Application of genotypic and phenotypic analyses to commercial probiotic strain identity and relatedness

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ABSTRACT

Aims: The objective of this study was to generate strain-specific genomic patterns of a bank of 67 commercial and reference probiotic strains, with a focus on probiotic lactobacilli.

Methods and Results: Pulsed-field gel electrophoresis (PFGE) was used as the primary method for strain differentiation. This method was compared with carbohydrate fermentation analysis. To supplement visual comparison, PFGE patterns were analysed quantitatively by cluster analysis using unweighted pair group method with arithmetic averages. *Sma*I, *Not*I and *Xba*I were found to effectively generate clear and easy-to-interpret PFGE patterns of a range of probiotic strains. Some probiotic strains from different sources shared highly similar PFGE patterns.

Conclusions: Results document the value of genotypic strain identification methods, combined with phenotypic methods, for determining probiotic strain identity and relatedness. No correlation was found between relatedness determined by carbohydrate fermentation profiles alone compared with PFGE analysis alone. Some commercial strains are probably derived from similar sources.

Significance and Impact of the Study: This approach is valuable to the probiotic industry to develop commercial strain identification patterns, to provide quality control of strain manufacturing production runs, to track use of protected strains and to determine the relatedness among different research and commercial probiotic strains.

Keywords: *Bifidobacterium*, *Lactobacillus*, probiotics, pulsed-field gel electrophoresis, strain differentiation.

INTRODUCTION

The increasing application of probiotics in food products and dietary supplements underscores the need to properly identify these beneficial bacteria. Probiotics are live micro-organisms which when administered in adequate amounts confer health benefits to the host (FAO/WHO 2001; ftp.fao.org/es/ESN/food/foodandfood_probio_en.stm). Probiotic activities, which confer human health benefits (such as modulating immune system function, reducing host colonization by pathogens and enhancing lactose digestion in lactose maldigesters), have been reviewed (Schiffrin and Blum 2001; Marteau *et al.* 2002). Previous studies have indicated that certain probiotic activities are strain-specific (Lee *et al.* 1993; Gupta *et al.* 1996; Jacobsen *et al.* 1999) and thus, identification of probiotics to the strain level is necessary. This conclusion is supported by FAO/WHO guidelines (http://www.fao.org/es/ESN/food/foodandfood_probio_en.stm) for the use of probiotics in food which stipulate that commercial probiotics be identified to the strain level. Further, the ability to identify specific probiotic strains provides manufacturers a useful quality control

tool and enables strains fed as probiotics to be tracked for efficacy and safety purposes. Strain identification also aids in surveillance and epidemiological studies (Rautio *et al.* 1999).

Current strain-specific techniques used for probiotics comprise multiple DNA-based methods such as pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) PCR, ribotyping and protein-based methods such as SDS-PAGE. The available literature indicates that for many genera, PFGE is more effective than ribotyping, SDS-PAGE or RAPD-PCR in discriminating between strains (O'Riordan and Fitzgerald 1997). Based on accumulating evidence from previous studies, PFGE is considered best for strain identification because of its sensitivity, consistency and accuracy.

The PFGE is a strain-specific DNA typing method that has been used widely for genomic analysis of various micro-organisms. This method has been used to differentiate members of different genera including *Lactococcus* (Tanskanen *et al.* 1990), *Clostridia* (Hielm *et al.* 1998), and *Streptomyces* (Leblond *et al.* 1990), and is considered to be a discriminating and reproducible method to differentiate strains of intestinal bacteria (O'Sullivan 1999). PFGE has been used in strain-typing of lactobacilli and bifidobacteria. Bourget *et al.* (1993) used this technique to compare the genomic restriction patterns of five *Bifidobacterium breve* strains. It has also been used for strain differentiation and chromosome size estimation in *Lactobacillus acidophilus* (Roussel *et al.* 1993; Sanders *et al.* 1996), *L. plantarum* (Daniel 1995), and other lactic acid bacteria (Tanskanen *et al.* 1990). In our previous study (Yeung *et al.* 2002), we applied partial 16S rDNA sequencing, carbohydrate fermentation and fatty acid methyl ester (FAME) analyses to determine the species identification of some commercial probiotic strains. In the present study, we continue this line of research by obtaining strain-specific identification of these commercial probiotic strains using PFGE. In addition, strain relatedness apparent from PFGE fingerprints were evaluated by comparing with carbohydrate fermentation profiles as indicators of phenotypic relatedness of tested strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains used in this study along with their sources are listed in Table 1. All *Lactobacillus* and *Bifidobacterium* strains were grown in MRS (Difco Laboratories, Sparks, MD, USA) or deMan, Rogosa and Sharpe (MRS) supplemented with 0·05% L-cysteine-HCl (Fisher Scientific, Pittsburgh, PA, USA) agars, respectively. All plates inoculated with cells were incubated anaerobically in GasPak® System with BBL® GasPak Plus™ disposable H_2 and CO_2 generator envelopes (BD Diagnostic Systems, Sparks, MD, USA). Upon receipt of the bacterial strains, frozen stocks (with the addition of glycerol, 10% final concentration) were immediately prepared from late log-phase cultures and kept at −80°C. Prior to every experiment, strains from the frozen stock were subcultured at least once in the appropriate medium. Bacterial cultures from probiotic-containing food products were isolated by streaking the product directly on MRS (for lactobacilli) or MRS supplemented with 0·05% L-cysteine-HCl (for bifidobacteria) agar and incubated anaerobically for 48 h at 37°C for single strain isolation. Gram stain was carried out on selected colonies to determine the Gram reaction and morphologies. Once purified, frozen stocks were prepared as described above. Species identification used throughout the text was determined in our previous study by partial 16S rDNA sequencing or carbohydrate fermentation analysis (Yeung *et al.* 2002).

DNA extraction

The *in situ* preparation of chromosomal DNA for PFGE was modified from the method of Tanskanen *et al.* (1990). An aliquot of overnight culture was transferred to fresh MRS or MRS and 0·05% L-cysteine-HCl broth and grown at 37°C for 12–16 h. Chloramphenicol was added to a final concentration of 100 μg ml^{-1} and the incubation was continued for 1 h. Cells from 1·5 to 3·0 ml samples of the culture were harvested by centrifugation for 30 s in a microcentrifuge at 11 000 *g* and washed with 1 ml of cell wash buffer (1 M NaCl, 10 mmol l^{-1} Tris-HCl, pH 7·6). The cell pellets were resuspended in 300 μl of the same buffer, warmed to 55°C and mixed with 300 μl of 2% (w/v) pulsed-field gel agarose (Sigma, St Louis, MO, USA) in cell wash buffer. The suspension was poured into a CHEF plug mould (Bio-Rad Inc., Hercules, CA, USA) and was allowed to solidify at room temperature for 15–20 min. Cells in the agarose blocks were lysed *in situ* with 10 ml of lysis buffer (6 mmol l^{-1} Tris–HCl, 1 mol l^{-1} NaCl, 100 mmol l^{-1} EDTA, 1 mg ml^{-1} lysozyme, pH 8·0) and 400 U mutanolysin solution (Sigma) at 37°C for 24 h. The reagents were drained and 10 ml of proteinase K solution [250 mmol l^{-1} EDTA, pH 8·0, 1% (w/v) sarkosyl, 100 μg ml^{-1} proteinase K] was added to hydrolyse cellular protein. The blocks were incubated at 50°C overnight. Subsequently, the blocks were treated twice for 12 h with 10 ml of 1 mmol l^{-1} phenylmethylsulphonyl fluoride in 1X TE (10 mmol l^{-1} Tris–HCl, pH 8·0, 1 mmol l^{-1} sodium EDTA) at 25°C. The blocks were washed three times with 10 ml of 1X TE for at least 2 h each at 25°C, and stored at 4°C in storage solution [0·5 mol l^{-1} sodium EDTA, pH 8·0, 1% (w/v) sarkosyl].

Table 1 Bacterial strains used in this study. The species identification used throughout this text is based on a previous study (Yeung *et al.* 2002). Discrepancies between species identification and designation by product or supplier have been discussed previously

Laboratory designation	Species identification	Designation by product or supplier	Source‡
ATCC 15696	ND	*B. bifidum*	ATCC (Manassa, VA, USA)
ATCC 15697	*Bifidobacterium suis**	*B. infantis*	ATCC
ATCC 15698	ND	*B. breve*	ATCC
ATCC 15700	*B. breve**	*B. breve*	ATCC
ATCC 15708	*B. longum**	*B. longum*	ATCC
ATCC 25302	*Lactobacillus paracasei*†	*L. paracasei*	ATCC
ATCC 25527	*B. lactis**	*B. animalis*	ATCC
ATCC 25962	ND	*B. infantis*	ATCC
ATCC 29521	ND	*B. bifidum*	ATCC
ATCC 33199	*L. crispatus**	*L. gallinarum*	ATCC
ATCC 33200	*L. johnsonii**	*L. johnsonii*	ATCC
ATCC 33323	*L. gasseri**	*L. gasseri*	ATCC
ATCC 33620	*L. gallinarum**	*L. amylovorus*	ATCC
ATCC 33820	*L. crispatus**	*L. crispatus*	ATCC
ATCC 4356	*L. acidophilus**	*L. acidophilus*	ATCC
ATCC 53103	*L. rhamnosus**	*Lactobacillus* GG	ATCC
ATCC 700396	*L. acidophilus**	*L. acidophilus*	ATCC
DPTC 001	*B. breve**	*B. breve* R-070	Institut Rosell Inc. (Montreal, QC, Canada)
DPTC 002	*B. lactis**	*B. lactis* BB12	Chr. Hansen, Inc. (Milwaukee, WI, USA)
DPTC 003	*B. lactis**	*B. longum* BBL	Chr. Hansen
DPTC 004	*B. longum**	*B. longum* BB46	Chr. Hansen
DPTC 005	*L. acidophilus**	*L. acidophilus* NCFM®	Rhodia Inc. (Madison, WI, USA)
DPTC 006	*L. acidophilus**	*L. acidophilus* NCFM®	NCSU (Raleigh, NC, USA)
DPTC 007	*L. acidophilus**	*L. acidophilus* PIM703	Chr. Hansen
DPTC 008	*L. acidophilus**	*L. acidophilus* SBT2062	Snow Yogurt + 2 (Snow Brand Milk Products Co., Ltd, Kawagoe, Japan)
DPTC 009	*L. crispatus**	*L. crispatus* BG2FO4	NCSU
DPTC 010	*L. crispatus**	*L. acidophilus* HP10	NN (Boston, MA, USA)
DPTC 011	*L. crispatus**	*L. acidophilus* HP100	NN
DPTC 012	*L. crispatus**	*L. acidophilus* HP101	NN
DPTC 013	*L. crispatus**	*L. acidophilus* HP102	NN
DPTC 014	*L. crispatus**	*L. acidophilus* HP103	NN
DPTC 015	*L. crispatus**	*L. acidophilus* HP104	NN
DPTC 016	*L. crispatus**	*L. helveticus* MR220	Rhodia
DPTC 017	*L. crispatus**	*L. helveticus* NCK388	NCSU
DPTC 018	*L. delbrueckii* subsp. *bulgaricus**	*L. lactis* San	Chr. Hansen
DPTC 019	*L. delbrueckii* subsp. *bulgaricus**	*L. delbrueckii* subsp. *bulgaricus* MR120	Rhodia
DPTC 020	*L. delbrueckii* subsp. *bulgaricus**	*L. delbrueckii* subsp. *bulgaricus* 2038	Yogurt (Meiji Milk Products Co. Ltd, Tokyo, Japan)
DPTC 021	*L. delbrueckii* subsp. *bulgaricus**	*L. delbrueckii* subsp. *bulgaricus* 2038	Yogurt (Meiji Milk Products)
DPTC 022	*L. delbrueckii* subsp. *bulgaricus**	*L. delbrueckii* subsp. *bulgaricus* PIM695	Chr. Hansen
DPTC 023	*L. delbrueckii* subsp. *bulgaricus**		Snow Yogurt + 2 (Snow Brand)
DPTC 024	*L. delbrueckii* subsp. *bulgaricus**		Snow Yogurt + 2 (Snow Brand)
DPTC 025	*L. gasseri**	*B. breve*, *B. bifidum* or *L. acidophilus*	Mil Mil fermented milk (Yakult, Tokyo, Japan)
DPTC 026	*L. gasseri**	*L. gasseri* ADH	NCSU
DPTC 027	*L. gasseri**	*L. acidophilus* DDS-1	Capsule supplement (Natren Inc., Westlake Village, CA, USA)
DPTC 028	*L. johnsonii**	*L. johnsonii* 11088 (NCK 088)	NCSU
DPTC 029	*L. johnsonii**	*L. johnsonii* La-1	Nestlé (Lausanne, Switzerland)
DPTC 030	*L. paracasei**	*L. casei* Shirota	Health drink produced by Yakult
DPTC 031	*L. paracasei**	*L. jugurti*	ACE fermented milk drink (Snow Brand)
DPTC 032	*L. paracasei**	*L. jugurti*	ACE fermented milk drink (Snow Brand)

Table 1 (*Contd*)

Laboratory designation	Species identification	Designation by product or supplier	Source‡
DPTC 033	*L. paracasei**	*L. casei*	Joie fermented milk drink (Yakult)
DPTC 034	*L. paracasei**	*L. casei* LC10	Rhodia
DPTC 035	*L. paracasei**	*L. casei* PIM661	Chr. Hansen
DPTC 036	*L. paracasei**	Unknown	Rolly fermented milk (Snow Brand)
DPTC 037	*L. reuteri**	*L. reuteri* 1063-S	Biogaia Biologics (Stockholm, Sweden)
DPTC 038	*L. reuteri**	*L. reuteri* 11284	Biogaia Biologics
DPTC 039	*L. reuteri**	*L. reuteri* SD2112	Biogaia Biologics
DPTC 040	*L. reuteri**	*L. reuteri* T-1	Biogaia Biologics
DPTC 042	*L. rhamnosus**	*L. rhamnosus* GR-1	UWO (London, ON, Canada)
DPTC 043	*L. rhamnosus**	*L. rhamnosus* R-011	Institut Rosell
DPTC 044	*L. rhamnosus**	*L. rhamnosus* R-049	Institut Rosell
DPTC 045	*L. rhamnosus**	*L. fermentum* RC-14	UWO
DPTC 046	*Streptococcus sanguis**	*L. acidophilus* AS-1	Oregon State University (Corvallis, OR, USA)
DPTC 047	*B. lactis**	*B. infantis* BBI	Chr. Hansen
DPTC 048	*L. crispatus**	*L. acidophilus* HP15	NN
DPTC 049	*L. acidophilus*†	*B. breve*, *B. bifidum* or *L. acidophilous*	Mil Mil fermented milk drink (Yakult)
DPTC 050	*L. helvaticus*†	*L. acidophilus* PIM883	Chr. Hansen
DPTC 052	ND	*B. longum* BB536	Lyophilized BB536 (Morinaga Milk Industries, Zama-City, Japan)

ND, Species not determined.

*Species determined by partial 16S rDNA sequencing (Yeung *et al.* 2002).

†Species determined by carbohydrate fermentation as sequencing data were unavailable (Yeung *et al.* 2002).

‡Strains sourced from North Carolina State University (NCSU), University of Western Ontario (UWO), Chr. Hansen, Inc., Rhodia Inc., Institute Rosell, Nestlé, Oregon State University, Biogaia Biologics, Northeast Nutraceuticals (NN) and ATCC were obtained directly from source. All other strains were isolated from retail products.

Restriction digestion

The agarose blocks were washed three times for at least 2 h each with 1X TE prior to restriction digestion. They were incubated for 24 h at 25°C with 10 U of *Sma*I in a 250 μl-solution consisting of the enzyme buffer and bovine serum albumin (Promega Corp., Madison, WI, USA) following manufacturer's recommendations for concentrations of each reagent. Digestion with other endonucleases was carried out in a similar manner, using buffers and temperatures recommended by the supplier.

Pulsed-field gel electrophoresis

Separation of DNA fragments was performed in a CHEF DRIII electrophoresis cell (Bio-Rad). Agarose gels were prepared using 1·2% pulsed-field running gel agarose (Sigma) in 0·5X TBE (45 mmol l^{-1} Tris, 45 mmol l^{-1} boric acid, 1 mmol l^{-1} EDTA, pH 8·0). Electrophoresis was performed at 8°C for 17 h at 6 V cm^{-1} at 120°-included angle with switching times of 1–20 s. These running conditions were optimized for the separation of DNA fragments of 40–200 kb. *Sma*I-digested DNA of *L. gasseri* ATCC 33323 was used as a standard. For each run, the standard was placed in the first, middle and last lane to allow alignment of the gel in the subsequent analysis of gel images. The standard was chosen based on: (i) good DNA quality, which would lead to discrete bands on the gel; (ii) sufficient amount of DNA, which would make the bands visible; and (iii) fairly even distribution of the bands on the gel. Gels were stained with ethidium bromide and photographed on a UV transilluminator.

Data analysis

Gel images were digitized with Gel Doc 1000 and Molecular Analyst Software version 1.4 (Bio-Rad). Normalization of densitometric traces with background subtraction and conversion were carried out with GelCompar version 4.2 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Clustering of strains was calculated using the unweighted pair group method with arithmetic averages (UPGMA).

RESULTS

Selection of restriction enzymes

The restriction enzyme, *Sma*I, was employed initially to digest DNA from 59 probiotic and research *Lactobacillus* and *Bifidobacterium* strains (Fig. 1). This enzyme, with the recognition sequence CCC/GGG, produced suitable PFGE patterns for most of the lactobacilli tested. However, for some probiotics such as *L. paracasei* and *Bifidobacterium* species that have greater frequencies of guanine and cytosine in their genome, *Sma*I cut the genomic DNA more extensively and the resulting patterns are difficult to visually evaluate. Consequently, several alternative restriction enzymes including *Xba*I, *Pst*, *Eco*RV, *Xho*, *Not*I and *Sfi*I were tested. It was not possible to identify one restriction enzyme that was effective for all probiotic strains. Overall, we found that *Sma*I, *Not*I and *Xba*I could effectively generate clear and easy-to-interpret PFGE patterns of a range of probiotic strains.

Evaluation of PFGE patterns

Direct, visual comparisons of PFGE patterns revealed that many strains shared similar or identical patterns. Most of these strains fall into one of the following categories: (i) they were isolated from the same product (e.g. *L. delbruecikk* subsp. *bulgaricus* DPTC 020 and DPTC 021); (ii) they were isolated from different products belonging to the same company (e.g. *L. paracasei* DPTC 031, DPTC 32 and DPTC 036); (iii) they were provided by the same supplier, although some identical strains were given different strain designations (e.g. *L. rhamnosus* DPTC 043 and DPTC 044, *L. crispatus* DPTC 011 and DPTC 015); or (iv) they were obtained from different sources (e.g. *L. acidophilus* DPTC 005, DTPC 006 and ATCC 700396). In addition, evaluation of the PFGE patterns also suggests that the four *L. reuteri* strains produced distinctly different patterns. It is worth noting that, with the exception of ATCC 4356 and ATCC 700396, all reference (ATCC) strains showed distinct patterns compared with the commercial strains of the same species. This suggests that most commercial probiotic strains are markedly different from the reference strains.

Cluster analysis

Clustering of 59 probiotic strains cut with *Sma*I using UPGMA yielded three major groups of >50% similarity (Fig. 1). The first group, with a similarity of 64·8 ± 4·6%, was composed mostly of *L. delbrueckii* subsp. *bulgaricus* (49–51 G + C mol%; Kandler and Weiss 1986) and *Bifidobacterium* strains (55–67 G + C mol%, Schleifer and Ludwig 1995). Group 2, with a similarity of 68·2 ± 10·2%, contained mostly *L. reuteri* (40–42 G + C mol%), *L. paracasei* (45–47 G + C mol%) and *L. rhamnosus* (45–47 G + C mol%), with a few *Bifidobacterium* strains. Group 3, with a similarity of 52 ± 11·1%, contained one *L. paracasei* strain and species from the *L. acidophilus* group, which comprises *L. acidophilus* (32–37 G + C mol%), *L. crispatus* (35–38 G + C mol%), *L. gasseri* (33–35 G + C mol%), *L. johnsonii* and *L. gallinarum*. Clustering using the alternative Ward linkage method gave slightly different groupings (data not shown). Three major clusters were still identified, but at higher similarity levels. The main discrepancy between the Ward and the UPGMA methods was that *L. johnsonii* ATCC 33200 was placed in group 1 with the Ward method, instead of group 3 with the UPGMA method.

Among the three groups, *Sma*I-PFGE patterns of group 3 are the most distinct. The quantitative cluster analysis is largely consistent with the qualitative direct, visual evaluation. For instance, *L. crispatus* DPTC 010, DPTC 011, DPTC 012, DPTC 013, DPTC 014, DPTC 015 and DPTC 048 shared highly similar PFGE patterns upon direct, visual comparison. They also grouped together in the cluster analysis. Similarly, *L. gasseri* DPTC 025 and DPTC 049, which shared identical PFGE patterns, also clustered together. A notable exception is *L. acidophilus* DPTC 006, which had a similar pattern to *L. acidophilus* DTPC 007, ATCC 4356 and ATCC 700396 upon qualitative evaluation. These strains, however, did not cluster closely and had only 52% similarity. The low similarity score suggests that actual and substantial differences exist among these strains – as discussed below, these strains have different carbohydrate fermentation patterns. Alternatively, the low similarity score could also be the result of the strong band intensities of DPTC 006 that made the cluster analysis less accurate (the bands were discrete when seen on gel, but appeared to smear in print).

In contrast to the results of Ferrero *et al.* (1996), *L. rhamnosus* and *L. paracasei* could not be differentiated using *Sma*I. More distinct PFGE patterns for *L. paracasei*, *L. rhamnosus* and *L. reuteri* were generated using *Not*I (Fig. 2). On the basis of visual judgment, DPTC 033 and DPTC 030 had highly similar patterns, whereas DPTC 036, DPTC 031 and DPTC 032 shared identical patterns. Cluster analysis based on PFGE patterns confirmed the close relationship of each group. The similarity score of each group was >97%.

Similar analysis was also performed on *Bifidiobacterium* strains that were cut with *Xba*I (Fig. 3). Fourteen *Xba*I-digested *Bifidobacterium* strains were divided into five clusters at similarity levels of >60%. With the exception of the strains in the *B. lactis* cluster (DPTC 002, DPTC 003 and DPTC 047), all strains showed rather dissimilar PFGE patterns, implying that the strains were distinctly different.

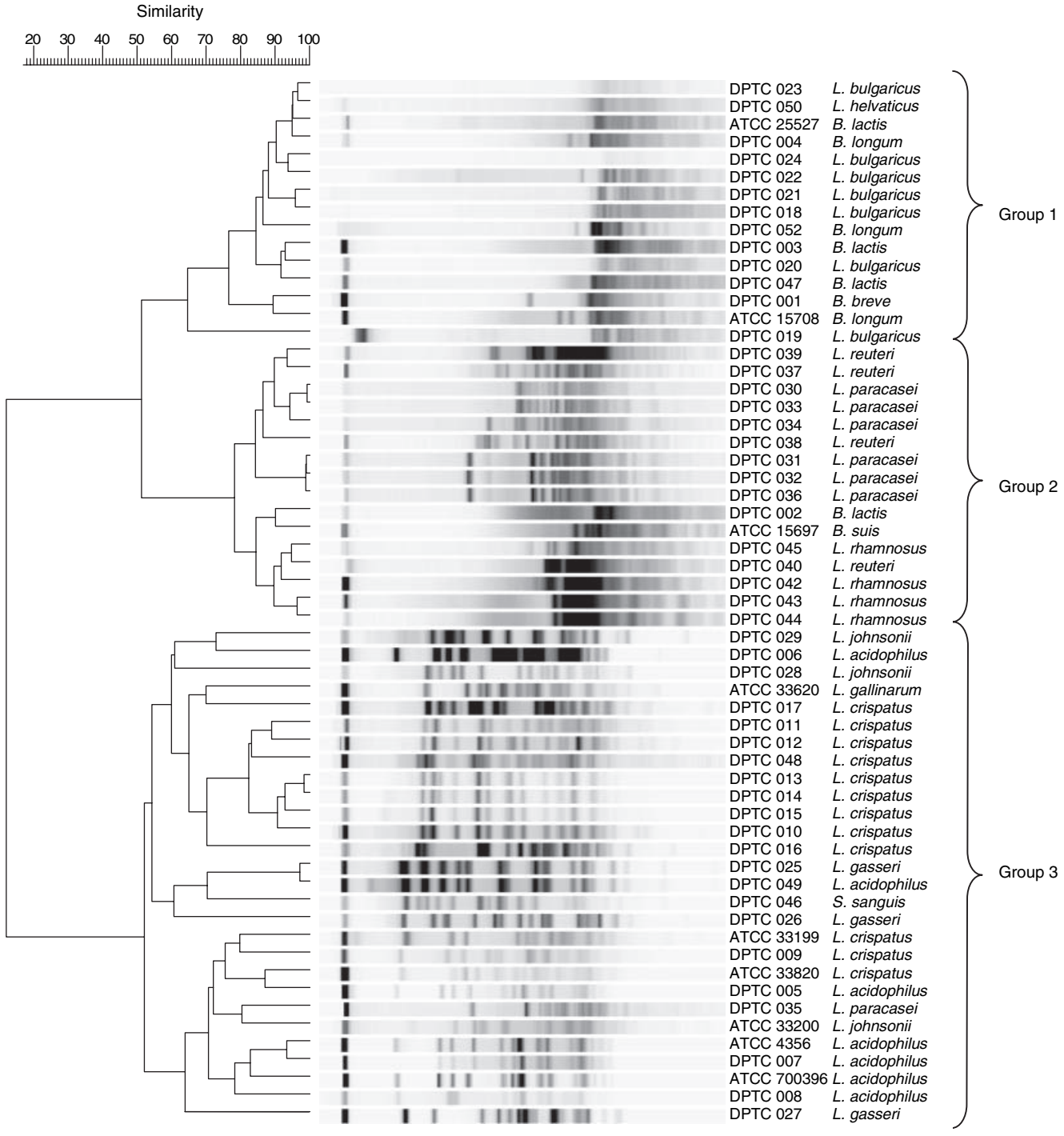

Fig. 1 Clustering of PFGE patterns following *Sma*I digestion using UPGMA method. *Lactobacillus delbrueckii* subsp. *bulgaricus* is abbreviated as *L. bulgaricus*

Comparison between PFGE and phenotypic analysis

As phenotypic expression of genes is more important to probiotic efficacy than DNA content, it is of interest to compare phenotypic with genotypic profiles. Strains used in this study had been previously characterized by carbohydrate fermentation analysis (Yeung *et al.* 2002). No correlation was found following comparison between the PFGE patterns determined in this study with the

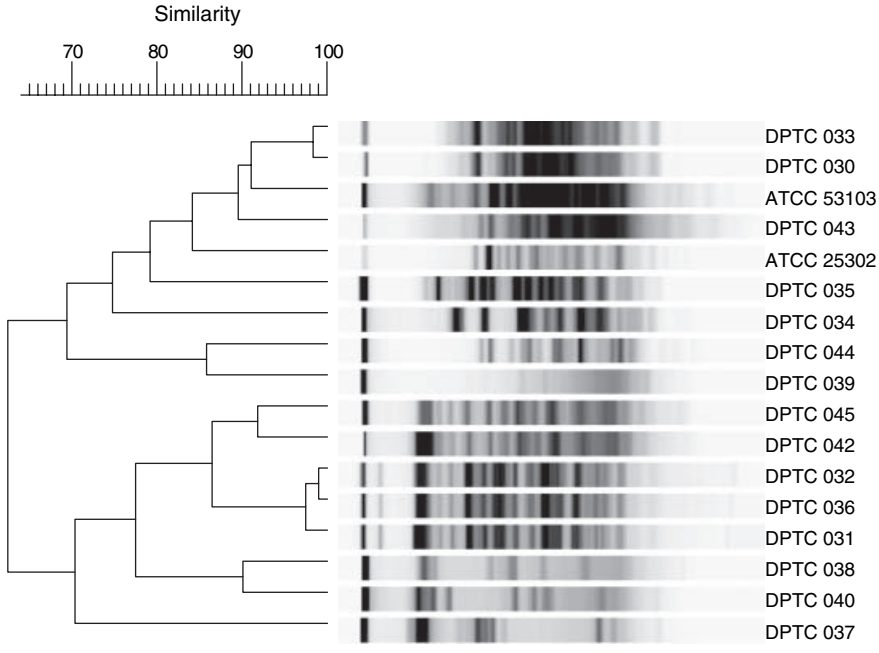

Fig. 2 Clustering, using UPGMA method, of PFGE patterns following *Not*I digestion of *Lactobacillus paracasei* and *L. rhamnosus* strains

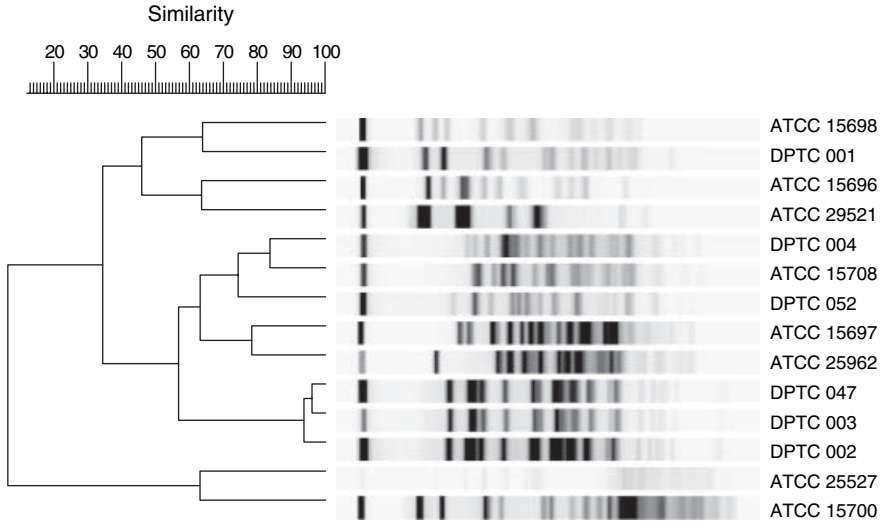

Fig. 3 Clustering, using UPGMA method, of PFGE patterns following *Xba*I digestion of *Bifidobacterium* strains

carbohydrate fermentation profiles. Some strains shown to be highly related by PFGE demonstrated identical carbohydrate fermentation profiles (Table 2), e.g. *L. gasseri* DPTC 025 and DPTC 049, and *L. paracasei* DPTC 031, DPTC 032 and DPTC 036. Different carbohydrate fermentation capability was evident for strains sharing high PFGE pattern similarity (e.g. *L. paracasei* DPTC 030 and DPTC 033). *L. reuteri* DPTC 039, DPTC 040 and ATCC 23272 appeared to have distinctly different PFGE patterns but shared identical carbohydrate fermentation profiles (data not shown). Finally, strains sharing less similar PFGE patterns (as indicated by generally <90% similarity score in the cluster analysis) demonstrated distinctly different carbohydrate fermentation profiles. For example, PFGE patterns of *L. acidophilus* ATCC 4356 and DPTC 007 showed *ca* 90% similarity by cluster analysis. This lower similarity score was consistent with their nonidentical carbohydrate fermentation profiles, in which ATCC 4356 did not ferment arbutine. *Lactobacillus acidophilus* DPTC 005, DPTC 006 and ATCC 700396 did not cluster closely with PFGE-generated data. Their phenotypic differences were apparent: ATCC 700396 fermented D-mannose while the other two did not; DPTC 005 fermented amygdaline and β-gentiobiose while the other two did not; and DPTC 006 did not ferment cellobiose while the other two did.

Table 2 Groupings of strains based on analysis of PFGE patterns and concomitant carbohydrate fermentation profiles

Genus species	Strain	Similarity score (%) of PFGE patterns	Carbohydrate fermentation comments
L. acidophilus	DPTC 005	52	ATCC 4356 and DPTC 007 galactose$^-$
	DPTC 006		DPTC 005 and DPTC 006 D-mannose$^-$
	DPTC 007		ATCC 700396 and DPTC 006 amygdaline$^-$
	ATCC 4356		and β-gentiobiose$^-$
	ATCC 700396		DPTC 007 arbutine$^+$
			DPTC 006 cellobiose$^-$
L. paracasei	DPTC 031	98·8	Strains shared identical profiles
	DPTC 032		
	DPTC 036		
L. paracasei	DPTC 030	99·3	DPTC 033 ribose$^-$
	DPTC 033		DPTC 030 amidon$^-$
L. rhamnosus	DPTC 043	96	DPTC 043 dulcitol$^-$, amygdaline$^-$,
	DPTC 044		cellobiose$^-$
L gasseri	DPTC 025	97·1	Strains shared identical profiles
	DPTC 049*		
L. crispatus	DPTC 012	82·2	DPTC 014 and DPTC 015 D-fructose$^-$
	DPTC 013		DPTC 012 and DPTC 013 D-mannose$^+$
	DPTC 014		DPTC 014 N-acetyl glucosamine$^-$
	DPTC 015		DPTC 013 esculine$^+$
	DPTC 011		
L. rhamnosus	DPTC 042	92	DPTC 042 inositol$^-$
	DPTC 045		

*Partial 16S rDNA sequencing data is not available for DPTC 049. This strain was identified as *L. acidophilus* by carbohydrate fermentation analysis. However, carbohydrate fermentation analysis could not differentiate *L. acidophilus* and *L. gasseri* (Yeung *et al.* 2002). This strain shared a highly similar PFGE pattern with *L. gasseri* DPTC 025 and thus is likely to be *L. gasseri*.

DISCUSSION

The ability to accurately differentiate among different strains of probiotic bacteria is important in the commercial application of probiotics. The strain-specific nature of functionality of different probiotic strains is suggested by the range of results generated on different strains *in vitro* (Lee *et al.* 1993; Gupta *et al.* 1996; Jacobsen *et al.* 1999), although few direct comparisons of strains for health effects have been conducted in humans. For this reason, it is important that probiotic products reveal the identity of specific strains on the labels. This practice would not only enable professionals to better evaluate the research base for substantiating a specific product's efficacy, but would also give emphasis to the importance of strain-specificity of health effects to consumers. Being able to conclusively identify strains in commercial probiotic products can be important if a product is suspected of contributing to an adverse incident in a consumer (Borriello *et al.* 2003). Finally, from the point of view of product manufacturers, using strain-specific patterns to verify phenotypic and genotypic consistency among production runs is a useful quality control technique. For these reasons it is critical that techniques such as PFGE become mainstream in the probiotic product industry.

The PFGE is a molecular strain-typing method that can be used in the identification of different strains within a species. Hence this method is useful in taxonomic grouping and epidemiological studies. PFGE not only plays an important role in foodborne outbreak investigations (http://www.cdc.gov/pulsenet), it also has become integral in studying probiotic strain relatedness (Klein *et al.* 1998; Mitterdorfer *et al.* 2002). To illustrate, PFGE has been used to complement and confirm other strain-typing methods and to study the dynamics of indigenous lactic acid bacteria and bifidobacteria in human feeding studies (McCartney *et al.* 1996; Kimura *et al.* 1997). Results from previous studies support the notion that PFGE is a powerful strain-typing method because of its superior discriminatory power over other methods (Kimura *et al.* 1997; O'Riordan and Fitzgerald 1997; FAO/WHO 2002, ftp.fao.org/es/ESN/food/foodandfood_probio_en.stm). However, the disadvantages of this method include its time-consuming nature (7–9 days) and the need to predetermine restriction enzymes which provide suitable fragment patterns.

In most cases, direct, visual evaluation of PFGE patterns among strains within the same species provides sufficient

information for strain differentiation purposes. More recently, statistical methods have been developed to analyse the genotypic 'fingerprints' that include PFGE patterns. For example, cluster analysis on patterns generated from RAPD-PCR and/or PFGE has been applied to probiotic lactobacilli (Roy *et al.* 2000) and *Saccharomyces* spp. (Mitterdorfer *et al.* 2002). Results from cluster analysis provide a quantitative measure that can be used to establish relatedness of a large number of strains. However, it is important to realize that PFGE is considered to be a strain- not a species-typing technique. To illustrate, taxonomically, *L. delbrueckii* subsp. *bulgaricus* strains are relatively distinct from the *Bifidobacterium* species, yet they share relatively similar PFGE patterns following *Sma*I digestion, and are clustered together in Fig. 1. In this study, the aim of the cluster analysis is to provide an overview on the relatedness and grouping among various strains. To definitively determine the species taxonomy, and/or to construct phylogeny relationships, it is more appropriate to apply techniques such as 16S rDNA sequencing.

Our results confirm that important differences existed between genotype and phenotype. Thus, as suggested by Vandamme *et al.* (1996), a polyphasic approach, in which a combination of genotypic and phenotypic analyses are employed, is required to ultimately delineate bacterial taxonomy. Although it is understandable that no single method alone can provide the complete picture of a strain's identity, PFGE is still a useful strain-typing technique as it is discriminatory, and unlike carbohydrate fermentation and FAME analyses, the resulting patterns are not significantly affected by experimental conditions such as incubation temperature and/or incubation time.

In conclusion, PFGE and cluster analysis of a library of commercial and research strains allowed for clear strain identification and showed several levels of relatedness among the strains tested. This approach is valuable to the probiotic industry in developing commercial strain identification patterns, providing quality control of strain manufacturing production runs, tracking use of protected strains and determining the relatedness among different research and commercial probiotic strains.

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