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Isolation and Characterization of *Bifidobacterium* Strains for Probiotic Formulation

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ABSTRACT

Twenty-five *Bifidobacterium* strains isolated from infant feces were identified by sugar fermentation patterns and whole-cell protein analysis. Using gradient SDS-PAGE, six characteristic protein bands of the genus were detected in 40 strains of bifidobacteria but not in lactobacilli. Computerized numerical analysis enabled strains to be grouped in two main clusters. Strains of *Bifidobacterium bifidum* belong to a well-differentiated cluster that joins the cluster of the remaining species at 0.582 similarity. The predominant species among isolated strains from infant feces were *B. bifidum, B. longum,* and *B. breve.* Probiotic and technological indicators such as surface properties, inhibitory capacity, resistance to bile and low pH, and ability to grow under aerobic conditions were studied. Not all desirable characteristics were present in a single strain. In general, adherent and inhibitory strains were not resistant to bile, low pH, and aerobic conditions. Only 10 of 40 strains were resistant to 0.5% bile.

Microorganisms of the genus *Bifidobacterium* are normal inhabitants of the human intestine. They are found at high concentrations in feces of breast-fed infants and play a role in resistance to infection. Placebo-controlled studies have shown that bifidobacteria prevent antibiotic-associated diarrhea and acute infantile diarrhea *(7).* It has been demonstrated that host resistance to infection is achieved in part by enhanced antibody production and proliferation of Peyer's patch cells stimulated by bifidobacteria *(36).* Protection of the mucosal epithelium against invasion by pathogenic bacteria could be a consequence of the adherent properties of some *Bifidobacterium* strains *(4).* In addition, the liberation of antimicrobial substances such as lactic and acetic acids by these bacteria causes a high rate of inhibition of gram-positive and gram-negative microorganisms in vitro *(12, 16, 22).* It seems that this metabolic activity also takes place in vivo, since infant feces with low pH have been associated with a high content of bifidobacteria in the intestinal lumen *(8).*

Consumption of cow's milk fermented experimentally and commercially with bifidobacteria cultures decreased nondesirable colonic microflora *(8, 22).* This displacement of intestinal flora was due to colonization and growth of bifidobacteria in the large intestine. Attempts to identify and use a single strain or a defined mixture of strains that would have the broad specificity of the normal microflora to resist pathogen invasion have not been successful *(7).*

New isolates are needed to improve the biotherapeutic action of the bifidobacteria included in several foods and pharmaceutical products; for this purpose, proper identification of new strains is required. In this regard, the use of molecular methods appears imperative, since the pattern of sugar fermentation could lead to mistakes in species identification. Strains that belong to *Bifidobacterium animalis* on the basis of sugar fermentation were identified as *Bifidobacterium longum* by DNA homology *(35),* and difficulties in distinguishing *Bifidobacterium adolescentis* from *Bifidobacterium dentium* have been reported *(23).* For these reasons more reliable techniques such as whole-cell protein analysis, enzymatic analysis, analysis of membrane fatty acids, determination of the electrophoretic mobility of enzymes, and DNA and RNA analysis have been employed for bacterial identification *(14, 18, 20, 26, 27, 33, 34).*

Numerical clustering of bifidobacterial strains has been reported *(9)* but, to our knowledge, numerical analysis based on whole-cell proteins of strains isolated exclusively from infant feces has not been performed.

The object of this study was to isolate bifidobacterial strains from humans for probiotic formulation. Strains were isolated from infant feces and identified by sugar fermentation and whole-cell protein analysis. Probiotic and technological indicators of each strain were studied.

MATERIALS AND METHODS

Bacterial strains. Type strains of *Bifidobacterium* (Table 1) were purchased from the American Type Culture Collection (Rockville, Md., USA) and from the collection of Morinaga Milk Industry Ltd., Japan. Isolation of wild strains is described below. *Lactobacillus delbrueckii* subsp. *bulgaricus* 334, Lactobacillus delbrueckii subsp. *lactis* 133, *Lactobacillus casei* subsp. *casei* 140 and *Lactobacillus acidophilus* 139 were isolated from fermented products and belong to the CIDCA collection (La Plata, Argentina). Bacterial cultures were maintained frozen at −80°C in 0.3 M sucrose. Bacteria were reactivated by two consecutive subcultures in TPY broth *(27)* containing (in grams per liter): tryptone, 10.0; soy peptone, 5.0; glucose, 5.0; yeast extract, 2.5; Tween 80, 1.0; cysteine hydrochloride, 0.5; K_2HPO_4, 2.0; $MgCl_2 \cdot 6H_2O$, 0.5; $ZnSO_4 \cdot 7H_2O$, 0.25; $CaCl_2$, 0.15; pH 7.0. Cultures were incubated at 37°C under anaerobic conditions.

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TABLE 1. *Origin of* Bifidobacterium *reference strains*

Source and strain	Species of *Bifidobacterium*
Morinaga Milk Industry	
MB-2	*B. bifidum*
MB-9	*B. infantis*
MB-19	*B. breve*
MB-56	*B. longum*
American Type Culture Collection	
ATCC 29521	*B. bifidum*
ATCC 15703	*B. adolescentis*
ATCC 15707	*B. longum*
ATCC 15697	*B. infantis*
ATCC 15700	*B. breve*
ATCC 29535	*B. angulatum*
ATCC 27919	*B. pseudocatenulatum*
ATCC 25865	*B. pseudolongum* subsp. *globosum*
ATCC 25527	*B. animalis*
ATCC 27534	*B. dentium*
ATCC 29539	*B. catenulatum*
ATCC 27540	*B. magnum*

Isolation procedures. Infant feces were obtained after spontaneous deposition, maintained in Stuart's medium *(31)* at room temperature for 10 to 20 h, and transferred to TPY agar. Stuart's medium contained (in grams per liter) sodium glycerophosphate, 10.0; sodium thioglycolate, 1.0; $CaCl_2$, 0.1; methylene blue, 0.002; and agar, 8.0; pH 7.4. Colonies appearing on TPY agar after 48 h at 37°C under anaerobic conditions were picked and streaked on TPY agar. White colonies with smooth edges were examined by Gram staining. Those showing typical microscopical morphology and unable to grow on TPY agar in aerobic conditions were subcultured in TPY broth and stored at −80°C for further analysis. Fructose 6-phosphate phosphoketolase activity was tested according to Scardovi et al. *(29)*.

Fermentation tests. Sugar fermentation tests leading to species differentiation were selected according to the procedure in *Bergey's Manual of Systematic Bacteriology (28)*. Glucose fermentation was included as a positive control. Strains were inoculated in TPY broth (5%, vol/vol) and incubated for 48 h at 37°C under anaerobic conditions. Cultures were centrifuged at 10,000 × *g* for 10 min, and cells were suspended in TPY medium without glucose. Sterile sugars were added to reach a final concentration of 1% (wt/vol) and bromcresol purple was used as indicator (0.006 g/liter). Color changes of the indicator and growth were recorded after 5 days of incubation at 37°C in anaerobic conditions. Controls without sugar did not change the indicator color.

Protein pattern fingerprint: preparation of bacterial suspension. After 48 hours of incubation, 10 ml of stationary-phase culture in TPY medium ($OD_{600\,nm}$ = 1.0 to 1.5) were harvested by centrifugation at 10,000 × *g* for 10 min in a Sorval RC-5B refrigerated super speed centrifuge at 4°C. Cells were washed once and suspended in 1 ml of sterile buffered solution (50 mM K_2HPO_4, 50 mM KH_2PO_4, pH 7.5).

Preparation of cell-free extracts. Bacterial suspensions (10^9 CFU/ml) were treated with lysozyme (Sigma Chemical Co., Saint Louis, Mo.) at a final concentration of 10 mg/ml. After incubation at 37°C for 3 h, cells were harvested by centrifugation at 15,800 × *g* for 10 min in an Eppendorf 5415C centrifuge (Hamburg, Germany), and sodium dodecyl sulfate (SDS) was added to a final concentration of 20% (wt/vol). The suspension was microwaved three times for 30 s each time at 2,450 MHz (Daenyx, Korea), diluted with 1 ml of distilled water, and heated at 100°C for 10 min. Samples were centrifuged for 25 min at 15,800 × *g* (Eppendorf 5415C). The supernatants were used as cell-free extracts.

SDS polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed as described by Laemmli *(13)* on vertical slab gels 16 by 12 by 0.75 cm in size (Hoefer Scientific Instruments, San Francisco, Calif.). Acrylamide, N-N′ methylene bisacrylamide, and SDS were purchased from Sigma Chemical Co. The polyacrylamide gel was composed of a stacking gel (4% acrylamide in 123 mM Tris-HCl buffer, pH 6.8) and a 10 to 18% acrylamide gradient separating gel in 368 mM Tris-HCl buffer, pH 8.8. The acrylamide gradient was achieved with solutions I (10% acrylamide-bisacrylamide) and II (18% acrylamide-bisacrylamide) in a gradient former (BioRad Laboratories, Calif.).

The cell-free extract was diluted in an equal volume of sample buffer to obtain a final concentration of 123 mM Tris HCl, pH 6.8, 0.1% SDS, 5% 2-mercaptoethanol, 25% glycerol, and 0.01% bromophenol blue; 30 μl of soluble protein fraction in sample buffer was applied to the gels. Electrophoresis was performed at 30 mA through the stacking gel and 60 mA in the separation gel until the tracking dye migrated to the bottom of the gel (approximately 4 h). Gels were stained in a solution of 1% (wt/vol) Coomassie blue R-250, 40% (vol/vol) methanol and 16.6% (vol/vol) acetic acid, and decolorized under continuous shaking in a 25% (vol/vol) ethanol solution containing 10% (vol/vol) acetic acid.

A low-molecular-weight marker (Pharmacia, Upsala, Sweden) composed of phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) was applied to each gel. Molecular weights were estimated using the linear relationship between the log of the molecular weight provided by the manufacturer of the marker and the log of relative mobility.

The reproducibility of the SDS-PAGE technique was estimated by including duplicate runs of a single protein extract in one gel and in separate gels, and by comparing duplicate extract preparations in different gels. The relative error was below 10%; it was calculated as $[(X_i - \overline{X})/\overline{X}] \times 100$, where $X_i = R_f$, $\overline{X} = \overline{R_f}$, and R_f is the distance between the top of the separating gel and each protein band, and $\overline{R_f}$ is the mean R_f for each band obtained in different gels.

Scanning of gels and data analysis. The stained protein patterns were scanned with a Shimadzu double-beam spectrodensitometer CS-910 (Kyoto, Japan) connected to an IBM computer. The gel scan was adjusted to the following settings: absorption wavelength 570 nm, slit width 0.03 mm, slit height 7 mm.

Normalization and compensation of the densitometer scans and computer-assisted calculation of the simple matching coefficient (S_{sm}) and clustering were performed according to the methods of Kersters and De Ley *(11)* and De Ley and Swings *(6)*.

For compensation of the normalized scans, the 100-kDa peak characteristic of the genus *Bifidobacterium (4)* and the lysozyme peak (molecular weight, 14 kDa) were selected and aligned in all scans to the same average position by stretching or narrowing the valley floors.

The normalized densitometric tracings were converted into a sequence of 146 numbers representing the distance between the top of the gel and each protein band *(10, 11)*. The simple-matching coefficient (S_{sm}) was calculated as $(a + d)/(a + b + c + d)$, where a and d correspond to the number of present and absent protein bands, respectively; b and c represent the number of nonmatching protein bands between pairs of strains. The S_{sm} between any pair of densitometric peak tracings of protein patterns was computed. The

matrix of S_{sm} correlation coefficients was clustered by unweighted average linkage method *(30)* using SYSTAT software (version 2.1).

Probiotic and technological characteristics: Hydrophobicity. Cell-surface hydrophobicity (*H*%) was measured as described *(19)* and calculated as $H\% = [(A_0 - A)/A_0] \times 100$, where A_0 and A are the absorbance at 600 nm of a bacterial suspension in 50 mM phosphate buffer, pH 7.0, before and after extraction with xylene, respectively.

Inhibitory power. Inhibition of *Bacillus subtilis* by supernatants of bifidobacteria cultures in stationary phase (pH between 4.03 and 4.45) was performed as described *(12)* by measuring the diameter of the inhibition halos (*D*) produced on solid medium. A standard linear curve (log *D* versus pH) was obtained with data from all strains *(12)*. Points on the curve corresponded to strains with normal inhibitory power (N). Points under and over the curve corresponded to strains with low (L), high (H) and very high (VH) inhibitory power, respectively.

Resistance to low pH. Revived strains were grown in TPY broth for 48 h at 37°C anaerobically and harvested by centrifugation at 10,000 × *g* for 10 min. The pellet was resuspended in the same volume of fresh TPY adjusted to pH 3.0 with 10% (wt/vol) HCl. Control cultures at pH 7.0 were included for each strain. Resuspended cells were incubated at 37°C for 1 h not under anaerobic atmosphere; control cultures incubated at pH 7.0 did not show any change in viability. After incubation, viable counts were determined by plating serial dilutions in 0.1% (wt/vol) tryptone on TPY agar. Plates were incubated at 37°C for 48 h anaerobically. Resistance to pH was defined by the percentage of surviving cells after the incubation at pH 3.0 for 1 h (% RpH).

Bile resistance. Revived strains were grown in TPY broth for 48 h at 37°C anaerobically. Viable colony counts were determined by plating serial dilutions in 0.1% (wt/vol) tryptone on TPY agar and TPY-bile agar, which contained 0.5% (wt/vol) ox bile (Merck, Darmstadt, Germany). Plates were incubated at 37°C for 48 h anaerobically. Resistance to bile was defined by the percentage of viable counts on TPY-bile agar (% R_{bile}).

Ability to grow aerobically in liquid medium. Revived strains grown in TPY broth for 48 h at 37°C anaerobically were diluted 1/100 in fresh medium and incubated aerobically in 16 by 1.5 cm tubes with 10 ml of TPY. Controls were incubated anaerobically in the same conditions. The $OD_{600\ nm}$ was determined after 48 h at 37°C. Results of the ability to grow in the presence of oxygen (G_{ox}) were recorded as high (H) for Δ OD > 1.0, medium (M) for $0.3 < \Delta\ OD < 1.0$ and low (L) for Δ OD < 0.3, where $\Delta\ OD = OD_f - OD_i$, OD_f being the absorbance after 48 h of incubation and OD_i the absorbance immediately after dilution.

Growth in anaerobic conditions. Revived strains grown in liquid TPY medium for 48 h at 37°C anaerobically were diluted 1/100 in fresh medium and incubated anaerobically. $OD_{600\ nm}$ was determined after 24, 48, and 72 h at 37°C. Growth was recorded as rapid (R), medium (M) and slow (S) for cultures that reached an OD higher than 1.0 after 24, 48, and 72 h, respectively.

RESULTS

Isolation of strains. Table 2 shows the *Bifidobacterium* strains isolated from feces of 10 infants aged from 6 days to 4 months (7 males and 3 females), mostly breast fed; samples "a" and "g" were from infants fed both breast milk and formula and samples "h" and "i" were from infants exclusively bottle fed with ultra-high-temperature-treated milk and formula, respectively.

TABLE 2. *Origin of* Bifidobacterium *strains isolated from infant feces*

Strains[a]	Source[b]	Age[c]	Feeding
CIDCA 531	Fermented milk		
CIDCA 532	a	2m 2d	Breast + formula
CIDCA 533	a	2m 2d	Breast + formula
CIDCA 536	b	1m 5d	Breast
CIDCA 537	b	1m 5d	Breast
CIDCA 538	c	21d	Breast
CIDCA 539	d	6d	Breast
CIDCA 5310	d	6d	Breast
CIDCA 5311	d	6d	Breast
CIDCA 5312	d	6d	Breast
CIDCA 5313	d	6d	Breast
CIDCA 5314	d	6d	Breast
CIDCA 5315	e	3m	Breast
CIDCA 5316	f	1m	Breast
CIDCA 5317	g	14d	Breast + formula
CIDCA 5318	h	4m	Formula
CIDCA 5319	h	4m	Formula
CIDCA 5320	h	4m	Formula
CIDCA 5321	h	4m	Formula
CIDCA 5322	i	4m	UHT milk
CIDCA 5323	i	4m	UHT milk
CIDCA 5324	i	4m	UHT milk
CIDCA 5325	i	4m	UHT milk
CIDCA 5326	j	3m	Breast
CIDCA 5327	j	3m	Breast

[a] All isolated strains were positive for fructose-6-phosphate phosphoketolase.
[b] Strains with the same letters were isolated from the same sample of infant feces.
[c] Age in months (m) and days (d).

White, dull, convex colonies with smooth edges were selected; under microscopic examination these were pleomorphic gram-positive bacilli in a typical arrangement and were also unable to grow aerobically in solid media. These isolated strains were positive in the fructose-6-phosphate phosphoketolase assay, characteristic of the genus *Bifidobacterium*.

Sugar fermentation profiles. Simple matching coefficients (S_{sm}) ranged from 1.000 to 0.706. Numerical analysis of sugar fermentation patterns did not lead to an unequivocal identification in 14 of 25 strains (56%). For these 14 strains, the same value for the S_{sm} coefficient was obtained for more than one species (Table 3). The sugar fermentation pattern of *B. bifidum* CIDCA 5321 (S_{sm} = 1.000) was coincident to that of the species reference strain ATCC29521.

Whole-cell proteins. Whole-cell protein electrophoretic patterns of different species of ATCC *Bifidobacterium* and *Lactobacillus* are compared in Figure 1. The reproducibility of the SDS-PAGE was studied by including duplicate runs of a single protein extract on one gel and on different gels and by comparing duplicate extract preparations on separate gels. Identification was undertaken by visual inspection as well as by means of densitograms of the electropho-

TABLE 3. *Sugar fermentation patterns of* Lactobacillus *spp. and* Bifidobacterium *strains and simple matching coefficients (S_{sm}) of* Bifidobacterium *strains*

	Ability of strain to ferment (+) or not (−) the sugar:[a]																	
Strain	L + ara	D-ara	cel	gln	glu	lac	mal	man	mne	mlz	raf	rib	sal	sor	tre	xyl	Species	S_{sm}
531	−	−	+	−	+	+	+	−	−	+	+	+	+	+	−	−	*B. infantis*	0.824
																	B. pseudocatenulatum	0.824
532	+	+	+	−	+	+	+	+	+	+	+	+	−	+	+	−	*B. breve*	0.765
533	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	*B. bifidum*	0.765
536	−	+	+	−	+	+	−	−	−	−	−	−	−	−	+	−	*B. bifidum*	0.765
537	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	*B. bifidum*	0.941
538	+	−	−	−	+	+	+	−	−	+	+	+	−	+	−	+	*B. longum*	0.765
																	B. animalis	0.765
																	B. pseudolongum	0.765
																	B. magnum	0.765
539	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	*B. bifidum*	0.941
5310	−	−	−	−	+	+	−	−	−	−	−	−	−	+	−	−	*B. bifidum*	0.882
5311	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	*B. bifidum*	0.941
5312	+	−	+	+	+	+	+	−	+	+	+	+	−	−	−	+	*B. catenulatum*	0.824
5313	+	−	−	−	+	+	+	+	+	−	−	−	−	−	−	+	*B. bifidum*	0.706
																	B. pseudolongum	0.706
																	B. magnum	0.706
5314	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	*B. longum*	0.706
																	B. animalis	0.706
																	B. dentium	0.706
																	B. adolescentis	0.706
5315	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*B. dentium*	0.824
																	B. adolescentis	0.824
5316	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*B. dentium*	0.824
																	B. adolescentis	0.824
5317	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*B. dentium*	0.824
																	B. adolescentis	0.824
5318	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−	*B. bifidum*	0.941
5319	+	−	−	−	+	+	+	−	+	+	+	+	−	−	+	+	*B. longum*	0.824
																	B. animalis	0.824
5320	+	−	−	−	+	+	+	−	+	+	+	+	−	−	+	−	*B. longum*	0.765
																	B. animalis	0.765
5321	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	−	*B. bifidum*	1.000
5322		−	−	−	+	+	+	−	+	+	+	+	−	−	−	+	*B. longum*	0.765
																	B. animalis	0.765
																	B. pseudolongum	0.765
																	B. magnum	0.765
5323	+	−	+	−	+	+	+	−	+	+	+	+	−	−	+	+	*B. longum*	0.765
																	B. animalis	0.765
5324	+	−	+	−	+	+	+	−	+	+	+	+	−	−	−	+	*B. catenulatum*	0.765
5325	+	−	+	−	+	+	+	−	+	+	+	+	−	−	+	−	*B. breve*	0.765
																	B. longum	0.706
																	B. animalis	0.706
5326	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*B. dentium*	0.824
																	B. adolescentis	0.824
5327	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*B. dentium*	0.824
																	B. adolescentis	0.824
MB-2	+	−	−	−	+	+	+	−	−	+	+	+	−	−	+	+	*B. longum*	0.882
																	B. animalis	0.882
MB-9	−	−	+	−	+	+	−	+	+	−	−	−	+	+	−	−	*B. bifidum*	0.706
																	B. breve	0.706
MB-19	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	*B. dentium*	0.824
																	B. adolescentis	0.824
MB-56	+	+	−	−	+	+	+	+	+	+	+	+	−	+	+	+	*B. longum*	0.765
																	B. dentium	0.765

Continued

TABLE 3. *(Continued)*

Strain	Ability of strain to ferment (+) or not (−) the sugar:[a]																Species	S_{sm}
	L + ara	D-ara	cel	gln	glu	lac	mal	man	mne	mlz	raf	rib	sal	sor	tre	xyl		
334[b]	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	−	*L. delbrueckii subsp. bulgaricus*	
133	−	−	−	−	+	+	+	−	+	−	−	−	−	−	+	−	*L. delbrueckii subsp. lactis*	
140	−	−	+	+	+	+	−	+	+	−	−	+	+	+	+	−	*L. casei subsp. casei*	
139	−	−	+		+	+	−	+	+	−	+	+	+	+	+	−	*L. acidophilus*	

[a] L + ara, L + arabinose; D-ara, D-arabinose; cel, cellobiose; gln, gluconate; glu, glucose; lac, lactose; mal, maltose; man, mannitol; mne, mannose; mlz, melizitose; raf, raffinose; rib, ribose; sal, salicin; sor, sorbitol; tre, trehalose; xyl, xylose. Sugar fermentation patterns of ATCC strains shown were coincident to published results *(2)*.

[b] Boldface indicates *Lactobacillus* spp. patterns.

retic patterns (data not shown). Bands of 69, 65, 56, 50, 48 and 43 kDa are evident in *Lactobacillus* protein profiles (Figure 1A). Profiles of *Bifidobacterium* ATCC strains show eleven different patterns but bands of aproximately 100, 85, 62, 49, 41, 36, and 31 kDa are present in all strains; these bands can be considered typical of the genus (Figure 1B). *L. delbrueckii* subsp. *lactis, L. delbrueckii* subsp. *bulgaricus, L. helveticus,* and *L. acidophilus* showed protein pattern fingerprints very different from those of *Bifidobacterium* strains. A typical protein pattern was obtained for *Streptococcus salivarius* subsp. *thermophilus* (data not shown) with bands of 103, 51, 46, 44.5, and 40.5 kDa as described previously *(32, 37). L. delbrueckii* subsp. *bulgaricus* has protein bands of 68, 46, 45, and 43 kDa as described previously *(32, 37).* It is also possible to differentiate *L. delbrueckii* subsp. *bulgaricus* from *L. delbrueckii* subsp. *lactis. L. delbrueckii* subsp. *bulgaricus* proteins greater in molecular mass than 100 kDa are absent in *L. delbrueckii* subsp. *lactis* protein profiles. None of the strains of *Bifidobacterium* have the protein bands described as characteristic of *Lactobacillus* and *Streptococcus* species.

Protein profiles of bifidobacteria isolated from human feces were compared with those of the respective type strain (Figure 2). All strains of *B. bifidum* (Figure 2A), including the type strain (Figure 1B), gave four blocks of bands, a, b, c, and d (Figure 2A). The width of all the bands were similar and no secondary bands were observed in any block. *B. longum* proteins (Figure 2B) resolved into bands of different widths equally distributed along the whole gel. Three wide bands were detected in the upper part of the gel (Figure 2B a); the upper one was the band of 100 kDa, described as characteristic of the genus *(1).* In the area between 67 and 43 kDa there were two bands not found in other species (Figure 2B b). *B. breve* strains (Figure 2C), including the type strain, displayed nine bands in the area between 94 and 67 kDa (Figure 2C a) and in the area between 67 and 43 kDa six bands (Figure 2C b). *B. adolescentis* strains, including the type strain, showed a wide band of approximately 90 kDa that was not found in the other species (Figure 2D). *B. infantis* displayed the characteristic bands of the genus, showing a different pattern than the other species (Figure 2E).

Strains belonging to *B. bifidum, B. longum, B. breve, B.*

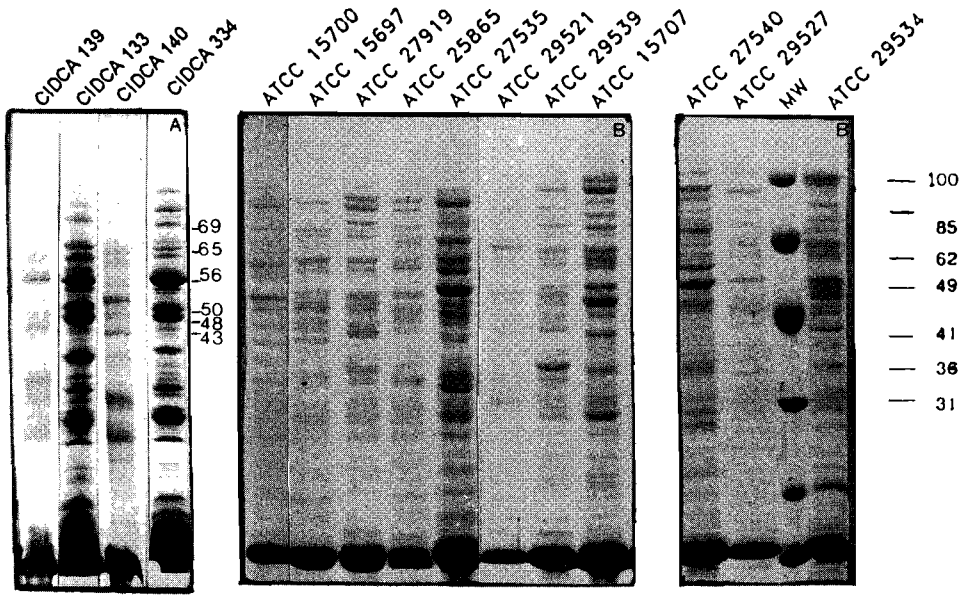

FIGURE 1. *SDS-PAGE of cell-free extracts from lactobacilli. (A)* L. acidophilus *CIDCA 139,* L. delbrueckii *subsp.* lactis *CIDCA 133,* L. casei *subsp.* casei *CIDCA 140,* L. delbrueckii *subsp.* bulgaricus *CIDCA 334. (B) SDS-PAGE of cell-free extracts from reference* Bifidobacterium *strains. MW, molecular weight marker in kDa (94, 67, 43, 30, 20.1 and 14.4 kDa) (B).*

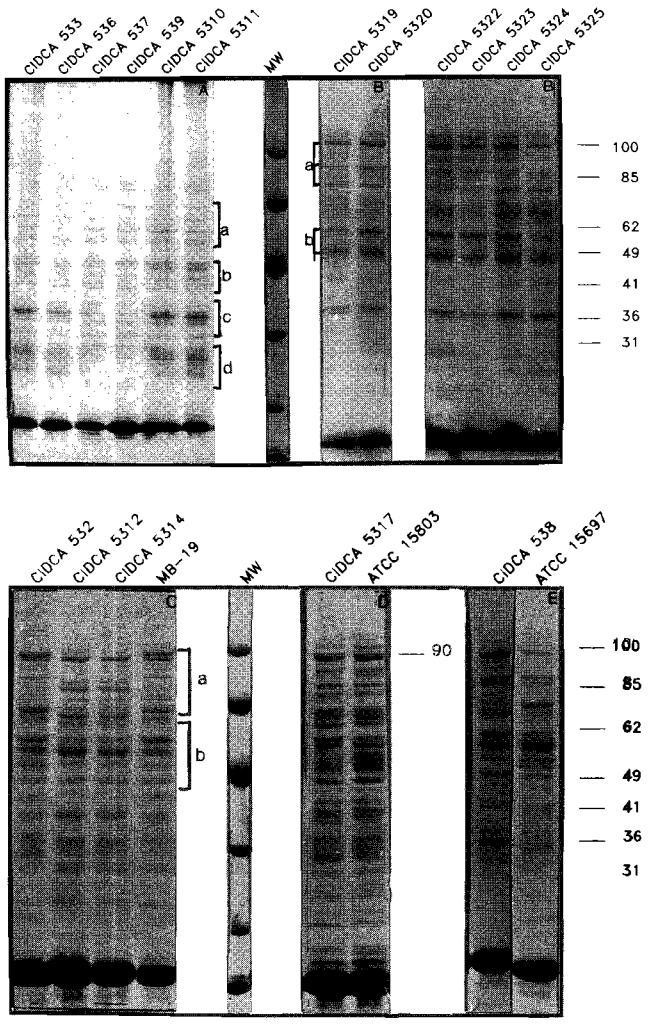

FIGURE 2. *SDS-PAGE of cell-free extracts from* Bifidobacterium bifidum *strains (A),* Bifidobacterium longum *strains (B),* Bifidobacterium breve *strains (C),* Bifidobacterium adolescentis *strains (D),* Bifidobacterium infantis *strains (E), MW, molecular weight marker in kDa (94, 67, 43, 30, 20.1 and 14.4 kDa).*

adolescentis, and *B. infantis* were identified among the isolated bifidobacteria. None of the isolated strains had protein profiles similar to those of the species *B. pseudocatenulatum* ATCC 27919, *B. pseudolongum* ATCC 25865, *B. angulatum* ATCC 27535, *B. catenulatum* ATCC 29539, *B. magnum* ATCC 27540, and *B. dentium* ATCC 29534 (Figure 1B).

A matrix of simple matching coefficients of whole-cell proteins and sugar fermentation of bifidobacteria was used to construct the dendrogram showed in Figure 3. Computerized numerical analysis enabled strains to be grouped in two main clusters. Strains of *B. bifidum* belong to a well-differentiated cluster that joins the cluster of the remaining species at 0.582 of similarity. The second cluster includes seven subclusters: 1, *B. catenulatum–B. magnum* (0.741); 2, *B. angulatum–B. pseudocatenulatum* (0.945); 3, *B. pseudolongum* (0.898); 4, *B. longum* (0.887); 5, *B. breve* (0.835); 6, *B. adolescentis–B. dentium–B. animalis* (0.781); and 7, *B. infantis* (0.782).

Probiotic characteristics. Table 4 shows that most of the strains of *B. bifidum* were highly hydrophobic. As described previously, all hydrophobic strains were autoagglutinating and had the ability to adhere to Caco-2 cells in vitro *(19).* In addition, most of these strains were extremely sensitive to low pH and bile and were unable to grow in aerobic conditions. *B. bifidum* ATCC 29521 showed characteristics different from those of the CIDCA strains of the same species: it was tolerant to oxygen, more resistant to low pH, not autoagglutinating, and less hydrophobic.

Resistance to low pH, bile, and oxygen was variable among strains of *B. longum.* The strains were not autoagglutinating and not adherent to Caco-2 cells in vitro *(19).* Strains of *B. breve* showed a variable degree of resistance to bile and were moderately to highly tolerant to oxygen. Only one strain of *B. breve* was autoagglutinating and had the ability to adhere to enterocytes *(19).* Strains of *B. infantis* were generally not tolerant to oxygen and showed variable resistance to bile and high resistance to low pH. Only MB-9 was autoagglutinating and hemagglutinating *(19).*

Table 4 and Figure 4 show that supernatants of all strains produced the expected inhibition of *Bacillus subtilis,* depending on the final pH of bifidobacterial cultures. The degree of inhibition was strain-dependent. *B. bifidum* 537,

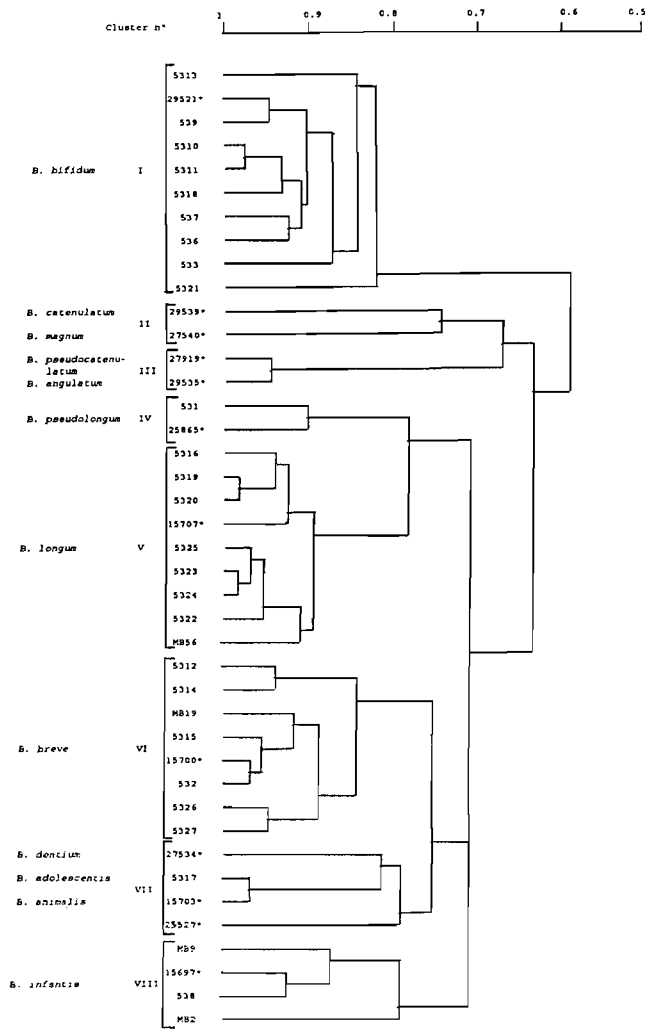

FIGURE 3. *Dendrogram obtained by UPGMA analysis of the simple matching coefficients (S_{sm}) among* Bifidobacterium *strains. ATCC strains are indicated by an asterisk*.*

TABLE 4. *Probiotic and technological characteristics of* Bifidobacterium *strains*

Species and strain	Assay[a]					
	% H (mean ± SD)	Inh	% RpH (mean ± SD)	% R_{bile}	G_{OX}	Gr
B. bifidum						
CIDCA 533	80 ± 17.1	N	1.84 ± 0.66	0.9	H	M
CIDCA 536	96 ± 3.9	N	0.092 ± 0.02	0.0	L	M
CIDCA 537	95 ± 4.0	H	0.40 ± 0.01	0.1	L	S
CIDCA 539	90 ± 0.4	N	2.86 ± 0.41	ND[b]	L	S
CIDCA 5310	95 ± 0.9	N	5.67 ± 0.28	ND	L	S
CIDCA 5311	50.2 ± 9.5	N	1.34 ± 0.24	0.2	L	S
CIDCA 5313	91 ± 4.2	N	0.005 ± 0.00	0.0	L	S
CIDCA 5318	98.2	ND	0.50 ± 0.03	8.0	L	S
CIDCA 5321	92.84 ± 12.3	ND	0.81 ± 0.04	13.0	L	S
ATCC 29521	8.09 ± 3.45	L	26.41 ± 8.34	ND	H	M
B. longum						
CIDCA 5316	1.8 ± 1.5	N	0.01 ± 0.00	0.0	L	R
CIDCA 5319	ND	ND	ND	0.0	H	R
CIDCA 5320	3.43 ± 1.4	ND	0.45 ± 0.06	0.0	L	R
CIDCA 5322	1.92 ± 0.32	N	40.01 ± 2.36	0.0	L	R
CIDCA 5323	4.14 ± 0.48	L	3.56 ± 0.15	0.0	H	R
CIDCA 5324	3.30	L	ND	0.8	L	R
CIDCA 5325	4.70	ND	1.01 ± 0.05	0.0	L	M
MB-56	2.0 ± 1.7	N	0.023 ± 0.01	4.0	L	R
ATCC 15707	16.5 ± 2.62	VH	100 ± 2.19	0.4	M	M
B. breve						
CIDCA 532	4.8 ± 3.0	L	0.105 ± 0.06	0.0	H	M
CIDCA 5312	0.1 ± 0.9	N	ND	0.9	L	M
CIDCA 5314	21.1 ± 1.8	N	0.31 ± 0.05	3.0	M	M
CIDCA 5315	2.2 ± 0.8	VH	100 ± 7.16	0.0	L	R
CIDCA 5326	1.13	H	0.40 ± 0.03	0.0	M	R
CIDCA 5327	0.71	ND	8.73 ± 2.46	5.0	H	M
MB-19	19.7 ± 12.2	N	0.3 ± 0.02	43.0	H	R
ATCC 15700	30.8 ± 5.6	N	1.01 ± 0.17	80.0	H	M
B. pseudolongum						
CIDCA 531	83	VH	100 ± 5.30	45.8	H	R
ATCC 25865	17.75	L	55.01 ± 6.85	0.0	L	M
B. infantis						
CIDCA 538	3.3 ± 2.3	VH	15.6 ± 5.17	0.8	L	M
MB-2	4.1 ± 0.2	H	31.2 ± 4.16	0.0	L	M
MB-9	93.6 ± 3.2	ND	ND	ND	M	S
ATCC 15697	3.1 ± 1.0	L	37.14 ± 1.55	23.0	L	S
B. adolescentis						
CIDCA 5317	3.0 ± 0.6	N	1.05 ± 0.05	4.0	M	M
ATCC 15703	2.93 ± 0.68	L	33.25 ± 5.23	ND	L	M
B. animalis						
ATCC 25527	27.31	N	28.8 ± 2.41	53.7	H	M
B. dentium						
ATCC 29534	2.42 ± 0.02	VH	0.80 ± 0.06	0.0	H	R
B. angulatum						
ATCC 27535	ND	L	ND	0.0	ND	S
B. catenulatum						
ATCC 27539	17.5	H	4.07 ± 1.77	0.0	H	S
B. pseudocatenulatum						
ATCC 27919	5.26 ± 2.52	L	18.93 ± 4.49	0.0	L	R

[a] % H, percent hydrophobicity; Inh, inhibition of *Bacillus subtilis* (agar diffusion assay); L, low; N, normal; H, high; VH, very high; % RpH, percent of survivors after 1 h at pH 3; % R_{bile}, percent of viable counts on solid media with 0.5% ox bile; the mean error in colony count was below 10%; G_{ox}, growth in aerobic atmosphere (liquid medium); L, low; M, medium; H, high; Gr, growth in anaerobic atmosphere (liquid medium); L, low; M, medium; S, slow.

[b] ND, not determined.

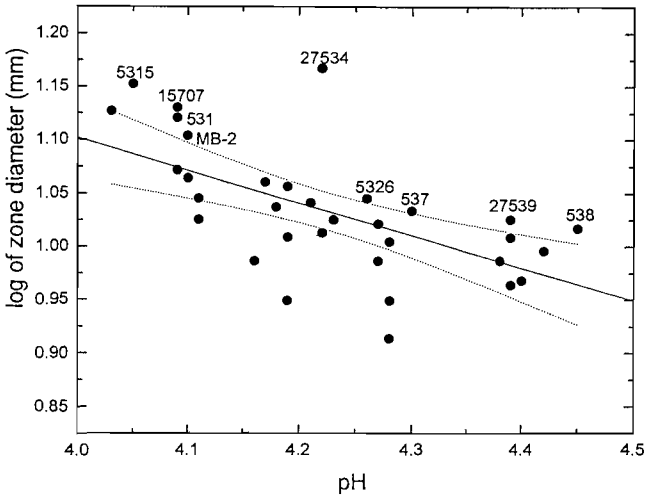

FIGURE 4. *Relationship between log of inhibition zone diameters and pH of supernatants of bifidobacterial cultures in TPY medium. Indicator strain was* B. subtilis *CIDCA 131. Each point corresponds to five experiments. Confidence limits (0.95) for the linear model are included.*

B. breve 5315, *B. pseudolongum* 531, *B. longum* ATCC 15707, *B. infantis* 538, and MB-2, *B. dentium* ATCC 27534 and *B. catenulatum* ATCC 27539 showed the highest inhibitory power.

Anaerobic growth of almost all *B. bifidum* strains was slow (with the exception of strains 533, 536, and ATCC 29521). The same characteristic was observed for *B. infantis* MB-9 and ATCC 15697, *B. angulatum* ATCC 27535, and *B. catenulatum* ATCC 27539. The remaining strains showed rapid or medium growth ($OD_{600\ nm} > 1$ after 24 to 48 h at 37°C).

DISCUSSION

In this research 25 strains of *Bifidobacterium* isolated from infant feces were identified by the presence of phosphoketolase, carbohydrate fermentation, and gradient SDS-PAGE whole-protein analysis. Carbohydrate fermentation patterns were not enough to identify bifidobacterial species, for which ambiguous results were obtained in some cases (Table 3).

The analysis of whole-cell protein profiles was successfully used in this study to resolve the taxonomic status of different strains of the genus *Bifidobacterium* isolated from infants' feces to the species level by comparison with reference strains from the ATCC (Figures 1 and 2). The presence of phosphoketolase and differences in typical bands allowed the differentiation among lactobacilli and bifidobacteria. In previous reports continuous SDS-PAGE had been used that lead to a poor resolution of protein bands *(4)*. The use of SDS-PAGE gel gradients was introduced in this study to improve the resolution of protein bands over a wide range of molecular weights. By SDS-PAGE and densitometric analysis it was possible to define six characteristic protein bands that were present in proteins of all strains of the genus *Bifidobacterium* studied. This study can be considered complementary to the study performed by Biavati et al. *(2, 4)* in which a peak of 100 kDa was described as characteristic of bifidobacteria. The protein patterns obtained are very different from those of *Propionibacterium (1), Lactobacillus,* and *Streptococcus* (Figure 1A) *(32, 37)*. Other methods, such as electrophoretic mobility of β-galactosidase, have been employed to identify *Bifidobacterium* strains. However, this method is laborious when a large number of strains needs to be analyzed *(24, 25)*. Protein profiles were successfully employed to distinguish between *B. adolescentis* and *B. dentium,* which are normally not distinguishable by sugar fermentation *(28)*. The predominant species among our isolates were *B. bifidum, B. longum,* and *B. breve*. The distribution of the species of bifidobacteria among the new isolates reported in Figure 3 is in only partial agreement with the results of other authors *(2, 15)*. This discrepancy could be due to differences in age, feeding, and environment that could change the ecological balance of the gut. Other authors *(2)* did not find *B. adolescentis* in infant feces. We found a low proportion of *B. infantis,* which otherwise has been reported *(2, 17, 23)* to be one of the prevalent species isolated from infant feces. The high prevalence of *B. breve* can be correlated with the origin of the strains, since the presence of this species accounts for the main difference between adult and infant bifid microbiota in the human intestine *(3)*.

As shown in Table 4, not all desirable characteristics for probiotic formulation are present in any single strain. One of the most important biotherapeutic actions is exerted through the colonization of the intestine by bifidobacteria. In this regard Table 4 shows that all *B. bifidum* strains tested were adherent to enterocyte-like cells. Three other adherent strains belonged to the species *B. breve, B. infantis,* and *B. animalis*. Supernatants of these strains produced higher inhibition of *B. subtilis* in vitro due to the production of acetic acid when compared with the action of supernatants of lactobacilli at the same pH *(12)*. This result could be due to the production of both lactic and acetic acid by bifidobacteria. *B. bifidum* 537 showed an inhibitory power significantly higher than the other strains of the same species.

Only 10 of 41 strains studied were resistant to 0.5% bile (Table 4). This concentration of bile was selected for the screening because it is equivalent to the physiological bile concentration in the duodenum. Several possible protection mechanisms may be responsible for the displacement of enteropathogens by bifidobacteria. Competitive binding could occur by means of adherent and inhibitory properties of bifidobacteria strains. For these reasons new natural probiotic strains should be selected taking into account these characteristics. However, one of the 41 strains studied, *B. breve* ATCC 15700, has nearly all the probiotic and technological characteristics desired for biotherapeutic use.

The results show that adherent and inhibitory strains were not resistant to bile and low pH and were unable to grow in aerobic conditions. However, these problems could possibly be overcome by encapsulation and optimization of growth conditions. Microencapsulation technology applied to bifidobacteria has been described and survival of encapsulated microorganisms in simulated gastric and intestinal juices has been demonstrated *(5, 21)*. Since each specific

strain may have a specific biotherapeutic action, a cocktail of diferent strains may be the best alternative.

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