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Accuracy of species identity of commercial bacterial cultures intended for probiotic or nutritional use

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Abstract

Independent studies have indicated that the microbiological composition of several commercial probiotic products does not correspond to the product label information. The present study set out to investigate to what extent these problems may be due to the use of misidentified cultures at the onset of production. For this purpose, 213 cultures of lactic acid bacteria (LAB) and propionibacteria intended for probiotic or nutritional use were collected from 26 manufacturers of probiotic products, three international culture collections and one research institute. The accuracy of the taxonomic identity provided by the strain depositor was assessed through a polyphasic approach based on validated and standardized identification methods including fluorescent amplified fragment length polymorphism (FAFLP) and repetitive DNA element (rep)-PCR fingerprinting, protein profiling and partial 16S rDNA sequencing. The majority of the cultures were received as members of the genera *Lactobacillus* (57%) and *Bifidobacterium* (22%); however, propionibacteria, enterococci, *Lactococcus lactis* (subsp. *lactis*), *Streptococcus thermophilus* and pediococci were also obtained. Upon reidentification, 46 cases of misidentification at the genus level ($n = 19$) or species level ($n = 27$) were recorded, including 34 commercial probiotic cultures deposited by 10 different companies. The finding that more than 28% of the commercial cultures intended for human and/or animal probiotic use were misidentified at the genus or species level suggests that many cases of probiotic product mislabeling originate from the incorporation of incorrectly identified strains. A large number of these discrepancies could be related to the use of methods with limited taxonomic resolution (e.g., API strips) or that are unsuitable for reliable identification up to species level (e.g., pulsed-field gel electrophoresis and randomly amplified polymorphic DNA analysis). The current study has again highlighted that reliable identification of LAB and propionibacteria requires molecular methods with a high taxonomic resolution that are linked to up-to-date identification libraries.

Keywords: Product labeling; FAFLP; Rep-PCR; *Lactobacillus*, *Bifidobacterium*

1. Introduction

The application of microorganisms as probiotics for healthy humans and specific patient groups has gained considerable interest amongst microbiologists, nutritionists and clinicians worldwide. In parallel with the growing number of probiotic applications ranging from food supplements to biotherapeutics, the biodiversity of strains exhibiting potentially probiotic functionalities has increased remarkably in recent years. The large majority of commercial probiotic products contain one or multiple strains of lactic acid bacteria (LAB) primarily belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Pediococcus*, *Enterococcus* and *Streptococcus*. In addition, other bacterial taxa such as *Propionibacterium* spp., *Bacillus* spp. and *Escherichia coli* and the yeast "*Saccharomyces boulardii*" (nom. inval.) have also been used in probiotic products [14,17,21].

The selection and evaluation of potential probiotic candidates is a multistep process focusing on functional, safety and

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technological aspects [30,32]. However, because most LAB are generally considered to have a long history of safe use, the safety assessment of probiotics for human use has long been ignored or considered irrelevant. Fortunately, there is a growing awareness that the correct identification of a probiotic strain is one of the first prerequisites documenting its microbiological safety. Given the complex and challenging process from strain selection to product development, taxonomic characterization of probiotic cultures ideally needs to be conducted before production (i.e., by analyzing pure cultures) as well as after production (i.e., by analyzing the products). In practice, however, studies that have assessed the identity of probiotic strains mainly relied on isolates recovered from commercial products and only rarely included the original probiotic cultures. Several of these surveys have revealed deficiencies in the microbiological quality and labeling of probiotic products [8,9,15,18,34,35,46]. From the taxonomic point of view, the reported discrepancies can be categorized according two main scenarios: (i) the product contains the correct number of species mentioned on the label but the identity of the detected species does not match with those indicated; or (ii) the product contains additional or fewer species than indicated on the label and the identity of the detected species does not necessarily match with the species designations mentioned. Clearly, these deficiencies can have public health implications, e.g., by undermining the efficacy of probiotic products and by affecting public confidence in functional foods [12,13]. However, at present, little is known about the possible causes leading to incorrect labeling of probiotic products or at which stages of the production process deficiencies are introduced.

In the framework of the EU-funded project PROSAFE dealing with the biosafety of LAB for human use, we established a collection of 213 bacterial cultures intended for probiotic or nutritional use which were deposited by 26 manufacturers of commercial probiotic products, three international culture collections and one research institute. The present study set out to assess the accuracy of the taxonomic identity provided by the depositor of the commercial or research strains by reidentifying the strains using a polyphasic approach based on validated and standardized identification methods. In this way, it could be determined in which bacterial taxa and at what taxonomic level (genus or species) misidentifications primarily occurred. Based on information provided by strain depositors through questionnaires, certain misidentifications could be correlated with the identification method used by the depositors.

2. Materials and methods

2.1. Strain collection

A total of 54 companies involved in the worldwide production and/or distribution of probiotics were contacted and requested to submit strains to the PROSAFE project. Among these, the following 26 companies responded positively and provided strains: Anidral-Probiotical (Italy), Arla Foods (Sweden), BioGaia Biologics (Sweden), Centro Sperimentale del Latte (Italy), Cerbios Pharma (Switzerland), Chr. Hansen (Denmark), CSK Food Enrichment (The Netherlands), Cultech Agriculture Limited (currently known as Cultech Ltd; UK), Danisco (Denmark), Danone Vitapole (France), Degussa Biosystems (Germany), DSM Food Specialities (Australia), Essum AB/Norrmejerier (Sweden), Friesland Coberco Research (The Netherlands), Gewürzmüller (Germany), Kerry Bio-Science (The Netherlands), Lallemand-Institut Rosell (Canada), Morinaga Milk Industry (Japan), Natren Inc. (USA), Numico Research (The Netherlands), Probi AB (one of the strains currently owned by Celac Sweden AB; Sweden), Rhodia Food (currently owned by Danisco; USA), Sacco SRL (Italy), Symbio Herborn Group (Germany), Techno High Technology (Belgium) and Veneto Agricoltura (Italy). In addition, probiotic and nutritional strains were also obtained from three international culture collections, i.e., BCCM^{TM}/LMG Bacteria Collection, Belgium (http://bccm.belspo.be/db/lmg_search_form.php), Culture Collection University of Göteborg, Sweden (http://www.ccug.se) and German Collection of Microorganisms and Cell Cultures, Germany (http://www.dsmz.de), and from the VTT Technical Research Center, Finland (http://www.vtt.fi). Descriptive and other relevant information on 213 strains was collected from the 30 depositors via a questionnaire [38]. This information enabled us to classify the strains included into three categories: probiotic strains (i.e., those that are effectively used in probiotic products; $n = 121$), research strains (i.e., under investigation as probiotic candidates; $n = 57$); and nutritional strains (i.e., used in food products as functional and/or starter cultures without a specific probiotic claim; $n = 35$) categories. Most strains were originally isolated in Italy, The Netherlands, the USA, France, Canada, Germany and Sweden [38].

Depending on the genus designation received from the depositor, cultures were recovered on MRS agar or in MRS broth (Oxoid CM361) and incubated aerobically or anaerobically at 28 or 37 °C for 1–4 days. Following purity check, cultures were stored on cryobeads at −80 °C using the Microbank (Pro-lab) system.

2.2. Polyphasic identification strategy

Based on taxonomic resolution at the species level, speed of performance and availability of an identification library, one specific method was chosen as the identification technique of first choice for each of the genera included in this study (Table 1). Taxonomic frameworks on which identification libraries for LAB are based were previously constructed for AFLP [10], $(GTG)_5$-PCR [11] and BOX-PCR [19] fingerprinting and for protein profiling [28].

Fluorescent amplified fragment length polymorphism (FAFLP) analysis was used as the first choice method for identification of enterococci, lactobacilli and lactococci, whereas repetitive DNA element (rep)-PCR fingerprinting was used as the first identification method for cultures received as members of *Bifidobacterium* and *Pediococcus*. For both methods, microscale DNA extraction was based on the method of Gevers and co-workers [11] or Pitcher and co-workers [27] with slight modifications [19]. FAFLP analysis was carried out essentially

Table 1
Identification methods used in this study

Genus or species	Identification method used	
	First choice	Optional
Bifidobacterium	BOX-PCR	Genus-specific PCR, protein profiling
Enterococcus	FAFLP	Protein profiling, $(GTG)_5$-PCR
Lactobacillus	FAFLP	Protein profiling, $(GTG)_5$-PCR
Lc. lactis	FAFLP	Protein profiling, $(GTG)_5$-PCR
Pediococcus	$(GTG)_5$-PCR	Protein profiling
Propionibacterium	Partial 16S rDNA sequencing	$(GTG)_5$-PCR
Streptococcus thermophilus	Protein profiling	$(GTG)_5$-PCR

as previously described [36] with slight modifications [20]. rep-PCR fingerprinting using the $(GTG)_5$ primer (5′-GTGGTG-GTGGTGGTG-3′) ($(GTG)_5$-PCR) and/or the BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′) (BOX-PCR) was performed as described previously [43] with modifications [11, 19]. Numerical analysis of rep-PCR and FAFLP data, including comparison with laboratory-based identification libraries, was performed with BioNumerics V4.1 software (Applied Maths). Similarities among digitized profiles were calculated using the Pearson product–moment correlation coefficient and the band-based Dice coefficient, respectively, and an average linkage (UPGMA) dendrogram was derived from the profiles.

Partial 16S rDNA sequence analysis was used as the first choice method for the identification of propionibacteria. Genomic DNA was prepared as previously described [25]. 16S rRNA genes were amplified by PCR using the primers 16F27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16R1522 (5′-AA-GGAGGTGATCCAGCCGCA-3′) targeting positions 8–27 and 1541–1522 according to *Escherichia coli* 16S rRNA gene sequence numbering, respectively. PCR amplified 16S rDNAs were purified using the NucleoFast® 96 PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). Sequencing reactions were performed using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and purified using the Montage™ SEQ_{96} Sequencing Reaction Cleanup Kit (Millipore, Bedford, MA, USA). The following sequencing primers were used: 16F358 (5′-CTCCTACGGGAGGCAGC-AGT-3′; position 339–358), 16R339 (5′-ACTGCTGCCTCCC-GTAGGAG-3′; position 358–339) and 16R519 (5′-GTATTAC-CGCGGCTGCTG-3′; 536–519). Sequencing was performed using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were assembled using the program AutoAssembler™ (Applied Biosystems, Foster City, CA, USA) and blasted into the international nucleotide sequence library EMBL. For identification of streptococci, one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) of whole-cell proteins was chosen as the method of first choice. Preparation of cell extracts and protein gel electrophoresis were carried out as previously described [28]. Normalized and digitized protein patterns were numerically analyzed and clustered with reference profiles of LAB type and reference strains stored in a user-generated laboratory database.

In cases in which the identification result obtained with the first choice method was difficult to interpret or where there was no agreement with the original species identity provided by the depositor, one or more additional methods were applied (Table 1). Cultures received as *Bifidobacterium* that could not be readily identified by BOX-PCR were first subjected to a genus-specific PCR assay with primers lm26 (5′-GATTCT-GGCTCAGGATGAACG-3′) and lm3 (5′-CGGGTGCTICCC-ACTTTCATG-3′) as previously described [16]. Cultures that were excluded from this genus based on a negative PCR result were then analyzed by protein profiling. Likewise, cultures deposited as members of *Enterococcus*, *Lactococcus*, *Propionibacterium* and *Streptococcus* that appeared to be misidentified at the genus level were subsequently subjected to $(GTG)_5$-PCR and/or protein profiling.

3. Results

3.1. Species diversity of commercial and research cultures with confirmed identities

Most of the 213 bacterial cultures received in this study was deposited as members of the genera *Lactobacillus* ($n = 122$; 57%) and *Bifidobacterium* ($n = 48$; 22%) (Table 2). The remaining consisted of strains received as propionibacteria ($n = 12$), enterococci ($n = 11$), *Lactococcus lactis* (subsp. *lactis*) ($n = 10$), *Streptococcus thermophilus* ($n = 6$) and pediococci ($n = 4$). Based on results of polyphasic identification strategy, Table 3 provides an overview of the taxonomic diversity of the 159 probiotic, nutritional and research cultures of which the original species identity was confirmed. The species that were by far most frequently deposited included *Lactobacillus paracasei* (15 depositors), *Lactobacillus acidophilus* (11 depositors), *Lactobacillus rhamnosus* (10 depositors) and *Lactobacillus plantarum* (9 depositors).

3.2. Identity confirmation at the genus level

Upon reidentification of the 213 cultures, it was found that 194 strains (91%) belonged to the correct genus. Proportionally, the accuracy of the original genus designations of the nutritional (100%) and research (95%) strains was somewhat higher in comparison with the probiotic category, in which 87 % were correctly identified at the genus level (Table 2). The 19 strains that were misidentified at the genus level originated from five different depositors. Remarkably, most of these strains ($n = 12$) were received from a single company and all belonged to the

Table 2
Identity confirmation rates at genus and species level for 213 probiotic, nutritional or research cultures

Genus/species (as received from depositor)	Number of strains investigated (number of different depositors)				Confirmation at the genus level (%)[a]				Confirmed at the genus level and species name supplied (%)[b]				Confirmation at the species level (%)[c]			
	P	N	R	Total	P	N	R	Total	P	N	R	Total	P	N	R	Total
Bifidobacterium	19 (10)	3 (3)	26 (3)	48 (14)	14	3	26	43 (90)	14	3	19	36	10	2	17	29 (80)
Enterococcus	11 (6)		11 (6)		10			10 (91)	10			10	9			9 (90)
Lactobacillus	72 (19)	26 (11)	24 (5)	122 (25)	72	26	24	122 (100)	72	26	24	122	59	23	21	103 (84)
Lc. lactis	4 (2)	6 (2)		10 (4)	1	6		7 (70)	1	6		7	1	6		7 (100)
Pediococcus	3 (1)		1 (1)	4 (2)	3		1	4 (100)	2		1	3	2		1	3 (100)
Propionibacterium	6 (1)		6 (1)	12 (2)	1		3	4 (33)	1		3	4	1		3	4 (100)
S. thermophilus	6 (6)			6 (6)	4			4 (67)	4			4	4			4 (100)
Total	121 (24)	35 (11)	57 (5)	213 (30)	105 (87)	35 (100)	54 (95)	194 (91)	104 (99)	35 (100)	47 (87)	186 (96)	86 (83)	31 (88)	42 (89)	159 (85)

N, nutritional strains; P, probiotic strains; R, research strains.
[a] Percentage of investigated strains of which the genus name supplied by the depositor was confirmed.
[b] Percentage of strains with confirmed genus name of which also the species name was supplied by the depositor.
[c] Percentage of strains with confirmed genus name and specified species name of which the species name was confirmed.

probiotic strain category. None of the 126 strains originally received as members of *Lactobacillus* or *Pediococcus* were misidentified at the genus level. Strikingly, eight of the 12 presumptive *Propionibacterium* strains representing both probiotic and research categories were in fact identified as *Lb. plantarum* ($n = 5$) or *Pediococcus acidilactici* ($n = 3$) (Table 4). Five probiotic strains previously assigned to the genus *Bifidobacterium* but that scored negative in the genus-specific PCR test were reidentified as lactobacilli ($n = 4$) or as *Enterococcus faecium* ($n = 1$) using protein profiling. Three other strains of the probiotic category received as *Lc. lactis* subsp. *lactis* were misidentified and in fact belonged to *Pc. acidilactici* based on FAFLP fingerprinting and protein profiling, whereas two probiotic *S. thermophilus* strains were reassigned to *Pc. acidilactici* or *Lb. rhamnosus* on the basis of protein profiling and $(GTG)_5$-PCR results.

3.3. Identity confirmation at the species level

Of the 194 cultures with confirmed genus identity, 186 (96%) were received with a species designation from the depositor (Table 2). For 159 out of the 186 strains (85%), the species name as supplied by the depositor was confirmed. Taking all strain categories into consideration, confirmation levels of the species identity varied from 83% (probiotic strains) to 89% (research strains). The 27 strains with confirmed genus identity but unconfirmed species identity originated from 13 different depositors. Most of these depositors accounted for 1–3 cases of misidentification at the species level except for one company, of which eight of the 16 deposited strains (including six probiotic cultures) were incorrectly identified at the species level. All cases of misidentification at the species level were situated in the genera *Lactobacillus* ($n = 19$), *Bifidobacterium* ($n = 7$) and *Enterococcus* ($n = 1$) (Table 4).

4. Discussion

To our knowledge, this is the first systematic survey that aimed to assess the accuracy of the species identity of commercial bacterial cultures intended for probiotic or nutritional use collected directly from the producer or distributor. Using a polyphasic identification approach, a total of 46 cases of misidentification were revealed at the genus level ($n = 19$) or at the species level ($n = 27$). Proportionally, the probiotic strain category included more strains with incorrect species designations (28.1%) than did the nutritional (11.4%) and research (14.0%) strains. The fact that the 34 misidentified strains in the probiotic category were deposited by 10 out of the 26 participating companies illustrates that inaccurate species identity of commercial bacterial cultures is a widespread problem in probiotic production. It is noteworthy that 18 of the 34 probiotic cultures with inaccurate species designations (52.9%) were deposited by only two companies.

Several lines of evidence suggest that most cases of misidentification stem from the use of inappropriate methods. Five depositors reported that species identification of a total of 27 strains was solely based on the use of API 50 CH and/or API 20

Table 3
Taxonomic diversity of 159 deposited probiotic, nutritional and research cultures with confirmed species identity

Identification obtained in this study[a]	Number of strains	Distribution per strain category							Number of depositors
		Probiotic[b]					Nutritional	Research	
		P	PH	PA	PHA	nq			
B. animalis subsp. *lactis*	11		4		1		2	4	6
B. bifidum	7							7	1
B. breve	7		2					5	3
B. longum biotype *infantis*	2		2						2
B. longum biotype *longum*	2		1					1	2
E. faecalis	4		4						1
E. faecium	5		3		2				5
Lb. acidophilus	14	1	8		1	1	3		11
Lb. casei (*zeae*)	3		2					1	3
Lb. curvatus	2						2		2
Lb. delbrueckii subsp. *bulgaricus*	7		3				4		5
Lb. fermentum	4							4	1
Lb. helveticus	2		2						2
Lb. johnsonii	2		1			1			2
Lb. paracasei	22	2	11				4	5	15
Lb. plantarum	17		6	5	1		1	4	9
Lb. reuteri	2		1				1		2
Lb. rhamnosus	17	2	4		1		5	5	10
Lb. sakei	2						2		1
Lb. salivarius	4	1	2					1	4
Lc. lactis subsp. *lactis*	7		1				6		3
Pc. acidilactici	2				1			1	2
Pb. avidum	3							3	1
S. thermophilus	4		3			1			4

B., Bifidobacterium; E., Enterococcus; Lb., Lactobacillus; Lc., Lactococcus; Pb., Propionibacterium; Pc., Pediococcus; S., Streptococcus.

[a] In addition to the species mentioned in the table, single strains with confirmed identity were deposited of the following species: *Lb. brevis*, *Lb. buchneri*, *Lb. gasseri*, *Pc. pentosaceus*, *Pc. freudenreichii* (all categorized as probiotic strains), *Lb. crispatus* (categorized as a research strain) and *Lb. paraplantarum* (categorized as a nutritional strain).

[b] Based on questionnaires received from the original depositors, probiotic strains were further categorized in the following subgroups: P, probiotic strain without specified use; PH, probiotic strain for human use; PA, probiotic strain for animal use; PHA, probiotic strain for human and animal use; nq, probiotic strain for which no questionnaire was received.

STREP carbohydrate fermentation strips. Among these, eight strains were incorrectly identified at the genus or species level (Table 4). In line with the recent study of Boyd and co-workers [2], these findings suggest that the use of API strips can lead to major misidentifications of LAB and *Propionibacterium* isolates up to the genus level. Molecular methods such as protein profiling, DNA fingerprinting and 16S rDNA sequencing are generally regarded as more powerful for reliably identifying LAB [17,33,41] and can be used in combination with biochemical characterization, but also here the choice of method is crucial. For instance, one of the depositors combined the use of API systems with pulsed-field gel electrophoresis (PFGE) of macrorestriction fragments and/or randomly amplified polymorphic DNA (RAPD) fingerprinting. Using this approach, six strains of the probiotic category were assigned to an incorrect genus (Table 4). While PFGE is a highly suited typing method for the differentiation of probiotic LAB at the strain level [45], its discriminatory potential generally is considered too high for species identification [17]. RAPD analysis, on the other hand, has mainly been used for typing and characterization of probiotic LAB strains [4,29] but holds only limited potential for long-term identification purposes due to low interassay reproducibility and standardization [22,26]. The combination of biochemical systems with molecular typing techniques such as PFGE and RAPD may thus be particularly useful for identification of probiotic LAB at the strain level, but not at the genus or species level. The DNA fingerprinting methods that were employed in this study, i.e., AFLP and rep-PCR, have been thoroughly validated for reproducible species identification of even closely related LAB [10,11,19]. The success of these methods strongly depends on the composition of their databases and, as in the case of non-LAB such as propionibacteria, they may need to be combined with universal 16S rDNA sequencing analysis which enables positioning any given probiotic strain in a specific genus or species. On the other hand, the use of 16S rDNA sequences does not reveal relationships at the intraspecific level and fails to reliably separate certain pairs of closely related taxa of bifidobacteria and lactobacilli [23,37].

At the species level, misidentifications were mostly situated in the taxonomically complex genera *Lactobacillus* and *Bifidobacterium*. The 19 strains of *Lactobacillus* that were deposited with an incorrect species designation mainly belonged to the *Lb. acidophilus* group, the *Lb. casei* group and the *Lb. plantarum* group (Table 4). Within the *Lb. acidophilus* group, strains of *Lb. acidophilus* sensu strictu, *Lb. johnsonii*, *Lb. gasseri*, *Lb. crispatus* and the phylogenetically related *Lb. helveticus* are being used as probiotics (Table 3). Some of these species are difficult to separate using phenotypic methods

Table 4
Detailed description of misidentification cases at the genus and species level among probiotic, nutritional or research cultures

Taxon name as received from depositor	Identification techniques used by depositor[a]	Taxon name obtained in this study	Identification methods used in this study[a]	Distribution over strain categories			Number of depositors
				P	N	R	
Misidentifications at genus level							
B. adolescentis	S-S PCR	*Lb. plantarum*	BOX-PCR, G-S PCR, SDS–PAGE	1			1
B. animalis	ns	*Lb. plantarum*	BOX-PCR, G-S PCR, SDS–PAGE	1			1
B. bifidum	PHENO, DNA FP	*Lb. plantarum*	BOX-PCR, G-S PCR, SDS–PAGE	1			1
B. breve	SS PCR	*Lb. rhamnosus*	BOX-PCR, G-S PCR, SDS–PAGE	1			1
B. infantis	PHENO, DNA FP	*E. faecium*	BOX-PCR, G-S PCR, SDS–PAGE	1			1
E. faecium	DNA FP	*Lb. plantarum*	FAFLP, SDS–PAGE, $(GTG)_5$-PCR	1			1
Lc. lactis subsp. *lactis*	PHENO, DNA FP	*Pc. acidilactici*	FAFLP, SDS–PAGE	3			1
Pb. acidipropionici	PHENO	*Pc. acidilactici*	16S rDNA, $(GTG)_5$-PCR	2			1
Pb. acidipropionici	PHENO, DNA FP	*Lb. plantarum*	16S rDNA, $(GTG)_5$-PCR	1		1	2
Pb. freudenreichii	PHENO	*Pc. acidilactici*	16S rDNA, $(GTG)_5$-PCR	1			1
Pb. freudenreichii	ns	*Lb. plantarum*	16S rDNA, $(GTG)_5$-PCR			1	1
Pb. jensenii	ns	*Lb. plantarum*	16S rDNA, $(GTG)_5$-PCR			1	1
Propionibacterium sp.	ns	*Lb. plantarum*	16S rDNA, $(GTG)_5$-PCR	1			1
S. thermophilus	PHENO, DNA FP	*Pc. acidilactici*	SDS–PAGE, $(GTG)_5$-PCR	1			1
S. thermophilus	PHENO	*Lb. rhamnosus*	SDS–PAGE, $(GTG)_5$-PCR	1			1
Misidentifications at species level							
B. bifidum	PHENO, 16S rDNA	*B. animalis* subsp. *lactis*	BOX-PCR, AFLP	2		1	3
B. breve	S-S PCR	*B. animalis* subsp. *lactis*	BOX-PCR	1			1
B. infantis	S-S PCR	*B. animalis* subsp. *lactis*	BOX-PCR	1			1
B. infantis	16S rDNA	*B. longum* bt. *longum*[b]	BOX-PCR			1	1
B. longum	PHENO	*B. animalis* subsp. *lactis*	BOX-PCR		1		1
E. faecium	PHENO, 16S rDNA	*E. faecalis*	FAFLP, SDS–PAGE, $(GTG)_5$-PCR	1			1
Lb. acidophilus	PHENO, DNA FP	*Lb. delbrueckii* subsp. *bulgaricus*	FAFLP, $(GTG)_5$-PCR	1			1
Lb. acidophilus	PHENO	*Lb. gasseri*	FAFLP, SDS–PAGE, $(GTG)_5$-PCR	2			2
Lb. acidophilus	PHENO, DNA FP, 16S rDNA	*Lb. helveticus*	FAFLP, $(GTG)_5$-PCR	2			2
Lb. acidophilus	PHENO	*Lb. johnsonii*	FAFLP, $(GTG)_5$-PCR		1		1
Lb. acidophilus	ARDRA	*Lb. paracasei*	FAFLP, $(GTG)_5$-PCR	1			1
Lb. bulgaricus[c]	16S rDNA	*Lb. helveticus*	FAFLP, SDS–PAGE, $(GTG)_5$-PCR	1			1
Lb. casei subsp. *rhamnosus*[d]	ns	*Lb. plantarum*	FAFLP, SDS–PAGE		1		1
Lb. delbrueckii subsp. *bulgaricus*	PHENO, DNA FP, ARDRA	*Lb. plantarum*	FAFLP, SDS–PAGE, $(GTG)_5$-PCR	1			1
Lb. paracasei	PHENO	*Lb. rhamnosus*	FAFLP, SDS–PAGE			2	1
Lb. plantarum	DNA FP, S-S PCR	*Lb. crispatus*	FAFLP, SDS–PAGE			1	1
Lb. plantarum	DNA FP, S-S PCR	*Lb. pentosus*	FAFLP, $(GTG)_5$-PCR	4			1
Lb. rhamnosus	16S rDNA	*Lb. paracasei*	FAFLP, $(GTG)_5$-PCR	1	1		2

B., Bifidobacterium; E., Enterococcus; Lb., Lactobacillus; Lc., Lactococcus; Pb., Propionibacterium; Pc., Pediococcus; S., Streptococcus. N, nutritional strains; P, probiotic strains; R, research strains.

[a] 16S rDNA, partial or complete 16S rDNA sequencing; ARDRA, amplified ribosomal DNA restriction analysis; BOX-PCR, rep-PCR method targeting the BOX element; DNA FP, DNA fingerprinting methods including RAPD (randomly amplified polymorphic DNA), ITS (internally transcribed spacer)-PCR, PFGE (pulsed-field gel electrophoresis), REP (repetitive extragenic palindromic)-PCR; FAFLP, fluorescent amplified fragment length polymorphism analysis; G-S PCR, genus-specific PCR assay for bifidobacteria [16]; $(GTG)_5$-PCR, rep-PCR method targetting the $(GTG)_5$ element; ns, identification method was not specified by depositor; PHENO, phenotypic characterization including API 50 CH and API 20 STREP systems and conventional biochemical tests; SDS–PAGE, protein profiling using sodium dodecyl sulfate–polyacrylamide gel electrophoresis; S-S PCR, species-specific PCR assay.

[b] Based on the proposal of Sakata and co-workers [31] to classify *Lb. infantis* as a biotype (bt.) of *Lb. longum*, this should be considered as a case of misidentification beyond species level.

[c] *Lb. bulgaricus* is a basonym of *Lb. delbrueckii* subsp. *bulgaricus* [44].

[d] *Lb. casei* subsp. *rhamnosus* is a basonym of *Lb. rhamnosus* [5].

alone [10,17], and a reliable differentiation among members of this group only seems possible with genomic fingerprinting methods such as AFLP and RAPD [10], ARDRA [40], $(GTG)_5$-PCR [11] and tDNA intergenic spacer PCR [1] or on the basis of partial 16S rDNA sequence analysis [46]. In the *Lb. casei* group, the controversial nomenclatural status of the species *Lb. casei* and *Lb. paracasei* and their affiliation with other members *Lb. rhamnosus* and *Lb. zeae* largely complicate the correct identification of probiotic strains belonging to this group [17]. Depending on conflicting taxonomic opinions about whether to reject [7] or to retain [6] the species name *Lb. paracasei*, it is thus possible that the same probiotic strain is named either *Lb. casei* or *Lb. paracasei*. On the other hand, the finding that some *Lb. rhamnosus* strains were referred to as *Lb. paracasei* or vice-versa (Table 4) are genuine cases of misidentification based on a previous nomenclatural change [5]. Likewise, differentiation of *Lb. plantarum*, *Lb. pentosus* and *Lb. paraplantarum* has proven to be problematic with phenotypic methods and on the basis of 16S rDNA sequences [37]. Along with FAFLP and $(GTG)_5$-PCR fingerprinting, partial *recA* sequencing has also been recommended for unambiguous identification of these taxa [37]. Most of the seven bifidobacterial strains assigned to an incorrect species were reidentified as *B. animalis* subsp. *lactis* (Table 4), which cannot be reliably differentiated from *B. animalis* subsp. *animalis* on the basis of 16S rDNA sequences [3]. Instead, other methods such as BOX-PCR and AFLP fingerprinting, SDS–PAGE of whole-cell proteins and partial sequencing of the housekeeping genes *tuf*, *recA*, *atpD* and *groEL* are recommended to discriminate between the two taxa [20,42]. The taxonomic positioning of probiotic *B. longum* strains has recently changed due to the definition of three biotypes in the latter species [31]. Reliable separation of biotypes longum and infantis is not possible based on 16S rDNA sequencing [23], which may explain why one probiotic research strain of *B. longum* biotype longum was initially identified as *B. infantis* (now *B. longum* biotype infantis) based on 16S rDNA sequencing (Table 4). However, there are several other molecular methods that enable discrimination between the three biotypes in *B. longum* including ribotyping, RAPD, BOX-PCR and partial *tuf* sequencing [19,31,39].

Based on the finding that more than 28% of commercial cultures intended for probiotic use were misidentified at the genus or species level, it is reasonable to assume that deficiencies in the microbiological quality and label correctness of probiotic products reported by several authors [8,9,15,18,34,35,46] may be largely due to the incorporation of incorrectly identified bacterial cultures. The present study has highlighted the fact that many of the observed discrepancies result from the use of methods with limited taxonomic resolution (e.g., API strips) or which are unsuitable for reliable identification up to the species level (e.g., PFGE and RAPD analysis). In our hands, species identities of LAB and propionibacterial cultures are best obtained by the use of pattern- and/or sequence-based molecular methods, provided that reproducibility, taxonomic resolution and availability of validated and updated identification databases are guaranteed. Based on these criteria, 16S rDNA sequencing analysis may be universally regarded as the best tool for the taxonomic positioning of probiotic cultures. However, it should be kept in mind that 16S rDNA sequences have a limited resolution for the discrimination of several closely related LAB species used in probiotic production. In this regard, first results obtained with partial sequencing of alternative taxonomic marker genes with a higher resolving capacity, such as *tuf* and *pheS*, show great promise for rapid and reliable sequence-dependent identification of probiotic bifidobacteria and lactobacilli [24,39].

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References

[1] M. Baele, M. Vaneechoutte, R. Verhelst, M. Vancanneyt, L.A. Devriese, F. Haesebrouck, Identification of *Lactobacillus* species using tDNA-PCR, J. Microbiol. Methods 50 (2002) 263–271.

[2] M.A. Boyd, M.A.D. Antonio, S.L. Hillier, Comparison of API 50 CH strips to whole-chromosomal DNA probes for identification of *Lactobacillus* species, J. Clin. Microbiol. 43 (2005) 5309–5311.

[3] Y. Cai, M. Matsumoto, Y. Benno, *Bifidobacterium lactis* Meile et al. 1997 is a subjective synonym of *Bifidobacterium animalis* (Mitsuoka 1969) Scardovi and Trovatelli 1974, Microbiol. Immunol. 44 (2000) 815–820.

[4] M.C. Collado, Y. Moreno, J.M. Cobo, M. Hernandez, Microbiological evaluation and molecular characterization of bifidobacteria strains in commercial fermented milks, Eur. Food Res. Technol. 222 (2006) 112–117.

[5] M.D. Collins, B.A. Phillips, P. Zanoni, Deoxyribonucleic acid homology studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov., Int. J. Syst. Bacteriol. 39 (1989) 105–108.

[6] F. Dellaglio, G.E. Felis, S. Torriani, The status of the species *Lactobacillus casei* (Orla-Jensen 1916) Hansen and Lessel 1971 and *Lactobacillus paracasei* Collins et al. 1989: Request for an Opinion, Int. J. Syst. Bacteriol. 52 (2002) 285–287.

[7] L.T. Dicks, E.M. Du Plessis, F. Dellaglio, F. Lauer, Reclassification of *Lactobacillus rhamnosus* ATCC 15820 as *Lactobacillus zeae* nom. rev., designation of ATCC 334 as neotype of *L. casei* subsp. *casei*, and rejection of the name *Lactobacillus paracasei*, Int. J. Syst. Bacteriol. 46 (1996) 337–340.

[8] J. Drisko, B. Bischoff, C. Giles, M. Adelson, R.-V.S. Rao, R. McCallum, Evaluation of five probiotic products for label claims by DNA extraction and polymerase chain reaction analysis, Digest. Dis. Sci. 50 (2005) 1113–1117.

[9] S. Fasoli, M. Marzotto, L. Rizzotti, F. Rossi, F. Dellaglio, S. Torriani, Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis, Int. J. Food Microbiol. 82 (2003) 59–70.

[10] A. Gancheva, B. Pot, K. Vanhonacker, B. Hoste, K. Kersters, A polyphasic approach towards the identification of strains belonging to *Lactobacillus acidophilus* and related species, Syst. Appl. Microbiol. 22 (1999) 573–585.

[11] D. Gevers, G. Huys, J. Swings, Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species, FEMS Microbiol. Lett. 205 (2001) 31–36.

[12] J.M.T. Hamilton-Miller, S. Shah, Deficiencies in microbiological quality and labeling of probiotic supplements, Int. J. Food Microbiol. 72 (2002) 175–176.
[13] J.M.T. Hamilton-Miller, S. Shah, J.T. Winkler, Public health issues arising from microbiological and labeling quality of foods and supplements containing probiotic microorganisms, Publ. Health Nutr. 2 (1999) 223–229.
[14] W.H. Holzapfel, P. Haberer, J. Snel, U. Schillinger, J.H. Huis in't Veld, Overview of gut flora and probiotics, Int. J. Food Microbiol. 41 (1998) 85–101.
[15] N.T. Hoa, L. Baccigalupi, A. Huxham, A. Smertenko, P.H. Van, S. Ammendola, E. Ricca, S.M. Cutting, Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders, Appl. Environ. Microbiol. 66 (2000) 5241–5247.
[16] P. Kaufmann, A. Pfefferkorn, M. Teuber, L. Meile, Identification and quantification of *Bifidobacterium* species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR, Appl. Environ. Microbiol. 63 (1997) 1268–1273.
[17] G. Klein, A. Pack, C. Bonaparte, G. Reuter, Taxonomy and physiology of probiotic lactic acid bacteria, Int. J. Food Microbiol. 41 (1998) 103–125.
[18] L. Masco, G. Huys, E. De Brandt, R. Temmerman, J. Swings, Culture-dependent and culture-independent qualitative analysis of probiotic products claiming to contain bifidobacteria, Int. J. Food Microbiol. 102 (2005) 221–230.
[19] L. Masco, G. Huys, D. Gevers, L. Verbrugghen, J. Swings, Identification of *Bifidobacterium* species using rep-PCR fingerprinting, Syst. Appl. Microbiol. 26 (2003) 557–563.
[20] L. Masco, M. Ventura, R. Zink, G. Huys, J. Swings, Polyphasic taxonomic analysis of *Bifidobacterium animalis* and *Bifidobacterium lactis* reveals relatedness at the subspecies level: Reclassification of *Bifidobacterium animalis* as *Bifidobacterium animalis* subsp. *animalis* comb. nov. and *Bifidobacterium lactis* as *Bifidobacterium animalis* subsp. *lactis* comb. nov., Int. J. Syst. Evol. Microbiol. 54 (2004) 1137–1143.
[21] A. Mercenier, S. Pavan, B. Pot, Probiotics as biotherapeutic agents: Present knowledge and future prospects, Curr. Pharm. Design 9 (2003) 175–191.
[22] J.R. Meunier, P.A.D. Grimont, Factors affecting reproducibility of random amplified polymorphic DNA-fingerprinting, Res. Microbiol. 144 (1993) 373–379.
[23] T. Miyake, K. Watanabe, T. Watanabe, H. Oyaizu, Phylogenetic analysis of the genus *Bifidobacterium* and related genera based on 16S rDNA sequences, Microbiol. Immunol. 42 (1998) 661–667.
[24] S.M. Naser, K.E. Hagen, M. Vancanneyt, I. Cleenwerck, J. Swings, T.A. Tompkins, *Lactobacillus suntoryeus* Cachat and Priest 2005 is a later synonym of *Lactobacillus helveticus* (Orla-Jensen 1919) Bergey et al. 1925 (Approved lists 1980), Int. J. Syst. Evol. Microbiol. 56 (2006) 355–360.
[25] S. Niemann, A. Puehler, H.-V. Tichy, R. Simon, W. Selbitschka, Evaluation of the resolving power of three different DNA fingerprinting methods to discriminate among isolates of a natural *Rhizobium meliloti* population, J. Appl. Microbiol. 82 (1997) 477–484.
[26] D.M. Olive, P. Bean, Principles and applications of methods for DNA based typing of microbial organisms, J. Clin. Microbiol. 37 (1999) 1661–1669.
[27] D.G. Pitcher, N.A. Saunders, R.J. Owen, Rapid extraction of bacterial genomic DNA with guanidium thiocyanate, Lett. Appl. Microbiol. 8 (1989) 151–156.
[28] B. Pot, P. Vandamme, K. Kersters, V.A.G. O'Donnell, Analysis of electrophoretic whole-organism protein fingerprints, in: M. Goodfellow (Ed.), Chemical Methods in Prokaryotic Systematics, Wiley, Chichester, 1994, pp. 493–521.
[29] G. Reuter, G. Klein, M. Goldberg, Identification of probiotic cultures in food samples, Food Res. Int. 35 (2002) 117–124.
[30] M. Saarela, G. Mogensen, R. Fondén, J. Mättö, T. Mattila-Sandholm, Probiotic bacteria: Safety, functional and technological properties, J. Biotechnol. 84 (2000) 197–215.
[31] S. Sakata, M. Kitahara, M. Sakamoto, H. Hayashi, M. Fukuyama, Y. Benno, Unification of *Bifidobacterium infantis* and *Bifidobacterium suis* as *Bifidobacterium longum*, Int. J. Syst. Evol. Microbiol. 52 (2002) 1945–1951.
[32] M.E. Sanders, J. Huis in't Veld, Bringing a probiotic-containing functional food to the market: Microbiological, product, regulatory and labeling issues, Antonie van Leeuwenhoek 76 (1999) 293–315.
[33] R. Temmerman, G. Huys, J. Swings, Identification of lactic acid bacteria: Culture-dependent and culture-independent methods, Trends Food Sci. Technol. 15 (2004) 348–359.
[34] R. Temmerman, B. Pot, G. Huys, J. Swings, Identification and antibiotic susceptibility of bacterial isolates from probiotic products, Int. J. Food Microbiol. 81 (2003) 1–10.
[35] R. Temmerman, I. Scheirlinck, G. Huys, J. Swings, Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis, Appl. Environ. Microbiol. 69 (2003) 220–226.
[36] F.L. Thompson, B. Hoste, K. Vandemeulebroecke, J. Swings, Genomic diversity amongst *Vibrio* isolates from different sources determined by fluorescent amplified fragment length polymorphism, Syst. Appl. Microbiol. 24 (2001) 520–538.
[37] S. Torriani, G.E. Felis, F. Dellaglio, Differentiation of *Lactobacillus plantarum, L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers, Appl. Environ. Microbiol. 67 (2001) 3450–3454.
[38] V.V. Vankerckhoven, T. Van Autgaerden, G. Huys, M. Vancanneyt, J. Swings, H. Goossens, Establishment of the PROSAFE collection of probiotic and human lactic acid bacteria, Microb. Ecol. Health Dis. 16 (2004) 131–136.
[39] M. Ventura, C. Canchaya, V. Meylan, T.R. Klaenhammer, R. Zink, Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification, Appl. Environ. Microbiol. 69 (2003) 6908–6922.
[40] M. Ventura, I.A. Casas, L. Morelli, M.L. Callegari, Rapid amplified ribosomal DNA restriction analysis (ARDRA) identification of *Lactobacillus* spp. isolated from fecal and vaginal samples, Syst. Appl. Microbiol. 23 (2000) 504–509.
[41] M. Ventura, D. van Sinderen, G.F. Fitzgerald, R. Zink, Insights into the taxonomy, genetics and physiology of bifidobacteria, Antonie von Leeuwenhoek 86 (2004) 205–223.
[42] M. Ventura, R. Zink, Comparative sequence analysis of the *tuf* and *recA* genes and restriction fragment length polymorphism of the internal transcribed spacer region sequences supply additional tools for discriminating *Bifidobacterium lactis* from *Bifidobacterium animalis*, Appl. Environ. Microbiol. 69 (2003) 7517–7522.
[43] J. Versalovic, M. Schneider, F.J. de Bruijn, J.R. Lupski Jr, Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction, Method. Mol. Cell. Biol. 5 (1994) 25–40.
[44] N. Weiss, U. Schillinger, O. Kandler, *Lactobacillus lactis*, *Lactobacillus leichmannii* and *Lactobacillus bulgaricus* subjective synonyms of *Lactobacillus delbrueckii* subsp. *lactis* comb. nov. and *Lactobacillus delbrueckii* subsp. *bulgaricus* comb. nov., Syst. Appl. Microbiol. 4 (1983) 552–557.
[45] P.S.M. Yeung, C.L. Kitts, R. Cano, P.S. Tong, M.E. Sanders, Application of genotypic and phenotypic analyses to commercial probiotic identity and relatedness, J. Appl. Microbiol. 97 (2004) 1095–1104.
[46] P.S.M. Yeung, M.E. Sanders, C.L. Kitts, R. Cano, P.S. Tong, Species-specific identification of commercial probiotic strains, J. Dairy Sci. 85 (2002) 1039–1051.