Effect of bile on the lipid composition and surface properties of bifidobacteria

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Aim: The changes produced on the bacterial surface of Bifidobacteria cells when they are grown in bile were compared with those provoked by bile added to bacteria grown in the absence of bile.

Methods and Results: The adhesive properties, the zeta potential and the lipid composition of Bifidobacterial strains, isolated from human faeces and grown in MRS medium, were determined. Bacteria grown in MRS with bile showed a loss of adherence and autoaggregation in correlation with a decrease in the surface hydrophobicity in comparison to those grown in MRS without bile, concomitant with the absence of two glycolipids, the increase of sugar content and minor changes in fatty acid composition. The surface changes caused by bile shock on bacteria grown in bile-free medium were much less pronounced and, in addition, no effect on the lipid composition was apparent.

Conclusions: The comparison of the results indicates that bile action on surface properties is related to metabolic changes.

Significance and Impact of the Study: Long-term exposure of bacteria to bile may cause metabolic changes affecting their adhesive properties irreversibly. This may be taken as a criterion to define the probiotic properties of different strains.

INTRODUCTION

Bifidobacteria are normal inhabitants of the intestinal tract, where they can exert a probiotic effect. The health-promoting action is related to the production of acetic, lactic and formic acid which decreases pH in the large intestine and thereby inhibits the growth of undesirable bacteria. Indirectly, the acidity increase prevents the production of harmful amines and nitrites produced by putrefactive bacteria (Rašić and Kurmann 1983). The probiotic effect is also related to the surface properties of the bacteria, which determines the ability of bacteria to adhere to enterocyte cells thus protecting the intestinal epythelium and enhancing the capability to sequest toxic compounds (Rašić and Kurmann 1983) Bifidobacterial cultures, used as dietary adjuncts, should contain strains

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able to survive conditions in the stomach and duodenum before arriving in the gut (Norris *et al*. 1950; Mitsuoka 1982; Ishibashi and Shimamura 1993). For this reason, bile tolerance is considered to be an important property of probiotic bacteria. This enables the bacteria to survive its transit along the duodenum and subsequently to grow and to colonize the gut epithelia by adhesion to enterocytes (Kociubinski *et al*. 1999; Ueda 1986).

Bifidobacterium bifidum strains adhere to enterocyte-like cells Caco–2 *in vitro*. In addition, these strains are also autoaggregating (Bibiloni *et al*. 2001). As bile resistance is considered an important parameter to select probiotic strains (Kociubinski *et al*. 1999, 2002), it is of interest to evaluate the way in which bile affects adherence.

Previous studies have shown bifidobacteria autoaggregating strains form clumps in liquid media that appear as large clusters of cells when viewed under the microscope (Pérez *et al*. 1998). In contact with bile, the aggregation and adherence of lactobacilli decreases in correlation with

changes in surface hydrophobicity and zeta potential (Kociubinski *et al.* 2002). However, it is not known how these surface properties may alter when strains are grown in bile.

In this regard, it would be of importance to know if the effect of bile on surface properties is reversible. In addition, it would be of interest to determine if the bacteria components might be affected when they are grown in bile. Therefore, the purpose of this study was to compare the changes produced on the lipid composition and bacterial surface after growing in bile with those produced by shocking the bacteria with bile. This comparison can give an indication as to whether the change induced by the surface active agent is of only physical nature, i.e. affects the physical chemical properties of the surface, or whether it is also dependent on the bacterial metabolism. With this in mind, the lipid composition of lactic acid of bacteria grown in the presence of bile or shocked with bile was taken as an indicator of metabolic changes and correlated with their adhesive properties under both conditions.

MATERIALS AND METHODS

Strains and culture conditions

Bacterial strains were isolated at the Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA) and identified as described (Gómez Zavaglia *et al.* 1998, 1999; Pérez *et al.* 1998). *Bif. bifidum* CIDCA 536 (now NCC 189, Nestlé culture collection), CIDCA 537 and CIDCA 5324 were isolated from infant faeces. *Bif. pseudolongum* CIDCA 531 was isolated from a dairy product. *Bif. bifidum* ATCC 15700 was obtained from the American Type Culture Collection (Rockville, MD, USA). Strains were maintained frozen at -80 °C in sterile 0.3 mol l^{-1} sucrose. They were reactivated by two consecutive subcultures in liquid media. Isolated strains were identified by morphology, Gram staining and biochemical characteristics according to Bergey's *Manual* (Scardovi 1986) and whole protein pattern by SDS-PAGE (Gómez Zavaglia *et al.* 1999). All strains were isolated from healthy human newborn faeces, with the exception of CIDCA 531, isolated from a dairy product. For details see Pérez *et al.* (1998) and Gómez Zavaglia *et al.* 1998, 1999).

Bacteria were grown in anaerobic conditions for 15 h at 37 °C in MRS (Man Rogosa Sharpe) medium (Difco, Detroit, MI, USA) at 37 °C. After harvesting in the stationary phase (15 h) by centrifugation at 14000 *g* for 5 min, cells were washed twice with 0.05 mol l^{-1} K_2HPO_4 (pH 7.0), and resuspended in the same buffer to a final concentration of 10^9 cfu ml^{-1} (bacterial suspension).

All strains under study were grown at 37 °C in MRS medium with the addition of 1 g l^{-1} bile (ox-bile Difco).

The cells were harvested in the stationary phase (after 24 h of incubation) following the procedure described above.

Shock with bile was carried out on cells grown in MRS. The bacterial suspension was washed in 0.05 mol l^{-1} K_2HPO_4 (pH 7.0) and incubated in the presence of 5 g l^{-1} bile for 1 h at 37 °C. No bacterial growth was apparent under these conditions.

Adhesion assays

Enterocyte-like Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in Eagle's minimum essential medium with Earle's salts supplemented with L-glutamine, non-essential amino acids (Sigma Chemical Co., St Louis, MO, USA) and 20% (w/v) inactivated fetal calf serum (General SA, Buenos Aires, Argentina). Streptomycin and penicillin G were added to give final concentrations of 50 μg ml^{-1} and 50 000 IU l^{-1}, respectively. Monolayers were prepared on glass coverslips and placed in 24-well tissue plates (Corning Glass Works, Corning, NY, USA). Cells were added at a concentration of 2×10^5 cells per well and were incubated at 37 °C in a 5% CO_2−95% air atmosphere. The culture medium was changed every 2 d. Cells were used between passages 23 and 34. Adherence assays were performed with cells at late post-confluence (15 d in culture).

Bacterial suspensions (0.5 ml) grown in the presence or in the absence of bile were added to each well of the tissue culture plate. After 1 h of incubation at 37 °C the monolayers were washed and adherence was examined microscopically after Gram staining (Pérez *et al.* 1998).

Autoaggregation assays

Autoaggregation was determined by following the change in optical density at 600 nm. Cultures were homogenized gently in a cuvette cell and the optical density was followed until a constant value was obtained. Autoaggregating strains formed clumps that flocculated to give a clear supernatant. In contrast, the turbidity of non-aggregating bacteria suspensions remained constant during at least 1 h.

Electrophoretic mobility and zeta potential

Electrophoretic mobilities of bacteria resuspended in 0.001 mol l^{-1} KCl were determined in a capillary H-cell with Ag/AgCl electrodes connected to a variable direct current source. The electrophoretic mobility of bacteria (ξ) was determined by measuring the rate of migration of the bacteria in the stationary layer when a constant electric field was applied. The effective electrical distance of the cell was calculated by using KCl solutions of known conductivity at 25 °C. The rate of migration was determined by microscopic

observation of the displacement of individual cells with rectilinear and uniform movement along a reticular lattice (length 1 mm). The optical distance of the microscope was fixed by determining the zeta potential (ξ) of phosphatidyl-serine liposomes whose value was approximately $- 120$ mV in 0·01 mol l^{-1} NaCl at pH 7·4 (Pérez *et al.* 1998).

The potential was fixed at 40 V and measurements were obtained by alternatively changing the polarity of the electrodes to avoid polarization. At least 10 determinations in each direction were made for each sample.

The temperature was maintained at 25 °C. The zeta potential was calculated with the equation $\times = 4\pi\eta\mu/\varepsilon$, where η and ε are the viscosity and the dielectric constant of the solution, respectively.

Hydrophobicity assays

Two millilitres of each bacterial suspension were mixed with 0·4 ml of xylene by vortexing for 120 s. Phases were separated by decantation. The aqueous phase was carefully removed and the absorbance was read at 600 nm (A_{600}). The decrease of absorbance in the aqueous phase was taken as a measure of the bacterial surface hydrophobicity (H%) and was calculated as $H\% = [(A_0–A)/A_0] \times 100$, where A_0 and A are the absorbance of the aqueous phase before and after the extraction with xylene, respectively (Bibiloni *et al.* 2001).

Lipid extraction

Lipids were extracted from bacteria harvested in the stationary phase (10^8 cfu ml^{-1}) adding 5 ml water and 18 ml methanol : chloroform solution (2 : 1 v/v) to 2 g of wet weight of cells (Marinetti 1993). The supernatants of two consecutive extractions were mixed with 12 ml of chloroform:water (1 : 1 v/v) and centrifuged. The chloroform phase was diluted with benzene to remove traces of water, and dried in a rotary evaporator at 30–35 °C.

Characterization of lipids by thin layer chromatography

Polar lipids were characterized by their migration on aluminium-backed Silica Gel 60 thin-layer chromatographic plates (Merck, Darmstadt, Germany) using chloroform : methanol : acetic acid : water (65 : 25 : 4 : 2 v/v/v/v) as a mobile phase and developed with iodine vapours (Marinetti 1993).

Glycolipids were characterized using 5 g l^{-1} α-naphtol solution, dried in air and sprayed with a sulphuric acid solution (10 mol l^{-1}) and heated at 120 °C until maximum colour was developed (Marinetti 1993). Choline was identified by using the Dragendorff stain (Marinetti 1993). The plate was sprayed with ninhydrin solution (2 g l^{-1} ninhydrin in methanol : acetic acid, 49 : 1 v/v) for chemical characterization of aminolipids (Marinetti 1993). Phosphates were characterized using Kostetsky's reagent (Marinetti 1993).

Determination of total sugar and phosphorous content

Sugars and phosphorous were determined on lipid samples according to Dubois and Fiske Subbarrow, respectively (Marinetti 1993; Gómez Zavaglia *et al.* 2000).

Characterization of fatty acids

Fatty acid methylesters were prepared by methanolysis; 2 ml of 20 ml l^{-1} H$_2$SO$_4$ in methanol were added to 3 mg lipid, and heated at 60 °C for 2 h. The fatty acid methyl esters were extracted with 1 ml CH Cl$_3$: H$_2$O (2 : 0·7, v/v) and washed twice with 0·7 ml water. Gas chromatography analysis was performed on a Hewlett-Packard 5980 using a 25-m HP-5 column (0·32 mm i.d.). Analysis conditions were: injection temperature, 260 °C; detector temperature, 300 °C; column temperature, 70 °C, then increased to 280 °C at 10 °C min^{-1} (Gómez Zavaglia *et al.* 2000). Data shown in Table 1 are expressed as the percentage of the ratio between area under each peak and the summation of areas for all the species.

RESULTS

Table 2 shows that when strains were grown in bile containing medium (1 g l^{-1} bile), two glycolipids of the lowest R$_f$ were absent (column C). However, the six different lipid species found in the strains studied did not

Table 1 Fatty acid composition of bacteria grown and shocked with bile

Strain	189	189 shock	189 grown	531	531 shock	531 grown	5310	5310 shock	5310 grown
16:0	36·80 ± 0·5	40·02 ± 1·6	48·2 ± 3·5	48·2 ± 3·5	30·6 ± 2·3	43·6 ± 3·3	38·2 ± 2·9	33·1 ± 1·0	45·5 ± 2·5
18:0	2·76 ± 1·0	13·72 ± 0·2	16·38 ± 1·4	9·5 ± 0·2	12·5 ± 0·9	13·4 ± 1·1	3·2 ± 0·3	10·0 ± 0·3	10·5 ± 0·5
16:1	2·19 ± 1·0	2·20 ± 1·0	1·41 ± 0·0	0	3·1 ± 0·2	1·1 ± 0·02	0·7 ± 0·02	2·5 ± 0·1	0
18:1	32·66 ± 0·9	27·74 ± 0·6	16·14 ± 2·3	50·5 ± 3·2	11·8 ± 1·0	31·7 ± 2·2	32·2 ± 2·1	31·3 ± 0·5	23·9 ± 1
Cyc 19	1·4 ± 0·7	0	0	9·0 ± 0·2	1·0 ± 0·1	0	5·2 ± 0·5	0	0

Table 2 Characterization of lipids from strains grown and shocked with bile

Lipid	R_f[1]	A	B	C
N	0·95 ± 0·0005	+	+	+
CL	0·88 ± 0·0089	+	+	+
GL	0·75 ± 0·0004	+	+	+
PG	0·66 ± 0·0005	+	+	+
GL	0·39 ± 0·0024	+	+	−
GL	0·22 ± 0·0080	+	+	−

N: neutral lipid; CL: cardiolipin, GL: glycolipid, PG: phosphatidyl-glycerol.

[1]Rf were calculated as mean values from at least two lipid extraction from independent cultures. Triplicates were performed with each sample. All assayed strains showed the same lipid composition. Strains: CIDCA 531, 5310, and NCC 189.

A: lipids were extracted from bacteria grown without bile.

B: lipids extracted form bacteria grown without bile and shocked with 5 g/l bile.

C: lipids were extracted from bacteria grown with 1 g/l bile.

+: presence of the lipid spot; −: absence of the lipid spot.

Table 3 Sugar and phosphorus content in lipids extracted from bacteria grown with and without bile

Strain	Grown without bile	Grown with bile
Sugar (nmol × 10^{-9}/microorganism)		
CIDCA 531	8·5 ± 0·4	125·7 ± 12·0
NCC 189	18·8 ± 0·3	152·3 ± 0·7
CIDCA 5310	20·9 ± 0·36	85·8 ± 0·6
ATCC 15700	32·0 ± 2·9	79·4 ± 6·8
Phosphorous (nmol × 10^{-9}/microorganism)		
CIDCA 531	5·6 ± 0·3	7·3 ± 0·4
NCC 189	6·9 ± 0·2	8·9 ± 0·1
CIDCA 5310	7·3 ± 0·2	6·2 ± 0·1
ATCC 15700	13·9 ± 3·4	6·8 ± 1·5

change when bacteria were shocked with a high concentration of bile after growing in MRS medium without bile (columns A and B). Interference of bile in thin layer chromatography (TLC) was discounted, because the addition of 50 g l^{-1} of bile to the extracted bacterial lipids did not affect the chromatographic patterns.

The sugar concentration in the lipids extract increased between two and 15 times when strains were grown in the presence of bile (Table 3). In contrast, phosphorous content was not markedly altered. Thus, the sugar/phosphorous ratio was between 12 and 18 when bacteria were grown in the presence of bile and between 1·5 and 3 when strains were grown in the absence of bile. It is noteworthy that strain NCC189 showed a 7·5-fold increase in sugar content, which was nearly twice the increment observed in strain CIDCA 5310. The presence of bile in the growth medium of strains NCC189 and 5310 promoted a net increase of the saturated

fatty acids 16 : 0 and 18 : 0, and a decrease of the unsaturated fatty acids 16 : 1, 18 : 1 and cyc 19 : 0 (Table 1). In contrast, non-autoaggregating strain 531 did not show changes in the 16 : 0 fatty acid residues.

Table 4 shows that strains CIDCA 5310, 537 and NCC 189 grown in bile-containing medium were less adhesive to Caco-2 cells (Adh) and less autoaggregating (Aag) in correlation with a decrease of surface hydrophobicity and a slight shift to negative values of zeta potential.

Also in Table 4 it can be seen that the effect on the hydrophobicity and autoaggregation of the same bacteria was less marked when they were grown in bile-free medium and then shocked with bile. However, the zeta potential reached more negative values than those observed in bacteria grown in bile-containing medium. Similar results were obtained with two non-autoaggregating and non-adherent strains (CIDCA 531 and 5324).

A rapid decrease in optical density values was observed for 5310 cells grown in bile-free medium denoting autoaggregation (Fig. 1a). The same bacteria grown in bile-containing medium showed no decrease in optical density values even after 30–40 min. Subsequent washing of these bacteria in medium without bile did not show significant differences.

Table 4 Surface hydrophocity, zeta potential and autoaggregation of bacteria grown or shocked with bile

Strains[a]	Grown in regular medium				Grown with bile 1 g/l				Shocked with bile 5 g/l			
	H%	ζ (mV)	Aag	Adh	H%	ζ (mV)	Aag	Adh	H%	ζ (mV)	Aag	Adh
NCC 189	90·2 ± 3·9	−25 ± 2	H	H	49 ± 10	−37 ± 2	L	L	93·3 ± 2	−43·7 ± 2·1	M	L
537	91·4 ± 3·9	−24 ± 1	H	H	40·8 ± 9·9	−38 ± 2	L	L	85·0 ± 1	ND	M	L
5310	93·5 ± 2·9	−23 ± 2	H	H	28·0 ± 8·9	−35 ± 2	L	L	89·0 ± 2	−46·2 ± 1·5	M	L
531	97·2 ± 2·9	−25 ± 2	−	−	47·1 ± 28	−37·4 ± 2	−	−	86·1 ± 3	−50·2 ± 5·4	−	−
5324	3·3 ± 0·6	−74 ± 3	−	−	12·3 ± 5·9	−101 ± 3	−	−	32·4 ± 8	−61·3 ± 3·0	−	−

[a]Isolated strains as described in Materials and methods.

Aag: autoaggregation; Adh: adherence to Caco–2 cells.; H%: percentage of hydrophobicity; ζ (mV): zeta potential.

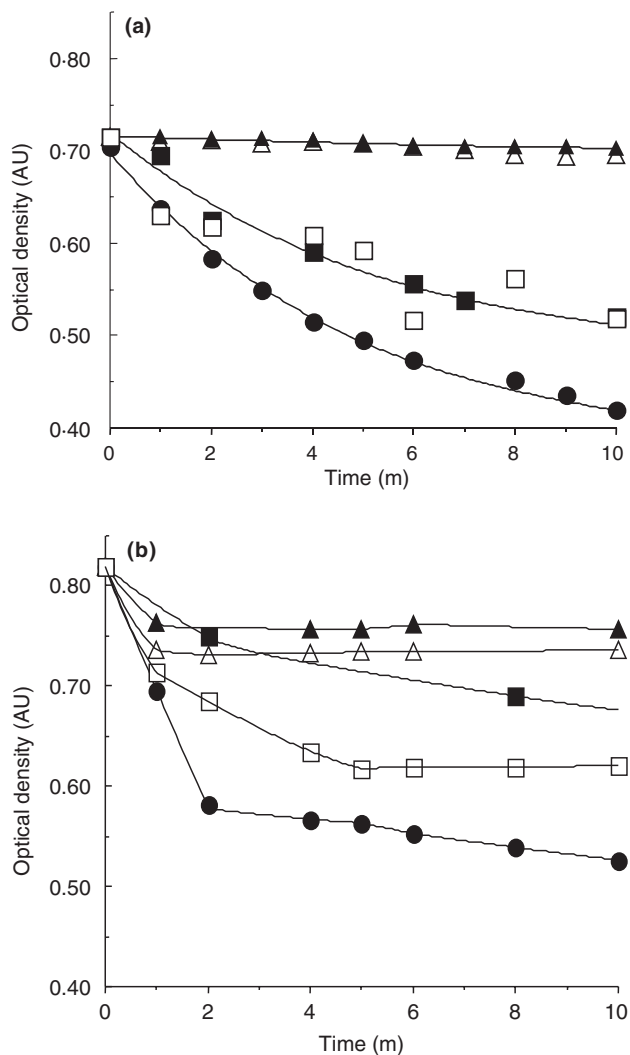(H) high adherence; (M) moderate adherence; (L) low adherence; (ND) not determined.

Fig. 1 Kinetics of aggregation followed by changes in optical density at 600 nm; (a) corresponds to strain CIDCA 5310 and (b) to strain NCC189. ●, Bacteria grown in MRS without bile (control). ▲, Bacteria grown in MRS with 1 g l^{-1} bile. △, Bacteria grown in MRS in 1 g l^{-1} and subsequently washed in media without bile. ■, Bacteria grown in MRS and shocked during 30 min with bile. □, Bacteria grown in MRS and shocked during 30 min with bile and washed subsequently in media without bile

When the same bacterial strain, grown in regular medium, was shocked with bile 5 g l^{-1} (1 h at 37 °C), the rate of autoaggregation was decreased in comparison to control without bile, but to a lower extent than those grown in bile. These autoaggregation properties were maintained after washing the bacteria with bile-free medium.

Changes in hydrophobicity and in the zeta potential promoted by bile on strain 5310 were not eliminated by washing. This occurred not only when bacteria were grown

in bile but also when they were shocked with bile after growing in regular medium (data not shown).

A slightly different response was obtained with strain NCC189. The autoaggregation was not lost completely after growing in the presence of bile. In addition, bacteria shocked with bile recovered to some extent autoggregation properties after washing (Fig. 1b).

DISCUSSION

The results presented here show clearly that growth in bile alters autoaggregation and adhesion to Caco-2 cells to a much more pronounced extent than when the same bacteria were shocked with bile after growing in a bile-free medium. The changes in adherence and autoaggregation were correlated with a significant decrease in the surface hydrophobicity, both changes being irreversible.

A slight decrease in adherence was observed in shocked bacteria, which appeared to be associated with an increase in the surface potential with little or no change in hydrophobicity. This effect was partially reversible in strain NCC 189, which is slightly less hydrophobic than strain 5310 (Fig. 1 and Table 4). The change of the surface charge produced by bile indicates that bile components may adsorb to both hydrophobic and non-hydrophobic strains. In hydrophobic strains this change was parallel to a decrease in the surface hydrophobicity, suggesting that bile adsorption could be due to hydrophobic interactions. As a result, cell–cell hydrophobic interactions decreased parallel to an increase in the electrostatic repulsion due to the charges of the bile components located at the surface.

Values of the zeta potential of bile-shocked bacteria can be ascribed to incorporation of bile molecules to the bacterial surface. In contrast, when bacteria are grown in bile, changes of zeta potential were less pronounced (Kociubinski *et al*. 2001). One possibility is that bile was washed out from the surface to the surrounding aqueous medium. However, as shown in Fig. 1 the adhesion properties remains unaltered in strain CIDCA 5310 and recovers slightly in strain NCC 189 after the shocked bacteria were washed in medium without bile. The drastic loss of adherence observed in strains grown in the presence of bile cannot be ascribed solely to physical adsorption of bile onto the external surface because the bile-shocked bacteria show a slight decrease in adhesion, due possibly to the increase in electrostatic repulsion imposed by charge adsorption. Moreover, bile shock produced a lower decrease in hydrophobicity and an increase in surface potential. If adherence was caused by electrostatic forces, adhesion should be more affected in shocked bacteria than in bile-grown bacteria. As this appears to be against the experimental evidence, non-electrostatic interactions may be the main driving forces for adhesion. Hence, changes in adherence induced by the

growth in bile-containing medium can be related apparently to metabolic changes in the bacteria, as suggested by the changes observed in the lipid composition.

The presence of bile during growth promotes a change in lipid metabolism affecting the glycolipid/phospholipid ratio, the relative concentration of saturated/unsaturated fatty acids and the presence of certain glycolipid species.

However, it must be borne in mind that adherent and non-adherent strains show the same lipid composition and, in addition, similar changes in the lipid composition are observed in hydrophobic, non-hydrophobic, adherent and non-adherent bifidobacterial strains after growing in bile-containing medium. This suggests that lipid composition is not related directly to adhesive properties. In the context of this work, changes in lipid composition can be taken as an indication of bacterial metabolism during growth in bile-containing medium. The changes in lipid composition and in the fatty acid species (Tables 1, 2 and 3) suggest that, after adsorption to the external surface of the cell, bile can induce changes in the bacterial metabolism. However, no differences can be ascribed to the fatty acid composition. Therefore, changes in the surface and adhesion properties might be correlated with a modification in sugar components induced by bile during growth.

REFERENCES

Bibiloni, R., Pérez, P., Garrote, G., Disalvo, E. and De Antoni, G. (2001) Surface characterization and adhesive properties of bifido-bacteria. In *Methods in Enzymology. Microbial Growth in Biofilms Part A* ed. Doyle R.J. vol 336. pp. 411–227. New York: Academic Press.

Gómez Zavaglia, A., Abraham, A., Giorgieri, S. and De Antoni, G. (1999) Application of polyacrylamide gel electrophoresis and capillary gel electrophoresis to the analysis of *Lactobacillus delbrueckii* whole-cell proteins. *Journal of Dairy Science* **82**, 870–877.

Gómez Zavaglia, A., Disalvo, E.A. and De Antoni, G.L. (2000) Fatty acid composition and freeze-thaw resistance in lactobacilli. *Journal of Dairy Research* **67**, 241–247.

Gómez Zavaglia, A., Kociubinski, G., Pérez, P. and De Antoni, G. (1998) Isolation and characterization of *Bifidobacterium* strains for probiotic formulation. *Journal of Food Protection* **61**, 865–873.

Ishibashi, N. and Shimamura, S. (1993) Bifidobacteria: research and development in Japan. *Food Technology* **47**, 126–135.

Kociubinski, G., Gomez Zavaglia, A., Pérez, P., Disalvo, E. and De Antoni, G. (2002) Effect of bile components on the surface properties of bifidobacteria. *Journal of Dairy Research* **69**, 294–303

Kociubinski, G., Pérez, P. and De Antoni, G.L. (1999) Screening of bile resistance and bile precipitation in lactic acid bacteria and bifidobacteria. *Journal of Food Protection* **62**, 905–912.

Marinetti, G.V. (1993) General Analytical procedures In *Techniques of Lipidology, Isolation, Analysis and Identification of Lipids* eds Kates, M., Burdon, H.R. and Knippemberg, P.H. pp. 112–185 Amsterdam: Elsevier.

Mitsuoka, T. (1982) Recent trends in research on intestinal bifidobacteria. *Bifid Microflora* **1**, 3–7.

Norris, R., Flanders, T., Tomarelli, R. and György, P. (1950) The isolation and cultivation of *Lactobacillus bifidus*: a comparison of branched and unbranched strains. *Journal of Bacteriology* **60**, 681–696.

Pérez, P.F., Minnaard, J., Disalvo, E. and De Antoni, G. (1998) Surface properties of bifidobacterial strains of human origin. *Applied and Environmental Microbiology* **64**, 21–26.

Rašić, J. and Kurmann, J. (1983) The human gastrointestinal flora and bifidobacteria In *Bifidobacteria and Their Role*. pp. 51–80. Basel: Birkhäuser-Verlag.

Scardovi, V. (1986) Irregular non-sporulating gram positive rods. Genus Bifidobacterium Orla-Jensen 1924. In *Bergey's Manual of Systematic Bacteriology* ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. vol. 2. pp. 1418–1424. Baltimore: Williams & Wilkins.

Ueda, K. (1986) Immunity provided by colonized enteric bacteria. *Bifid Microflora* **5**, 67–72.