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EVALUATION OF PRIMERS APPLIED FOR PCR IDENTIFICATION OF *BIFIDOBACTERIUM* SPP.

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The level of detection and the specificity of primers have a great influence on examination results. The aim of the study was to evaluate the primers applied for identification of *B. longum, B. breve, B. infantis* and *B. bifidum* – the species most commonly inhabiting the gastrointestinal tract of infants, and also *B. lactis* strains commonly used in bioyogurt manufacturing. Ten primer pairs were applied in the study, nine of them were previously described by Kok *et al*. [1996], Roy *et al.* [1996], Matsuki *et al.* [1998, 1999], and Ventura *et al.* [2001], and one pair was original. The specificity and sensitivity of the PCRs were tested using pure reference bacterial strains, followed by studying the freshly isolated strains, preliminary classified due to phenotypic features to *B. longum, B. breve, B. bifidum* and *B. lactis* species.

The tested primer sets enabled classification of the human-derived strains to *B. longum, B. breve,* and *B. bifidum*, as well as *B. lactis* species with all the primers applied, except for BreU3/BreL5 primer set, which did not give any amplification product with any tested *B. breve* strain. The primers Bflact2/Bflact5 applied for *B. lactis* identification yielded additional PCR products with all the strains originating from adults, and belonging to *B. longum* or *B. bifidum* species. It was impossible to classify any strain originated from rats with the applied primer sets. The performed PCR amplifications revealed highly significant differences in the detection level, which amounted from 1 pg to 10 ng of the template DNA in the reaction mixture.

The performed evaluation can facilitate the examination of complex microecosystems with the tested primers.

INTRODUCTION

An increased interest in the *Bifidobacterium* genus has been observed over the last few decades, caused by their beneficial impact on a host body. Bifidobacteria constitute a very important functional group of intestinal bacteria playing a significant role in stimulating the immune response, suppressing putrefactive bacteria, and preventing intestinal diseases, including cancer [Mitsuoka, 1990]. The most interest is focused on human-derived *Bifidobacterium* species, such as *B. infantis, B. breve, B. longum* and *B. bifidum* [Benno *et al.,* 1984; Biavati *et al*., 1986], as well as recently investigated *B. lactis* species isolated from fermented milk products. Therefore, the proper identification of these bifidobacterial species is still required.

For many years, the identification of *Bifidobacterium* isolates was carried out on the basis of phenotypic features and DNA-DNA homology [Scardovi, 1986]. However, a very close relationship between some species made such identification very laborious and time-consuming, whereas the results obtained were often ambiguous. Fast development of molecular biology methods in recent years, significantly facilitated and accelerated the classification of even so closely related species as *B. infantis* and *B. longum* or *B. animalis* and *B. lactis*. Molecular identification of *Bifidobacterium* isolates can be carried out using very conservative sequences universally present in bacterial genomes, *e.g*. L-lactate dehydrogenase gene *ldh* [Roy & Sirois, 2000], *recA* gene [Kullen *et al*., 1997] or partially 60 kDa heat-shock protein (HSP60) gene [Jian *et al*., 2001]. 16S rRNA and 23S rRNA encoding genes, as well as 16S-23S internal transcribed spacer (ITS) are the most frequently used sequences for bifidobacteria identification at the genus and species level. Especially, 16S rDNA sequence, which contains nine very variable regions, appeared to be exceptionally appropriate to design genus-, species- or group--specific oligonucleotides. The advantage of using rRNA genes as targets in the experiments is their high copy numbers within one single bacterial cell, exceeding by far the number of chromosomally encoded genes.

In recent years, Roy *et al.* [1996] designed oligonucleotides based on 16S rDNA sequences and used them with the probes described previously by Yamamoto *et al.* [1992] and Frothingham *et al.* [1993] as the primers specific to *B. breve*, *B. infantis,* and *B. longum*. Next, Matsuki *et al.* [1998, 1999] reported on the primer pairs targeted for most *Bifidobacterium* species inhabiting the human intestinal tract: *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum* group, *B. longum*, *B. infantis*, *B. dentium* and *B. gallicum*. Also, Wang *et al.* [1996] and Dong *et al.* [2000] reported the sequences applied for genetic identification of bifidobacteria.

The aim of the study was to evaluate the specificity and sensitivity of the primer pairs designed for the identification of most common human bifidobacterial species: *B. longum*, *B. breve*, *B. infantis* and *B. bifidum,* and also *B. lactis* species most frequently used in food industry. The majority of the primers used in this study were formerly described and cited by other scientists [Kok *et al*., 1996; Roy *et al.,* 1996; Matsuki *et al.,* 1998, 1999; Ventura *et al.,* 2001], but there is a lack of any

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TABLE 1. The *Bifidobacterium* strains used in the study.

Reference strains		Examined strains		
strain	origin	strain	origin	phenotypic classification
B. adolescentis ATCC 15703	intestine of adult	Bi 11	bioyoghurt	*B. animalis*
B. angulatum ATCC 27535	human faeces	Bi 30	bioyoghurt	*B. animalis*
B. animalis DSM 20104	rat faeces	Bi 50	bioyoghurt	*B. animalis*
B. bifidum ATCC 29521	stool of breast-fed infant	KNA1	infant faeces	*B. longum*
B. breve ATCC 15700	intestine of infant	KN2	infant faeces	*B. breve*
B. catenulatum ATCC 27539	human faeces	KN3	infant faeces	*B. breve*
B. pseudocatenulatum ATCC 27919	infant faeces	KN10	infant faeces	*B. breve*
B. globosum DSM 20092	rumen	KD4	adult faeces	*B. longum*
B. infantis ATCC 15697	intestine of infant	KD5	adult faeces	*B. longum*
B. lactis DSM 10140	yoghurt	KD6	adult faeces	*B. bifidum*
B. longum ATCC 15707	intestine of adult	KD7	adult faeces	*B. bifidum*
B. pseudolongum DSM 20092	pig faeces	KSI-9	rat faeces	*B. pseudolongum*
		PS14	intestine of rat	*B. pseudolongum*
		KS7	rat faeces	*B. animalis*

reports on the differences in the specificity, as well as the sensitivity between the primers targeted to the same *Bifidobacterium* species. The level of detection and the specificity of primers have a great influence on examination results, especially for the samples containing PCR inhibitors, *e.g.* from faeces.

MATERIALS AND METHODS

Strains and culture conditions. All bacterial strains used in this study are listed and described in Table 1. Bifidobacterial strains were grown in Garche's broth [Teraguchi *et al.,* 1982] in anaerobic jars (Gas Pak anaerobic system H_2+CO_2, Oxoid, UK) at 37ºC for 24 h.

Isolation of DNA. Genomic DNA of the tested strains was isolated using Wizard® Genomic Purification Kit (Promega, USA) with the modification for Gram-positive bacteria. Briefly: 1 mL of freshly multiplied culture was centrifuged, next 450 μL of 50 mmol/L EDTA, pH 8.0, and 100 μL lysozyme (10 mg/mL; Sigma Cat. No. L6751) was mixed with the pellet of bacterial cells, and the whole lot was incubated at 37ºC for 60 min. Further, it was proceeded according to the kit manufacturer's recommendation. The DNA content was measured using Eppendorf BioPhotometer (Germany). The extracted DNA was stored at -20ºC.

In order to determine the sensitivity of the primers tested, the dilutions of isolated genomic DNA were prepared. First, the mixture of chromosomal DNA obtained after isolation was standardized to the concentration of 100 μg/mL, and then decimal dilutions were made to the final concentration of 0.1 ng/mL.

Primers and PCR conditions. All the primers tested are described in Table 2. In order to compare the primers, and also to confirm the membership of the bacterial strains to the *Bifidobacterium* genus, the 16S rDNA- and 16S-23 ITS-based polymerase chain reactions were carried out. The sequences of the primers BiBIF-1N and BiBIF-2N were retrieved from GeneBank database and aligned using BLASTA algorithm. All primers were synthesized by TIB MOLBIOL (Poznań, Poland). Amplification reactions were prepared in the total volumes of 20 μL, containing: 2 μL of 10x PCR buffer (500 mmol KCl and 100 mmol Tris-HCl, pH 8.8, at 25°C, 0.8% of Nonidet P40), each deoxynucleoside triphosphate at the concentration of 250 μmol, a pair of specific primers at the concentration of 0.25 μmol each primer, 0.5 U of *Taq* DNA polymerase (Fermentas, Lithuania), and 100 ng of template DNA. The final concentrations of magnesium chloride were optimized and chosen among 1.5, 2.5 and 5.0 mmol for each primer pair, and are shown in Table 2. 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, primer annealing – at temperature dependent on the pair of primers used for 15 s, DNA extension – 72°C for 15 s and the final cycle of 72°C for 4 min. The temperature of annealing was previously optimized from 58ºC to the final temperature shown in Table 2. The amplification products were separated in 1.5% (wt/vol) agarose gel electrophoresis (at 100 V) followed by ethidium bromide staining.

Sensitivity of PCR. The assessment of detection limit for the primers was carried out under the PCR conditions described above with the quantity of template DNA amounting to 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg and 0.1 pg. As a template DNA of *B. bifidum* ATCC 29521*, B. breve* ATCC 15700, *B. infantis* ATCC 15697, *B. lactis* DSM 10140 and *B. longum* ATCC 15707 were used.

RESULTS

The membership of all tested bacterial strains to the *Bifidobacterium* genus was confirmed using the lm26/lm3 ge-

TABLE 2. The primers applied in the study.

Identification result	Name of primer	Sequence 5' – 3'	Temperature of annealing[a]	Concentration of $MgCl_2$ (mmol/L)[b]	Product size (bp)	Target Sequence	Reference
B. bifidum	BiBIF-1 BiBIf-2	CCACATGATCGCATGTGATTG CCGAAGGCTTGCTCCCAAA	67	5.0	278	16S rDNA	Matsuki *et al.*, [1998]
	BiBIF-1N BiBIF-2N	ATGATCGCATGTGATTGTGG GGAAACGCCATCTCTGG	60	2.5	842	16S rDNA	This study
B. breve	BiBRE-1 BiBRE-2	CCGGATGCTCCATCACAC ACAAAGTGCCTTGCTCCCT	63	2.5	288	16S rDNA	Matsuki *et al.*, [1998]
	BreU3 BreL4	CTCCAGCTCGACTGTCGC GCACTTTGTGTTGAGTGTACCTTTCG	65	2.5	811	16S rDNA	Roy *et al.*, [1996]
B. infantis	BiINF-1 BiINF-2	TTCCAGTTGATCGCATGGTC GGAAACCCCATCTCTGGGAT	63	1.5	828	16S rDNA	Matsuki *et al.*, [1999]
	InfU5 InfL6	CCATCTCTGGGGATCGTCGG TATCGGGAGCAAGCGTGA	62	1.5	565	16S rDNA	Roy *et al.*, [1996]
B. lactis	LW420C LW420D	GGATGCTCCGCTCCATCG GGGAAACCGTGTCTCCAC	66	2.5	846	16S rDNA	Kok *et al.*, [1996]
	Bflact2 Bflact5	GTGGAGACACGGTTTCCC CACACCACACAATCCAATAC	62	5.0	680	16S rDNA 16S-23S ITS	Ventura *et al.*, [2001b]
B. longum	BiLON-1 BiLON-2	TTCCAGTTGATCGCATGGTC GGGAAGCCGTATCTCTACGA	65	5.0	831	16S rDNA	Matsuki *et al.*, [1999]
	LonU7 LonL8	GCCGTATCTCTACGACCGTCG TATCGGGGAGCAAGCGAGAG	65	2.5	567	16S rDNA	Roy *et al.*, [1996]
Bifido-bacterium spp.	lm26 lm3	GATTCTGGCTCAGGATGAACG CGGGTGCTNCCCACTTTCATG	60	5.0	1400	16S rDNA	Kaufmann *et al.*, [1997]

[a] Temperature of species-specific primers annealing optimized by authors; [b] Concentration of $MgCl_2$ in PCR mixture optimized by authors.

nus-specific primers set described by Kaufmann *et al.* [1997] (Table 3). Similarly, most of the species-specific primer sets tested yielded an amplification product only with suitable reference strain. The exceptions were BiLON-1/2 primer pair which additionally gave PCR product with *B. pseudolongum* DSM 20092 strain (data not shown), and both *B. lactis*-specific primers sets (LW420C/LW420D and Bflact2/Bflact5) that gave a weak product with *B. animalis* DSM 20104. Nevertheless, after the optimisation of PCR conditions (temperature of annealing and concentration of magnesium chloride), none PCR product with *B. pseudolongum* DSM 20092 with BiLON-1/2 primers was obtained, whereas both *B. lactis*-specific products were still present with *B. animalis* DSM 20104 strain (Table 3). Regarding the freshly isolated strains, the results of PCR amplifications did not reveal any differences between the specificities of two primer sets assigned to identify *B. bifidum.* Similarly, no differences were stated between the primers assigned to identify *B. longum*. Thus, to *B. bifidum* KD6 and KD7 strains were classified, and to *B. longum* – KNA1, KD4 and KD5, though, most of them gave additional PCR products with *B. lactis*-specific primers (Table 3). Both *B. lactis*-specific primer sets tested (LW420C/\LW420D and Bflact2/Bflact5) yielded PCR amplicons with all the bioyoghurt-originated strains (Bi11, Bi30 and Bi50) and with human-derived KD6 strain. The Bflac2/Bflact5 set gave additionally a weak PCR product for three other human--originated strains isolated from adults (KD4, KD5 and KD7; Table 3; Figure 1). The performed Bflact2/Bflact5 PCRs for these strains with decimal-diluted DNA templates (identically as in the sensitivity determination) revealed that the 680 bp specific amplicons were obtained at the amount of 100 ng/ /20 µL of DNA template in PCR mixture in the case of KD4 and KD5 strains (Figure 2), and 10 ng of DNA template of KD7 strain. The KD6 strain only showed a band also at 1 ng of the template DNA (Figure 2). More evident discrepancies in the primer specificity occurred in the case of *B. breve*-specific sets. The BiBRE-1/2 set yielded PCR products with DNA extracted from KN2, KN3 and KN10 strains, whereas the BreU3/BreL5 primers gave PCR negative results with all these strains. Similarly, as in our phenotypic classification [Wasilewska *et al.*, 2003] no *B. infantis* strain was affirmed among the tested isolates. The applied primer sets did not enable classification of any strain originated from rats.

The results of PCR amplifications with different amount of template DNA revealed very considerable differences in the detection level of the primers used. The Bflact2/Bflact5 and LonU7/LonL8 sets revealed the highest sensitivity; the PCR product was still visible after amplification of 1 pg of template DNA in 20 µL of the reaction mixture (Figure 3). Ten pg of extracted DNA was detected with BiLON-1/BiLON-2 primers (Figure 3). The lowest detection level was demonstrated by InfU5/InfL6 and LW420C/LW420D primers (100 pg of the DNA template in a reaction mixture), as well as by BiINF-1/ /BiINF-2 and all *B. bifidum*- and *B. breve*-specific primer sets (1 ng of the template DNA).

DISCUSSION

All strains used in the study were previously phenotypically classified to five *Bifidobacterium* species: *B. longum*,

TABLE 3. The results of PCR amplifications with the primer sets tested[a].

Bifidobacterium strains/ Primer pairs	lm26 lm3	LW420C LW420D	Bflact2 Bflact5	BiBIF-1 BiBIF-2	Bbif1N Bbif2N	BiBRE-1 BiBRE-2	BreU3 BreL4	BiINF-1 BiINF-2	InfU5 InfL6	BiLON-1 BiLON-2	LonU7 LonL8
B. adolescentis ATCC 15703	+	-	-	-	-	-	-	-	-	-	-
B. angulatum ATCC 27535	+	-	-	-	-	-	-	-	-	-	-
B. animalis DSM 20104	+	+-	+-	-	-	-	-	-	-	-	-
B. bifidum ATCC 29521	+	-	-	+	+	-	-	-	-	-	-
B. breve ATCC 15700	+	-	-	-	-	+	+	-	-	-	-
B. catenulatum ATCC 27539	+	-	-	-	-	-	-	-	-	-	-
B. pseudocatenulatum ATCC 27919	+	-	-	-	-	-	-	-	-	-	-
B. globosum DSM 20092	+	-	-	-	-	-	-	-	-	-	-
B. infantis ATCC 15697	+	-	-	-	-	-	-	+	+	-	-
B. lactis DSM 10140	+	+	++	-	-	-	-	-	-	-	-
B. longum ATCC 15707	+	-	-	-	-	-	-	-	-	+	+
B. pseudolongum DSM 20092	+	-	-	-	-	-	-	-	-	-	-
Bi 11	+	+	+	-	-	-	-	-	-	-	-
Bi 30	+	+	+	-	-	-	-	-	-	-	-
Bi 50	+	+	+	-	-	-	-	-	-	-	-
KNA1	+	-	-	-	-	-	-	-	-	+	+
KN2	+	-	-	-	-	+	-	-	-	-	-
KN3	+	-	-	-	-	+	-	-	-	-	-
KN10	+	-	-	-	-	+	-	-	-	-	-
KD4	+	-	-+	-	-	-	-	-	-	+	+
KD5	+	-	-+	-	-	-	-	-	-	+	+
KD6	+	+-	+	+	+	-	-	-	-	-	-
KD7	+	-	-+	+	+	-	-	-	-	-	-
KSI-9	+	-	-	-	-	-	-	-	-	-	-
PS14	+	-	-	-	-	-	-	-	-	-	-
KS7	+	-	-	-	-	-	-	-	-	-	-

[a] The results were evaluated as follows: '++' - very strong band, '+' - strong band, '+-' - weak product, '-+' - very weak product, '-' - no product.

B. breve, B. bifidum, B. animalis, and *B. pseudolongum* [Wasilewska *et al.*, 2003] (Table 1). The performed genetic identification was generally in agreement with the phenotypic classification of the human-derived strains. The discrepancies in the specificity occurring between the results obtained with *B. breve*-specific primers could be an effect of a mistake in the sequence of BreU3 primer. This oligonucleotide, passed by Roy *et al.* [1996] and cited after Frothingham *et al.* [1993], shows differences at 3' end of the sequence, in comparison to that described by the latter authors. The PCR amplifications carried out with BreU3 primer described by Frothingham *et al.* [1993] revealed that KN2 and KN3 strains belong to the *B. breve* species, and KN10 does not (data not shown). Further alignment of the sequences of BreU3 primer described by Roy *et al.* [1996] and BreU3 described by Frothingham *et al.* [1993] with DNA sequences available in GenBank database revealed the lack of complementarity of the first primer to any bacterial DNA sequences and full complementarity of the latter one to *B. breve* 16S ribosomal RNA gene. Therefore, taking into consideration the results of PCR and sequence alignment, as well as phenotypic classification, the use of *B. breve*-specific primers described by Matsuki *et al.* [1998] seems to be more adequate.

As regards the results obtained with the Bflact2/Bflact5 primer set for two *B. bifidum* and two *B. longum* strains, the presence of the *B. lactis* specific band at a lower or equal concentration of template DNA, in comparison to sensitivity of *B. longum*- and *B. bifidum*-specific primers, and a very high detection level for the Bflac2/Bflact5 primers seem to indicate that these strains may be contaminated with *B. lactis*. However, after isolation, the tested strains were purified by fivefold subculturing in Garche's medium [Teraguchi *et al.,* 1982], every time inoculated with an isolated single colony. Nevertheless, highly adherent strains difficult to separate exist, therefore, further studies, *e.g.* cloning and sequencing of full 16S rRNA gene of these strains, are needed to solve this problem.

The *B. lactis* or *B. animalis* nomenclature used is very disputable. The *Bifidobacterium lactis* taxon was specified to "honour the source of isolation" [Meile *et al.*, 1997] and is used with reference to bifidobacterial strains most commonly used

in probiotic dairy products and infant formulas (*e.g. B. lactis* Bb12 strain). Meile *et al.* [1997] were the first to characterise this species, Kok *et al.* [1996] and Ventura *et al.* [2001] designed *B. lactis*-specific primers. Our studies confirmed the separation of *B. animalis* and *B. lactis* species only partially, due to the presence of a weak PCR band with reference to *B. animalis* DSM 20104 strain. The authors of *B. lactis*-specific primer sets, however, did not claim any specific band with this strain. Nevertheless, the classification of all bioyoghurt-originated strains to *B. lactis* was very clear and consistent with the foregoing assumptions, although contradictory to the former phenotypic classification to *B. animalis* species [Wasilewska *et al*., 2003]. On the other hand, the KS7 strain isolated from rat and phenotypically classified to *B. animalis* has not been classified to any species using PCR technique, which seems to confirm the differences between food- and animal-derived *B. lactis/B.animalis* strains. Similarly, rat-originated KSI-9 and PS14 strains have not been classified to any tested *Bifidobacterium* species using PCR, but they were phenotypically determined as *B. pseudolongum* strains. The lack of cross-reactions in the case of these strains with both *B. longum*-specific primer pairs confirms the proper optimisation of PCR conditions, and also the specificity of these primers. The results obtained for all rat-originated strains, however, are in agreement with the authors' expectations and with the description of all primer sets tested, as they were designed only for human-derived *Bifidobacterium* species. Until now one did not find any published *B. pseudolongum*-specific primers, which may occur due to the low interest of researchers in investigating the intestinal microflora of rats, and which follows a relatively small number of reports of the sequences encoding 16S rRNA of *B. pseudolongum* strains registered in the GenBank database. Widening of databases with the sequences of intestinal bacteria of so commonly used animal model as rat, would effectively influence the knowledge about the composition of their intestinal ecosystem, and thereby would enable more precise characterisation of the influence of different factors on rat's intestinal bacterial population.

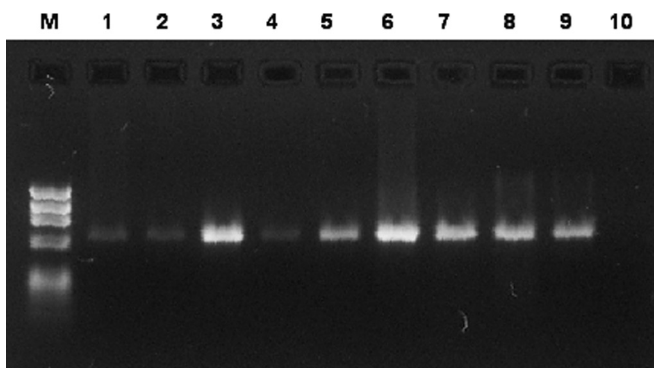

FIGURE 1. Bflact2/Bflact5 PCR amplification results. M – DNA molecular weight marker (ΦX174 DNA/*Bsu*RI, Fermentas), lanes 1-4 – human-originated strains: KD4, KD5, KD6 and KD7, lane 5 – *B. animalis* DSM 20104, lane 6 – *B. lactis* DSM 10140, lanes 7-9 – bioyoghurt-derived strains: Bi11, Bi30 and Bi50, lane 10 – negative control (with no DNA template).

The detection limit of primers is a very important factor influencing the route of PCR. Especially, as the presence of inhibitors in faecal samples results in false negative PCR result. Schwiertz *et al.* [2000] revealed that the detection limit for *Eubacterium limosum* primers was by several orders of magnitude lower in autoclaved faeces in comparison to pure cultures. Also Brigidi *et al.* [2003] made the inability to detect *B. infantis* and *B. breve* species in purified faecal samples partially dependent on the sensitivity of the primer sets used. Our evaluation revealed relatively low sensitivity of *B. bifidum-, B. breve-* and *B. infantis*-specific primer sets. However, the ability to detect 1-10 pg of the template DNA in a single PCR using *B. longum-* (LonU7/LonL8, BiLON-1/BiLON-2) and *B. lactis*-specific primers (Bflact2/Bflact5) seems to be a good achievement with reference to the results obtained by Ventura *et al.* [2001], who received visible PCR product for Bflact2/Bflact5 primers after amplification of 3.4 pg of the mixture template by nested PCR approach.

CONCLUSIONS

Among ten primer pairs specific to five *Bifidobacterium* species tested in this study, only *B. breve*-specific primer pairs (BreU3/BreL4 and BiBRE-1/BiBRE-2) revealed the differences in the specificity. *B. bifidum*-specific primers pairs did not vary either in specificity or sensitivity of

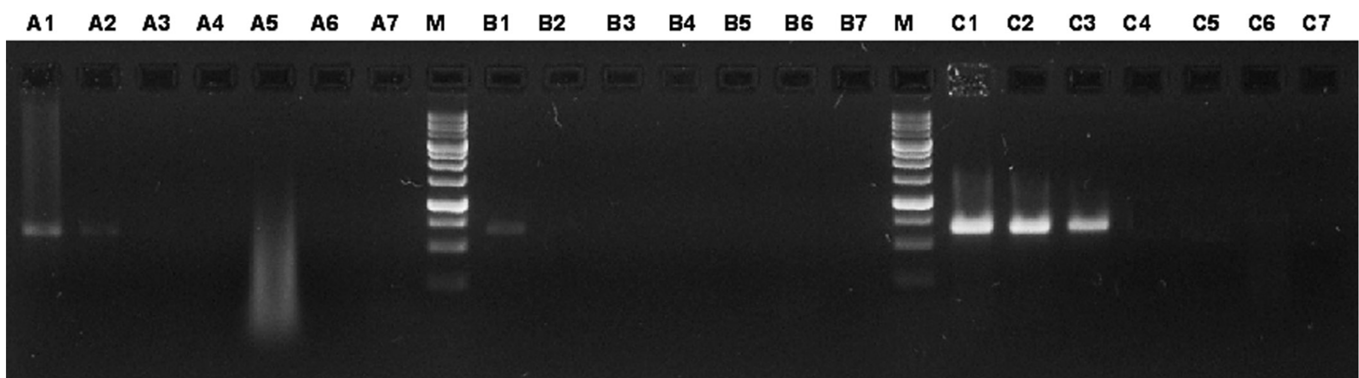

FIGURE 2. Bflact2/Bflact5 PCR products obtained with decimal diluted DNA of KD4 (A1-A7), KD5 (B1-B7) and KD6 (C1-C7) strains. The amounts of the template DNA in PCR mixtures are marked with digits: 1 – 100 ng, 2 – 10 ng, 3 – 1 ng, 4 – 100 pg, 5 – 10 pg, 6 – 1 pg, 7 – 0.1 pg. M – DNA molecular weight marker (1kb DNA ladder, TIB MOLBIOL, Poland).

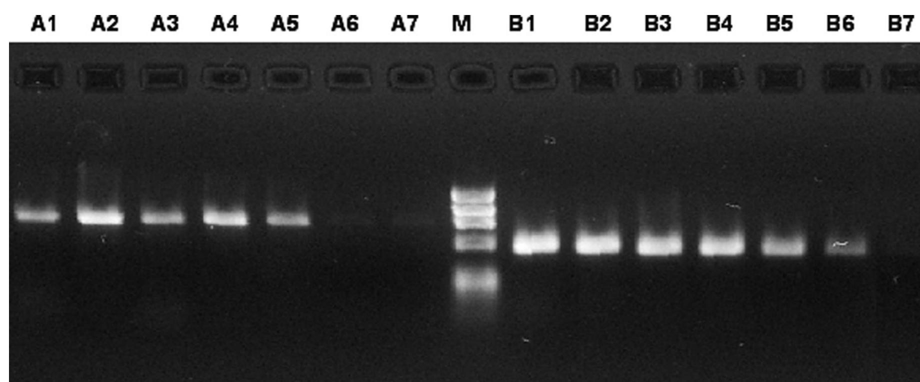

FIGURE 3. Sensitivity of *B. longum*-specific primer sets, lanes from A1 to A7 – amplicons obtained with BiLON-1/2 primers, lanes from B1 to B7 – with LonU7/LonL8 primers. The amount of DNA template of *B. longum* KNA1 strain is marked with digits: 1 – 100 ng, 2 – 10 ng, 3 – 1 ng, 4 – 100 pg, 5 – 10 pg, 6 – 1 pg, 7 – 0.1 pg. M – DNA molecular weight marker (ΦX174 DNA/*Bsu*RI, Fermentas).

the PCR amplification. The other primer sets tested differed in the detection level. According to the performed evaluation we suggest InfU5/InfL6, Bflact2/Blact5, BiBRE-1/BiBRE-2, LonU7/LonL8, and both *B. bifidum*-specific primers pairs as more suitable for investigating mixed bifidobacterial population.

REFERENCES

1. Benno Y., Sawada K., Mitsuoka T., The intestinal microflora of infants: composition of fecal flora in breast-fed and bottle-fed infants. Microbiol. Immunol., 1984, 28, 975-86.
2. Biavati, B., Castagnoli P., Trovatelli L.D., Species of the genus *Bifidobacterium* in the faeces of human adults. Appl. Environ. Microbiol., 1986, 62, 3668-3672.
3. Brigidi P., Swennen E., Vitali B., Rossi M., Matteuzzi D., PCR detection of *Bifidobacterium* strains and *Straptococcus thermophilus* in feces of human subjects after oral bacteriotherapy and yogurt consumption. Int. J. Food Microbiol., 2003, 81, 203-209.
4. Dong X., Cheng G., Jian W., Simultaneous identification of five *Bifidobacterium* species isolated from human beings using multiple PCR primers. Syst. Appl. Microbiol., 2000, 23, 386-90.
5. Frothingham R., Duncan A.J., Wilson K.H., Ribosomal DNA sequences of bifidobacteria: implications for sequence-based identification of the human colonic flora. Microb. Ecol. Health Dis., 1993, 6, 23-27.
6. Jian W., Zhu L., Dong X., New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial HSP60 gene sequences. Int. J. Syst. Evol. Microbiol., 2001, 51, 1633-1338.
7. Kaufman P., Pfeffernkorn A., Teuber M., Meile L., Identification and quantification of *Bifidobacterium* species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR. Appl. Environ. Microbiol., 1997, 63, 1268-1273.
8. 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, 3668-3672.
9. Kullen M.J., Brady L.J., O'Sullivan D.J., Evaluation of using a short region of the *recA* gene for rapid and sensitive speciacion of dominant bifidobacteria in the human large inestine. FEMS Microbiol. Lett., 1997, 154, 377-393.
10. 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.
11. 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.
12. Meile L., Ludwig W., Rueger U., Gut C. Kaufmann P., Dasen G., Wenger S., Teuber M., *Bifidobacterium lactis* sp. nov., a moderately oxygen tolerant species isolated from fermented milk. Syst. Appl. Microbial., 1997, 20, 57-64.
13. Mitsuoka T., Bifidobacteria and their role in human health. J. Indust. Microbiol., 1990, 6, 263-268.
14. Roy D., Sirois S., Molecular differentiation of *Bifidobacterium* species with amplified ribosomal DNA restriction analysis and alignment of short regions of the *ldh* gene. FEMS Microbiol. Lett., 2000, 191, 17-24.
15. Roy D., Ward P., Campagne G., Differentiation of bifidobacteria by use of pulsed-field gel electrophoresis and polymerase chain reaction. Int. J. Food Microbiol., 1996, 29, 11-29.
16. Scardovi, V. Genus *Bifidobacterium Orla-Jensen 1924, 472*[AL], 1986, *In*: Bergey's Manual of Systematic Bacteriology, (eds. P.H.A. Sneath, N.S. Mair, M.E. Sharpe, J.G. Holt), vol. 2., Williams & Wilkins, Baltimore, Md., pp. 1418-1434.
17. Schwiertz A., Le Blay G., Blaut M., Quantification of different *Eubacterium* spp. in human fecal samples with species-specific 16S rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol., 2000, 66, 375-382.
18. Teraguchi S., Kawashima T., Kuboyama M., Selective cell counting of bifidobacteria in commercial dairy and pharmaceutical products. Mat. XXI Dairy Congr. Moscow., 1982, 1(2), 2, 375.
19. Ventura M., Reniero R., Zink R., Specific identification

and targeted characterization of *Bifidobacterium lactis* from different environmental isolates by a combined multiplex-PCR approach. Appl. Envirom. Microbiol., 2001, 67, 2760-2765.

20. Wang R.F., Cao W.W., Cerniglia C.E., PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl. Envir. Microbiol., 1996, 62, 1242-1247.
21. Wasilewska E., Bielecka M., Markiewicz L., Numerical analysis of biochemical and morphological features of bifidobacteria as a tool for species characteristic and identification. Pol. J. Food Nutr. Sci., 2003, 12/53, SI 2, 149-156
22. Yamamoto T., Morotomi M., Tanaka R., Species-specific oligonucleotide probes for five *Bifidobacterium* species detected in human intestinal microflora. Appl. Envir. Microbiol., 1992, 58, 4076-4079.