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NUMERICAL ANALYSIS OF BIOCHEMICAL AND MORPHOLOGICAL FEATURES OF BIFIDOBACTERIA AS A TOOL FOR SPECIES CHARACTERISTIC AND IDENTIFICATION

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Key words: *Bifidobacterium*, identification, numerical analysis, PCR

Identification of bifidobacterial species is still problematic because of phenotypic and genetic heterogeneities. We described a method of the *Bifidobacterium* identification to species based on the numerical analysis of morphological and biochemical features.

Seventy five *Bifidobacterium* strains isolated from infants, adults, laboratory rats and bioyogurts were tested for the presence of species-characteristic cellular morphology and their biochemical patterns using the phase contrast microscopy and the API 50 CHL test, respectively. The obtained results were analysed numerically with IdBact v. 1.1 computer program (copyright by G. Kronvall, Sweden) in order to classify strains to the species. The matrices for the identification, including fermentation patterns of 20 carbohydrates, species-characteristic cellular morphology and the natural occurrence of 24 *Bifidobacterium* species, were created on the basis of the Bergey's Manual identification key and the data published after 1986. PCR technique applying 16S rRNA-gene-targeted species-specific primers described by Matsuki *et al.* [1999] and Kok *et al.* [1996] were used for confirmation of the results of phenotypic identification. Type strains from American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) were used as reference.

The results obtained showed that bifidobacteria species significantly differ in morphology and biochemical activity. The majority of the species revealed characteristic and stable morphological traits, which in combination with their fermentation patterns enabled distinction and identification of most of the *Bifidobacterium* strains tested. The results of the phenotypic classification were in correlation with the performed genetical identification based on 16S rRNA-gene-targeted species-specific sequences. Finally, the tested strains were assigned to species: *B. breve, B. longum, B. bifidum, B. angulatum, B. adolescentis, B. catenulatum, B. pseudocatenulatum, B. lactis, B. animalis,* and *B. pseudolongum*.

INTRODUCTION

Since its first isolation from human breast-fed infants' faeces [Tissier, 1899] and its designation as *Bacillus bifidus communis*, bifidobacteria has been the object of numerous nutritional, biochemical, ecological, taxonomical and genetical studies designed either to elucidate their role in the host body or to find a way of their implantation to the intestine, especially in the ill and the elderly subjects, when the number of bifidobacteria drastically decline.

They are Gram-positive, strictly anaerobic rods, which do not reduce nitrate; are nonspore-forming; do not produce catalase; and can ferment lactose, glucose, galactose, and fructose with the production of acetic and lactic acids in an approximate molar ratio of 1.5:1, without CO_2 production [Gorbati *et al*., 1995]. Bifidobacteria contain fructose-6-phosphate phosphoketolase, the key enzyme of glycolytic fermentation, which serves as a taxonomic character in the genus identification. The G+C contents of DNA vary from 55 to 67 mol%.

According to the most recent classification, the genus consists of 32 species, 12 of which are of human origin, 15 of warm-blooded animals, 3 of honeybees, 2 of wastewater and 1 of fermented milk [Gorbati *et al*., 1995; Meile *et al*., 1997]. The occurrence of the various bifidobacterial species is very habitat-specific, and in fact, the genus *Bifidobacterium* can most likely be subdivided into four subgenera based on the habitat of their component species: human, animal, insect, and sewage. Differentiation of species within the genus has traditionally consisted in DNA-DNA homology or various phenotypic characteristics [Lauer & Kandler, 1983].

The recognized therapeutic value of these microorganisms has resulted in their incorporation into many functional foods. Because of this, considerable effort has been devoted to the application of various molecular techniques for rapid identification of these strains, especially these genus-specific [Kaufmann *et al.,* 1997], species-specific [Matsuki *et al.,* 1998, 1999], and strain-specific [Kok *et al.,* 1996] probes based on appropriate 16S rRNA sequences. Phenotypic characteristics, however are still of great importance because they provide a rational approach to the selection of probiotic bacterial strains. An assessment of the phenotypic characteristic of endogenous intestinal bifidobacteria can provide insight into the traits necessary for bacterial colonisation and survival in the intestine. Furthermore, some scientific controversy still exists concerning the classification and identification of bifidobacteria and, in the case of several species, identification on the basis of phenotypic characteristic could give additional information regarding taxonomical differences.

The aim of the study was to characterize biochemical and morphological properties of a large number of bifidobacteria originated from different sources as well as to refine the method for identification of the *Bifidobacterium* strains to species based on the numerical analysis of the tested phenotypic features. According to Bergey's Manual [Scardovi, 1986],

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TABLE 1. Matrices for numerical identification of the *Bifidobacterium* species.

Species	Matrix FPME[a)]																														
	matrix FPM																													natural occurrence	
	matrix FP - fermentation profile																				characteristic cellular morphology										
	ARA	RIB	XYL	GAL	FRU	MNE	MAN	SOR	SAL	CEL	MAL	LAC	MEL	SUC	TRE	INU	MLZ	RAF	S	GNT	VLC	VSC	CSC	SCP	LCP	VFP	CPE	ALG	CGS	HGT	AGT
B. bifidum	10	10	10	90	90	10	10	10	10	10	10	90	50	50	10	10	10	10	10	10	0	0	0	0	0	0	0	100	0	100	0
B. longum	90	50*	90**	90	90	50	10	10	10	10	90	90	90	90	10	10	50*	90	10	10	0	0	0	0	0	0	0	0	0	100	0
B. infantis	10	90	50	90	90	50	10	10	10	10	90	90	90	90	10	50	10	90	10	10	0	0	0	0	0	0	0	0	0	100	0
B. breve	10	90	10	90	90	90	50	50	90	50	90	90	90	90	50	50	50	90	10	10	0	0	0	0	0	0	0	0	0	100	0
B. adolescentis	90	90	90	90	90	50	50	50	90	90	90	90	90	90	50	90**	50*	90	90	90	0	0	0	0	0	0	0	0	0	100	0
B. angulatum	90	90	90	90	90	10	10	50	90	10	90	90	90	90	10	90	10	90	90	50	0	0	0	0	0	100	0	0	0	100	10
B. catenulatum	90	90	90	90	90	10	50	90	90	90	90	90	90	90	50	50	10	90	10	50	0	0	0	100	0	0	0	0	0	100	10
B. pseudocatenulatum	90	90	90	90	90	90	10	50	90	50	90	90	90	90	50	10	10	90	90	50	0	0	0	10	0	0	0	0	0	50	50
B. dentium	90	90	90	90	90	90	90	10	90	90	90	90	90	90	90	10	90	90	90	90	0	0	0	0	0	0	0	0	0	100	0
B. globosum	50	90	50	90	90	10	10	10	10	10	90	90	90	90	10	10	10	90	90	10	0	0	100	0	0	0	0	0	0	10	100
B. pseudolongum	90	90	90	90	90	90	10	10	10	50	90	50	90	90	10	10	50	90	90	10	0	0	100	0	0	0	0	0	0	0	100
B. cuniculi	90	10	90	90	10	10	10	10	10	10	90	10	90	90	10	10	10	10	90	10	0	0	100	0	0	0	0	0	0	0	100
B. choerinum	10	10	10	90	10	10	10	10	10	10	90	90	90	90	10	10	10	90	90	10	0	0	100	0	0	0	0	0	0	0	100
B. animalis	90	90	90	90	90	50	10	10	90	50	90	90	90	90	50	10	50	90	90	10	0	0	0	0	0	0	100	0	0	0	100
B. thermophilum	10	10	10	90	90	10	10	10	50	50	90	50	90	90	50	50	50	90	90	10	0	0	0	0	0	0	0	0	0	0	100
B. boum	10	10	10	90	90	10	10	10	10	10	90	50	90	90	10	90	10	90	90	10	0	0	0	0	0	0	0	0	0	0	100
B. magnum	90	90	90	90	90	10	10	10	10	10	90	90	90	90	10	10	10	90	10	10	100	0	0	0	0	0	0	0	0	0	100
B. pullorum	90	90	90	90	90	90	10	10	90	10	90	10	90	90	90	90	10	90	10	10	0	0	0	0	100	0	0	0	0	0	100
B. suis	90	10	90	90	50	50	10	10	10	10	90	90	90	90	10	10	10	90	10	10	0	0	0	0	0	0	0	0	0	0	100
B. minimum	10	10	10	10	90	10	10	10	10	10	90	10	10	90	10	10	10	10	90	10	0	100	0	0	0	0	0	0	0	50***	50***
B. subtile	10	90	10	90	90	10	10	90	50	10	90	10	90	90	50	50	90	90	90	90	0	100	0	0	0	0	0	0	0	50***	50***
B. coryneforme	90	90	90	50	90	10	10	10	90	90	90	10	90	90	10	50	10	90	10	90	0	100	100	0	0	0	0	0	0	0	0
B. asteroides	90	90	90	50	90	10	10	10	90	90	50	10	90	90	10	10	10	90	10	50	0	100	0	0	0	0	0	0	100	0	0
B. indicum	10	90	10	50	90	50	10	10	90	90	50	10	90	90	10	10	10	90	10	90	0	100	100	0	0	0	0	0	0	0	0

[a)] The table shows percentages of strains belonging to the taxa (species) and revealing analyzed tests: ARA - L-arabinose, RIB - ribose, XYL - D-xylose, GAL - galactose, FRU - fructose, MNE - mannose, MAN - mannitol, SOR - sorbitol, SAL - salicine, CEL - cellobiose, MAL - maltose, LAC - lactose, MEL - melibiose, SUC - sucrose, TRE - trehalose, INU - inuline, MLZ - melezitose, RAF - D-raffinose, S – starch, GNT – gluconate; VLC – very long cells present (usually 2x10-20 μm), VSC – very small cells present (usually 0.3x1.3-1.5 μm), CSC – cells are generally short, coccoid or almost spherical to curved or tapered, arranged singly or doubly or rarely in short chains, SCP - cells are characteristically arranged in chains of three, four or more elements, the distal ends of the chains are usually tapered, LCP – the long chains of regular cells, FVP – cells characteristically disposed in V or palisade arrangements, CPE – cells show characteristically the central portion slightly enlarged, ALG – groupings of 'amphora-like' cells are characteristic, CGS – star-like arrangement of cells is characteristic; HGT – human gastrointestinal tract, AGT – animal gastrointestinal tract.

*) Data according to Yaeshima *et al.* [1991, 1992] (according to Bergey's Manual [Scardovi, 1986] 90% of strains are positive in taxa); **) Data according to Yaeshima *et al.* [1991, 1992] (according to Bergey's Manual [Scardovi, 1984] 50% of strains are positive in taxa); ***) So far, strains of this species were isolated only from sewage.

some bifidobacterial species had distinct shapes or arrangements which might be helpful in their recognizing. Numerical clustering of bifidobacterial strains based on biochemical properties has been reported [Gavini *et al*., 1991], but their numerical identification on the basis of biochemical, together with morphological features, has not been done yet. Genetical identification based on species-specific 16S rRNA sequences was also performed.

MATERIAL AND METHODS

Bacteria. The bifidobacterial strains were isolated from infant (strains signed as KN), adults (KD), laboratory rats (KS and PS) and bioyogurts (BI) [Wasilewska *et al.*, under preparation]. All strains were assigned to the genus *Bifidobacterium* on the basis of fructose-6-phosphate phosphoketolase activity, and 16S rRNA genus-specific sequences and PCR technique described by Kaufmann *et al.* [1997]. The reference strains derived from American Type Culture Collection (ATCC) and from Deutsche Sammlung von Mikroorganismen (DSMZ).

Storage and culture condition. Bacterial stock cultures were maintained frozen at –70°C in reconstituted skim milk (5% dry wt.) supplemented with sucrose (10%) in the ratio 1:2. Before every experiment, strains from frozen stocks were subcultured twice in Garche's broth [Teraguchi *et al.,* 1982] in anaerobic jars (Gas Pak anaerobic system H_2+CO_2, Oxoid, UK) at 37°C. Each time five-percentage inoculum was used (vol/vol).

Cellular morphology. Active cultures of the tested and reference strains were grown on Garche's agar stabs for 48 h under anaerobic conditions, next morphology of live cells was examined at phase contrast microscopy (Microphot FXA, Nikon, Japan).

Carbohydrate fermentation. Carbohydrate fermentation patterns of *Bifidobacterium* strains were determined using the API 50 CHL system (BioMerieux, France). Active cells were multiplied anaerobically on Garche's agar slants during 48 h at 37°C, washed out with Garche's broth without lactose and with the addition of bromocresol purple (0.5 g/L) and standardized to about $9x10^8$ cells/mL using McFarland scale. The test procedure was carried out following the manufacturer's guidelines. Results were checked after 24, 48 and for confirmation after 96 h. Each strain was tested in duplicate.

Numerical taxonomy. Numerical identification of the *Bifidobacterium* strains to species was performed with Idbact v. 1.1 computer program (copyright by G. Kronvall, Sweden). The program compares the test results of an unknown isolate with the known test result percentages in these tests for a collection of related bacterial species, using established numerical methods. The identification matrices including fermentation patterns of 20 carbohydrates, species-characteristic cellular morphology and the natural occurrence of 24 *Bifidobacterium* species were created on the basis of the Bergey's Manual identification key and the data published after 1986 [Scardovi, 1986; Yaeshima *et al.,* 1991, 1992]. All matrices are set in Table 1. To evaluate a usefulness of the newly created matrices for *Bifidobacterium* species differentiation, numerical identification of all taxa (species) was performed on the basis of the characters analysed; described for them in Bergey's Manual [Scardovi, 1986] (Table 2).

PCR procedure. Phenotypic classification of the strains was confirmed with PCR technique using the species--specific primers described by Matsuki *et al.* [1998, 1999] and Kok *et al.* [1996]. The primers were synthesized by TIB MOLBIOL (Poland). DNA was isolated using Wizard® Genomic Purification Kit (Promega, USA). Each PCR mixture (20 μL) was composed of 2 μL MasterAmp *Taq* 10x PCR buffer (500 mM KCl and 100 mM Tris-HCl pH 8.3 at 22°C), 5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 250 μM, a pair of specific primers at a concentration of 1.0 μM each, 0.8 U of MasterAmp™ *Taq* DNA Polymerase (Epicentre, USA), and 100 ng of template DNA. The PCR was carried out in Eppendorf Mastercycler gradient (Germany) applying the following PCR temperature profiles: denaturation - 1 cycle of 94°C for 4 min, followed by 30 cycles of 94°C for 15 s, primer annealing – 66°C for 15 s, DNA extension - 72°C for 15 s and final cycle of 72°C for 2 min. 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). Gels were documented using KODAK DC4800 Software.

RESULTS AND DISCUSSION

According to Bergey's Manual [Scardovi, 1986], *B. bifidum, B. angulatum, B. catenulatum, B. pullorum, B. animalis, B. minimum, B. asteroides, B. globosum, B. pseudolongum, B. cuniculi* and *B. choerinum* grown anaerobically in agar stabs showed distinct cell shapes or arrangement which could be helpful in species identification (Table 1). Species-characteristic bifidobacterial morphology has been also reported in some latest works [Bezkorovainy, 1989; Yaeshima *et al.,* 1991; Tamime *et al.,* 1995]. Our comparison of cellular morphology of type bifidobacterial strains proved the presence of such features in *B. angulatum* ATCC 27535, *B. pseudolongum* DSM 20099 and *B. globosum* DSM 20092, *B. catenulatum* ATCC 27539, *B. animalis* ATCC 25527 and *B. bifidum* ATCC 29521. Such characteristics were not observed in *B. infantis* ATCC 15697, *B. breve* ATCC 15700, and *B. longum* ATCC 15707 strain. However, *B. pseudocatenulatum* ATCC 27919 strain showed similar cellular morphology to *B. catenulatum* ATCC 27539, and also *B. adolescentis* ATCC 15703 seemed to be similar to *B. angulatum* ATCC 27535. As for the isolated bifidobacterial strains, cellular morphology characteristic for *B. bifidum, B. angulatum, B. catenulatum, B. globosum, B. pseudolongum,* and *B. animalis* as well as populations without clearly visible cellular traits were observed.

Of 49 analyzed carbohydrates and carbohydrate derivative compounds as many as 34 were fermented by the tested bifidobacterial strains. Pentoses (L-arabinose, ribose, D-xylose), hexoses (D-galactose, D-glucose, D-fructose, D-mannose, esculine, salicine) and some di- (maltose, lactose, melibiose,

TABLE 2. Evaluation of the created matrices as a result of identification of the *Bifidobacterium* species described in Bergey's Manual [Scardovi, 1986].

Species	Matrix								
	FP			FPM			FPME		
	ID SCORE[a)]	other similar species [b)]	identification result	ID SCORE	other similar species	identification result	ID SCORE	other similar species	identification result
B. bifidum	1.00	ns[c)]	excellent						
B. longum	0.29	*B. suis* (0.52), *B. magnum* (0.19)	poor	0.35	*B. suis* (0.64)	poor	0.99	ns	very good
B. infantis	0.77	*B. globosum* (0.15), *B. magnum* (0.06)	poor	0.97	ns	very good	0.99	ns	very good
B. breve	0.59	*B. infantis* (0.38)	poor	0.60	*B. infantis* (0.39)	poor	0.60	*B. infantis* (0.39)	poor
B. adolescentis	0.56	*B. angulatum* (0.36), *B. coryneforme* (0.04)	poor	0.97	ns	very good	0.99	ns	excellent
B. angulatum	0.94	*B. animalis* (0.03)	good	1.00	ns	excellent			
B. catenulatum	0.93	*B. asteroides* (0.02)	good	1.00	ns	excellent			
B. pseudocatenulatum	0.43	*B. animalis*. (0.43), *B. pseudolongum* (0.09)	poor	0.99	ns	excellent			
B. dentium	0.99	ns	excellent						
B. globosum	0.88	*B. infantis* (0.05), *B. choerinum* (0.04)	poor	0.96	*B. choerinum* (0.04)	very good	0.96	ns	very good
B. pseudolongum	0.94	*B. globosum* (0.02)	good	0.98	*B. globosum* (0.02)	very good	0.98	*B. globosum* (0.02)	very good
B. cuniculi	1.00	ns	excellent						
B. choerinum	0.99	ns	very good	1.00		excellent			
B. animalis	0.50	*B. angulatum* (0.18), *B. globosum* (0.18), *B. magnum* (0.06), *B. pseudocaten.* (0.06)	poor	1.00	ns	excellent			
B. thermophilum	0.27	*B. boum* (0.56), *B. choerinum* (0.11), *B. globosum* (0.04),	poor	0.32	*B. boum* (0.67)	poor	0.32	*B. boum* (0.68)	poor
B. boum	0.95	*B. thermophilus* (0.05)	good	0.95	*B. thermophilum* (0.05)	good	0.95	*B. thermophilum* (0.05)	good
B. magnum	0.79	*B. longum* (0.16), *B. globosum* (0.03)	poor	1.00	ns	excellent			
B. pullorum	1.00	ns	excellent						
B. suis	0.90	*B. longum* (0.06), *B. magnum* (0.04)	poor	0.94	*B. longum* (0.06)	good	1.00	ns	excellent
B. minimum	1.00	ns	excellent						
B. subtile	1.00	ns	excellent						
B. coryneforme	0.64	*B. asteroides* (0.36)	poor	0.99	ns	excellent			
B. asteroides	0.98	*B. coryneforme* (0.02)	very good	1.00	ns	excellent			
B. indicum	0.99	ns	very good	1.00	ns	excellent			

[a)] Identification score: amounting 1.00 means identical. ID SCORE amounting 0.02 means no similarity; [b)] ID SCORE was announced in parenthesis; [c)] not stated.

TABLE 3. Classification of the *Bifidobacterium* strains to species.

Strain	Numerical method						PCR technique
	result of identification	ID SCORE in matrices			evaluation	similar species	
		FP	FPM	FPME			
KNA1	*B. longum*	0.58	0.96	0.99	excellent	ns[a)]	*B. longum*
KN2	*B. breve*	0.09*	0.23*	0.48	poor	*B. pseudocat.* (0.36), *B. adoles.* (0.10), *B. infantis* (0.03)	*B. breve*
KN3	*B. breve*	0.09*	0.23*	0.48	poor	*B. pseudocat.* (0.36), *B. adoles.* (0.10), *B. infantis* (0.03)	*B. breve*
KN4	*B. longum*	0.81	0.96	0.98	very good	ns	*B. longum*
KN5	*B. longum*	0.09*	0.57	0.80	poor	*B. pseudocat.* (0.10), *B. dentium* (0.06), *B. adoles.* (0.03)	*B. longum*
KN10	*B. breve*	0.46	0.66	0.86	poor	*B. pseudocat.* (0.07), *B. infantis* 0.06)	*B. breve*
KN11	*B. breve*	0.09*	0.23*	0.48	poor	*B. pseudocat.* (0.36), *B. adoles.* (0.10), *B. infantis* (0.03)	*B. breve*
KN13	*B. longum*	0.64	0.75	0.88	poor	*B. infantis* (0.10)	nt[b)]
KN14	*B. breve*	0.46	0.66	0.86	poor	*B. pseudocat.* (0.07), *B. infantis* 0.06)	*B. breve*
KN20	*B. longum*	0.09*	0.57	0.80	poor	*B. pseudocat.* (0.10), *B. dentium* (0.06), *B. adoles.* (0.03)	*B. longum*
KN38	*B. longum*	0.81	0.96	0.99	very good	ns	*B. longum*
KN43	*B. breve*	0.86	0.93	0.93	good	*B. infantis* (0.07)	*B. breve*
KN45	*B. breve*	0.46	0.66	0.86	poor	*B. pseudocat.* (0.07), *B. infantis* 0.06)	*B. breve*
KN48	*B. breve*	0.86	0.93	0.93	good	*B. infantis* (0.07)	*B. breve*
KN62	*B. breve*	0.86	0.93	0.93	good	*B. infantis* (0.07)	*B. breve*
KN65	*B. breve*	0.86	0.93	0.93	good	*B. infantis* (0.07)	*B. breve*
KD1	*B. angulatum*	0.94	1.00		excellent	ns	*B. adolescentis*
KD2	*B. adolescentis*	0*	0.12*	0.19*	poor	*B. angul.* (0.69), *B. pseudocat.* (0.06), *B. longum* (0.04)	*B. adolescentis*
KD3	*B. adolescentis*	0*	0.12*	0.19*	poor	*B. angul.* (0.69), *B. pseudocat.* (0.06), *B. longum* (0.04)	*B. adolescentis*
KD4	*B. longum*	0.11*	0.34*	0.90	poor	*B. infantis* (0.10)	*B. longum*
KD5	*B. longum*	0.14*	0.72	0.90	poor	*B. infantis* (0.10)	*B. longum*
KD6	*B. bifidum*	1.00			excellent	ns	*B. bifidum*
KD7	*B. bifidum*	1.00			excellent	ns	*B. bifidum*
KD8	*B. longum*	0.58	0.96	0.99	very good	ns	*B. lactis*
KD9	*B. animalis*	0.33	0.99		very good	ns	*B. lactis*
KD10	*B. animalis*	0.33	0.99		very good	ns	*B. lactis*
KD11	*B. adolescentis*	0*	0.12*	0.19*	poor	*B. angul.* (0.69), *B. pseudocat.* (0.06), *B. longum* (0.04)	nt
KD12	*B. animalis*	0.33	0.99		very good	ns	nd[c)]
KD13	*B. pseudocatenulatum*	0.43	0.99		excellent	ns	*B. caten. group*
KD14	*B. catenulatum*	0.06*	0.52	0.80	poor	*B. pseudocatenulatum* (0.18)	*B. caten. group*
KD15	*B. pseudocatenulatum*	0.43	0.99		excellent	ns	*B. caten. group*
KD16	*B. catenulatum*	0.06*	0.52	0.80	poor	*B. pseudocatenulatum* (0.18)	*B. caten. group*
KD17	*B. pseudocatenulatum*	0.43	0.99		excellent	ns	*B. caten. group*
PS11	*B. animalis*	0.24*	0.98		very good	ns	nd
PS14	*B. pseudolongum*	0.65	0.83	0.85	poor	*B. globusom* (0.15)	nd
PS34	*B. pseudolongum*	0.65	0.83	0.85	poor	*B. globusom* (0.15)	nt
PS36	*B. pseudolongum*	0.65	0.83	0.85	poor	*B. globusom* (0.15)	nd
PS37	*B. animalis*	0.07*	0.97	1.00	excellent	ns	nd
PS46	*B. animalis*	0.07*	0.97	1.00	excellent	ns	nd

Table 3 is continued on next page

Continuation of Table 3

PS85	*B. pseudolongum*	0.65	0.83	0.85	poor	*B. globusom* (0.15)	nd
KSp4	*B. animalis*	0.72	1.00		excellent	ns	nd
KSp5	*B. animalis*	0.07*	0.97	1.00	excellent	ns	nd
KSp6	*B. animalis*	0.07*	0.97	1.00	excellent	ns	nd
KS7	*B. animalis*	0.07*	0.97	1.00	excellent	ns	nd
KSI-9	*B. pseudolongum*	0.65	0.83	0.85	poor	*B. globusom* (0.15)	nd
KS1b2	*B. animalis*	0.45	0.99		excellent	ns	nd
KS7d3	*B. animalis*	0.49	0.99		excellent	ns	nd
KS20a1	*B. animalis*	0.45	0.99		excellent	ns	nd
KS29a3	*B. animalis*	0.09*	0.92	0.98	very good	*B. pseudocatenulatum* (0.02)	nd
Bi11	*B. animalis*	0.17*	0.97	0.80	poor	*B. pseudocatenulatum* (0.18)	*B. lactis*
Bi24	*B. animalis*	0.17*	0.97	0.80	poor	*B. pseudocatenulatum* (0.18)	*B. lactis*
Bi30	*B. animalis*	0.27*	0.99	0.81	poor	*B. pseudocatenulatum* (0.19)	*B. lactis*
Bi31	*B. animalis*	0.17*	0.97	0.80	poor	*B. pseudocatenulatum* (0.18)	nt
Bi36	*B. animalis*	0.07*	0.97	0.95	very good	*B. pseudocatenulatum* (0.02)	*B. lactis*
Bi45	*B. animalis*	0.17*	0.97	0.80	poor	*B. pseudocatenulatum* (0.18)	*B. lactis*
Bi50	*B. animalis*	0.07*	0.97	0.95	very good	*B. pseudocatenulatum* (0.02)	*B. lactis*
Bi52	*B. animalis*	0.07*	0.97	0.95	very good	*B. pseudocatenulatum* (0.02)	*B. lactis*
Bi55	*B. animalis*	0.07*	0.97	0.95	very good	*B. pseudocatenulatum* (0.02)	*B. lactis*
BiG	*B. animalis*	0.17*	0.97	0.80	poor	*B. pseudocatenulatum* (0.18)	*B. lactis*
BiH	*B. animalis*	0.33	0.99	0.81	poor	*B. pseudocatenulatum* (0.19)	*B. lactis*
BiO	*B. animalis*	0.17*	0.97	0.80	poor	*B. pseudocatenulatum* (0.18)	*B. lactis*
J38	*B. animalis*	0.33	0.99	0.81	poor	*B. pseudocatenulatum* (0.19)	*B. lactis*
BE	*B. animalis*	0.17*	0.97	0.80	poor	*B. pseudocatenulatum* (0.18)	*B. lactis*
BL	*B. longum*	0.58	0.96	0.96	very good	*B. suis* (0.02)	*B. longum*
ATCC 29521	*B. bifidum*	0.98	1.00		excellent	ns	*B. bifidum*
ATCC 15697	*B. infantis*	0.46	0.95	0.98	very good	ns	*B. infantis*
ATCC 15700	*B. breve*	0.04*	0.12*	0.14*	poor	*B. infantis* (83)	*B. breve*
ATCC 15703	*B. adolescentis*	0.10*	0.95	0.96	very good	ns	*B. adolescentis*
ATCC 15707	*B. longum*	0.55	0.94	0.97	very good	ns	*B. longum*
DSM 20099	*B. pseudolongum*	0.48	0.83	0.84	poor	*B. globusom* (0.15)	nd
ATCC 27535	*B. angulatum*	0.97	1.00		excellent	ns	*B. angulatum*
ATCC 27539	*B. catenulatum*	0.15*	0.99	1.00	excellent	ns	*B. caten.* group
DSM 20092	*B. globusom*	0.64	0.94	0.94	good	*B. pseudolongum* (0.06)	nd
ATCC 25527	*B. animalis*	0.09*	0.92	0.98	very good	ns	nd
ATCC 27919	*B. pseudocatenulatum*	0.84	1.00		excellent	ns	*B. caten.* group

*) Other species revealed higher ID SCORE; a) not stated; b) not tested; c) not determined in the applied system of PCR primers.

sucrose) and oligosaccharides (D-raffinose, D-turanose) belonged to the more frequently metabolized compounds. Of carbohydrates fermented by bifidobacteria only D-galactose, D-glucose, lactose and melibiose were used by all strains. The ability of the tested strains to utilize the other carbohydrate compounds was very much differentiated. A few researchers made an attempt to identify bifidobacteria on the basis of broad fermentation profiles [Gavini *et al.,* 1991; Yaeshima *et al.,* 1991], however, they appeared to be insufficiently selective to distinguish *Bifidobacterium* species. Our cluster analysis of newly isolated bifidobacterial strains and the features tested, showed that there are significant differences in the fermentation profiles and cellular morphology between the strains belonging to various species of the genus *Bifidobacterium* [Wasilewska *et al.,* under preparation].

Numerical identification based on matrices including ability of the *Bifidobacterium* species to ferment 20 carbohydrates, as well as characteristic cellular morphology of individual bifidobacterial species enabled appropriate differentiation of 21 from 24 species described in Bergey's Manual [Scardovi, 1986] (Table 2). Exceptions were *B. longum, B. breve* and *B. thermophilum* species in the case of which separation was poor, due to a similarity of the analysed features of these species to *B. suis, B. infantis* and *B. boum*, respectively. Similarly, numerical identification of the type strains confirmed very good distinction of *B. bifidum* ATCC

29521, *B. angulatum* ATCC 27535, *B. pseudocatenulatum* ATCC 27919 and *B. catenulatum* ATCC 27539, as well as good distinction of *B. infantis* ATCC 15697, *B. adolescentis* ATCC 15703, *B. animalis* ATCC 25527 and *B. globosum* DSM 20092 strains (Table 3). However, *B. longum* ATCC 15707 strain was well identified, whereas, as in the case of differentiation of individual bifidobacterial species, *B. breve* ATCC 15700 and *B. pseudolongum* DSM 20099 strains were identified poor, owing to the general similarity of the analysed features of these strains to *B. infantis* and *B. globosum* species, respectively.

Regarding the tested bifidobacterial isolates, they were classified with good results to *B. bifidum, B. angulatum* and *B. pseudocatenulatum,* and partially to *B. longum, B. breve* and *B. animalis* species (Table 3). Both, the analyzed features and evaluation of the identification results were in accordance with the results obtained for the reference strains. As in the case of type strains, the isolates classified to *B. pseudolongum* showed a great similarity to *B. globosum.* Similarly, it was difficult to differentiate some *B. longum* and *B. breve* strains, because as in the case of type strains they were similar to *B. infantis.* However, significant similarity of some strains of *B. breve, B. catenulatum* and *B. animalis* to other species, mainly to *B. pseudocatenulatum* as well as *B. adolescentis* to *B. angulatum* was also observed.

More than half isolates tested (36 from 64) were classified on the basis of fermentation profiles, but only in the cases of *B. bifidum* and *B. angulatum* the identification results were excellent and good, respectively. In the remaining cases, the identification was poor. When characteristic cellular morphology was analysed together with fermentation patterns, the identification significantly improved, since strains with correct fermentation profile, but atypical for the taxa (species) morphology, were eliminated. Consequently, identification of most of the previously indicated strains was confirmed with good, very good or excellent result, and additionally 23 strains were classified with equally satisfying result.

In the case of 5 strains of human origin, classification was accomplished only when natural occurrence of individual *Bifidobacterium* species was additionally taken into consideration. These strains were classified to *B. breve* and *B. longum* species. In accordance with the performed numerical identification, three strains isolated from adults (KD2, KD3 and KD11) showed the highest similarity to *B. angulatum* species, however owing to the lack of characteristic cellular morphology for this species, the following *B. adolescentis* result was taken as positive.

The results of phenotypic classification were in correlation with the performed genetic identification (Table 3). The membership of most strains isolated from human to the species pointed in the phenotypic classification was confirmed. Exceptions were KD8 and KD1 strains phonotypically assigned to *B. longum* and *B. angulatum*, but while using PCR technique and the species-specific primers described by Matsuki *et al.* [1999] and Kok *et al.* [1996] to *B. lactis* and *B. adolescentis,* respectively. However, the KD8 strain gave positive result for *B. longum* when using Lon U7/Lon L8 primers described by Roy *et al.* [1996] (unpublished data). So, in these cases further studies including additional strains of these species are needed using both phenotypic as well as genetic characteristics.

All strains isolated from bioyogurts and a few from rats were classified as members of *B. lactis*, although they were grouped with *B. animalis* in phenotypic clustering. Meile *et al.* [1997] classified *Bifidobacterium* strain freshly isolated from bioyogurt as a new species *Bifidobacterium lactis* (named such to honour source of isolation) because despite of the great similarity of sequence of 16S rRNA of the isolated strain to reference strain of *B. animalis* both strains showed relatively low homology of genomic DNA. However, it has not been settled so far whether they are two different bifidobacterial species, as some scientists claim, or *B. lactis* is only a subspecies of the *B. animalis* [Cai *et al.,* 2000; Ventura & Zink, 2002]. The *B. lactis*-specific primer set LW420C/LW420D applied in the study was primarily designed for detection of probiotic *Bifidobacterium* LW420 strain originated from food [Kok *et al.*, 1996]. Also the presence of a considerable group of rat-originated strains phenotypically assigned to *B. animalis,* which did not give any positive results with the applied primer sets, seems to confirm the existence of considerable differences within the *B. animalis* species. The authors have not found any published *B. animalis* nor *B. pseudolongum*-specific PCR primers. It may be due to a low interest of researches in investigating the intestinal microflora of animals, which follows a relatively small number of reports of the sequences encoding 16 S rRNA of *B. animalis* and *B. pseudolongum* strains registered in the GenBank database. However, widening databases with sequences of intestinal bacteria of widely used animal models would effectively influence bacterial taxonomy, especially of the closely related strains, such as *B. lactis* and *B. animalis.*

CONCLUSIONS

The carried out studies proved that bifidobacteria species differ significantly in morphology and biochemical activity. The majority of bifidobacterial species revealed characteristic morphological traits, which in combination with their fermentation patterns enabled distinction and identification of most of the *Bifidobacterium* strains tested.

The results of phenotypic classification were in correlation with the performed genetic identification, which seems to confirm the usefulness of the applied numerical method for identification of the *Bifidobacterium* species. Taking into consideration species-characteristic, cellular morphology would effectively improve identification using the API 50 CHL test, which is still commonly used for bacterial identification.

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