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# Effect of bile components on the surface properties of bifidobacteria

By GUILLERMO KOCIUBINSKI[1], ANDREA GÓMEZ ZAVAGLIA[1,2], PABLO F. PÉREZ[1], EDGARDO A. DISALVO[2] and GRACIELA L. DE ANTONI[1,*]

[1] *Centro de Investigación y Desarrollo en Criotecnología de Alimentos, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 1900 La Plata, Argentina*
[2] *Cátedra de Química General e Inorgánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, 1113 Buenos Aires, Argentina*

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Summary. The action of bile on bifidobacteria was studied by measuring changes in zeta potential, hydrophobicity and adherence to enterocyte-like cells *in vitro*. Highly hydrophobic strains shocked with bile displayed more negative zeta potential values and a decrease in adherence. When a non-hydrophobic non-adherent strain (CIDCA 5324) was shocked with bile, an increase in hydrophobicity was observed. However, no changes of zeta potential or adherence properties were apparent. The action of the bile components was different from the action of whole bile. Cholate and deoxycholate produced a decrease in the negativity of zeta potential values of all strains studied whereas taurocholate displayed a shift in zeta potential of hydrophobic strains to more negative values, thus explaining the decrease in the autoaggregation by charge repulsion. However, the decrease in zeta potential caused by cholate and deoxycholate did not increase autoaggregation in a hydrophobic non-adherent strain (CIDCA 531). This suggests that other forces are contributing to autoaggregation.

KeyWords: Bile, bacterial surface, adhesive properties, bifidobacteria.

Bifidobacteria are normal inhabitants of the human intestinal tract where they exert health-promoting effects, such as the prevention of diarrhoea caused by pathogenic bacteria, reduction of colon cancer-risk, decrease of serum cholesterol levels and stimulation of the immune response (Mitsuoka, 1982; Tojo *et al*. 1986; Ueda, 1986; Naidu *et al*. 1999).

Probiotic bacteria administered orally should resist a high bile concentration that increases in the duodenum after a meal and progressively decreases in the ileum (Klaenhammer, 1982; Klaver & Van der Meer, 1983; Salminen & Von Wright, 1993; Fuller, 1997; Marteau *et al*. 1997). Therefore, bile tolerance could be an essential criterion in the selection of microbial strains for probiotic use.

Another criterion for strain selection is the interaction with intestinal epithelial cells. Recently, a survey of the surface properties of bifidobacteria (Gómez Zavaglia *et al*. 1998; Pérez *et al*. 1998) showed that most of the strains that are able to adhere to human enterocytes in culture (Caco-2 cells) are also highly hydrophobic, autoagglutinating and hemagglutinating.

Bile salts abolish the ability of Gram negative bacteria to adhere to hydrophobic

* For correspondence; e-mail: gdantoni@nahuel.biol.unlp.edu.ar

plastic surfaces (Hills *et al*. 1983). However, although the resistance to bile has been tested in several probiotic strains (Kociubinski *et al*. 1999), the effect of bile on both the surface properties and adherence of probiotic bacteria to enterocytes, have not been so far studied.

Since bacteria pass through the duodenum before reaching the intestine, it may happen that the surface properties, and hence the adherence to enterocytes, change after their exposure to bile and bile components.

The main physicochemical forces involved in the interaction between bacteria and different surfaces are both electrostatic (repulsion) and hydrophobic (attraction) forces (Busscher & Weerkamp, 1987). In general, the surface and adhesive properties can be modified by tensioactive agents according to their ability to change the repulsive and attractive contributions. Bile is composed of bile salts (in varying ratios) which have different critical micellar concentrations and charges. In addition, the steroid groups of bile salts will strongly interact with hydrophobic patches on the bacterial surface. Therefore, they may affect the surface properties of lactic bacteria due to differences in adsorption rate and amphiphilic balance.

Microorganisms orally administered would suffer changes in surface properties during the passage across the small bowel. These changes might lead to interactions with the host that are very different from those found using models without bile. Therefore, in the selection of probiotic strains by means of adhesive properties, the study of the surface in the presence of bile, gives a better indication of the response of bacteria to near physiological conditions.

The aim of this work is to determine the effect of bile and bile components on the surface charges and hydrophobicity of different strains of bifidobacteria and to determine their consequences for the adherence to enterocyte-like cells and autoaggregation.

## materials and methods

### *Strains and culture conditions*

Bacterial strains (Table 1) were isolated at the Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA) and identified as published (Gómez Zavaglia *et al*. 1998; Pérez *et al*. 1998). ATCC strains were purchased from the American Type Culture Collection (Manassas, VA 20108, USA). Strains were maintained frozen at −80 °C in sterile 0·3 M-sucrose. They were reactivated by two consecutive subcultures in liquid media.

Bacteria were cultured in anaerobic conditions for 24 h at 37 °C in MRS medium (Difco, Detroit, MI 48232, USA). In all cases, bacteria were harvested in stationary phase. Cells were collected by centrifugation at 14000 ***g*** for 1 min, washed twice with 50 M-sodium phosphate buffer, pH 7·0±0·2, and finally resuspended in the same buffer (bacterial suspensions). The final concentration was $10^9$ cfu/ml.

Shock with bile was carried out by adding 0·1 ml of an ox-bile stock solution in water (500 g/l) (Merck & Co., Rahway, NJ 08873, USA) to the bacterial suspensions neutralised to pH 6·8–7·0 with NaOH (100 g/l). A final bile concentration of 5 g/l was achieved. The mixture was incubated for 1 h at 37 °C. Cells were collected by centrifugation at 14000 ***g*** for 1 min, washed twice with 50 mM-sodium phosphate buffer, pH 7·0±0·2, and finally resuspended in the same buffer (shocked bacterial suspensions).

Table 1. *Effect of bile on the hydrophobicity adhesive and autoaggregating properties of Bifidobacterium strains of different origin*

Values are means ± SD for $n = 3$

| Strains[a] | Bile resistance g/l[b] | Non-bile shock | | | Shock with bile 5 g/l | | |
|---|---|---|---|---|---|---|---|
| | | Aag[c] | Adh[d] | H % | Aag[c] | Adh[d] | H % |
| *Bifid. bifidum* NCC 189 (formerly CIDCA 536) | 1 | ++ | ++ | 90·2 ± 2·9 | + | + | 93·3 ± 2·5 |
| *Bifid. pseudolongum* CIDCA 531 | 5 | − | − | 97·2 ± 2·4 | − | − | 86·1 ± 3·3 |
| *Bifid. bifidum* CIDCA 537 | 1 | ++ | ++ | 91·4 ± 3·9 | + | + | 85·0 ± 1·6 |
| *Bifid. bifidum* CIDCA 5310 | 1 | ++ | ++ | 93·5 ± 1·7 | + | + | 89·0 ± 2·0 |
| *Bifid. adolescentis* CIDCA 5317 | 1 | − | − | 3·0 ± 0·6 | − | − | 15·7 ± 10 |
| *Bifid. longum* CIDCA 5324 | 1 | − | − | 3·3 ± 0·6 | − | − | 32·4 ± 8·0 |

[a] Strains were identified by morphology, Gram staining and biochemical characteristics according to the Bergey's (Scardovi, 1986) and whole protein pattern by SDS-PAGE (Gómez Zavaglia *et al*. 1998). All strains were isolated from healthy human new-born faeces, with the exception of strain CIDCA 531 isolated from a dairy product. Strains were grown in MRS without bile. For details see references (Gómez Zavaglia *et al*. 1998; Pérez *et al*. 1998).

[b] Bile resistance was determined as the ability to grow in MRS agar supplemented with 1–5 g bile/l. Values represent the maximum bile concentration for growth (Kociubinski *et al*. 1999).

[c] Aag: autoaggregation. ++ strongly positive; + positive; − negative.

[d] Adh: adherence to Caco-2 cells, ++ >100 bacteria/microscopic field; +around 50 bacteria/microscopic field; −1 bacteria/10 microscopic fields.

*Adhesion assays*

Enterocyte-like Caco-2 cells were purchased from the American Type Culture Collection. 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 63178, USA) and 20% inactivated foetal calf serum (Gen SA, Buenos Aires, Argentina). Streptomycin and penicillin G were added to final concentrations of 0·05 g/l and 50 IU/ml respectively. Monolayers were prepared on glass coverslips, which were placed in 24-well tissue plates (Corning Glass Works, Corning, NY 14831, USA). Cells were added at a concentration of $2 \times 10^5$ cells/well and were incubated at 37 °C in a 5% $CO_2$-95% air atmosphere. Culture medium was changed every 2 d. Cells were used between passages 23 and 34. Adherence assays were performed with cells at late postconfluence (15 d in culture).

Bacterial suspensions and shocked bacterial suspensions (0·5 ml) 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 by counting attached bacteria after Gram stain (Pérez *et al*. 1998). Concentration was expressed as bacteria per microscopic field. For each sample, at least ten fields were examined.

*Hydrophobicity assays*

Two millilitres of bacterial suspensions and shocked bacterial suspensions were mixed with 0·4 ml of xylene by vortexing for 120 s. Phases were allowed to separate by decantation. The aqueous phase was carefully removed and the absorbance was read at 600 nm ($A_{600}$). Absorbance decrease 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 before and after extraction with xylene, respectively (Doyle & Rosenberg, 1995; Pérez *et al*. 1998).

*Electrophoretic mobility and zeta potential*

Electrophoretic mobilities of bacteria resuspended in 0·0745 g KCl/l were determined in a capillary H-cell with Ag/AgCl electrodes connected to a variable direct current source. The electrophoretic mobility ($\mu$) 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. Rate of migration was determined by microscopic observation of displacement of individual cells with rectilinear and uniform movement along a reticular slide (length 1 mm). The optical distance of the microscope was fixed by determining the zeta potential ($\xi$) of phosphatidylserine liposomes whose value was around −120 mV in 0·585 g NaCl/l at pH 7·4 (Pérez *et al*. 1998).

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

Temperature was maintained at 25 °C. Zeta potential was calculated with the equation $\xi = 4\,\pi\,\eta\,\mu/\epsilon$, where $\eta$ and $\epsilon$ are the viscosity and the dielectric constant of the solution, respectively.

The electrophoretic mobilities of the different strains were performed with bacterial suspensions diluted to a final concentration of $10^3$ cfu/ml. Different concentrations of sodium cholate, sodium deoxycholate, sodium taurocholate and sodium glycocholate (Sigma) were added in order to measure the electrophoretic

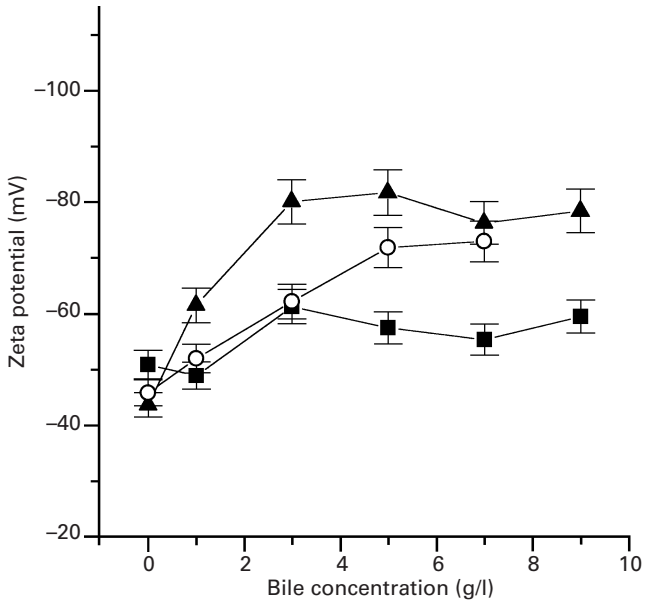


Fig. 1. Changes in the zeta potential of hydrophobic and non-hydrophobic strains of Bifidobacteria with bile. Titration of: hydrophobic strains, NCC 189 (▲) and CIDCA 531 (○) and non-hydrophobic strain CIDCA 5324 (■). Vertical bars show SD for $n = 3$.

mobility of the strains as a function of the bile salt concentration. Titrations were carried out in a range below the critical micellar concentrations (cmc) of the bile components (taurocholate cmc, 10 mM; glycocholate cmc, 8 mM; deoxycholate cmc, 2 mM; cholate cmc, 3 mM).

*Autoaggregation assays*

Autoaggregation was determined, initially, by visual inspection of broth-bacterial cultures in stationary phase. Cultures were gently homogenised with vortex and examined after a few minutes. Autoaggregating strains formed clumps that separated from the suspension media by sedimentation giving a clear supernatant. Non-aggregating bacteria remain dispersed showing a turbid solution.

After shock treatment with a pulse of concentration of bile, sodium cholate, sodium deoxycholate, sodium taurocholate, or sodium glycocholate, suspensions were immediately placed in a spectrophotometric cuvette and turbidity at 600 nm was followed through time until no further change was observed. The rate of relative absorbance decrease was taken as a degree of aggregation as described before (Bibiloni *et al*. 2001).

## RESULTS

The effect of bile on the surface properties of bile-resistant bifidobacteria of different origins was determined in non-growing conditions. Table 1 shows the correlation between autoaggregation, adherence and hydrophobicity for different strains before and after bile shock. Highly hydrophobic strains were autoaggregating (Aag) and also adherent to Caco-2 cells, with exception of CIDCA 531. In contrast, non-hydrophobic bacteria were non-autoaggregating and non-adherent to Caco-2.

Hydrophobic strains (such as NCC 189, CIDCA 537 and CIDCA 5310) shocked with bile (5 g/l) showed no changes of surface hydrophobicity whereas a decrease of

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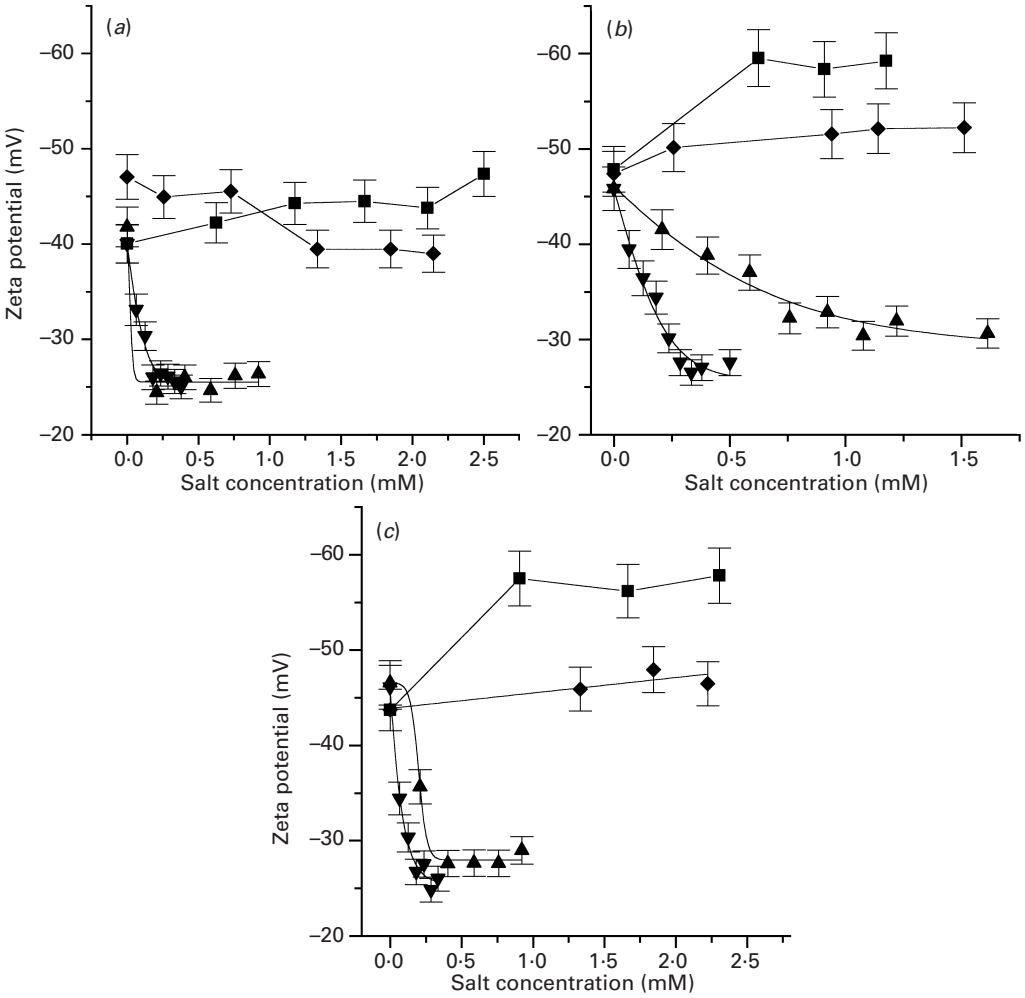


Fig. 2. Effect of bile salts on the zeta potential of Bifidobactria (*a*) the non-hydrophobic strain CIDCA 5324; (*b*) the hydrophobic, non-adherent strain CIDCA 531 and (*c*) the hydrophobic adherent strain NCC189. The strains were titrated with (▲) sodium cholate; (▼) sodium deoxycholate; (◆) sodium glycocholate; and (■) sodium taurocholate. Vertical bars show SD for $n = 3$ for cholate and deoxycholate and $n = 2$ for glycocholate and taurocholate. MW of bile salts are 430·57, 432·6, 537·7 and 487·6 for cholate, deoxycholate, taurocholate and glycocholate respectively.

autoaggregation and adherence to Caco-2 cells was observed. In contrast, the non-hydrophobic strain CIDCA 5324 showed an increase in hydrophobicity. As shown also in Table 1, this increase in hydrophobicity was not high enough to modify autoaggregation or adhesion to enterocyte-like cells.

Bile also produced changes in the net surface charge of the bacteria. As shown in Fig. 1, the hydrophobic strains NCC 189 and CIDCA 531, showed a shift of the zeta potential to more negative values (from −40 mV to c.a. −80 mV) at around 3–5 g bile/l. In contrast, the non-hydrophobic strain CIDCA 5324 showed a lower change in zeta potential in the same range of bile concentrations.

When taurocholate was added to a non-hydrophobic strain, such as CIDCA 5324, no changes of the zeta potential were observed (Fig. 2*a*). On the other hand, cholate, and glycocholate produced a decrease in zeta potential to less negative values.

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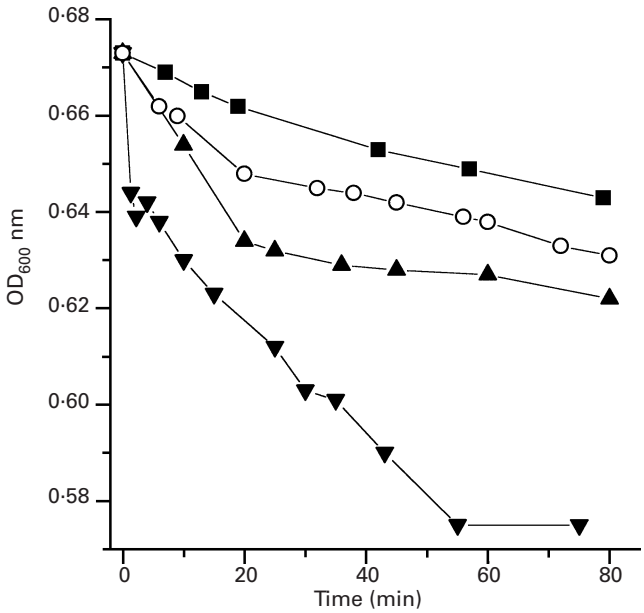


Fig. 3. Autoaggregation of a hydrophobic adherent Bifidobacteria strain induced by bile components. Strain NCC189 was titrated with (▲) sodium cholate; (▼) sodium deoxycholate; and (■) sodium taurocholate. Autoaggregation of the strain in the absence of bile salts (○, control).

As shown in Fig. 2*b*, the zeta potential of strain CIDCA 531 (hydrophobic non-adherent strain) was strongly dependent on the bile salt added. Cholate and deoxycholate led to less negative values of zeta potential whereas taurocholate increased the negative charge of the bacterial surface. In contrast, glycocholate showed no effect.

In Fig. 2*c* the effect of bile salt components on the zeta potential of a hydrophobic-adherent strain (NCC 189) was compared. The response was the same as that found for the strain CIDCA 531 (Fig. 2*b*), for all the bile components.

Fig. 3 displays the effect of bile salts on the aggregation properties of strain NCC 189. According to this assay, taurocholate was the only component able to damp the aggregation as observed with bile (Fig. 3 and Table 1). Cholate did not strongly affect aggregation and deoxycholate further enhanced it with respect to the control without bile.

No changes in autoaggregation plots were apparent when bile salts were added forming micelles with phosphatidylcholine and cholesterol (data not shown).

## DISCUSSION

The changes observed in the zeta potential values and in the hydrophobic index indicate that bile and bile components affect the surface properties of bifidobacteria. Since in the present study, low bile concentrations and mild conditions were used, the effect of viscosity and/or extraction of bacterial surface components on zeta potential can be ruled out. Major changes were observed in the zeta potential of hydrophobic strains. In the case of strain NCC189, the increase in the negative surface potential in the presence of bile (Fig. 1) could explain the decrease in

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autoaggregation and adherence to Caco-2 cells (Table 1). The increase of net charge would make the bacteria repel each other hindering the interaction by hydrophobic forces, which in turn are not affected.

Addition of bile to a non-hydrophobic strain (CIDCA 5324), that has been shown to be non-autoaggregating and non-adherent to Caco-2 cells, did not affect the zeta potential to a great extent indicating a lower change in the surface properties (Fig. 1). The slight increase in the negative values from −50 to −60 mV indicates an increase in the surface charge. However, the addition of bile caused an increase in surface hydrophobicity, denoting that bile also affects the surface of non-hydrophobic strains. We could argue that the interaction between bile components and the bacterial surface of strain CIDCA 5324 can take place by means of the charged moiety. Therefore, the hydrophobic portion of the bile salts molecules will be in contact with the external medium, thus increasing hydrophobicity index. However, the hydrophobicity increase caused by bile (Table 1) was not high enough to modify the interaction with Caco-2 cells. As reported previously, bifidobacteria with hydrophobicities below 30% do not adhere to enterocyte-like cells in culture (Pérez *et al*. 1998).

In the case of strain CIDCA 531, the hydrophobicity is high. However, this does not directly determine autoaggregation nor adhesion to Caco-2 cells (Table 1). Bile and its different components changed the surface of this strain in a same way as in the hydrophobic adherent strain NCC 189. These findings suggest that the interaction between bacteria and intestinal cells is a multifactorial process.

The effect of bile on the bacterial surface could be attributed to the adsorption of one or more bile components. Human and animal bile contain a complex and variable mixture of bile salts and phospholipids. The major bile acids present in bile have structures based on the sterol backbone. They differ in the degree of hydroxylation and type of functional group. Based on this structure, three major groups of bile acids can be identified: 1) free bile acids or cholic acids with hydroxyl group, 2) glycocholic acids with aminocarboxylate groups, and 3) taurocholic acids with aminosulphonate groups (Yarabe *et al*. 1998). The cmc of the bile salts follows the sequence deoxycholate < cholate < < glycocholate < taurocholate (see Materials and methods and Sung *et al*. 1994). These differences in the hydrophilic-hydrophobic balance seem to be correlated with their effects on the bacterial net charge (Fig. 2). When cholate or deoxycholate were added to bacteria, a decrease in the surface negative charges was observed (Fig. 2). This means that these salts are masking or diluting the surface electrical charges on the bacteria. In this condition, an increment in the autoaggregation of the autoaggregating adherent strains was observed, according to the reduction of the electrostatic repulsion and the stability of the hydrophobic index. When glycocholate was added, no significant changes of zeta potential were observed in hydrophobic strains. This finding might indicate either that glycocholate does not interact with the bacterial surface or that the overall effect remains masked. On the other hand, the slight decrease in zeta potential for the strain CIDCA 5324 could be related to more basic (Lewis base) bacterial surfaces in the strain under study (Pelletier *et al*. 1997).

Taurocholate, the most hydrophilic compound studied, increased the surface potential in the hydrophobic strains. This could be due to the insertion of the net charge of the sulphonate group at neutral pH. The effect of taurocholate is similar to that produced by bile, probably due to the high proportion of this salt in ox-bile (Que *et al*. 2000).

The increase in hydrophobicity produced by the bile on non-hydrophobic strains,

would suggest a predominance of the adsorption of bile components with the highest hydrophobicity i.e. cholate and deoxycholate.

In conclusion, the mechanism by which bile affects the surface of the strains appears to be complex since the interaction of bile components affects the surface charges in different ways.

To our knowledge, all the *in vitro* studies on the interaction between probiotic bacteria and eucaryotic cells have been performed with bacteria not exposed to bile. Our results are the first obtained *in vitro* in which bacteria were exposed to bile.

In addition to bile, during the passage across the intestinal tract, the bacteria have to cope with several stress factors such as the exposure to the low pH of the stomach followed by neutralisation in the upper intestinal tract. As reported previously (Pérez *et al*. 1998), extreme pH values have a dramatic effect on surface properties of bifidobacteria. In the present study, we show that bacteria in contact with bile would be drastically affected in their surface properties. Therefore, it could be important to examine the interaction with bile, in the selection of probiotic strains based on the surface properties (e.g. adhesion to enterocyte like cells, zeta potential, hydrophobicity). Study of the effect of bile on the surface of intestinal bacteria could be useful not only for the selection of probiotic strains but also for a better understanding of the host-microflora interactions.

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