

IDENTIFICATION OF *LACTOBACILLUS* AND *BIFIDOBACTERIUM* SPECIES WITH PCR APPLIED TO QUALITY CONTROL OF FERMENTED DAIRY BEVERAGES

Lidia Markiewicz, Elżbieta Biedrzycka

Department of Food Microbiology, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn

Key words: PCR, identification, *Bifidobacterium*, *Lactobacillus*, microflora, yoghurt, kefir

The broadening of fermented milk beverage assortment with probiotic products containing bacteria of *Bifidobacterium* and *Lactobacillus* genera prompts to develop reliable and fast methods for the quantitative and qualitative control. The aim of the present study was, therefore, to apply Polymerase Chain Reaction (PCR) carried out on DNA template extracted directly from beverages (a step of strain isolation excepted) for the detection and identification of *Lactobacillus* and *Bifidobacterium* cultures to evaluate commercial kefir and yoghurts. Bacterial DNA was extracted from 3 kefir and 5 yoghurt samples of 5 producers. Bacterial species were identified with reference to the type strains using primers specific to the *Lactobacillus* and *Bifidobacterium* genera; *L. casei* group; *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus/lactis*, *L. fermentum*, *L. johnsonii*, *L. plantarum*, *L. rhamnosus*, *B. animalis/lactis*, *B. bifidum*, *B. breve*, *B. longum* species. On the basis of positive PCR results, the presence of *Lactobacillus* cultures was stated in all yoghurts and kefir, and that of *Bifidobacterium* cultures – in those with appropriate declaration. The applied primer sets enabled detection of the species of *L. acidophilus*, *L. casei*, *L. johnsonii*, and *B. animalis/lactis* in kefir, and those of *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *L. johnsonii*, *L. acidophilus*, *L. fermentum*, and *B. animalis/lactis* in yoghurts. Identification of *Lactobacillus* species was satisfactory, whereas that of *Bifidobacterium* species was sporadically unsuccessful, which indicates that the determination of low-number *Bifidobacterium* cultures demands more efficient DNA extraction and/or more sensitive detection methods to be applied in the fermented milk control.

INTRODUCTION

Probiotic strains of *Lactobacillus* and *Bifidobacterium* genera recognised as non-pathogenic are increasingly used in dairy production [Reuter *et al.*, 2002]. Yet, they require a strict control due to the uncritical strain selection and false declarations of producers, misleading the consumer [McKevith, 2002]. FAO/WHO [2002] recommendations, which define probiotics as precisely identified, characterised and described strains deposited in international culture collections, with efficacy proved in double blind, randomised, placebo-controlled (DBPC) phase 2 human trial, provide detailed guidelines for the labelling of probiotic food, with stress put on genus, species and strain designation as well as the minimum numbers of viable bacteria at the end of the shelf-life claimed. Legal regulations concerning the control of probiotic foods are currently discussed on the European forum, indicating the need for development of reliable analytical methods. Commonly used culturing methods of determination enable the quantitative evaluation of bacteria present in the product on a genus level, which makes the distinction of technological cultures from the probiotic ones impossible, *e.g.* numerous *Lactobacillus* species widely present in fermented dairy products and their probiotic strains supplied additionally. To identify species of dairy cultures with traditional methods, the isolation of pure strains is commonly required, followed by

unreliable and time-consuming phenotypic assays, including biochemical capabilities, fermentation profile, or profile of proteins extracted from bacterial cells using an SDS-PAGE (Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis) technique [Temmerman *et al.*, 2003; Witthuhn *et al.*, 2005]. Only few molecular tools have recently been available for the rapid and precise identification of species [Ventura *et al.*, 2000]. Of these methods, a partial sequence analysis of DNA fragments containing variable regions V1 and V2 of the 16S rRNA coding gene [Gueimonde *et al.*, 2004] and genus-specific PCR [Coeuret *et al.*, 2004] have proven to be useful tools of identification, however both were preceded by pure strain isolation considerably prolonging the time of analyses. An alternative approach to direct analyses of DNA extracted from even more complex and multi-strain material as human faeces appeared to be successful in the studies of Matsuki *et al.* [1999] and Schwiertz *et al.* [2000]. Therefore, the aim of the present study was to apply polymerase chain reaction on DNA template extracted directly from the product for the detection and identification of *Lactobacillus* and *Bifidobacterium* cultures to evaluate commercial kefir and yoghurts.

MATERIAL AND METHODS

Fermented milk products. The identification of *Lactobacillus* and *Bifidobacterium* to the species level was carried

out in five commercially available yoghurts and three kefir (plain products all) produced by leading manufacturers of dairy products on the Polish market. The presence of live yoghurt bacteria or kefir cultures was declared by all the producers, and that of *L. acidophilus* and *Bifidobacterium* sp. – additionally in two yoghurts and two kefir. All products were tested before the expiry date.

Reference strains. The identification was performed with reference to the following type strains: *B. animalis* subsp. *lactis* DSM 10140, *B. bifidum* ATCC 29521, *B. breve* ATCC 15700, *B. longum* ATCC 15707, *L. delbrueckii* subsp. *bulgaricus* DSM 20081, *L. fermentum* DSM 20052, *L. johnsonii* DSM 10533, *L. acidophilus* DSM 20079, *L. rhamnosus* DSM 20021, *L. plantarum* DSM 20174, and *L. casei* DSM 20011. These strains were also used for the optimisation of PCR conditions and as a positive control in the species-specific PCRs conducted on complex DNA template of dairy product cultures. Additional strains, used for the determination of *L. casei*- and *L. plantarum*-species-specificity of newly-designed primers, were: *L. helveticus* DSM 20075, *L. salivarius* subsp. *salicinius* DSM 20554, *L. salivarius* subsp. *salivarius* DSM 20555, *L. paracasei* subsp. *paracasei* DSM 5622, *L. reuterii* DSM 20016, *L. crispatus* DSM 2058, and *L. gasseri* DSM 20243.

Isolation of bacterial DNA. Chromosomal DNA of bacteria was extracted directly from fermented milks without the step of strain isolation. The sample (1 g) was suspended in 9 mL of PBS buffer (137 mmol/L NaCl, 2.7 mmol/L KCl,

10 mmol/L Na₂HPO₄, 2 mmol/L KH₂PO₄; pH 7.1), mixed and centrifuged at 1700x g for 10 min at 4°C (a 5804R centrifuge, Eppendorf, Germany). The supernatant was collected and bacterial cells were harvested by centrifugation (10620x g, 10 min), washed twice with PBS buffer, spinned and resuspended in 0.5 mL of TE buffer (Tris-EDTA, 10 mmol/L Tris-Cl, 1 mmol/L EDTA; pH 8.0). Next, about 0.3 g of sterile glass beads (1 mm diameter) was added and the mixture was vortexed for 2 min at the maximal speed (3D Uniprep Gyrotor, Germany). A bead/cell mixture was then centrifuged and the upper phase was transferred to a 1.5 mL tube containing proteinase K solution (50 µg/300 µL of 2x T&C Lysis Solution, Epicentre, USA). After incubation (65°C, 15 min), the proteins were removed with MPC Protein Precipitation Reagent (Epicentre, USA). Nucleic acids were precipitated with isopropanol, centrifuged (10 min, 4°C), washed three times with 70% ethanol and after removing the alcohol – suspended in 100 µL of TE buffer. DNA solutions were stored at -20°C until examination. Genomic DNA of the reference strains was extracted according to the method described by Bielecka *et al.* [2003].

PCR conditions. Amplification was performed with primer pairs described by other authors (Table 1) or designed in the present study. Sequences of *L. casei* group- and *L. plantarum*-specific oligonucleotides were retrieved from GenBank database and their complementarity to the target species was confirmed by the Blast algorithm. Amplification reactions were prepared in the total volume of 15 µL, containing: 1.5 µL of 10x PCR buffer (500 mmol/L

TABLE 1. Primers used in the study.

Nomenclature	Sequence	Target genus/species	Annealing temp. (°C)	MgCl ₂ concentration (mmol/L)	Target sequence	Reference
L R	tggaaacaggtgctaataccg ccattgtggaagattccc	<i>Lactobacillus</i> spp.	58	1.5	16S rRNA	McOrist <i>et al.</i> [2002]
Aci I Aci II	tctaaggaagcgaaggat ctcttctcgtcgctcta	<i>L. acidophilus</i>	62	3.0	16S-23S rRNA	Tilsala-Timisjarvi <i>et al.</i> [1997]
LB1 LLB1	aaaaatgaagttgtttaaagtagta aagtctgtcctctgctgg	<i>L. delb. subsp. bulgaricus</i> / <i>lactis</i>	60	2.5	<i>pepIP</i>	Torriani <i>et al.</i> [1999]
Joh 16S I 16S II	gagcttgcctagatgatttta actaccagggtatctaatcc	<i>L. johnsonii</i>	60	1.5	16S-23S rRNA	Walter <i>et al.</i> [2000]
Lfpr Ferm II	gccgctaaggtgggacagat ctgatcgtagatcagcaag	<i>L. fermentum</i>	60	5.0	16S-23S rRNA	Walter <i>et al.</i> [2000]
Rha II Pr I	gcatgccaatttctattatt cagactgaaagtctgacgg	<i>L. rhamnosus</i>	60	5.0	16S-23S rRNA	Tilsala-Timisjarvi <i>et al.</i> [1997]
Lpl-1N Lpl-2N	taggaaccagccgctaag cggtgttctcggttcatta	<i>L. plantarum</i>	67	5.0	16S-23S rRNA	this study
Lcas-1N Lcas-2N	gcccttaagtgggggataac tagagtttggccgtgtctc	<i>L. casei</i> group	64	1.5	16S rRNA	this study
Bif-662 Bif-164	ccaccgttacccgggaa gggtgtaatgccgatg	<i>Bifidobacterium</i> spp.	66	5.0	16S rRNA	Langendijk <i>et al.</i> [1995]
LW420C LW420D	ggatgctccgctccatcg gggaaaccgtgtctccac	<i>B. animalis</i> / <i>lactis</i>	66	5.0	16S rRNA	Kok <i>et al.</i> [1996]
BiBIF-1 BiBIF-2	ccacatgatcgatgtgattg ccgaaggcttgcctccaaa	<i>B. bifidum</i>	67	5.0	16S rRNA	Matsuki <i>et al.</i> , [1998]
BiBRE-1 BiBRE-2	ccgatgctccatcacac acaagtgccttgcctcct	<i>B. breve</i>	63	2.5	16S rRNA	Matsuki <i>et al.</i> [1998]
BiLON-1 BiLON-2	ttccagttgatcgatggtc gggaagccgtatctctacga	<i>B. longum</i>	65	5.0	16S rRNA	Matsuki <i>et al.</i> [1999]

KCl and 100 mmol/L Tris-HCl, pH 8.8, at 25°C, 0.8% of Nonidet P40; Fermentas, Lithuania), each deoxynucleoside triphosphate at a concentration of 250 µmol/L, a pair of the specific primers at a concentration of 1.0 µmol/L of each primer, 0.4 U of *Taq* DNA polymerase (Fermentas, Lithuania), magnesium chloride at a concentration ranging from 1.5 to 5.0 mmol/L (Table 1), and 0.5 µL of template DNA. PCR amplifications were carried out in Eppendorf Mastercycler Gradient (Germany) applying the following PCR temperature profile: denaturation – 1 cycle of 94°C for 4 min, followed by 30 cycles of 94°C for 15 s, annealing – at temperature dependent on the pair of primers used for 15 s (Table 1), elongation - 72°C for 15 s, and the terminal elongation at 72°C for 4 min.

All primers were commercially synthesised by TIB Molbiol Poznań (Poland). The concentration of magnesium chloride and the temperature of annealing were optimised for all primer pairs specific to *Lactobacillus* species with reference to DNA of the type strains. The PCR parameters applied to *Bifidobacterium* species-specific primers were optimised as described previously [Bielecka *et al.*, 2003]. The amplification products were separated in 2.0% (w/v) agarose gel electrophoresis (100 V) in 0.5x TAE buffer, followed by ethidium bromide staining.

Determination of bacterial counts. The sample of yoghurt or kefir (1 g) was suspended in 9 mL of 1.0% peptone water (0.5% Pepton Tryptone, 0.5% Peptobak, BTL, Łódź, Poland), and the sequence of decimal dilutions was prepared. Appropriate dilutions were plated using the following bacteriological media: MRS [DeMan *et al.*, 1960] (double layer) for determination of *Lactobacillus*, M17 [IDF Standard 146A:1998] – for *Streptococcus* and *Lactococcus*, and TOS medium (Yakult Pharmaceutical Ind. Co. Ltd., Japan) – for *Bifidobacterium* (anaerobic conditions, BD BBL GasPak Anaerobic System Envelopes, USA). Bac-

terial counts were determined after 48-h incubation at 37°C and presumptive colonies were confirmed with phase contrast microscopy (magnitude of 500x, Microphot FXA, Nikon, Japan).

RESULTS AND DISCUSSION

Traditional quantitative evaluation of fermented milk products with culturing methods

The quantitative determination of cultures is indispensable for the evaluation of fermented dairy products, although time-consuming, laborious, and requiring experience. Bacterial colony forming unit (cfu) numbers grown after 48-h incubation of Petri-dishes were verified by the microscopic evaluation of bacterial cell morphology. Colonies of yoghurt cultures obtained in MRS medium had their cells morphologically characteristic for rods or cocci and were classified to the species of *Lactobacillus* or *Streptococcus*, respectively. Cells of all colonies counted on M17 medium had the form of cocci that is typical of *Streptococcus* and *Lactococcus* (for yoghurt and kefir, respectively). Two types of colonies considerably differing in size appeared on TOS-agar, however cells of merely larger ones were characterised by the morphology typical of bifidobacteria [Scardovi, 1986].

Cocci predominated in both the examined products – *Lactococcus* in kefir (2.0×10^6 – 2.3×10^8 cfu/g), and *Streptococcus* in yoghurt (1.0×10^8 – 3.1×10^9 cfu/g) (Table 2). Naturally lower counts of *Lactobacillus* ($<10^5$ – 6.9×10^7 cfu/g) were assessed in kefir, whereas a considerably lower count of *Lactobacillus* compared to that of streptococci in yoghurts (1.5×10^5 – 2.6×10^6 cfu/g or even $<10^5$) is likely to indicate incorrectly selected yoghurt cultures of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* that did not form synergistic sets [Bielecka *et al.*, 1994], the presence of antibiotic or inhibitory compound residues or problems

TABLE 2. Results obtained using cultivation methods and PCR technique.

Product	Manufacturer's declaration	Live cell number (cfu/g)			The presence of <i>Bifidobacterium</i> and <i>Lactobacillus</i> species detected using PCR technique															
Manufacturer	Before the expiry date (days)	Live kefir/yoghurt cultures	<i>L. acidophilus</i> , <i>Bifidobacterium</i> sp.	<i>Lactobacillus</i>	<i>Bifidobacterium</i>	<i>Lactococcus</i> / <i>Streptococcus</i> *	<i>Lactobacillus</i> spp.	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	<i>L. acidophilus</i>	<i>L. casei</i> group	<i>L. johnsonii</i>	<i>L. fermentum</i>	<i>L. delbrueckii</i> subsp. <i>lactis</i>	<i>L. rhamnosus</i>	<i>L. plantarum</i>	<i>Bifidobacterium</i> spp.	<i>B. animalis/lactis</i>	<i>B. bifidum</i>	<i>B. breve</i>	<i>B. longum</i>
Kefir	A	13	x	x	6.9×10^7	6.1×10^5	2.3×10^8	+	-	+-	+-	-	-	-	-	-	+	-	-	-
	B	8	x	o	$< 1 \times 10^5$	nt**	2.2×10^8	+	-	-	+-	-	-	-	-	nt	nt	-	-	-
	D	5	x	x	1.5×10^5	7.5×10^5	2.0×10^6	+	-	+-	+-	+	-	-	-	+	-	-	-	-
Yoghurt	A	17	x	x	2.6×10^6	1.2×10^6	3.1×10^9	+	+	+-	+-	-	-	-	-	+	+	-	-	-
	B	17	x	o	$< 1 \times 10^5$	nt	7.4×10^8	+	+	-	+-	-	+	-	-	nt	nt	-	-	-
	C	24	x	o	1.5×10^5	nt	1.0×10^8	+	+	-	+-	+	-	-	-	nt	nt	-	-	-
	D	17	x	x	5.5×10^5	3.7×10^5	1.0×10^8	+	-	+	+	+	-	-	-	+	-	-	-	-
	E	7	x	o	3.5×10^5	nt	2.2×10^8	+	+	-	+	+	-	-	-	nt	nt	-	-	-

* *Lactococcus* in kefir, *Streptococcus* in yoghurts; x/o - presence/absence of bacterial cultures according to the producer's declaration; nt - not tested; Strength of PCR product: + strong, +- weak, ++ very weak, - no product

with survival during shelf-life. *Bifidobacterium* counts in kefir, 6.1×10^5 and 7.5×10^5 cfu/g, were slightly lower than the minimal probiotic level assumed (1×10^6 cfu/g). However, taking into consideration that *Bifidobacterium* cells are able to multiply during kefir production in the presence of carbon dioxide produced by yeasts [Usajewicz *et al.*, 2002], manufacturers might cautiously increase their populations because of unfavourable influence of bifidobacteria on sensory attributes (the formation of an unacceptable 'acetic' aftertaste). *Bifidobacterium* count in yoghurt was determined at a level of 1.2×10^6 and 3.7×10^5 cfu/g, hence one product did not meet the assumed minimum probiotic level. Short time of yoghurt incubation determines the amount of *Bifidobacterium* supplementation, applied directly in the amount required at the end of the expiry date [Bielecka *et al.*, 2000]. The highest counts of *Lactobacillus* were stated in 'A' and 'D' yoghurts with declared *L. acidophilus*-supplementation (Table 2). The quantitative evaluation of fermented milks demonstrated that the kefir and yoghurt of manufacturer A were distinguished by high counts of bacterial cultures. The latter product, as the only one of all the yoghurts studied, fulfilled the criteria of the Polish Standard [2002], whereas the others failed to meet recommendations for the count of *Lactobacillus*.

Qualitative evaluation with molecular technique (PCR)

The presence of *Lactobacillus* in all the products tested was confirmed using genus-specific primers (Table 2). With the primer sets applied, the species of *L. acidophilus*, *L. johnsonii*, *L. casei* and *B. animalis/lactis* were detected in kefir, and those of *L. delbrueckii* subsp. *bulgaricus*, *L. johnsonii*, *L. acidophilus*, *L. casei*, *L. fermentum* and *B. animalis/lactis* – in yoghurts. One, two, or three *Lactobacillus* species were present in individual kefir. Four of the five yoghurts contained typical yoghurt cultures of *L. delbrueckii* subsp. *bulgaricus* and two other *Lactobacillus* species – of *L. casei*, *L. johnsonii*, *L. acidophilus* or *L. fermentum*, whereas one – the species of *L. johnsonii*, *L. acidophilus*, *L. casei*, but not *L. delbrueckii* subsp. *bulgaricus*. The presence of *Bifidobacterium* was confirmed with genus-specific primers in these yoghurts and kefir (A and D) whose producers declared their presence. In two products, the species of *B. animalis/lactis* were determined, but not those of *B. breve*, *B. bifidum* and *B. longum*. In single kefir and yoghurts, the detection of *Bifidobacterium* species was unsuccessful (Table 2).

To recapitulate, with the approach established for the qualitative evaluation of fermented milks, three *Lactobacillus* species were detected in each yoghurt of different producers, and one to three species – in kefir. In the investigations of commercial fermented milks or probiotic dairy products available on the Western Europe market, the isolates (strain isolation step applied) were classified to one or two *Lactobacillus* species and to one *Bifidobacterium* species (when declared) [Temmerman *et al.*, 2003; Gueimonde *et al.*, 2004]. In those and our studies, the same species of *Lactobacillus* and *Bifidobacterium* were detected, as the experiments are designed in a manner enabling the detection of species of *B. animalis/lactis* (the only prevalent *Bifidobacterium* species), *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus/lactis*, *L. johnsonii*, *L. casei*, *L. fermentum* *L. plan-*

tarum, and *L. rhamnosus* broadly used in starter cultures and present in fermented milks [Andrighetto *et al.*, 1998; Schillinger *et al.*, 1999; Bielecka *et al.*, 2000; Simova *et al.*, 2002; Gueimonde *et al.*, 2004; Witthuhn *et al.*, 2005]. In traditional kefir produced using grains, other lactobacilli than those detected in this study were also stated, like the species of *L. delbrueckii* subsp. *bulgaricus* that together with *L. fermentum* constituted up to 98.2% of the *Lactobacillus* population [Witthuhn *et al.*, 2005], or *L. helveticus* which together with *L. delbrueckii* subsp. *bulgaricus* and *L. casei* constituted 24-33% of the microflora [Simova *et al.*, 2002], which confirms the high diversity of kefir microflora.

The results of studies by Temmerman *et al.* [2003] and Gueimonde *et al.* [2004] have indicated that the declared bacterial composition of commercial fermented milks or probiotic dairy products did not always reflect the actual content. The Polish Standard [2002] regulates the labelling of additional microflora of yoghurt by demanding species or genus announcement, e.g. *L. acidophilus*, or *Bifidobacterium*. Taking into consideration these liberal rules, it should be stated that bacterial species composition of all the yoghurts tested was consistent with the demands of the Polish Standard [2002] and with the producers' declarations as well.

PCR approach – advantages, faults, critical points

The broadening of probiotic product assortment prompts to work out the adequate standards of quantity and quality control which entail the necessity to develop the reliable and fast methods for the determination of probiotic bacteria, especially those belonging to the most commonly used *Bifidobacterium* and *Lactobacillus* genera. In the present study, an attempt was made to apply PCR on DNA template extracted directly from beverages (a step of strain isolation excepted) for the detection and identification of *Lactobacillus* and *Bifidobacterium* cultures. In PCR analyses, the critical factors affecting the number of the species detected are the number and specificity of primer pairs applied, their detection threshold, and adequate amplification conditions (temperatures of primer annealing and con-

TABLE 3. Specificity of *L. plantarum*- and *L. casei* group-specific primers.

Reference strains	Primer pairs	
	Lpl-1N Lpl-2N	Lcas-1N Lcas-2N
<i>L. acidophilus</i> DSM 20079	-	-
<i>L. casei</i> DSM 20011	-	+
<i>L. paracasei</i> subsp. <i>paracasei</i> DSM 5622	-	+
<i>L. crispatus</i> DSM 2058	-	-
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> DSM 20081	-	-
<i>L. fermentum</i> DSM 20052	-	-
<i>L. gasseri</i> DSM 20243	-	-
<i>L. helveticus</i> DSM 20075	-	-
<i>L. johnsonii</i> DSM 10533	-	-
<i>L. plantarum</i> DSM 20174	+	-
<i>L. reuteri</i> DSM 20016	-	-
<i>L. rhamnosus</i> DSM 20021	-	+
<i>L. salivarius</i> subsp. <i>salicinius</i> DSM 20554	-	-
<i>L. salivarius</i> subsp. <i>salivarius</i> DSM 20555	-	-

centration of magnesium chloride). The specificity of the primers reported previously was proved by the respective authors (Table 1), whereas that of the two pairs of hereby designed ones was confirmed in relation to the reference strains (Table 3). The newly-designed Lpl-1N and Lpl-2N primers were solely *L. plantarum*-specific, whereas Lcas-1N and Lcas-2N primers gave positive results with *L. casei* DSM 20011, *L. paracasei* subsp. *paracasei* DSM 5622 and *L. rhamnosus* DSM 20021 strains. The positive results obtained for *L. rhamnosus* strain with *L. casei*-specific primers can be explained by close affinity of these two species [Reuter *et al.*, 2002]. The identification of the cultures to the *L. casei* group and to the *L. rhamnosus* species has been performed in two-step PCR, *i.e.* when positive amplification with Lcas-1N and Lcas-2N primers occurred, the amplification with Pr I and Rha II primers was conducted. A lack of PCR product in the first step makes the second step pointless and proves the absence of both *L. casei* and *L. rhamnosus* species. Regarding all the products tested, the positive PCR results with Lcas-1N/2N primers and subsequent negative ones with Pr I/Rha II were obtained, so that the presence of members of *L. casei*-group but not *L. rhamnosus* species was stated. The detection limit of 16S rRNA-specific PCR method applied for the detection of bacteria has been evaluated at a level of 10^3 cfu/mL [Kok *et al.*, 1996; Matsuki *et al.*, 1999; Furet *et al.*, 2004]. The technique enables, therefore, detecting bacteria present in minority in a mixed population – low-number cultures are hard to determine in the presence of accompanying bacteria when cultivated using bacteriological media, whereas directly detectable using the PCR technique. Considering the last critical factor mentioned, the conditions of amplification were optimised for the thermocycler and polymerase applied, in relation to the reference strains.

The crucial factor affecting the results obtained with the PCR technique is the sufficient quality and quantity of template DNA which, in turn, depends on the efficiency of DNA isolation method [McOrist *et al.*, 2002]. The applied method of DNA isolation appeared to be excellent for *Lactobacillus* genus-specific PCR ('strong' product) and sufficient for detection of *Lactobacillus* species ('weak' or 'very weak' product). The identification of *Lactobacillus* species was, therefore, satisfactory, however that of *Bifidobacterium* cannot be regarded as entirely successful. The characteristic products of genus-specific PCR were obtained, however two 'weak', and two 'very weak' ones. In consequence, when 'weak' *Bifidobacterium*-specific products appeared, the positive results of PCR species-specific solely to *B. animalis/lactis* were obtained ('strong' and 'very weak' product), but in the cases of 'very weak' genus-specific products – no further species-specific amplicons were formed. The results indicate that the determination of low-number *Bifidobacterium* cultures demands more efficient DNA extraction or more sensitive detection methods for the fermented milk control. Modification of the present method towards more efficient *Bifidobacterium* identification is the subject of an ongoing investigation.

CONCLUSIONS

Generally, the usefulness of the PCR method applied to

the template DNA extracted directly from fermented milk for the qualitative evaluation of *Lactobacillus* cultures in kefir and yoghurts was confirmed. Further optimisation of *Bifidobacterium* detection method in a combination with plate counting will enable the complex characteristics of fermented dairy product microflora.

ACKNOWLEDGEMENTS

We are grateful to Prof. Maria Bielecka for valuable suggestions and criticism of the manuscript.

REFERENCES

1. Andrighetto C., De Dea, P., Lombardi A., Neviani E., Rossetti L., Giraffa G., Molecular identification and cluster analysis of homofermentative thermophilic lactobacilli isolated from dairy products. *Res. Microbiol.*, 1998, 149, 631–634.
2. Bielecka M., Majkowska A., Biedrzycka E., Biedrzycka E.I., Microbiological changes in modified yoghurts during manufacture and storage. 2000, *in*: Food Biotechnology. Progress in Biotechnology 17 (eds. S. Bielecki, J. Tramper, J. Polak), pp. 283–289.
3. Bielecka M., Majkowska A., Biedrzycka E., Synergistic yogurt cultures with antibacterial properties. *Pol. J. Food Nutr. Sci.*, 1994, 3/44, 63–73.
4. Bielecka M., Markiewicz L., Wasilewska E., Evaluation of primers applied to PCR identification of the *Bifidobacterium* spp. *Pol. J. Food Nutr. Sci.*, 2003, 12/53 SI 2, 10–15.
5. Coeuret V., Gueguen M., Vernoux J.P., Numbers and strains of lactobacilli in some probiotic products. *Int. J. Food Microbiol.*, 2004, 97, 147–156.
6. DeMan J.C., Rogosa M., Sharpe M.E., A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.*, 1960, 23, 130–135.
7. FAO/WHO, Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food, London, Ontario, Canada, April 30 and May 1, 2002.
8. Furet J-P., Quenee P., Tailliez P., Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. *Int. J. Food Microbiol.*, 2004, 97, 197–207.
9. Gueimonde M., Delgado S., Mayo B., Ruas-Madiedo P., Margolles A., de los Reyes-Gavilan C.G., Viability and diversity of probiotic *Lactobacillus* and *Bifidobacterium* populations included in commercial fermented milks. *Food Res. Int.*, 2004, 37, 839–850.
10. IDF Standard 146A:1998. Yoghurt. Identification of characteristic microorganisms (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*).
11. Kok R.G., de Waal A., Schut F., Welling G.W., Weenk G., Hellingwerf K.J., Specific detection and analysis of a probiotic *Bifidobacterium* strain in infant feces. *Appl. Environ. Microbiol.*, 1996, 62, 3368–3672.
12. Langendijk P.S., Schut F., Jansen G.J., Raangs G.C., Kamphuis G.R., Wilkinson M.H., Welling G.W., Quantitative fluorescence *in situ* hybridization of *Bifidobacte-*

- rium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Appl. Environ. Microbiol.*, 1995, 61, 3069–3075.
13. Matsuki T., Wanatabe K., Tanaka R., Fukuda M., Oyaizu H., Distribution of *Bifidobacterium* species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. *Appl. Environ. Microbiol.*, 1999, 65, 4506–4512.
 14. Matsuki T., Wanatabe K., Tanaka R., Fukuda M., Oyaizu H., Rapid identification of human intestinal bifidobacteria by 16S rRNA-targeted species- and group-specific primers. *FEMS Microbiol. Lett.*, 1998, 167, 113–121.
 15. McKeivith B., EU legislation for health claims on the way. *British Nutrition Foundation Nutr. Bull.*, 2002, 27, 185–186.
 16. McOrist A. L., Jackson M., Bird A., A comparison of five methods for extraction of bacterial DNA from human faecal samples. *J. Microbiol. Methods*, 2002, 50, 131–139.
 17. Polish Standard No PN-A-86061:2002. Milk and milk products. Fermented milk (in Polish).
 18. Reuter G., Klein G., Goldberg M., Identification of probiotic cultures in food samples. *Food Res. Int.*, 2002, 35, 117–124.
 19. Schillinger U., Isolation and identification of lactobacilli from novel-type probiotic and mild yoghurts and their stability during refrigerated storage. *Int. J. Food Microbiol.*, 1999, 47, 79–87.
 20. Scardovi V., Genus *Bifidobacterium* Orla-Jensen 1924, 472AL. 1986, in: *Bergey's Manual of Systematic Bacteriology* (eds. Holt J.G., Sneath P.H.A., Mair N.S., Sharpe M.E.). Williams & Wilkins, Baltimore, USA, pp. 1418–1434.
 21. Schwiertz A., Le Bay P.F., Zakrzewska E.I., Quantification of different *Eubacterium* spp. in human fecal samples with species-specific 16S rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.*, 2000, 60, 375–382.
 22. Simova E., Beshkova D., Angelov A., Hristova Ts., Frenogova G., Spasov Z., Lactic acid bacteria and yeasts in kefir grains and kefir made from them. *J. Ind. Microbiol. Biotechnol.*, 2002, 28, 1–6.
 23. Temmerman R., Pot B., Huys G., Swings J., Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *Int. J. Food Microbiol.*, 2003, 81, 1–10.
 24. Tilsala-Timisjärvi A., Alatossava T., Development of oligonucleotide primers from the 16S-23S rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. *Int. J. Food Microbiol.*, 1997, 35, 49–56.
 25. Torriani S., Zapparoli G., Dellaglio F., Use of PCR-based methods for rapid differentiation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus delbrueckii* subsp. *lactis*. *Appl. Environ. Microbiol.*, 1999, 65, 4351–4356.
 26. Usajewicz I., Bielecka M., Kimborowicz B., Usefulness of bifidobacteria strains in biokefir production. 2002, in: *Materials Congrilaite*, 26th IDF World Dairy Congress, September 24–27, 2002, Paris, France.
 27. Ventura M., Callegari M.L., Morelli L., S-layer gene as molecular marker for identification of *Lactobacillus helveticus*. *FEMS Microbiol. Lett.*, 2000, 189, 275–279.
 28. Walter J., Tannock G. W., Tilsala-Timisjarvi A., Rodtong S., Loach D. M., Munro K., Alatossava T., Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl. Environ. Microbiol.*, 2000, 66, 297–303.
 29. Witthuhn R.C., Schoeman T.J., Britz T.J., Characterization of the microbial population at different stages of kefir production and kefir grain mass cultivation. *Int. Dairy J.*, 2005, 15, 383–389.

Received June 2005. Revision received August and accepted September 2005.

**IDENTYFIKACJA GATUNKOWA *LACTOBACILLUS* I *BIFIDOBACTERIUM* TECHNIKĄ PCR
W JAKOŚCIOWEJ OCENIE MLECZNYCH NAPOJÓW FERMENTOWANYCH***Lidia Markiewicz, Elżbieta Biedrzycka**Zakład Mikrobiologii Żywności, Instytut Rozrodu Zwierząt i Badań Żywności PAN w Olsztynie*

Rozszerzenie asortymentu mlecznych napojów fermentowanych o produkty prozdrowotne zawierające dodatkowo bakterie z rodzajów *Bifidobacterium* i *Lactobacillus* powoduje konieczność opracowania niezawodnych i szybkich metod kontroli ilościowej i jakościowej. Włączając się w nurt tych badań, celem niniejszej pracy było zastosowanie łańcuchowej reakcji polimerazy (PCR) przeprowadzonej na matrycy DNA wyizolowanego bezpośrednio z produktów (z pominięciem etapu izolacji szczepów) do wykrywania i identyfikacji kultur *Lactobacillus* i *Bifidobacterium* w ocenie kefiru i jogurtu komercyjnego. Bakteryjne DNA izolowano z 3 wyrobów kefiru i 5 wyrobów jogurtu pochodzących od 5 producentów. Gatunki bakterii identyfikowano w odniesieniu do szczepów referencyjnych z zastosowaniem starterów specyficznych do rodzajów *Lactobacillus* i *Bifidobacterium*; grupy *L. casei*; oraz do gatunków *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus/lactis*, *L. fermentum*, *L. johnsonii*, *L. plantarum*, *L. rhamnosus*, *B. animalis/lactis*, *B. bifidum*, *B. breve* i *B. longum*. Na podstawie pozytywnych wyników PCR stwierdzono obecność kultur *Lactobacillus* we wszystkich wyrobach jogurtu i kefiru oraz kultur *Bifidobacterium* w wyrobach dla których deklarowano ich obecność. Zastosowany układ starterów pozwolił na oznaczenie w kefirach obecności gatunków *L. acidophilus*, *L. casei*, *L. johnsonii* oraz *B. animalis/lactis*, a w jogurtach – *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *L. johnsonii*, *L. acidophilus* i *L. fermentum* oraz *B. animalis/lactis* (tab. 2). Identyfikacja *Lactobacillus* była zadowalająca, podczas gdy identyfikacja *Bifidobacterium* sporadycznie nie powiodła się, co wskazuje na konieczność zastosowania bardziej wydajnej metody izolacji DNA lub bardziej czułych metod oznaczania kultur *Bifidobacterium* o niskiej liczebności w stosunku do pozostałej mikroflory w jakościowej kontroli fermentowanych produktów mlecznych.