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ISOLATION AND IDENTIFICATION OF BIFIDOBACTERIA FROM INFANT GUT

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The research comprises isolation and identification of *Bifidobacterium* strains from faeces of breast-fed infant. Modified Garche's agar medium, containing selective (penicillin and lithium chloride) or stimulating substances (maltodextrine instead of lactose), and anaerobic culture conditions (Gas Pak H_2 + CO_2, Oxoid) were used for strain isolation. Identification of isolates to the genus was based on fructose-6-phosphate phosphoketolase activity, lack of ability to produce catalase, indol from tryptophan, gas from glucose, and to reduce nitrate. Species identification was performed using PCR technique applying 16S rDNA-gene-targeted primers specific to *B. infantis, B. breve* and *B. longum*. Type strains from American Type Culture Collection (ATCC) were used as reference.

As a result of the studies, 14 *Bifidobacterium* strains were isolated, among which 7 were classified to *B. breve* and 6 to *B. longum* species. *B. infantis* was not isolated. It was also impossible to identify *Bifidobacterium* sp. N10 in the applied system of primers.

INTRODUCTION

Bifidobacteria are intestinal bacteria which play a major role in the most complex and diverse ecosystem of the intestinal tract of humans as well as of warm-blooded animals and honeybees. Since their first isolation from human breast-fed infants' faeces [Tissier, 1899], they have been the object of numerous nutritional, biochemical, ecological, taxonomical and genetic studies. It has been demonstrated that bifidobacteria have several health-promoting effects, including immunomodulation, elimination of procarcinogens, production of vitamin, prevention of diarrhoea and intestinal infections, alleviation of constipation, production of antimicrobials against harmful intestinal bacteria, and protection of the mucosal epithelium against invasion by pathogenic bacteria [Gibson & Roberfroid, 1995; Naidu *et al.*, 1999; Marteau *et al.*, 2001].

The probiotic effectiveness of bifidobacteria seems to be related with the ability to colonize the intestine and interact with the host. Despite the numerous studies performed, it has not been stated so far, what factors decide that bifidobacteria settle the gut. There are only few studies regarding probiotic effectiveness of exogenous bifidobacteria administered with food, confirmed in clinical trials [Salminen *et al.*, 1996 a, 1996 b]. Recent analyses of faecal population of bifidobacteria using new genetic methods suggest that each subject harbour a unique collection of bifidobacterial strains [Kimura *et al.*, 1997; Tannock *et al.*, 2000]. Thus, more detailed studies of bifidobacterial populations harbouring the gastrointestinal tract of different subjects applying new identification methods are needed.

Bifidobacteria are Gram-positive, non-sporing, non-motile, strictly anaerobic intestinal rods of variable morphology, which possess fructose-6-phosphate phosphoketolase (F6 PPK), the key enzyme of glycolytic fermentation which serves as a taxonomic character in the genus identification [Scardovi *et al.*, 1986]. Differentiation of species within the genus has traditionally relied on DNA-DNA homology or various phenotypic characteristics. In recent years, the attention of many taxonomically interested scientists has been drawn towards specific DNA probes and rRNA genes. The most frequently used sequences for bifidobacteria identification at the genus and species level are 16S rRNA and 23S rRNA encoding genes, as well as 16S-23S internal transcribed spacer (ITS). Especially, 16S rDNA sequence, which contains nine very variable regions, appeared to be exceptionally appropriate to design genus-, species- or group-specific oligonucleotides and verified bifidobacteria identification.

The aim of the study was to isolate and identify bifidobacterial strains harbouring the gut of 3-month-old breast-fed infant. For this purpose, two newly modified Garche's [Teraguchi *et al.*, 1982] media containing stimulating or selective substances were used. Classification of the strains to species was performed with PCR technique applying 16S rDNA-gene-targeted species-specific primers described by Frothingham *et al.* [1993] and Roy *et al.* [1996]. All isolated strains will be used as the material for further studies on selection of probiotic *Bifidobacterium* strains able to settle the human gut.

MATERIAL AND METHODS

Bacteria. The bacterial strains were isolated from faeces of 3-month-old healthy breast-fed infant. The reference strains originated from American Type Culture Collection (ATTC).

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Culture and storage conditions. Bacterial strains were multiplied in Garche's broth (G) [Teraguchi *et al.*, 1982] containing in grams per litter: bacto peptone (BTL, Poland) - 20.0, yeast extract - 2.0, L-cysteine hydrochloride - 0.4, lactose - 10.0, CH_3COONa - 6.0, $MgSO_4$ x 7 H_2O - 0.12, Na_2HPO_4 x 12 H_2O - 2.5, KH_2PO_4 - 2.0, and agar - 1.0 (pH after sterilization - 6.4) in anaerobic jars with Gas Pak anaerobic system H_2 + CO_2 (Oxoid, UK) at 37°C until stationary phase was achieved (pH ~4.4).

Bacterial stock cultures were maintained frozen at -70°C in reconstituted skim milk (5% d.m.) supplemented with saccharose (10%) in the ratio of 1:2. Before each experiment, strains from frozen stocks were subcultured twice in Garche's broth. Each time, five-percentage inoculum was used (vol/vol).

Isolation. Two modified Garche's agar media - one containing stimulating bifidobacteria growth maltodextrine (Hortimex, Poland) instead of lactose (MG), and the other with the addition of lithium chloride (3 g/L) and penicillin G sodium salt (50 U/L) (LPG), were used for bifidobacteria isolation. Ringer solution diluted (1:4) and supplemented with (in gram per litter): bacto peptone - 1.0, L-cysteine hydrochloride - 0.5, and agar - 1.0 (pH after sterilization - 6.4) was used for sample dilution.

Genus identification. Gram-positive, catalase-negative, unable to produce indol from tryptophan and gas from glucose as well as to reduce nitrate, but possessing fructose-6-phosphate phosphoketolase (EC 4.1.2.22) anaerobic isolates with irregular cellular morphology were identified as the *Bifidobacterium* genus [Scardovi *et al.*, 1986]. The F6PPK activity was assessed according to the method described by Scardovi *et al.* [1986]. Briefly: cells harvested from 10 mL Garche's broth were washed twice in phosphate buffer (0.05 mol/L, pH 6.5), resuspended in 1 mL of the same buffer and disrupted by sonication in the cold. A solution containing NaF (6 g/L) and sodium iodoacetate (10 g/L) and other containing fructose-6-phosphate (80 g/L in water) were added to the sonicate in the amount of 0.25 mL each. After 30 min of incubation at 37°C, the reaction was stopped with 1.5 mL of hydroxylamine hydrochloride (139 g/L of water, freshly neutralized with 5 mol/L NaOH to pH 6.5). After 10 min at room temperature, 1 mL of 15% (w/v) trichloroacetic acid, next 1 mL of 4 mol/L HCl and finally 1 mL of the colour developing 5% (w/v) $FeCl_3$ x $6H_2O$ in 0.1 mol/L HCl were added. Any reddish violet colour that developed immediately was taken as a positive result.

Species identification. The classification of the strains to the species was confirmed with PCR technique using species-specific primers described in Table 1. Primers BreU3, InfU5, and LonU7 are 5'-3' nucleotides sequences complementary to the partial 16S rDNA sequences. Primer BreU7 was previously described by Frothingham *et al.* [1993]. Primers InfU5 and LonU7 were selected by comparison of 25 partial 16S rDNA sequences of *Bifidobacterium* spp. obtained from Genbank and from the Ribosomal Database Project. Primers BreL4, LonL8 and InfL6 are sequences complementary to the respective probes PBR, PIN and PLO described by Yamamoto *et al.* [1992]. All primes were previously applied by Roy *et al.* [1996]. The synthetic oligonucleotide primers were purchased from DNA GDAŃSK (Poland). DNA was isolated using Wizard® Genomic Purification Kit (Promega, USA). The PCR was curried out in Hot-Shot 25 thermal cycler (DNA Gdańsk, Poland) applying the following PCR temperature profile: denaturation - 1 cycle of 94°C for 180 s, followed by 40 cycles of 94°C for 30 s, primer annealing - 57°C for 30 s, DNA extension - 72°C for 120 s followed by the final cycle of 72°C for 120 s. Each PCR mixture (50 μL) was composed of 5 μL Delta 2 Pwo 10x PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 1% Triton X-100, and 100 mM Tris-HCL, pH 8.8 at 25°C), each deoxynucleoside triphosphate at a concentration of 200 μM, a pair of specific primers at a concentration of 0.8 μM each primer, 2 U of Delta 2 Pwo DNA Polymerase (DNA Gdańsk, Poland), and 2 μL of template DNA. The amplification products (7 μL of each) were separated in 1.5% (wt/vol) agarose gel electrophoresis (at 85 mA), followed by ethidium bromide staining (1 μg/mL).

RESULTS AND DISCUSSION

A number of selective media for *Bifidobacterium* isolation and enumeration have been described, but none is satisfactory for all species [Munon & Pares, 1988; Rašić, 1990]. We isolated bifidobacteria from infant faeces using two newly modified Gerche's media. In GM medium, maltodextrine - preferentially utilized by bifidobacteria, was the source of carbon and energy. The LPG was supplemented with small amounts of penicillin and lithium chloride to suppress growth of the other intestinal microflora. The bacterial population in the faeces assessed using GM and LPG medium was high and amounted to $1.1x10^{10}$ and $2.9x10^{10}$ cfu in gram, respectively. Further studies revealed that mainly bifidobacteria grew on the media used. Of 20 isolated anaerobic bacterial cultures (ten per each medium), 16 were characterized by cellular morphology typical of the genus *Bifidobacterium* and 14 were also Gram-positive, among them 8 derived from

TABLE 1. Pairs of synthesized primers, their nomenclature and hybridization temperature.

Primer	Sequence	PCR product [bp]	Tm [°C]	Target species
BreU3	5'-CTCCAGCTCGCACTGTC	811	57	*B. breve*
BreL4	5'-GCACTTTGTGTTGAGTGTACCTTTCG			
InfU5	5'-CCATCTCTGGGATCGTCGG	565	57	*B. infantis*
InfL6	5'-TATCGGGGAGCAAGCGTGA			
LonU7	5'-GCCGTATCTCTACGACCGTCG	567	59	*B. longum*
LonL8	5'-TATCGGGGAGCAAGCGAGAG			

TABLE 2. Characteristic of the isolated bacterial cultures.

MG[1)]	LPG[2)]
- irregular rods G(+): 9 - irregular rods G(-): 2 - the strains selected for identification: N1, N3, N4, N5, N6, N7, N8, N10 - identified species: *B. breve* (4 strains), *B. longum* (4 strains)	- irregular rods G(+): 6 - irregular rods G(-): 2 - cocci: 2 - the strains selected for identification: N11, N13, N14, N16, N18, N20 - identified species: *B. breve* (4 strains), *B. longum* (2 strains)

[1)] Garches medium with 10 g/L maltodextrine instead of lactose; [2)] Garche's medium with the addition of 3 g/L lithium chloride and 50 U/L penicilin G sodium salt.

MG and 6 from LPG medium (Table 2). All Gram-positive irregular rods exhibited fructose-6-phosphate phosphoketolase activity (F6PPK) (EC 4.1.2.22). Bifidobacteria can be distinguished from other intestinal microflora by their ability to breakdown glucose through the so-called "bifid shunt" *via* erythrose-4-phosphate and acetyl-phosphate [Scardovi, 1986; Bezkorovainy & Miller-Catchpole, 1989]. The phosphoketolase converting the fructose-6-phosphate to erytrose-4-phosphate and acetyl phosphate is absent in other anaerobic, Gram-positive intestinal bacteria with "pseudobifid" morphology such as: *Arthrobacter, Propionibacterium, Corynebacterium* and *Actinomyces* [Scardovi *et al.*, 1986]. Since F6PPK positive strains were also catalase--negative, and unable to produce indol from tryptophan and

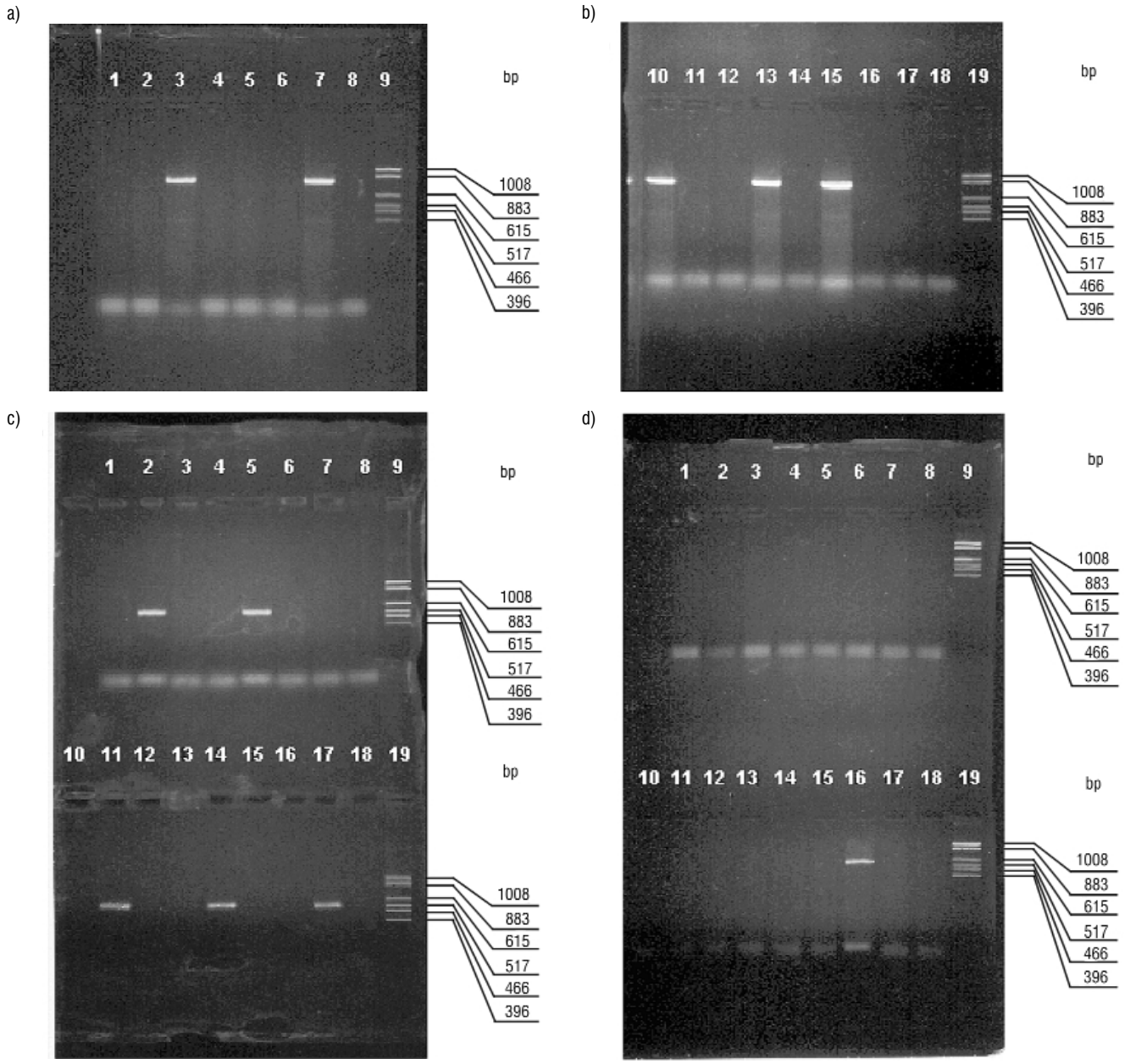

FIGURE 1. PCR products for *Bifidobacterium* species with primers specific to: a and b - *B. breve*, c - *B. longum*, d - *B. infantis*. Lines: 1 - N10, 2 - N8, 3 - N14, 4 - *B. adolescentis* ATCC 15703, 5 - *B. longum* ATCC 15707, 6 -*B. catenulatum* ATCC 27539, 7 - *B. breve* ATCC15700, 8 - *B. bifidum* ATCC 29521, 9 and 19 - DNA size marker M2 (pKO3/Hinfl), 10 - N16, 11 - N4, 12 - F6PPK(-) not identified isolate, 13 - N3, 14 - N13; 15 - N6, 16 - *B. infantis* ATCC15697, 17 - N1, 18 - negative control.

TABLE 3. Identification of *Bifidobacterium* strains to species based on 16S rDNA-gene-targeted primers specific to *B. infantis, B. breve* and *B. longum.*

Strain	Species
N3	*B. breve*
N6	*B. breve*
N7	*B. breve*
N10	nt[a])
N11	*B. breve*
N14	*B. breve*
N16	*B. breve*
N18	*B. breve*
N1	*B. longum*
N4	*B. longum*
N5	*B. longum*
N8	*B. longum*
N13	*B. longum*
N20	*B. longum*
B. longum ATCC 15707	*B. longum*
B. infantis ATCC 15697	*B. infantis*
B. breve ATCC 15700	*B. breve*

[a]) Not identified in the applied system of primers.

gas from glucose as well as to reduce nitrogen, we assigned them to the *Bifidobacterium* genus.

Identification of the isolated bifidobacterial strains to species was performed using PCR technique applying 16S rDNA-gene-targeted primers specific to *B. infantis, B. breve* and *B. longum*. As a result of the studies performed, 13 of 14 tested *Bifidobacterium* strains were identified, among which 7 were classified to *B. breve* and 6 to *B. longum* species (Table 3). Nevertheless, it was impossible to identify *Bifidobacterium* sp. N10 in the applied system of primers, so in this case further studies are necessary (Figure 1). Strains belonging to both species were isolated on MG as well as LPG medium; however the number of *B. longum* strains isolated on MG medium was twofold higher in comparison to LPG medium. It seems to confirm the stimulating effect of maltodextrine on bifidobacteria growth.

As regards the bifidobacterial species distribution in the infant gut, the obtained results revealed that *B. breve* and *B. longum* are the most common taxons inhabiting the gut of the tested breast-fed infant. *B. infantis* strains were not isolated, although some authors consider them as predominant in the infant gut. From the extensive data collected by Bezkorovainy and Miller-Catchpole [1989] it appeared however that only 54 of 115 breast-fed infants had *B. infantis*, and yet only 8 had *B. bifidum* strains. Other species found in various quantities in all infant faeces were the strains of *B. breve* and *B. longum*. Similarly, Matsuki *et al.* [1998, 1999], using a new identification method a16S rRNA-gene-targeted species-specific PCR technique, affirmed that *B. breve* is the most commonly found taxon followed by *B. longum* and *B. infantis* in the intestinal tract of 27 breast-fed infants. As in classifying bifidobacteria by classical methods, in some cases the authors reported the presence of *B. bifidum*, and *B. catenulatum*/*pseudocatenulatum* group and hardly ever of *B. adolescentis, B. angulatum*, and *B. dentium*. On the other hand, Zavaglia *et al.* [1998] classified 25 *Bifidobacterium* strains freshly isolated from infant faeces mainly to *B. bifidum*, *B. longum* and *B. breve*, whereas only single strains to *B. adolescentis, B. infantis* and *B. pseudolongum* on the basis of numerical analysis of whole-cell protein electrophoretic patterns. Thus, the results obtained, both our and these of other researches, showed a great diversity and specificity of the environment such as the infant gut.

CONCLUSIONS

Fourteen bifidobacterial strains were isolated using two modified Garche's media containing a small amount of selective or stimulating substances. There were no important differences between the strains isolated from the media used. According to the performed genetic identification of the strains it appeared that the tested microecosystem was dominated by various *B. breve* and *B. longum* strains.

The isolated strains will be used as the material for further studies on selection of probiotic *Bifidobacterium* strains able to settle the human gut.

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