially inhibited on media hitherto proposed like NPNL-MRS [Dave and Shah, 1996, Pacher and Kneifel, 1996]. Other key issues for the suitability of the method under practical conditions are its inexpensiveness and easy application. Hence, time-consuming media preparation procedures were not to be considered. Although the so-called Bif-agar medium enables a proper and selective enumeration of a wider spectrum of bifidobacteria [Pacher and Kneifel, 1996], its relatively complicated preparation limits its use for routine purposes. In Table 1, an updated overview on agar-media for bifidobacterial enumeration is given, taking into account the mainly used different agar-bases as well as several combinations of supplements conferring selectivity.

Table 1. Survey of agar media

	Medium	Supplements	Type	Reference
MRS	DeMan Rogosa Sharpe	-	Basic	DeMan et al., 1960
	MRS agar (N)	Cystein-HCl, LiCl, sodium propionate	Elective	IDF-Guideline - Group E104, 1999
	"Bif"-Medium	Cystein-HCl, Lactulose, sheep`s blood, vitamin-mixture, human milk whey, antibiotic mixture[f]	Selective	Pacher and Kneifel, 1996
	BSM (*Bifidobacterium* selective medium)	Cystein-HCl, Mupirocin	Selective	Leuschner et al., 2003 Simpson et al., 2004
	MRS-X-α-Gal	X-α-Gal[a]	Elective	Chevalier et al., 1991
	NPNL-MRS	NPN[b], LiCl	Selective	Dave and Shah, 1996 Vinderola Reinheimer 1999 Roy, 2001, Tharmaraj and Shah, 2003 Van de Casteele, 2005
	MRS-LP	LiCl, sodium propionate	Elective	Vinderola Reinheimer 1999 Van de Casteele et al., 2005
	BIM-25 modified	Antibiotic mixture[d], TTC, iodoacetic acid	Selective	Silvi et al., 1996
TPY	Tryptone Phytone Yeast	-	Basic	Scardovi, 1986
	TPY-MUP	Mupirocin	Selective	Rada and Petr, 2000 Vlkova et al., 2004
	TPY-NPNL	NPN[b], LiCl	Selective	Ghoddusi and Robinson, 1996
TOS	TOS propionate agar medium	Includes: Cystein-HCl, sodium propinate, TOS-S[c]	Elective	Sonoike et al., 1986 Jap. Ass. Fermented Milks, 2000
	TOS-NPNL	LiCl, NPN[b]	Selective	Wijsman et al., 1989
BL	Glucose-Blood-Liver	-	Basic	Mitsuoka et al., 1965
	NPNL Agar	LiCl, NPN[b]	Selective	Teraguchi et al., 1978 Laroia and Martin, 1991
	BL-OG	Oxgall, Gentamycin	Selective	Lim et al., 1995

	Medium	Supplements	Type	Reference
LCL	Liver-Cystine-Lactose	-	Basic	Blaurock, 1939
	LP	Sodium propionate, LiCl	Elective	Lapierre et al., 1992
RCM	Reinforced Clostridial Medium	-	Basic	Hirsch and Grinsted, 1954
	BIM-25	Antibiotic mixture[d], TTC, iodoacetic Acid	Selective	Munoa and Pares, 1988
	AMC Agar	Antibiotic mixture[e], TTC, iodoacetic Acid LiCl, sodium propionate	Selective	Arroyo et al., 1994, 1995
	AMC Agar modified	LiCl, sodium propinate	Elective	Payne et al., 1999
	RCPB pH 5	Prussian Blue	Elective	Rybka Kaiasapathy 1996
CAB	Columbia Agar Base (CAB)	Includes: Cystein-HCl	Basic	Beerens, 1991
	mCAB, Columbia Propionic Acid Medium	Cystein-HCl propionic Acid	Elective	Beerens, 1990
	mCAB plus raffinose	LiCl, sodium propinate	Elective	Roy et al., 1997
	DP	Dicloxacillin, propionic acid	Selective	Bonaparte, 1997
WCA	Wilkings-Chalgren agar	-	Basic	Wilkins and Chalgren, 1976
	NPNL-Agar	LiCl, NPN[b]	Selective	Rada and Koc, 2000
	Mupirocin-Agar	Mupirocin	Selective	Rada and Koc, 2000
	m WCA	Mupirocin glacial acetic acid	Selective	Rada et al., 1999 Rada and Petr, 2000
Rogosa	Rogosa agar	-	Basic	Rogosa et al., 1951
	RMS	Sodium propionate, LiCl, Neomycin, Paramomycin	Selective	Samona and Robinson, 1991
RB	Raffinose-Bifidobacterium agar	Includes: LiCl, propionate raffinose	Elective	Hartemink et al., 1996

[a] X-α-Gal = 5-bromo-4-chloro-3-indolyl-alpha-D-galactopyranoside
[b] NPN = Neomycin, Paromomycin, Nalidixic acid
[c] TOS-S = Transglalactosylated Oligo Saccharides
[d] Antibiotic mixture = Nalidixic acid, Polymyxin B sulphate, Kanamycin sulphate
[e] Antibiotic mixture = Nalidixic acid, Polymyxin B
[f] Antibiotic mixture = Aztreonam, Nalidixic acid, Netilmycin, Paromomycin sulphate

2. The development of a method for bifidobacteria enumeration – an IDF task

2.1 Former activities

Within International Dairy federation (IDF) a group of experts (lactic acid bacteria E 104) was involved in the development and the standardisation of a useful method to enumerate bifidobacteria. For this purpose, a joint action team was established to address this subject.

Based of the monography of Rasic (1990), Prof. R. Negri had already initiated this task. Unfortunately, no suitable selective agar-medium could be found. However, in 1999, a "Guideline for the Enumeration of Bifidobacteria in Fermented Dairy Products" was published as a position paper (IDF).

2.2 Current activities

In 2002, a new IDF-working group was established under the chairmanship of Dorthea Ellekaer. Taking into consideration recent methodological developments, a second initiative was taken to seek opportunities for an applicable standard method, and Prof. W. Kneifel was nominated as a group leader.

Based on the study of Rada and Koc (2000), Mupirocin (MUP) was discovered as a selective agent suppressing the growth of commonly used lactic acid bacteria. Since this antibiotic did not influence bifidobacterial growth, it could be considered as a relevant compound for a selective medium. Subsequently, the selectivity of MUP was confirmed in a series of tests carried out by eight laboratories of the IDF working group in 2003. Experiences were collected on the growth properties of a representative number of bifidobacterial reference strains and isolates. In addition, the growth performance of the typical yogurt microflora (S. thermophilus, L. delbrueckii spp. bulgaricus) as well as of mesophilic cultures (e.g. L. lactis), and of L. acidophilus, L. casei and L. rhamnosus were examined. As a result of these trials, the selectivity of MUP as a supplement could be confirmed. Among the microorganisms considered only the bifidobacteria were able to tolerate the MUP supplement.

Based on these findings, it was decided to identify an elective basic medium, which can be easily prepared. TPY-Agar [Scardovi, 1986] served as basal medium for bifidobacteria in the presence of MUP. Collaborative tests were carried out in 2004 to screen the growth performance of seven selected bifidobacterial cultures (B. animalis, B. breve, B. longum, B. sp.). TPY medium with and without MUP was used. It could be shown that the MUP-supplement did not interfere the growth of the bacterial strains.

Although most of the bifidobacterial strains grew on TPY, parallel efforts were made by the Japanese members of the action team to investigate the potential of TOS (transgalactosylated oligosaccharide mixture) propionate agar, which had originally been developed by the Japanese Association of Fermented Milks and Fermented Milk drinks, under the chairmanship of Dr. Toshiki Morichi [Expert Group on Selective Enumeration of Bifidobacteria, 2000]. Already in 1993, the selective utilisation of TOS had been studied [Tanaka et al., 1983]. These authors have described the ability of eight strains of bifidobacteria to be stimulated by this oligosaccharide. The trials performed by the members of the IDF team revealed that most strains exhibited bigger colonies on TOS-propionate than on TPY agar. Thus it was agreed that TOS propionate agar is highly suited. A third collaborative IDF-trial was carried out examining three commercially available probiotic yogurt products also containing S. thermophilus and L. acidophilus. Six laboratories used the TPY-MUP agar in comparison with the TOS-MUP agar. It was further decided, based on earlier findings, to use the pour plate method instead of the surface spread technique, since a higher precision was obtained. In conclusion, the third trial clearly showed that faster individual growth, bigger bifidobacterial colonies and higher colony counts resulted on TOS-MUP than on TPY-MUP medium.

3 Strategy and performance of collaborative trials

As the TOS-MUP medium yielded optimum performance, a series of additional tests were carried out by the central laboratory BOKU, Vienna to continue with the methodological developments. Emphasis was placed on the steps of media preparation and sample handling. These activities led to the final collaborative trial which was performed in 2005 and 2006.

3.1 Preparatory phase and establishment of study design

One of the most important steps during the planning phase was to create an applicable study design, and also to assure that all participating laboratories possess the qualification to apply the method on a comparable level. It is a pre-requisite for performing collaborative trials to estimate the actual margin of uncertainty of each laboratory reflecting the performance level of each analyst. The performance of each participating analyst was assessed based on ISO Standard 14461-1 / IDF 169-1 (test series "phase A", see also 3.1.3). Reliable plate count results are only obtained if homogeneity of the sample material (e.g., yogurt), exactness of the dilution steps and proper techniques of inoculation and counting are ensured. Another very important criterion is to guarantee the independence of the analyses. For this purpose, coded counting of the colony counts according to given randomization plans is advisable.

In the light of the above -mentioned requirements, the following steps, indicated in note form, were to be considered in the preparatory phase for the development of the standard method:

- Recruitment of qualified partner laboratories and organisation of the collaborative trial: To establish a commitment among participating partners; to assess analysts for his/her performance; to instruct each laboratory to strictly follow the defined procedure (SOP) and to document each deviation from the SOP (Counting of the plates according to randomization plans, data collection of all colony counts produced by all partners, careful documentation of all activities, involved/responsible persons, parameters, etc.).
- Selection of probiotic milk products: To define the quality and quantity of samples containing bifidobacteria, relevant lactobacilli and S. thermophilus cultures reflecting the spectrum of cultures used worldwide.
- Homogeneity of the products
- Finding suitable transport conditions: To guarantee that no changes occur in the sample material during shipment and storage until the day of investigation (stability of the products); transportation mode and shipment logistics; suitable sample packaging; time and temperature conditions; to simulate transportation under model conditions to identify and clarify possible problems
- Assessment of methodological parameters of the method in pre-trials: To define the required quality parameters and to certify their fitness of purpose according to CODEX STAN 243-2003 [Masco et al., 2005]. The following criteria are to be included:
  - Proven linearity within the targeted working range
  - Ruggedness of the method expressed as significant influencing factors
  - Repeatability of the method
  - Stability of the method
- Definition of experimental design and project plan for the final collaborative trial: To be able to detect various sources of variances, the use of a hierarchical study plan is recommended; to estimate the work load under practical conditions including the time-schedule to carry out the trials.

The documents listed underneath were helpful for the preparation as well as for the performance of the collaborative trials: ISO 707/ IDF 50C:1997, ISO 6887-1:1999, ISO 7218:1996, ISO 7889/ IDF 117:2003, ISO 8261/ IDF 122:2001 and the IDF-"Bifido"-Guideline, 1999; IDF Standard 135B:1991, IDF Standard 128A:1999, ISO 14461-1/ IDF 169-1:2005, ISO 14461-2/ IDF 169-2:2005, ISO 5725-1:1994, ISO 5725-2:2002, ISO 5725-3:2001.

3.1.1 Participants

The laboratories listed in Table 2 were members in the final IDF-bifidobateria collaborative trial.

Table 2. Participating laboratories and analysts

European Laboratories*	**Responsible Person(s)**	**Analyst(s)***
Bundesforschungsanstalt für Ernährung und Lebensmittel, Abteilung Mikrobiologie, Kiel, Germany	G. Engel	N. Rösch
Christian Hansen A/S, R&D Dept. of Applied Biotechnology, Hørsholm, Denmark	D. Ellekaer	G.G. Hansen
CNR – ISPA Sede di Milano, Milano, Italy	R. Lodi, M. Brasca	M. Brasca
Czech University of Agriculture, Prague, Department of Microbiology, Nutrition and Dietetics, Prague, Czech Republic	V. Rada	E. Vlkova
Danone S.A., Investigación y Desarollo, Barcelona, Spain	C. Alvarez	C. Alvarez
Danone Vitapole, Analytical Support Division, Palaiseau, France	S. Thompson	K. Bertrand, J.M. Prostak
Degussa BioActives Deutschland, Bönen, Germany	K. Holtmann	I. Firnrohr
Milchwirtschaftliche Lehr- und Untersuchungsanstalt Krefeld, Landwirtschaftskammer NRW, Krefeld, Germany	U. Hackl	U. Hackl
Milchwirtschaftliche Lehr- und Untersuchungsanstalt Oranienburg e.V., Oranienburg, Germany	B. Bartel	R. Lukowsky
MUVA Kempten, Qualitäts- und Laborzentrum, Kempten, Germany	K.Friedrich, M. Seidl	C. Schütze
Nestlé PTC – Konolfingen, Technology Center, Konolfingen, Switzerland	W. Sybesma, S.Knippenberg	D. Lusuardy
Teagasc Moorepark, Moorepark Food Research Centre, County Cork, Ireland	P. Simpson	P. Simpson
Tierärztliche Hochschule Hannover, Zentrum für Lebenswissenschaften, Institut für Lebensmittelqualität und -sicherheit, Hannover, Germany	G. Klein, C. Bonaparte	B. Ahlfeld
University of Natural Resources and Applied Life Sciences, Vienna (BOKU), Department of Food Science and Technology, Division of Food Quality Assurance, Vienna, Austria**	W. Kneifel, U. Zitz	U. Zitz, I. Gretner
University of Verona, Dipartimento Scientifico e Tecnologico, Verona, Italy	F. Dellaglio, M. Marzotto	M. Marzotto
Laboratories in Japan and New Zealand	**Responsible Person**	**Analyst**
Fonterra Innovation Palmerston North, Palmerston North, New Zealand	S. Miller	L. Ward
Meiji Dairies Corporation, Division of Research and Development, Food Technology Research Institute, Kanagawa, Japan	H. Kamikado	Y. Tsujimoto
Morinaga Milk Industry Co., Ltd., Analytical Research Center, Chemical & Microbiological Inspection Section, Kanagawa-pref., Japan	Y. Yano	Y. Yano
Kyodo Milk Industry Co., Ltd., Research Laboratories, Tokyo, Japan	M. Matsumoto	M. Matsumoto
Snow Brand Milk Products Co., Ldt., Technology and Research Institute, Kawagoe, Saitama, Japan	Y. Seto	E. Mochiduki
Yakult Central Institute for Microbiological Research, Tokyo, Japan	Y. Shimakawa	Y. Shimakawa

*Twentythree analysts from 21 laboratories participated in the collaborative trial leading to 23 datasets. For reasons of simplification these datasets were treated like individual laboratories.
** Central laboratory

3.1.2 Samples

Each of the samples originated from the same lot. For test series "phase B" (see 3.1.3), a probiotic spray-dried infant milk powder containing bifidobacteria was selected (Nestlé, Konolfingen, Switzerland). For "phase C" (see 3.1.3), a selection of probiotic yogurt products containing different bifidobacterial species and strains was used. In this context, the market relevance for Europe and Japan was considered. The European yogurts originated from different dairy companies (in alphabetical order: Danone, Vienna, Austria; NOEM AG, Baden, Austria; Zott GmbH & Co KG, Mertingen, Germany). Each sample was coded according to a given study plan (Figures 3 and 4). Samples were packaged by the central laboratory in Europe (BOKU, Vienna) and shipped to the partners taking into account the stability results of the transport simulation tests (3.1). All yogurts were shipped under comparable conditions at a temperature between 0°C and +4°C, in accordance with ISO Standard 7218:1996. A second centre for sample distribution was established in Japan (Yakult, Tokyo), also ensuring shipment conditions under analogous conditions ("phase C" test series, for details see 3.1.3). A survey of all samples is presented in Table 3.

Table 3. Samples used in the collaborative trial; 1-6: yogurts; 7: infant milk formula

Samples	Description	Type	Code
1	Commercial European probiotic yogurt containing B.animalis (BB12®), L.acidophilus (LA5®), S.thermophilus	liquid	A, B
2	Commercial European probiotic yogurt containing B.animalis (Digestivum essensis), L.delbrueckii subsp.bulgaricus, S.thermophilus	firm	C, D
3	Commercial European probiotic yogurt containing B.animalis, L.casei (belactiva 3), S.thermophilus	liquid	E, F
4	Commercial Asian probiotic yogurt containing B.breve (SBR3212), L.casei (AST-8), S.thermophilus	liquid	A, B
5	Commercial Asian probiotic yogurt containing B.longum (SBT2928), L.gasseri, L.delbrueckii subsp.bulgaricus, S.thermophilus	firm	C, D
6	Commercial Asian probiotic yogurt containing B.animalis (BB12®), L.acidophilus, S.thermophilus	firm	E, F
7	Commercial probiotic infant milk formula containing B.animalis (BB12®)	powder	K, L, N

3.1.3 Study design for the "IDF-Bifido"- collaborative trial

A graphical survey of the studies performed is shown in Figure 1. The "IDF-Bifido"- collaborative trial was carried out in 3 phases. Starting with the assessment of the analyst performance of all participating analysts in December 2005 (phase A), phases B and C were performed with probiotic products, throughout ensuring that the same analysts be involved. After sample examination (phases B and C) the data were collected and statistical analyses carried out to calculate the precision parameters of the method.

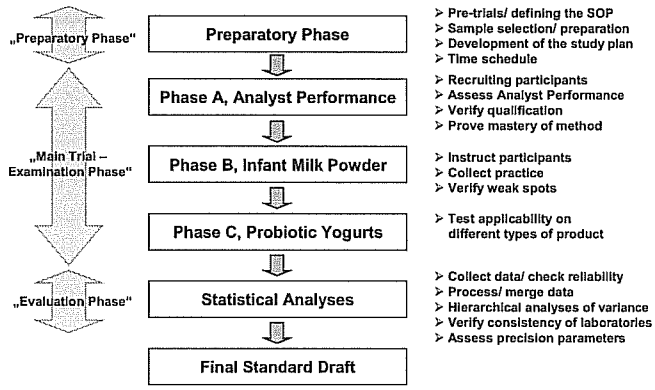

Figure 1. General structure of the working programme; SOP = standard operation procedure

3.1.3.1 Phase A

It was agreed among all partners to assess the analyst performance using the so-called "half-fraction" mode, in accordance with ISO Standard 14461-1 / IDF 169-1. For this purpose, the culture technique with a pre-grown strain of Lactobacillus (e.g. L. casei or L. delbrueckii subsp. bulgaricus) in MRS broth was applied and pour plates with MRS-medium were produced followed by anaerobic incubation for 48 hours at 37°C (Fig. 2). The colony counts (coded plates) obtained were registered in data entry forms.

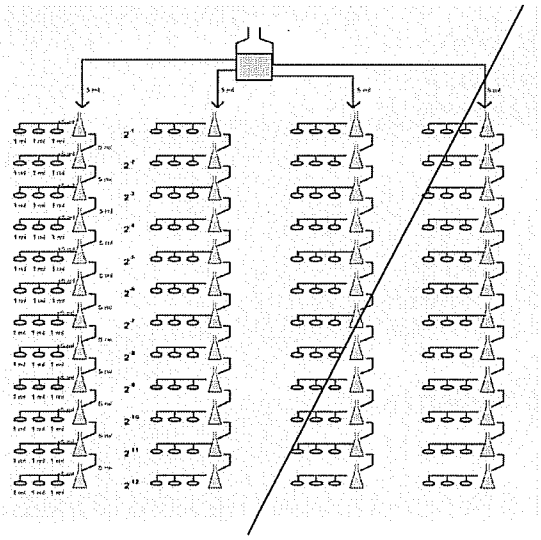

Figure 2. Study plan of analyst performance assessment [ISO 14461-1 / IDF 169-1:2005] – Phase A

3.1.3.2 Phase B

A commercially available infant milk formula (spray-dried powder) containing bifidobacteria was used, as this low-water-activity product guarantees a constant viable count level even during long-distance transportation. This sample was delivered to all participating analysts (for explanation see also footnote to Table 2). According to the hierarchical study plan, three sample units were subjected to analysis, followed by the preparation of two sub-samples. Two dilutions series of each sub-sample were prepared. The study protocol of phase B is shown in Fig. 3.

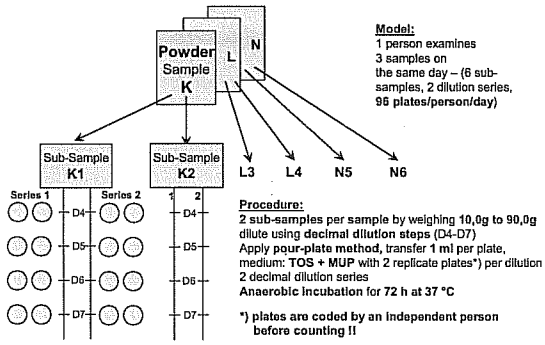

Figure 3. Study plan of Phase B (examination of infant milk powder containing bifidobacteria)

3.1.3.3 Phase C

Owing to the fact that the stability of the viable count in the samples can only be guaranteed if short distance shipping is facilitated under cooled conditions, two regional centres (Europe, Asia-Pacific) were defined. Both centres chose three locally relevant yogurts with different bifidobacterial strains. The homogeneity of these yogurt products was tested in preceding trials. Simulation of transportation at different temperatures and duration was made in order to find out optimum packaging and shipment conditions, thereby avoiding changes in the viable counts of the products. The examination of the yogurt products was agreed to be performed on a fixed date. Two sub-samples of each selected product were analysed (Fig. 4).

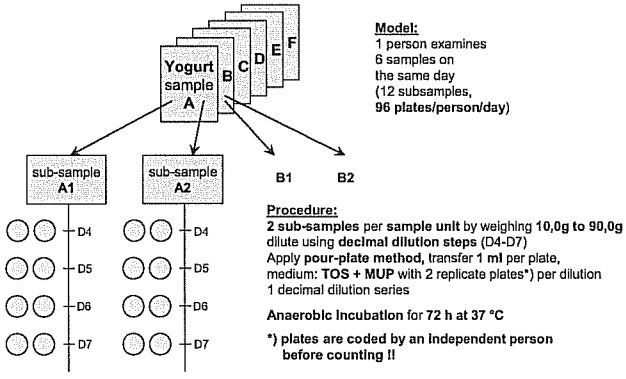

Figure 4. Study plan of Phase C (examination of probiotic yogurts)

3.1.3.4 Statistical evaluation

Using the data from phase A statistical analysis was performed with a software programme developed by the Institute for Biometrics and Data Processing (http://web.fu-berlin.de/glp-analyse/). Based on this calculation, the mastery of this method by each analyst was evaluated.

The hierarchical structure of the study phases B and C enabled the assessment of each participating laboratory based on descriptive argumentation. This aided in the verification of the validity of the datasets and possible deficiencies could be identified. It was decided to proceed according to following steps:

- Data collection of the randomized colony counts of all partners
- Decoding of all colony count results
- Determination of the reliability of colony counts of parallel plates and subsequent dilution steps according to ISO 14461-2 / IDF 169-2
- Exclusion of outlier data
- Calculation of the weighted arithmetical mean values of CFU`s counted for all dilution steps within

the countable range
- Calculation of the logarithms there from
- Merging of all logarithmic values of each phase (phase B and C) each into a separate Excel-data-file
- Performance of statistical tests to assess the consistency between and within all laboratories (Mandel`s h and Mandel`s k statistics), application of the classical [ISO 5725-2] as well as the robust mode [Wilrich, 2007].
- Detection of sources of additional deviations based on variance component analyses according to ISO 5725-3
- Evaluation of the laboratory results, aiming at finding explanations for non-conformities, decision on the considering or non-considering the involved laboratories
- Preparation of diagrams depicting the individual results of both study phases.
- Performance of the one-way Analysis of Variance
- Calculation of the Intra- and Inter laboratory precision data, repeatability r, reproducibility R, considering the classical [ISO 5725-2] as well as the robust mode [Wilrich, 2007].
- Calculation of the precision data for each product
- Calculation of collective precision data for similar product groups
- Final discussion

3.2 Results and Discussion

3.2.1 Phase A – Analyst Performance

For each participating analyst randomized colony counts were subjected to statistical analysis. Based on variance component analysis, the overall variance was found to be acceptable and the individual variation reflecting typical parts of the procedure was used to trace variabilities. Fig. 5 shows the results of this evaluation.

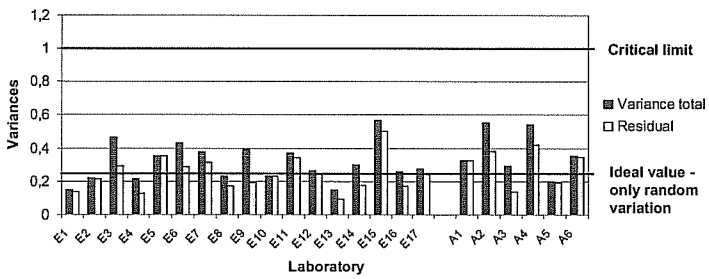

Figure 5. Analyst performance assessment of involved laboratories – Phase A; the numbering of the laboratories does not correspond with the order appearing in Tab 2

It could be demonstrated that all laboratories were capable of meeting the requirements. This lead to the assumption that all analysts worked on a comparable level of qualification. None of the total variances exceeded the critical value of 1. Most of the residual variances, including the variance component of the parallel plates, were predominantly found around an ideal level of 0,25. Variances may have their sources in the pouring technique as well as in visual counting, and are to be seen in addition to the existing random variation. Higher differences observed with some laboratories may be caused by outliers or slight irregularities of the procedure.

3.2.2 Phase B – Examination of a probiotic infant milk formula

This phase of the ring-trial was performed by 23 laboratories according to 3.1.3.2. The product was provided and shipped by Nestlé (Switzerland). The TOS-medium originated from Yakult Pharmaceuticals (Japan), Li-Mupirocin was from GlaxoSmithKline (UK).

Raw data were verified according to the ISO Standard 14461-2 / IDF 169-2, and the reliability of the colony counts of parallel plates as well as of subsequent ten-fold dilution steps was assessed. Data exceeding the limits of agreement were excluded before the calculation of the logarithms of the weighted arithmetical mean values.

In Fig. 6 the variability of the mean values is shown with reference to all participating laboratories. The graphical consistency technique was applied to evaluate the individual laboratories. The

Mandel`s h and k statistics indicates that some laboratories produced some deviating results. Possible sources of error were located by using hierarchical variance analyses of noticeable data sets (Table 4). Based on this method, non-conformities could be identified and subsequently discussed with the laboratories.

Table 4. Exemplary data of variance component analyses expressed as their individual contributions of variances (examination of the infant milk formula)

Laboratory	1		13		14		15		6	
	variance	%	variance	%	variance	%	variance	%	variance	%
Sample	0,038	66,2	0,071	93	0,069	92,6	0,018	88,1	0,000	0
Sub-sample	0,016	27,7	0,003	4,3	0,000	0	0,000	0	0,000	0
Error	0,004	6,1	0,002	2,7	0,006	7,4	0,002	11,9	0,002	100

It is evident from Figures 6 and 7 that the findings from two laboratories (13 and 14) were differing from the others and thus led to higher standard deviations (Fig. 7). However, as far as the consistency between the laboratories is considered (Mandel`s h values in Fig. 6), these two laboratories fitted well into the community, albeit their within-laboratory consistency (Mandel`s k values in Fig. 6) markedly differed from the others. We could not trace a systematic error because there were only minor differences between the Mandel`s h values.

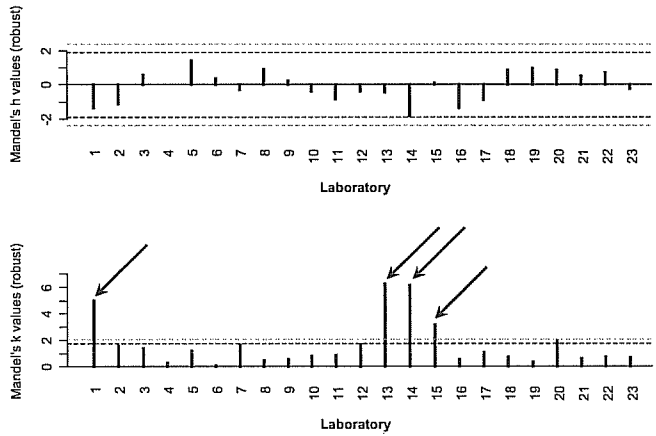

Figure 6. Mandel`s h and k values calculated from the mean values obtained for infant milk formula containing bifidobacteria (robust analysis according to [Wilrich, 2007]) - Higher variances are marked with arrows. The numbering of the laboratories does not correspond with the order appearing in Tab 2

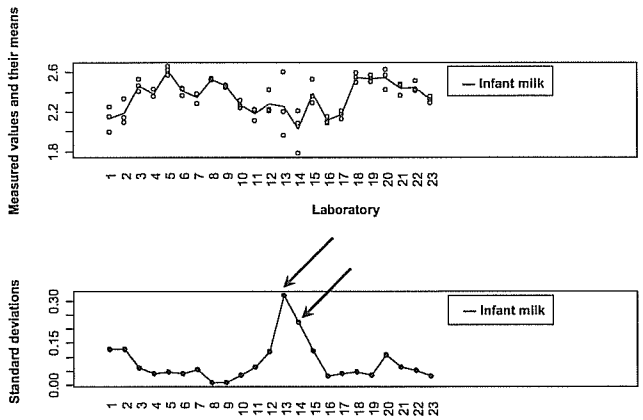

Figure 7. Bifidobacterial counts [log CFU / g] of infant milk formula – Results and corresponding standard deviations with reference to all laboratories – Higher deviations are marked with arrows. The numbering of the laboratories does not correspond with the order appearing in Tab 2

To detect the sources of additional variances, a variance component analysis was performed. In Table 4, the results of the laboratories with significant deviations are shown in comparison with Lab 6, where definitely no additional deviations occurred.

Variances due to the "sample" were identified to be highest at laboratory 13. However, this was not due to inhomogeneities of the sample material, since this property had been monitored throughout the ring-trial. The reason was more likely resulting from deviations in the sample treatment, e.g., during the preparation of sub-samples. Nevertheless, the preparation of dilutions and parallel plates as well as counting of the colonies basically did not influence too much the variance components for most of the laboratories. Laboratory 6 had variations within the parallel plate components only. This is also visualized by the calculated random error. However, both "error-values" were low (0,002), demonstrating that these laboratories are well trained to apply this technique. This was in agreement with the results of the analyst performance assessment. Based on these findings, the following reasons were identified in cooperation with laboratory 13.

Deviating from the original protocol, the trials were carried out on two subsequent days and the working order of sample treatment was not according to the protocol. Obviously, the same explanation might have been valid for laboratory 14.

Marked deviations were also observed with the results from laboratory 1. It could be verified that differing sample treatment led to the variations.

3.2.3 Phase C – Examination of selected probiotic yogurts

These trials were performed at two centres and with different yogurt products of local significance (for details see 3.1.3.3). Both test series were run under analogous conditions. Raw data assessment as well as statistical analysis were carried out at the European centre, in cooperation with the Institute for Biometrics and Data Processing. Seventeen laboratories participated in the European study, while six laboratories were coordinated by the Japanese centre.

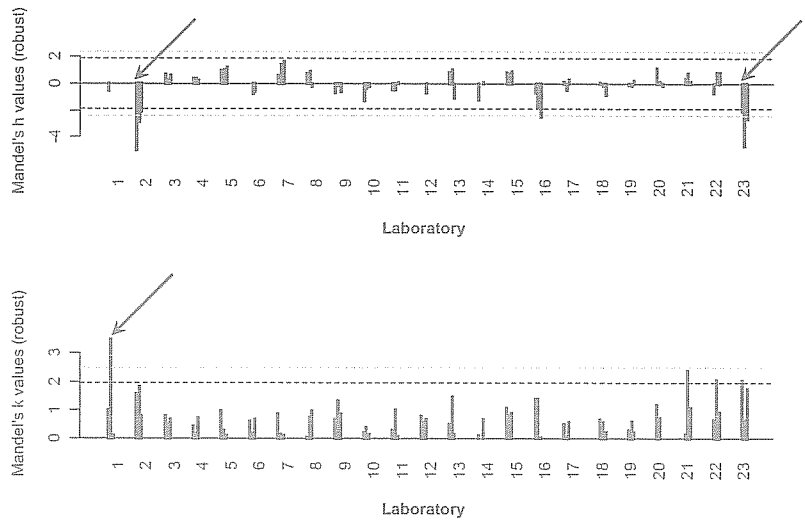

Figure 8. Mandel`s h and k values calculated from the mean values obtained for all probiotic yogurts containing bifidobacteria (robust analysis according to [Wilrich, 2007]) – Higher variances are marked with arrows. The numbering of the laboratories does not correspond with the order appearing in Tab 2

The results of the yogurt ring test are shown in Figure 8. In this graph, the consistency between and within the laboratories is demonstrated based on the Mandel`s h and the Mandel`s k statistics. Almost all laboratories were within the given limits and exhibited comparable patterns of results. Negative h value deviations were allocated to the findings of laboratories 2 and 23. These were probably of systematic nature and not caused by additional within-variances. Regarding the k values, most of the laboratories exhibited comparable variances, meeting the corresponding limits. If the results from the European laboratories (1 – 17) are considered only, a significantly higher within-laboratory variance (Mandel`s k values) is evident when including the results of Lab 1. In analogy to the findings in Phase B, it was assumed that the deviation again resulted from some non-conformities in the

procedure. The results obtained from the Asian laboratories (18 - 23) exhibited a higher within-laboratory variance when including the results of Lab 21 and Lab 22.

Table 5. Variance component analysis of yogurt 2, exemplarily shown for the European laboratories.

Laboratory	1		5		6	
	variance	%	variance	%	variance	%
Sample	0,010	39,5	0,000	0	0,000	0
Error	0,015	60,5	0,004	100	0,008	100

The variance component analyses results of the European laboratories showed a relatively high sample variance component if the results obtained for yogurt 2 at laboratory 1 were considered (see Table 5). Inhomogeneities of the sample lot were checked and not evident. This information was also supported by the low variance components detected with laboratories 5 and 6. Laboratory 1 produced results with a variance of 0.01 log CFU/g, possibly resulting from deviations from the protocol. The higher variance component identified as "error" fraction includes all deviations resulting from the whole procedure (sample dilution, counting of the plates). In Lab 1 this value (0,015) was somewhat higher than in the other labs.

Table 6. Variance component analyses of yogurt 5, exemplarily shown for the Asian laboratories.

Laboratory	21		22		18	
	variance	%	variance	%	variance	%
Sample	0,032	93,1	0,023	85,5	0,002	98,1
Error	0,002	6,9	0,004	14,5	0,000	1,9

Variance component analyses of the results obtained in the Asian laboratories for yogurt 5 (see Table 6) differed from those of yogurt 2 of the European centre. The largest variance component assigned to the "sample" could be explained as a lot-dependent inhomogeneity. The between-laboratory consistency among the laboratories (Mandel`s h values) was acceptable, with the following exception: considering all products tested, the values of laboratory 23 were lower than of the others. Laboratory 23 (located in Europe) also analyzed the samples disseminated in the Asian region. However, owing to the long distance, the samples had to be shipped on dry ice and this was different from the other shipment conditions. For this reason, deviating results were expected.

In Figure 9, individual results are shown whereas the different yogurts are marked in different patterns. It is evident from the graphs that within the European centre higher deviations are only located at laboratory 1 (yogurt 2). Within the Asian centre the laboratories 21 and 22 had higher deviations only with yogurt 5.

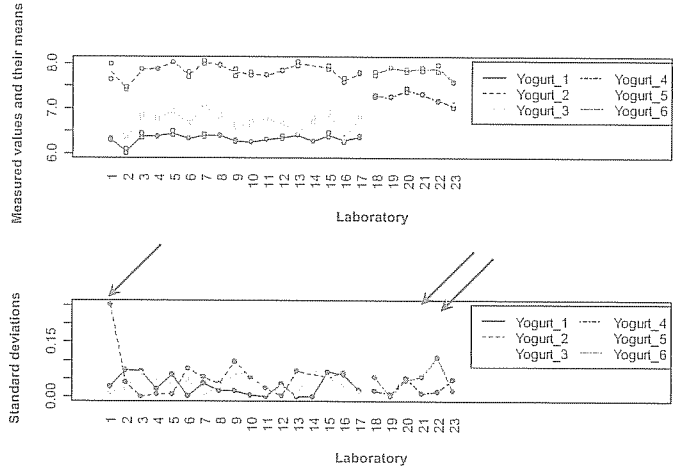

Figure 9. Bifidobacterial counts [log CFU / g] of probiotic yogurts – Results and corresponding standard deviations with reference to the different centres, yogurts 1-3 were examined by laboratories 1-17 (Europe), yogurts 4-6 by laboratories 18-23 (Asia) – Higher deviations are marked with arrows. The numbering of the laboratories does not correspond with the order appearing in Tab 2

3.2.4 Precision data

Repeatability and reproducibility limits were determined using the results of the examination of infant milk powder containing bifidobacteria as well as of six different probiotic yogurt products containing different bifidobacterial strains. The viable count levels of the bifidobacteria in the selected products were found to be similar to other products marketed worldwide [Masco et al., 2005] being in accordance with the Codex Standard regulation [CODEX STAN 243-2003].

Taking into account the results from phases B and C, the standard deviations of the repeatability and of the reproducibility were calculated according to the classical [ISO 5725-2] as well as the robust analysis approach [Wilrich, 2007]. All variations reflecting practical conditions were considered.

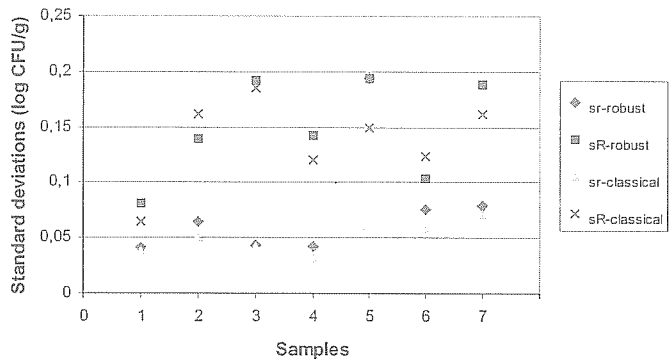

Figure 10. Repeatability (r) and reproducibility (R) expressed as standard deviations calculated by classical and robust methods with reference to all samples (for product details see Tab 3)

In Figure 10, the precision data are summarized for each probiotic product. Corresponding robust variation data can be seen in Table 7. The repeatability expressed as standard deviation was quite similar within the liquid products and also within the firm products, with the exception of yogurt 5, which exhibited irregularities due to inhomogeneity. Hence it was excluded from the final calculation of the precision data according to the type of product (Tab. 8). Based on the similarities, combined precision data were defined characterising each product type.

Table 7. Precision data, expressed as repeatability limit (r) and reproducibility limit (R) as well as their standard deviations (s_r, s_R respectively) calculated by robust method with reference to different product types (for product details see Tab 3)

Sample	Type	Datasets	s_r	s_R	r	R
1	liquid	17	0,041	0,081	0,115	0,227
2	firm	17	0,065	0,139	0,182	0,389
3	liquid	17	0,044	0,192	0,123	0,538
4	liquid	6	0,042	0,143	0,118	0,400
5	firm	6	0,194	0,194	0,543	0,543
6	firm	6	0,076	0,104	0,213	0,291
7	powder	23	0,079	0,189	0,221	0,529

Table 8. Precision data combined for each product type

Type	s_r collective	s_R collective	r (log CFU/g)	R (log CFU/g)
liquid	0,042	0,139	0,12	0,39
firm	0,071	0,144	0,20	0,40
powder	0,079	0,189	0,22	0,53

The differences observed can be of diverse origin: It is known that the texture of a yogurt may influence the viable count of a fermented milk product. This effect may be due to incomplete pipetting or residues on the glassware, in particular with set-style and creamy products. Moreover, steps of sample preparation before dilution (e.g., a powdered sample has to be dissolved and reconstituted) may also bear sources of variation and cause differing individual results. It is, therefore, of importance to strictly follow a given procedure if such deviations are to be avoided. After incubation of the inoculated plates, the size and appearance of the colonies may also influence the counting. In particular, pin point colonies usually may cause under-estimation of the counts. The reason for appearing as pin point colonies may be found in the specific nature of the individual microorganisms, but also in the mode of incubation. For example, anaerobic jars may produce colonies differing in size from those obtained by incubation in an anaerobic cabinet. Furthermore, "running colonies" with haloes can be observed with some strains, especially when jars are used. Another influence on colony performance may be exerted by different atmospheric conditions. Sample inhomogeneities are lot-specific and also depend on the status of the shelf-life of a yogurt. In this context, syneresis can be a source of irregular distribution and density of the bacteria in the product. Last but not least, the analyst can be identified as being responsible for deviating results, especially when the procedure is not strictly followed.

4 Conclusions

In general, the results of the ring test have demonstrated a successful application of the developed method, which is recommended to be subjected to standardisation. However, further practical steps will be needed to accomplish this. The availability of the medium and of the supplements is of crucial importance, before a standard method can be approved [ISO / IDF Standard, Draft-Version 1].

The study has also shown that besides a series of pre-trials where experience on the method as well as on the investigators can be collected, a comprehensive preparation phase is necessary for planning, discussing and establishing a useful analytical design for the collaborative trials. The statistical approach is of high relevance from the very beginning, since depending on the field of application, the quality parameters of the method need to be defined. Another important criterion is the proper selection of the sample material for collaborative trials. In addition, the documentation of all relevant details throughout the trials is a pre-requisite for being able to optimize the procedure and to solve emerging problems. The methodological developments and the collaborative trials have shown that not only the ripeness of the method influences the outcome but also the integration of the participating laboratories and persons in the whole development process.

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6 References

Arroyo, L., Cotton, L.N., Martin, J.H. Evaluation of media for enumeration of Bifidobacterium adolescentis, B. infantis and B. longum from pure culture. Cult. Dairy Prod. J. 29: 20-24 (1994)

Arroyo, L., Cotton, L.N., Martin, J.H. AMC Agar – A composite medium for selective enumeration of Bifidobacterium longum. Cultural Dairy Products Journal 30: 12-15 (1995)

Beerens, H. An elective and selective isolation medium for Bifidobacterium spp. Letters in Applied Microbiology 11: 155-157 (1990)

Beerens, H. Detection of Bifidobacteria by Using Propionic Acid as a Selective Agent. Applied and Environmental Microbiology 57: 2418-2419 (1991)

Blaurock, G. Bifiduszüchtung auf zystinhaltigem Nährböden. Zbl. Bakt. Hyg I. Abt. Orig. 144: 75-79 (1939)

Bonaparte, C. Selective isolation and taxonomic position of bifidobacteria isolated from commercial fermented dairy products in central Europe. PhD Thesis, Münster, Berlin Technical University 199p. (1997)

Chevalier, P., Roy, D., Savoie, L. X-α-Gal-based medium for simultaneous enumeration of bifidobacteria and lactic acid bacteria in milk. Journal of Microbiological Methods 13: 75-83 (1991)

Codex Stan 243-2003. Codex Standard for Fermented Milks. Codex Alimentarius 13: 1-5 (2003)

Dave, R.I., Shah, N.P. Evaluation of Media for Selective Enumeration of Streptococcus thermophilus, Lactobacillus delbrueckii ssp. bulgaricus, Lactobacilus acidophilus, and Bifidobacteria. J. Dairy Sci. 79: 1529-1536 (1996)

DeMan, J.C., Rogosa, M., Sharpe, M.E. A medium for the cultivation of lactobacilli. Journal of Applied Bacteriology 23: 130-135 (1960)

Expert Group on Selective Enumeration of Bifidobacteria. Method of Enumeration of Bifidobacteria in Fermented Milks and Fermented Milk Drinks. Japanese Association of Fermented Milks and Fermented Milk Drinks (2000)

Ghoddusi, H.B., Robinson, R.K. Enumeration of starter cultures in fermented milks. Journal of Dairy Research 63: 151-158 (1996)

Hartemink, R., Kok, B.J., Weenk, G.H., Rombouts, F.M. Raffinose-Bifidobacterium (RB) agar, a new selective medium for bifidobacteria. Journal of Microbiological Methods 27: 33-43 (1996)

Hirsch, A., Grinstead, E. Reinforced clostridial medium. J. Dairy Res. 21: 101-110 (1954)

Holzapfel, W.H., Haberer, P., Snel, J., Schillinger, U., Huis in`t Veld, J.H.J. Overview of gut flora and probiotics. Int. J. Food Microbiology 41: 85-101 (1998)

Holzapfel, W.H., Schillinger, U. Introduction to pre-and probiotics. Food Research International 35: 109-116 (2002)

IDF-"Bifido"-Guideline; IDF Group E104 – Lactic acid bacteria and starters. Guideline for the Enumeration of Bifidobacteria in Fermented Dairy Products. Bull. Int. Dairy Fed. 340: 19-23 (1999)

IDF Int. Standard 128A. Milk: Definition and evaluation of the overall accuracy of indirect methods of milk analyses – Application to calibration procedure and quality control in the dairy laboratory (1999)

IDF Int. Standard 135B. Milk and Milk Products – Precision characteristics of analytical methods – Outline of collaborative study procedure (1991)

ISO / IDF Standard, Draft-Version 1. – Selective Enumeration of Bifidobacteria in Dairy Products. (Under preparation)

ISO 707 / IDF 50C, Milk and milk products – Guidance on sampling (1997)

ISO 5725-1. Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions (1994)

ISO 5725-2. Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method (1994) / Cor 1: 2002

ISO 5725-3. Accuracy (trueness and precision) of measurement methods and results – Part 3: Intermediate measures of the precision of a standard measurement method (1994) / Cor 1: 2001

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 (1999)

ISO 7218, Microbiology of food and animal stuffs – General rules for microbiological examinations (1996) / Amd 1: 2001

ISO 7889 / IDF 117. Yogurt – Enumeration of characteristic micro-organisms – Colony-count technique at 37°C. (2003)

ISO 8261 / IDF 122, Milk and milk products – General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examinations (2001)

ISO 14461-1 / IDF 169-1. Milk and Milk Products – Quality control in microbiological laboratories – Part 1: Analyst performance assessment for colony counts (2005)

ISO 14461-2 / IDF 169-2. Milk and Milk Products – Quality control in microbiological laboratories – Part 2: Determination of the reliability of colony counts of parallel plates and subsequent dilution steps (2005)

Lapierre, L.P., Undeland, P., Cox, L.J. Lithium chloride - sodium propionate agar for the enumeration of bifidobacteria in fermented dairy products. Journal of Dairy Science 75: 1192-1196 (1992)

Laroia, S., Martin, J.H. Methods for Enumeration and Propagating Bifidobacteria. Cultured Dairy Products Journal 32-33 (1991)

Leuschner, R.G.K., Bew, J., Simpson, P., Ross, P.R., Stanton, C. A collaborative study of a method for the enumeration of probiotic bifidobacteria in animal feed. Internat. Journal of Food Microbiology 83: 161-170 (2003)

Lim, K.S., Huh, C.S., Baek, Y.J. A selective Enumeration Medium for Bifidobacteria in Fermented Dairy Products. J. Dairy Sci. 78: 2108-2112 (1995)

Masco, L., Ventura, M., Zink, R., Huys, G., Swings, J. Polyphasic taxonomic analyses of Bifidobacterium animalis and Bifidobacterium lactis reveals relatedness at the subspecies level: reclassification of Bifidobacterium animalis as Bifidobacterium animalis subsp. animalis subsp. nov. and Bifidobacterium lactis as Bifidobacterium animalis subsp. lactis subsp. nov. Int. Journal of Systematic and Evolutionary Microbiology 54: 1137-1143 (2004)

Masco, L., Huys, G., De Brandt, E., Temmerman, R., Swings, J. Culture-dependent and culture-independent qualitative analyses of probiotic products claimed to contain bifidobacteria. Int. Journal of Food Microbiology 102: 221-230 (2005)

Mayer, H.K., Bonaparte, C., Newart, M., Kneifel, W. Authentication of probiotic bifidobacteria using protein and DANN fingerprinting techniques. IDF-Bulletin S.I. 0301, 62-84 (2003)

Mitsuoka , T., Sega, T., Yamamoto, S. Improved methodology of qualitative and quantitative analyses of the intestinal flora of man and animals. Zbl. Bact. Hyg., I Abt.Orig. 195: 455-465 (1965)

Munoa, F.J., Pares, R. Selective Medium for Isolation and Enumeration of Bifidobacterium spp. Applied and Environmental Microbiology 54: 1715-1718 (1988)

Pacher, B., Kneifel, W. Development of a Culture Medium for the Detection and Enumeration of Bifidobacteria in Fermented Milk Products. Int. Dairy Journal 6: 43-64 (1996)

Payne, J.F., Morris, A.E.J., Beers, P. Evaluation of selective media for the enumeration of Bifidobacterium sp. in milk. Journal of Applied Microbiology 86: 353-358 (1999)

Rada, V., Sirotek, K., Petr, J. Evaluation of Selective Media for Bifidobacteria in Poultry and Rabbit Faecal Samples. J. Vet. Med. B. 46: 369-373 (1999)

Rada, V., Koc, J. The use of mupirocin for selective enumeration of bifidobacteria in fermented milk products. Milchwissenschaft 55: 65-67 (2000)

Rada, V., Petr., J. A new selective medium for the isolation of glucose non fermenting bifidobacteria from hen caeca. Journal of Mikrobiological Methods 43: 127-132 (2000)

Rasic, J.Lj. Culture media for detection and enumeration of bifidobacteria in fermented milk products. Bull. Int. Dairy Fed. 252: 24-34 (1990)

Rogosa, M., Mitchell, J. A., Wijsman, R.F. A selective medium for the isolation of oral and faecal lactobacilli. Journal of Bacteriology 62: 132-133 (1951)

Roy, D., Mainville, I., Mondou, F. Selective Enumeration and Survival of Bifidobacteria in Fresh Cheese. Int. Dairy Journal 7: 785-793 (1997)

Roy, D. Media for the isolation and enumeration of bifidobacteria in dairy products. Int. Journal of Food Microbiology 69: 167-182 (2001)

Rybka, S., Kailasapathy, K. Media for the Enumeration of Yoghurt Bacteria. Int. Dairy Journal 6: 839-850 (1996)

Samona, A., Robinson, R.K. Enumeration of bifidobacteria in dairy products. Journal of the Society of Dairy Technology 44: 64-66 (1991)

Scardovi, V. Genus Bifidobacterium Orla-Jensen 1924, 472AL, p. 1418-1434. In P.H.A.Sneath, N.S.Mair, M.E.Sharpe, and J.G.Holt (ed.), Bergey`s manual of systematic bacteriology, Vol. 2. The Williams & Wilkings Co., Baltimore. (1986)

Silvi, S., Rumney, C.J., Rowland, I.R. An assessment of three selective media for bifidobacteria in faeces. Journal of Applied Bacteriology 81: 561-564 (1996)

Simpson, P.J., Fitzgerald, G.F., Stanton, C., Ross, R.P. The evaluation of a mupirocin-based selective medium for the enumeration of bifidobacteria from probiotic animal feed. Journal of Microbiological Methods 57: 9-16 (2004)

Sonoike, K., Mada, M., Mutai, M. Selective Agar Medium for Counting Viable Cells of Bifidobacteria in Fermented Milk. J. Food Hyg. Soc. Japan. 27: 238-244 (1986)

Tanaka et al., Effects of Administration of TOS and Bifidobacteriaum breve 4006 on the Human Faecal Flora. Bifidobacteria Microflora 2: 17-24 (1983)

Teraguchi, S., Uehara, M., Ogasa, K., Mitsuoka, K. Enumeration of bifidobacteria in dairy products. Japanese Journal of Bacteriology 33: 753-761 (1978)

Tharmaraj, N., Shah, N.P. Selective Enumeration of Lactobacillus delbrueckii ssp. bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Bifidobacteria, Lactobacillus casei, Lactobacillus rhamnosus, and Propionibacteria. J. Dairy Sci. 86: 2288-2296 (2003)

Van de Casteele, S., Vanheuverzwijn, T., Ruyssen, T., Van Assche, P., Swings, J., Huys, G. Evaluation of culture media for selective enumeration of probiotic strains of lactobacilli and bifidobacteria in combination with yoghurt or cheese starters. Int. Dairy Journal 16: 1470-1476 (2006)

Vinderola, C.G., Reinheimer, J.A. Culture media for the enumeration of Bifidobacterium bifidum and Lactobacillus acidophilus in the presence of yoghurt bacteria. Int. Dairy Journal 9: 497-505 (1999)

Vlkova, E., Rada V., Trojanova, I. Enumeration, Isolation and Identification of Bifidobacteria from Dairy Products. Original Scientific Paper. (2004)

Wijsman, M.R., Hereijgers J.L.P.M., de Groote, J.M.F.H. Selective enumeration of bifidobacteria in fermented dairy products. Neth. Milk Dairy J. 43: 395-405 (1989)

Wilkins, T.D., Chalgren, S. Wilkins-Chalgren Anaerobe Agar. Antimicrob. Agents Chemother. 10: 926-928 (1976)

Wilrich, P.-Th. Robust estimates of the theoretical standard deviation to be used in interlaboratory precision experiments. Accredit. Qual. Assur. In press (2007)

SELECTIVE ENUMERATION OF BIFIDOBACTERIA IN DAIRY PRODUCTS: DEVELOPMENT OF A STANDARD METHOD

ABSTRACT

Bifidobacteria play an important role as probiotic microorganisms in fermented dairy products. Usually, culture methods are used for routine examination of these products. As far as bifidobacterial enumeration is considered, several methodologies have been proposed during the last two decades, however, with limited applicability due to laborious procedures and/or lacking selectivity. According to experience, the accompanying microflora often negatively influences the discrimination among the different groups of bacteria contained in a product. On this account, a project team within the IDF "Lactic acid bacteria" group elaborated a procedure for bifidobacterial enumeration and a draft standard method. This report describes in detail, how the method was developed and assessed for its performance based on selectivity, reproducibility and repeatability. It can be used as a roadmap for developing a microbiological standard method.

Keywords: Bifidobacteria, probiotics, dairy products, colony count technique, collaborative trials, standardization

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" ..... Usually double quotes and not single quotes
? ! ..... Half-space before and after question marks, and exclamation marks
± ..... Half-space before and after
microorganisms ..... Without a hyphen
Infra-red ..... With a hyphen
et al. ..... Not underlined nor italic
e.g., i.e.,... ..... Spelled out in English - for example, that is
litre ..... Not liter unless the author is American
ml, mg,... ..... Space between number and ml, mg,...
skimmilk ..... One word if adjective, two words if substantive
sulfuric, sulfite, sulfate ..... Not sulphuric, sulphite, sulphate (as agreed by IUPAC)
AOAC International ..... Not AOACI
programme ..... Not program unless a) author is American or b) computer program
milk and milk product ..... rather than "milk and dairy product" - Normally some latitude can be allowed in non scientific texts
-ize, -ization ..... Not -ise, -isation with a few exceptions
Decimal comma ..... in Standards (only) in both languages (as agreed by ISO)
No space between figure and % - i.e. 6%, etc.
Milkfat ..... One word
USA, UK, GB ..... No stops
Figure ..... To be written out in full
1000-9000 ..... No comma
10 000, etc. ..... No comma, but space
hours ..... ø h
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