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Molecular discrimination of new isolates of *Bifidobacterium animalis* subsp. *lactis* from reference strains and commercial probiotic strains

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Abstract

Fifteen new isolates from pig faeces were identified as *Bifidobacterium animalis* subsp. *lactis* by polymerase chain reaction (PCR) using primers specific for this subspecies. Ten of the isolates could be differentiated at strain level by randomly amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis (PFGE). These new strains were different from the type strain and other reference strains of this subspecies, and from all commercial dairy strains with probiotic functionality. Thus, possibly some of the isolates are potential candidates for new probiotic *Bifidobacterium* strains that can be unambiguously identified by RAPD-PCR and PFGE, which could be of interest for the food industry. In contrast, reference strains and commercial dairy strains revealed great genetic homogeneity, showing almost identical DNA fingerprints using RAPD-PCR and PFGE. Some reference strains and all commercial probiotic strains that had been originally designated as *B. animalis* or *B. lactis* have to be assigned to *B. animalis* subsp. *lactis* to be correctly labelled.

Keywords: *Bifidobacterium animalis* subsp. *lactis*; Identification; Probiotics; PCR; RAPD-PCR; PFGE

1. Introduction

The importance of bifidobacteria as probiotic components of the human intestinal microflora and their use in health-promoting foods is generally accepted (Holzapfel, Haberer, Snel, Schillinger, & Huis int Veld, 1998; Fooks, Fuller, & Gibson, 1999; Gomes & Malcata, 1999; Rial, 2000; Mättö et al., 2004; Ventura, Van Sinderen, Fitzgerald, & Zink, 2004b). Strains of *Bifidobacterium animalis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. lactis* and *B. longum* are used in the production of fermented dairy products and seem to be involved in the maintenance of the intestinal micro-flora balance and health (McCartney, Wenzhi, & Tannock, 1996; Sanders, Walker, Walker, Aoyama, & Klaenhammer, 1996; Bonaparte & Reuter, 1997; Kimura, McCartney, McConnell, & Tannock, 1997; Kneifel & Bonaparte, 1998).

Identification of bifidobacteria based on phenotypic characteristics does not always provide clear results and is sometimes unreliable since bifidobacterial cells can change their morphology depending on respective media, growth and culture conditions (Scardovi, 1984; Bonaparte & Reuter, 1997). In recent years, several molecular tools have been proposed for the identification of bifidobacterial strains, e.g., ribotyping (Mättö et al., 2004), hybridisation techniques using 16S rRNA, *recA* and other gene probes (Yamamoto, Morotomi, & Tanaka, 1992; Langendijk et al., 1995; Kaufmann, Pfefferkorn, Teuber, & Meile, 1997; Kullen, Brady, & O'Sullivan, 1997), pulsed-field gel electrophoresis (PFGE) (Roy, Ward, & Champagne, 1996; Engel, Rösch, & Heller, 2003), randomly amplified polymorphic DNA (RAPD) analysis (Vincent, Roy, Mondou, & Déry, 1998; Mayer, Bonaparte, Newart, & Kneifel, 2003), amplified ribosomal DNA restriction

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analysis (ARDRA) (Roy & Sirois, 2000; Ventura, Elli, Reniero, & Zink, 2001a), denaturing gradient gel electrophoresis (DGGE) (Satokari, Vaughan, Akkermans, Saarela, & deVos, 2001; Temmerman, Masco, Vanhoutte, Huys, & Swings, 2003), and various polymerase chain reaction (PCR) methods (Prasad, Gill, Smart, & Gopal, 1998; Matsuki, Watanabe, Tanaka, & Oyaizu, 1998; Ventura, Reniero, & Zink, 2001b; Dong, Cheng, & Jian, 2000; Mullié, Odou, Singer, Romond, & Izard, 2003; Delcenserie, Bechoux, China, Daube, & Gavini, 2005).

Currently, over 30 species are recognised within the genus *Bifidobacterium* and the phylogenetic position of some of these species has been controversial for many years (Bonaparte & Reuter, 1997; Holzapfel, Schillinger, Du Toit, & Dicks, 1997; Ventura et al., 2004b). The taxonomic position of *B. lactis* has been much debated since its description by Meile et al. (1997), and several studies have investigated its affiliation with the closely related but earlier described *B. animalis* (Scardovi & Trovatelli, 1974). Meile et al. (1997) described *B. lactis* as a new species, based on 16S rDNA sequence analysis and DNA–DNA hybridisation results. As they found a weak DNA homology of only 27% between the new strain UR1 and the type strain of *B. animalis*, they concluded that strain UR1, an isolate from a French yoghurt, had to be regarded as a new species, which they named *B. lactis*, honouring the fact that strains of this species showed an elevated oxygen tolerance and were able to grow in milk-based media. However, 2 years later the members of the International Committee on Systematic Bacteriology (2001) declared that *B. lactis* is not a valid species, because there was a significant evidence that the type strain of *B. lactis* had a high DNA homology with the *B. animalis* type strain, although the reported level of 81–86% was based on unpublished data, only. In accordance, Cai, Matsumoto and Benno (2000) found the level of DNA–DNA hybridisation between the two strains ranging from 85.5% to 92.3%. They proposed rejection of the name *B. lactis* suggesting that *B. lactis* should be considered as a junior subjective synonym of *B. animalis*. However, by using molecular techniques (*B. lactis* or *B. animalis* PCR primers, enterobacterial repetitive intergenic consensus PCR), Ventura and Zink (2002) demonstrated that *B. lactis* and *B. animalis* form two main groups. They suggested a revision of the strains assigned to *B. animalis* and proposed that *B. lactis* should be separated from *B. animalis* at the subspecies level. This was confirmed by new genotypic evidence, recently reported by Mayer et al. (2003), Ventura and Zink (2003) and Ventura, Canchaya, van Sinderen, Fitzgerald and Zink (2004a), suggesting that *B. lactis* and *B. animalis* should still be considered to be two separate taxonomic entities, not at the species level but at the subspecies level. Consequently, Masco, Ventura, Zink, Huys and Swings (2004) proposed reclassification of *B. animalis* as *B. animalis* subsp. *animalis* subsp. nov., and *B. lactis* as *B. animalis* subsp. *lactis* subsp. nov. based on a polyphasic taxonomic approach using protein fingerprinting, BOX-PCR fingerprinting, fluorescent-amplified fragment length polymorphism (FALP), and *atpD* and *groEL* gene sequence analysis.

In recent years, growing interest for application of bifidobacteria in many fermented dairy foods has prompted starter industry to screen for new isolates from culture collections or human colonic flora. Although strains of other species are also used for the production of fermented milk products, most commercial dairy strains with probiotic relevance have been designated as *B. animalis* or *B. lactis* (Bonaparte & Reuter, 1997; Gomes & Malcata, 1999; Engel et al., 2003; Ventura et al., 2004b; Mayer, Philippi, Steinegger, & Amtmann, 2005). As properties of probiotics are highly strain-dependent, correct identification and unambiguous authentication of probiotic bifidobacteria at strain level is needed urgently.

The objective of the present study was to identify and characterise new *B. animalis* subsp. *lactis* strains isolated from pig faeces by PCR using primer-pairs specific at different taxonomic levels as well as by DNA fingerprinting using RAPD-PCR and PFGE. Moreover, molecular discrimination of these new isolates from reference strains and commercial dairy strains currently used as probiotics in fermented food products was studied to enable the authentication of new potentially probiotic *B. animalis* subsp. *lactis* strains.

2. Materials and methods

A high number of bifidobacteria had been isolated from the intestines of living or freshly slaughtered pigs and calves in Austria recently. Out of them, 15 isolates from pig faeces had been originally identified as presumptive *B. animalis* strains based on the results of biochemical tests (API-50-CHL and API rapid ID 32; Biomerieux, France). The following strains were included in this study: B35, B50, B52, B55b, B56a, B56b, B65a, B65b, B67, B71, B72a, B73a, B74, B104a, and B108. In addition, type and reference strains of *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* strains were purchased from different strain culture collections. Commercial dairy strains with probiotic functionality were isolated from yoghurt or were obtained directly from the supplier (see Table 1). Bifidobacterial strains were grown in 10 mL brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) under anaerobic conditions at 37 °C for 24–36 h.

Cells were harvested by centrifugation at $4000 \times g$ and 4 °C for 10 min. The pellets were washed twice with physiological saline. The cells were re-suspended in EDTA solution (50 mM, pH 8.0) and portioned into tubes. Tubes were centrifuged, supernatants were discarded, and cell masses were stored at −20 °C until further use.

2.1. Polymerase chain reaction using specific primers

Genomic DNA was isolated using the standard protocol described by Ausubel et al. (1990). Bacteria were lysed with

Table 1
List of *Bifidobacterium* strains used in this study

Code	Designation of strains	Origin	Received from
Bf 5^T	*B. animalis*^{T}, DSM 20104, ATCC 25527	Rat faeces	DSMZ[a]
Bf 8^T	*B. animalis*^{T}, CUETM 89/13, NCFB 2242, DSM 20104, ATCC 25527	Rat faeces	CUETM[b]
Bf 6	*B. animalis*, DSM 20105, ATCC 27536	Chicken faeces	DSMZ
Bf 1	*B. animalis*, CB 120, SKW Bio-Systems	Starter culture	SKW[c]
Bf 4^T	*B. lactis*^{T}, DSM 10140	Yoghurt	DSMZ
Bf 12^T	*B. lactis*^{T}, CUETM 98/1, DSM 10140	Yoghurt	CUETM
Bf 2	*Bifidobacterium* sp., 420, Wisby	Starter culture	Wisby[d]
Bf 3	*B. animalis*, Bb12, Chr. Hansen	Starter culture	Isolate
Bf 3a	*B. animalis*, Bb12, Chr. Hansen	Starter culture	Chr. Hansen[e]
Bf 45	*B. lactis*, HOWARU^{TM} Bifido, HN019, DR10^{TM}, Danisco	Starter culture	Danisco[f]
Bf 46	*B. animalis*, LMG 18906, ATCC 27674	Rabbit faeces	LMG[g]
Bf 44	*B. animalis*, DN-173010, Danone	Starter culture	Isolate
Bf 47	*B. lactis*, DELVO-PRO^{TM} LAFTI^{TM} B94	Starter culture	DSM[h]
Bf 7^T	*B. longum*^{T}, CUETM 89/11, NCTC 11818, DSM 20219, ATCC 15707	Adult intestine	CUETM
Bf 14	*B. longum*, BB 536, Wisby	Starter culture	Wisby

[a]Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.
[b]Collection Unité Ecotoxicologie, Villeneuve d'Ascq, France.
[c]SKW Biosystems GmbH, Germany (now: Degussa BioActives, Bönen, Germany).
[d]Wisby, Niebüll, Germany (now: Danisco, Niebüll, Germany).
[e]Chr. Hansen, Hørsholm, Denmark.
[f]Danisco, Niebüll, Germany.
[g]BCCM^{TM}/LMG, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium.
[h]DSM Food Specialities, Delft, The Netherlands.

lysozyme, and proteins were removed by digestion with proteinase K. Cell wall debris, polysaccharides and remaining proteins were removed by selective precipitation with CTAB, and high-molecular-weight DNA was recovered from the resulting supernatant by isopropanol precipitation. PCR was carried out using primer-pairs specific for *Bifidobacterium* spp., *B. animalis*, *B. animalis* subsp. *animalis* or *B. animalis* subsp. *lactis*, respectively, which had been published by different authors recently (see Table 2). Primers were synthesised by MWG Biotech, Ebersberg, Germany.

DNA amplification was performed in a total volume of 25 μL containing a final concentration of 0.5 units of Dynazyme^{TM} II DNA polymerase (Finnzymes OY, Espoo, Finland), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% (w/v) Triton X-100, 10 mM Tris-HCl (pH 8.8), 200 μM of each dNTP, 0.4 μM of each specific primer, and approximately 50 ng of DNA template.

PCR amplification in the thermocycler (PCR Sprint, Hybaid, Ashford, UK) was run under the following conditions: an initial denaturation step of 5 min at 95 °C; 35 cycles of 1 min at 95 °C, 1 min at the appropriate annealing temperature (see Table 2), and 1 min (time increment 2 s) at 72 °C; and a final extension step of 8 min at 72 °C, followed by cooling to 4 °C.

Electrophoretic separation of amplified PCR products was performed in 2% agarose gels (Seakem LE, FMC, Rockland, ME, USA) in 0.5× TBE buffer using a horizontal HE 100 SuperSub Submarine Unit (Amersham Pharmacia, Uppsala, Sweden) with magnetic buffer circulation and an integrated heat exchanger. Electrophoresis was performed at constant voltage (5 V cm^{-1}) for 3 h at 20 °C. Agarose gels were stained with ethidium bromide (1 mg L^{-1}), and destained with used TBE buffer. DNA fragments were visualised on a UV transilluminator and photographed (Polaroid 667, Polaroid Europe, Enschede, NL).

2.2. Randomly amplified polymorphic DNA -PCR

Amplification of DNA was performed in a thermal cycler (PCR Sprint, Hybaid) using a single primer of arbitrary oligonucleotide sequence. Fifty-seven different arbitrary primers were used for screening purposes (Steinegger, 2005); four primers yielding RAPD profiles appropriate to discriminate strains are listed in Table 3. DNA amplification was performed in a total volume of 25 μL containing 50 ng of DNA, 25 pmol of primer and 1 unit of Dynazyme^{TM} DNA polymerase (Finnzymes OY). The cycler programme consisted of an initial denaturation time of 5 min at 95 °C, followed by amplification for 45 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 36 °C, and extension for 2 min at 72 °C. Reactions were finished with an 8-min elongation period at 72 °C, followed by cooling to 4 °C.

Electrophoretic separation of amplified DNA products was performed in 2% agarose gels, which were then stained with ethidium bromide as described above.

2.3. Pulsed-field gel electrophoresis

High-molecular-weight DNA for PFGE was prepared in agarose plugs as described by Hung and Bandziulis (1990) with some modifications. Strains were grown in 10 mL BHI

Table 2
List of primer-pairs specific for *Bifidobacterium* species, *B. animalis*, *B. animalis* subsp. *animalis* or *B. animalis* subsp. *lactis*, respectively

Primer	Nucleotide sequence	Anneal. temp. (°C)	Target gene	PCR product (bp)
Bif164	5′-GG TGG TAA TGC CGG ATG-3′	60	16S rRNA	523[b]
Bif662	5′-CCA CCG TTA CAC CGG GAA-3′			
Pbi F1	5′-CCG GAA TAG CTC C-3′	50	16S rRNA	914[c]
Pbi R2	5′-GAC CAT GCA CCA CCT GTG AA-3′			
Ban F2	5′-AAC CTG CCC TGT G-3′	61	16S rRNA	925[d]
Pbi R1	5′-GCA CCA CCT GTG AAC CG-3′			
Csp3a	5′-GGT TYA AYG CNG ARA ARG GNT T-3′	50	Cold shock protein	380, 520[e]
Csp3b	5′-GTN ACR TTN GCN GCY TGN GG-3′			
Ban2	5′-CAT ATT GGA TCA CGG TCG-3′	63	ITS region[a]—23S rRNA	467[f]
23Si	5′-CAT TCG GAC ACC CTG GGA TC-3′			
Lw-A	5′-GCA CGG TTT CGG CCG TG-3′	55	16S rRNA—ITS region	567[g]
Lw-B	5′-GGG AAA CCG TGT CTC CAC-3′			
Bflact2	5′-GTG GAG ACA CGG TTT CCC-3′	64	16S rRNA—ITS region	680[h]
Bflact5	5′-CAC ACC ACA CAA TCC AAT AC-3′			

[a]16S-23S ribosomal RNA intergenic spacer region.
[b]Specific for *Bifidobacterium* species (according to Kok et al., 1996).
[c]Specific for *Bifidobacterium* species (according to Roy & Sirois, 2000).
[d]Specific for *B. animalis* subsp. *animalis* and *lactis* (according to Roy & Sirois, 2000).
[e]Specific for *B. animalis* subsp. *animalis* (according to Kim et al., 1998).
[f]Specific for *B. animalis* subsp. *animalis* (according to Ventura & Zink, 2002).
[g]Specific for *B. animalis* subsp. *lactis* (according to Kok et al., 1996; Prasad et al., 1998).
[h]Specific for *B. animalis* subsp. *lactis* (according to Ventura et al., 2001b).

Table 3
List of selected arbitrary primers used for RAPD-PCR

RAPD primer	Nucleotide sequence
Primer 13 (CRA 23)	5′-GCG ATC CCC A-3′
Primer 24 (OPV-07)	5′-GAA GCC AGC C-3′
Primer 43 (OPV-08)	5′-GGA CGG CGT T-3′
Primer 53 (OPR-13)	5′-GGA CGA CAA G-3′

broth to an OD_{600} of 0.4–1.0. Cells were harvested by centrifugation (4000 × *g* for 10 min at 4 °C), washed twice and suspended in 10 mL 50 mM EDTA (pH 8.5). After centrifugation, the cells were resuspended in 150 μL EDTA per OD_{600}. Low melting agarose (1%) was added to each cell suspension (1:7 dilution) and the mixture (150 μL per plug) was allowed to solidify in plug molds (Bio-Rad, Hercules, CA, USA). The embedded cells were lysed in situ by incubating the plugs in lysis buffer (50 mM EDTA, pH 8.5; 0.05% lauroyl sarcosine; 2 mg mL^{-1} lysozyme) overnight at 37 °C with gentle shaking. The agarose plugs (4–6) were placed in 4 mL NDS solution (0.5 M EDTA, pH 8.5; 10 mM Tris-HCl, pH 8.0; 1% SDS; 2 mg mL^{-1} proteinase K) and incubated for 18 h at 50 °C. The plugs were washed eight times with 50 mM EDTA (pH 8.5) for 30 min each time at room temperature, and were finally stored in fresh solution at 4 °C. Restriction enzymes (*Apa*I, *Not*I, *Sma*I, *Spe*I, *Xba*I) were from New England BioLabs (Beverly, MA, USA). One agarose plug was washed twice with 400 μL TE buffer (30 min at 4 °C), then equilibrated twice for 1 h at 4 °C in 300 μL of the restriction enzyme buffer recommended by the manufacturer. For digestion, each plug was incubated overnight in 300 μL of the reaction buffer with 20 U of restriction enzyme at the appropriate temperature. Prior to electrophoresis, agarose plugs were equilibrated in 400 μL TE buffer for 30 min at 4 °C.

DNA restriction fragments were subjected to electrophoresis in 1.1% agarose (Seakem LE, FMC) gels in 0.5 × TBE buffer by using the Gene Navigator System (Amersham Pharmacia) with a hexagonal electrode generating a homogeneous field. The running conditions were 175 V for 24 h at 13 °C, pulse times were increased with linear ramping from 5 to 20 s (*Spe*I and *Xba*I) or decreased 6 to 2 s (*Apa*I, *Not*I, *Sma*I), respectively. Gels were stained with ethidium bromide (1 mg mL^{-1}), destained with used 0.5 × TBE buffer and photographed under UV light.

2.4. Cluster analysis

Photographs were scanned (Sharp JX-330/F6; Vice Versa Scan software 2.23c, Krystek EDV-Beratung, Hamburg), and digitalised images were saved in TIFF format files. Cluster analyses were performed by an image analysis software (Phoretix 1D Advanced 4.01; Non Linear Dynamics Ltd., Newcastle upon Tyne, UK) using the UPGMA (unweighted pair group method with arithmetic averages) algorithm. Based on the PFGE patterns, UPGMA dendrograms (tree diagrams) were generated.

3. Results and discussion

3.1. Commercial dairy strains of Bifidobacterium animalis subsp. lactis

The taxonomic position of commercial dairy bifidobacterial strains (Table 1) that had been designated as

Bifidobacterium sp., *B. animalis* or *B. lactis* was studied by PCR (for primers see Table 2). By means of genus-specific PCR according to Roy and Sirois (2000), the specific amplification product (914 bp) was obtained for all *Bifidobacterium* strains included in this study (results not shown). Assignment of the strains to the genus *Bifidobacterium* was also proven (results not shown) by the genus-specific primers Bif164/662 published by Kok, Waal, Schut, Welling, Weenk and Hellingwerf (1996). Using primer-pair Ban F2/Pbi R1 designed for *B. animalis* subsp. *animalis* and *lactis* (Roy & Sirois, 2000), all strains of this species yielded the amplicon of 925 bp, whereas no PCR product could be observed testing strains of other species (results not shown). Only the type strain of *B. animalis* subsp. *animalis* (Bf 5^T and Bf 8^T) was confirmed using primer-pair Ban2/23Si, reported to be specific for this subspecies (Ventura & Zink, 2002). All other strains that had originally been designated as *B. animalis* or *B. lactis* did not yield an amplicon (Fig. 1). This was also true using primers Csp3a/b published by Kim, Khunajakr, Ren and Dunn (1998), where the two PCR products specific for *B. animalis* were obtained just for the type strain of *B. animalis* subsp. *animalis* (DSM 20104; ATCC 25527; results not shown). In good accordance with this assignment, all the other strains that had been designated as *B. animalis* or *B. lactis* (see Table 1) yielded an amplicon (Fig. 2) using the primer-pair Lw-A/B (Kok et al., 1996; Prasad et al., 1998). As this result could also be confirmed (results not shown) using the primer-pair Bflact2/5 published by Ventura et al. (2001b), the newly described subspecies *B. animalis* subsp. *lactis* (Masco et al., 2004) could be easily distinguished from *B. animalis* subsp. *animalis* by using subspecies-specific PCR. Consequently, not only the commercial probiotic strains Bf 1 (*B. animalis* CB 120, SKW), Bf 2 (*Bifidobacterium* sp. 420, Wisby), Bf 3 (*B. animalis* Bb12, Chr. Hansen) and Bf 44 (*B. animalis* DN-173010, Danone), but also the reference strains Bf 6 (*B. animalis*, DSM 20105, ATCC 27536) and Bf 46 (*B. animalis*, LMG 18906, ATCC 27674)

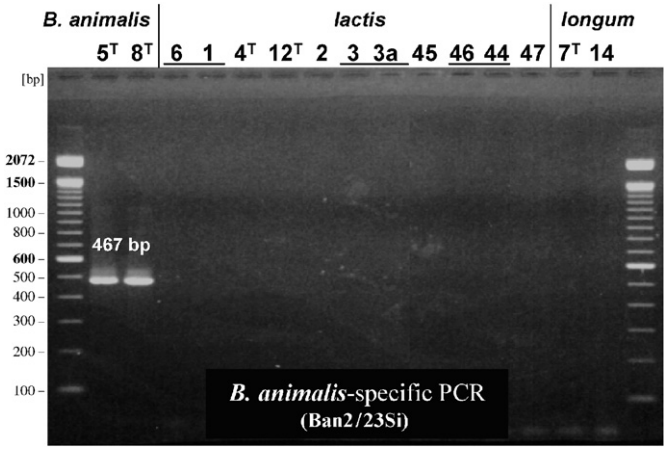

Fig. 1. Subspecies-specific PCR using a primer-pair specific for *B. animalis* subsp. *animalis* (Ban2/23Si) (according to Ventura & Zink, 2002). Underlined strains had been originally assigned to the species *B. animalis*.

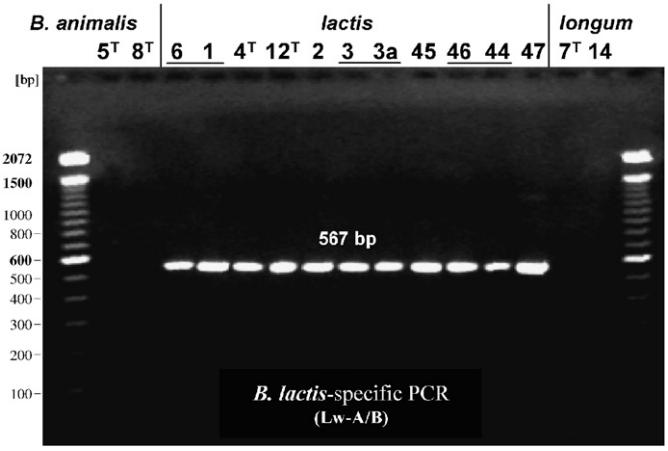

Fig. 2. Subspecies-specific PCR using a primer-pair specific for *B. animalis* subsp. *lactis* (Lw-A/Lw-B) (according to Kok et al., 1996; Prasad et al., 1998). Underlined strains had been originally assigned to the species *B. animalis*.

should be correctly assigned to *B. animalis* subsp. *lactis* to avoid mislabelling in the future.

Interestingly, the *B. animalis* subsp. *lactis* strains from different commercial probiotic dairy cultures as well as the type strain and other reference strains revealed great genetic homogeneity showing identical DNA fingerprints by RAPD-PCR using about 50 arbitrary primers (as example see Fig. 3). Despite their high degree of genetic relationship, at least one strain (Bf 47: DELVO-PRO^{TM} culture LAFTI^{TM} B94, DSM Food Specialities, Delft, NL) could be discriminated from all other strains of this subspecies by one additional band in its RAPD profiles using primer 53 (Fig. 4) and primer 24 (see Fig. 7).

In good accordance with the results obtained by RAPD-PCR, the *B. animalis* subsp. *lactis* strains revealed great genetic homogeneity showing the same identical pattern after restriction with *Apa*I, *Not*I and *Sma*I (results not shown). These results were in good agreement with those of Grand, Kuffer and Baumgartner (2003), who found identical PFGE patterns for different strains of bifidobacteria (*B. lactis* Bb12, Chr. Hansen; *B. animalis* DN 173010, Danone; and *Bifidobacterium* sp. 420, Wisby), corresponding to Bf 3, Bf 44 and Bf 2 in the present study. Accordingly, Engel et al. (2003) reported that bifidobacterial isolates from starter cultures and probiotic fermented milk products showed PFGE patterns identical to that of strain *B. animalis* (DSM 20105, ATCC 27536), which had been originally isolated from chicken faeces (corresponding to strain Bf 6 in this study). However, as shown in Fig. 5, the PFGE pattern of genomic DNA from strains Bf 2 (*Bifidobacterium* sp. 420, Wisby) and Bf 45 (HOWARU^{TM} Bifido, HN019, Danisco) after digestion with *Spe*I showed an additional band each, discriminating these strains from all other *B. animalis* subsp. *lactis* strains included in this study. Moreover, these strains showed a weaker band each: Bf 2 a weak upper band of doublet at 97 kbp, and Bf 45 a sharper band with less total intensity immediately above the additional one. This is especially surprising, as strain

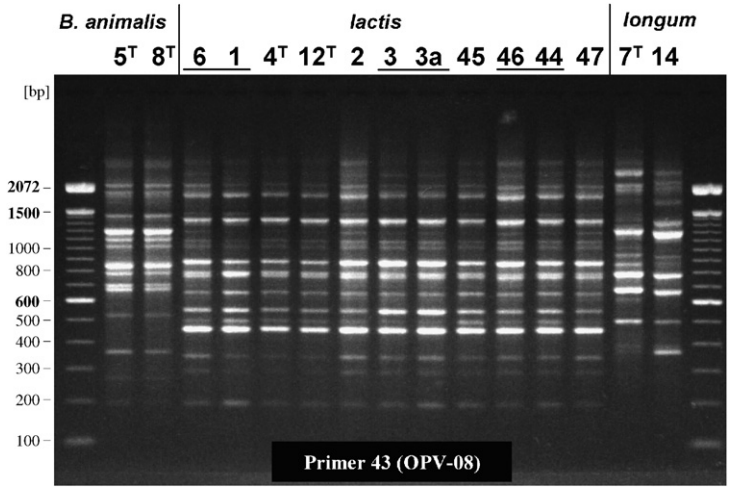

Fig. 3. RAPD-PCR profiles of *Bifidobacterium* strains using arbitrary primer 43 (OPV-08). Underlined strains had been originally assigned to the species *B. animalis*.

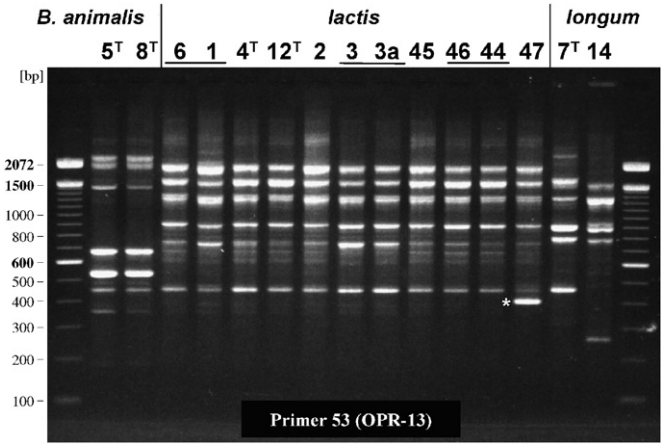

Fig. 4. RAPD-PCR profiles of *Bifidobacterium* strains using arbitrary primer 53 (OPR-13). Underlined strains had been originally assigned to the species *B. animalis*.

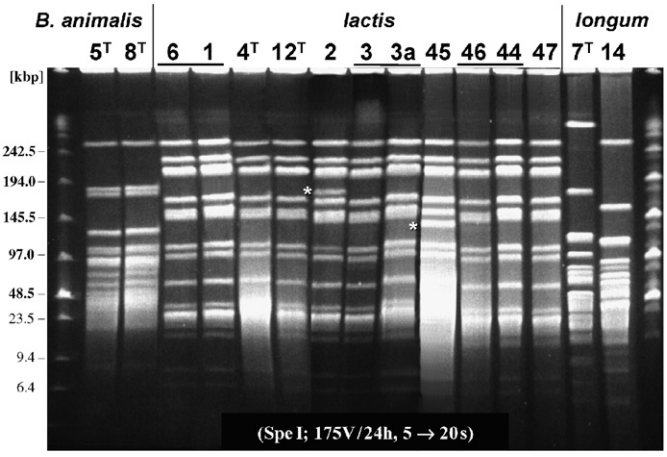

Fig. 5. PFGE patterns of genomic DNA from *Bifidobacterium* strains after restriction with Spe I (gel run at 175 V for 24 h with pulse time ramped from 5 to 20 s). Underlined strains had been originally assigned to the species *B. animalis*.

Bifidobacterium sp. 420 (Wisby) could not be discriminated from other *B. lactis* strains in the studies of Grand et al. (2003) and Mayer et al. (2003). A possible explanation for the additional band in the PFGE pattern of strain Bf 2 is the occurrence of a point mutation, creating thereby a new *Spe*I restriction site in the chromosomal DNA of this strain. Accordingly, the additional band of strain Bf 45 using *Spe*I (at 120 kbp; Fig. 5) and *Xba*I (at 170 kbp; results not shown) may also be considered an artefact unless other authors are able to confirm these distinct PFGE profiles.

Since PFGE of genomic DNA after restriction with several rare-cutting restriction enzymes has been reported to be the "gold standard" in DNA fingerprinting showing strain-specific profiles appropriate for the authentication of distinct strains (Farber, 1996), the extremely close genetic relationship between all these commercial probiotic dairy strains of *B. animalis* subsp. *lactis* from different starter culture companies represents quite a surprising phenomenon (Ventura & Zink, 2002; Engel et al., 2003; Mayer et al., 2003).

3.2. New strains of *Bifidobacterium animalis subsp. lactis* isolated from pig faeces

Assignment of the 15 new bifidobacterial isolates from pig faeces to the genus *Bifidobacterium* was proven (results not shown) by genus-specific PCR according to Kok et al. (1996) and Roy and Sirois (2000). Using primer-pair Ban F2/Pbi R1 designed for *B. animalis* subsp. *animalis* and *lactis* (Roy & Sirois, 2000), all isolates yielded the amplicon of 925 bp (results not shown). Only the type strain of *B. animalis* subsp. *animalis* (Bf 5^T) was confirmed using primer-pair Ban2/23Si, reported to be specific for this subspecies (Ventura & Zink, 2002), whereas the new isolates did not show an amplicon (results not shown). However, all 15 isolates yielded an amplicon (Fig. 6) using

primer-pair Lw-A/B (Kok et al., 1996; Prasad et al., 1998), which had been reported to be specific for *B. animalis* subsp. *lactis*. As this result could also be confirmed (results not shown) using primer-pair Bflact2/5 published by Ventura et al. (2001b), the 15 new bifidobacterial isolates from pig faeces were assigned to the recently described subspecies *B. animalis* subsp. *lactis* (Masco et al., 2004).

Surprisingly, these new isolates could be discriminated from the type strain, other reference strains and all commercial probiotic *B. animalis* subsp. *lactis* strains by using arbitrary primer 24 (OPV-07) (Fig. 7), although very similar RAPD profiles were obtained using other primers indicating a close relationship with these strains (as example see Fig. 8).

At least eight of the isolates could be discriminated at strain level by PFGE showing unique PFGE fingerprints after restriction with Spe I: B65a, B55b, B52, B71, B74, B104a, B56a, B35 (Fig. 9). UPGMA cluster analysis of PFGE profiles showed clearly (Fig. 9) that the type strain of *B. animalis* subsp. *animalis* (Bf 5^T) was delimited from all strains of *B. animalis* subsp. *lactis* (type strain and new isolates from pig faeces). As seen in the dendrogram, isolate B35 had nearly the same PFGE pattern as isolate B56b, but could be additionally discriminated by its RAPD-PCR profiles (e.g., using primers 24 and 13; Figs. 7 and 8). The remaining six isolates (B50, B65b, B67, B72a, B73a, B108) clustered together and could not be differentiated from each other either by PFGE or RAPD-PCR.

Thus, in total 10 unique strains of *B. animalis* subsp. *lactis* were found in this study: B35, B50, B52, B55b, B56a, B56b, B65a, B71, B74, B104a. As these strains were discriminated unambiguously, not only from the type strain and other reference strains of this subspecies, but also from the commercial probiotic strains, authentication of the new potentially probiotic strains can be easily performed using RAPD-PCR and PFGE. The availability

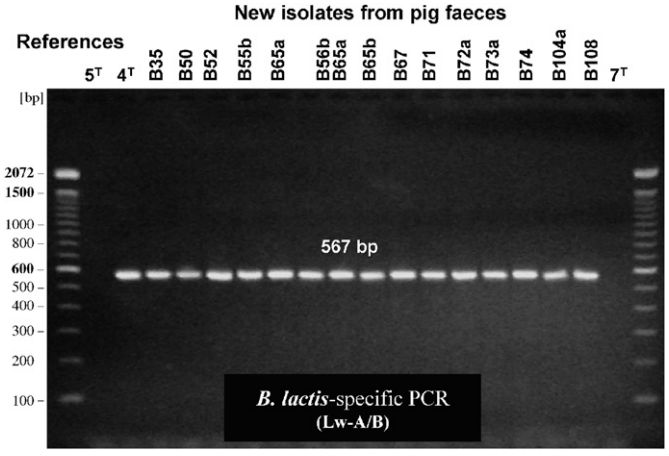

Fig. 6. Subspecies-specific PCR of reference strains and new bifidobacterial isolates from pig faeces using a primer-pair specific for *B. animalis* subsp. *lactis* (Lw-A/Lw-B) (according to Kok et al., 1996; Prasad et al., 1998).

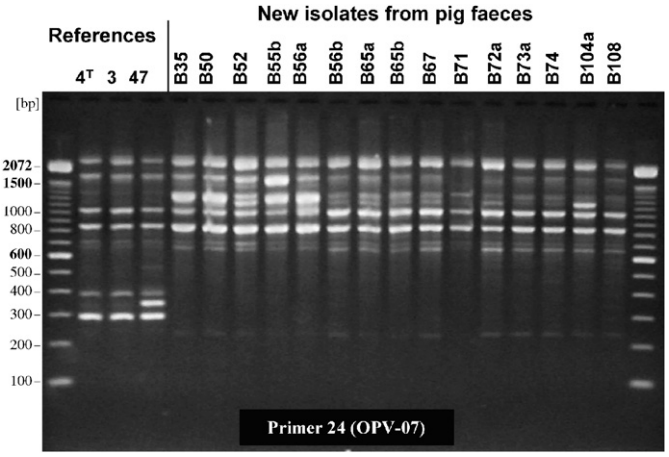

Fig. 7. RAPD-PCR profiles of reference strains and new strains of *B. animalis* subsp. *lactis* isolated from pig faeces using arbitrary primer 24 (OPV-07).

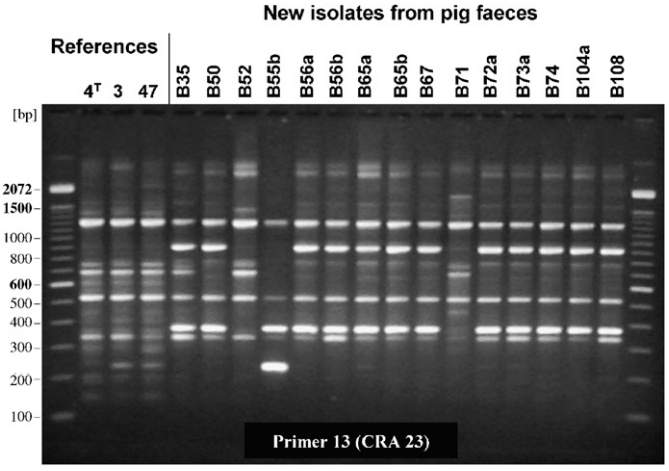

Fig. 8. RAPD-PCR profiles of reference strains and new strains of *B. animalis* subsp. *lactis* isolated from pig faeces using arbitrary primer 13 (CRA 23).

of simple molecular tools to identify these strains represents a prerequisite for their application as potential probiotics in food (Stanton et al., 2001; Suet al., 2005).

4. Conclusions

Some reference strains (Bf 6, *B. animalis*, DSM 20105, ATCC 27536; and Bf 46, *B. animalis*, LMG 18906, ATCC 27674) and all commercial probiotic strains from different starter culture companies that had been designated as *B. animalis* or *B. lactis* have to be assigned to the subspecies *B. animalis* subsp. *lactis*. Surprisingly, these strains revealed great genetic homogeneity, showing nearly identical DNA fingerprints by RAPD-PCR (using 57 arbitrary primers) and also by PFGE (using several rare-cutting restriction enzymes), quite a remarkable phenomenon.

Despite their high degree of genetic relationship, at least three strains (Bf 2: *Bifidobacterium* sp. 420, Wisby; Bf 45: HOWARU™ Bifido, Danisco; Bf 47: DELVO-PRO™

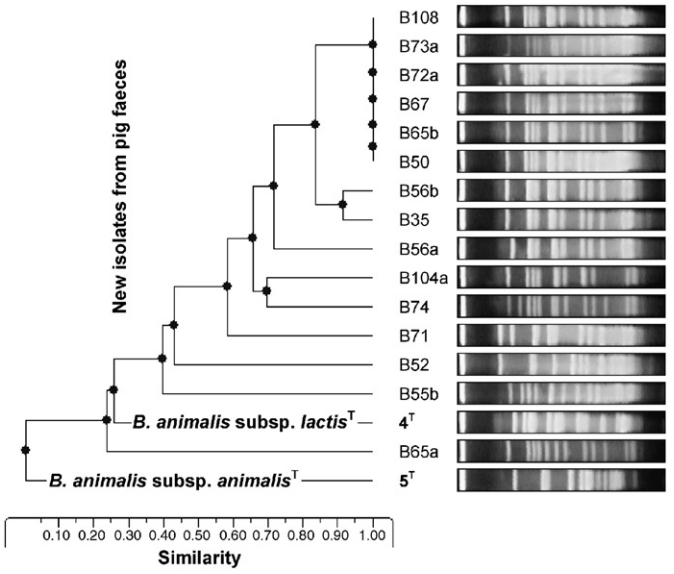

Fig. 9. Dendrogram based on UPGMA cluster analysis of PFGE patterns of genomic DNA from reference strains and new strains of *B. animalis* subsp. *lactis* isolated from pig faeces after restriction with *Spe*I (gel run at 175 V for 24 h with pulse time ramped from 5 to 20 s).

culture LAFTI^{TM} B94, DSM Food Specialities, Delft, NL) could be differentiated from the other commercial probiotic strains by just one additional band in their PFGE patterns or RAPD profiles, respectively. However, the confirmation of these unique patterns remains a subject for further studies to enable unambiguous authentication of these commercial probiotic strains.

The new strains of *B. animalis* subsp. *lactis* that had been isolated from pig faeces in Austria recently were different from all of the commercial dairy strains with probiotic functionality as well as from the type strain and other reference strains of this subspecies using PFGE and RAPD fingerprinting.

Thus, possibly some of the new isolates are potential candidates for new probiotic *Bifidobacterium* strains. As the increasing number of commercial bifidobacterial strains used in the food industry requires reliable methods for the identification and discrimination in particular at strain level, the successful authentication of these *B. animalis* subsp. *lactis* strains could be of highly relevant interest for the food industry. However, a number of technological aspects (e.g., stability and viability in food commodities and the human gastrointestinal tract), safety criteria (e.g., absence of transferable acquired antibiotic resistance genes), and especially the ability of these bifidobacterial strains to induce beneficial effects in the sense of health claims remain a subject for further studies.

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