RESEARCH LETTER

Cell envelope changes in *Bifidobacterium animalis* ssp. *lactis* as a response to bile

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Abstract

Bifidobacterium animalis ssp. *lactis* is a probiotic frequently used as adjunct culture in fermented dairy products. In order to ensure its proper function at the intestinal level, this bacterium has to be tolerant to physiological concentrations of bile. This study examined the influence of bile on the fatty acid composition and the membrane characteristics of *B. animalis* IPLA 4549 and its mutant with acquired resistance to bile, *B. animalis* 4549dOx. Bile adaptation triggers in *B. animalis* 4549dOx a decrease in membrane fluidity and in the protein : phospholipid ratio, as well as a shift in the fatty acid composition of the cell. Remarkably, the presence of bile in the growth medium induced similar changes in both *B. animalis* cells. Furthermore, transmission electron microscopy analysis showed that bile promotes a severe distortion of the cell surface. This study provides new insights of the action of bile on the cell envelope of bifidobacteria.

Introduction

Microorganisms of the genus *Bifidobacterium* are common inhabitants of the GI tract of humans, and they represent one of the most abundant microbial gut populations in the colon during the early stage of life (Harmsen *et al.*, 2000). Some strains are considered probiotics on the basis of their health-promoting effects (Araya *et al.*, 2002). In particular, strains of *Bifidobacterium animalis* ssp. *lactis* have been extensively used in functional dairy products (Masco *et al.*, 2005) and their health benefits have been documented and supported by clinical studies (Salminen *et al.*, 2005).

The functionality of probiotics depends on their ability to survive and remain in the human intestine. During digestion, bile is secreted into the intestine in order to solubilize liposoluble nutrients and facilitate their absorption. Besides its physiological function, bile salts are detergent-like biological compounds with a strong antimicrobial activity, which induces membrane damage and causes oxidative stress to the DNA (Begley *et al.*, 2005). Therefore, natural tolerance to bile is strictly necessary for *Bifidobacteria* in order to colonize the gut.

The bacterial cell surface is the first physical target of bile action. Bile salts modulate the expression of membrane proteins in enteric bacteria (Bron *et al.*, 2004; Sánchez *et al.*, 2006). They also affect the lipid composition of *Bifidobacterium* (Gómez-Zavaglia *et al.*, 2002) and the cell membrane functionality of *Lactobacillus* (Taranto *et al.*, 2003, 2006). Its mechanism of growth inhibition is likely to be carried out through the dissipation of the proton motive force (Kurdi *et al.*, 2006). Additionally, *Bifidobacteria* could display other mechanisms to counteract bile toxicity, such as active extrusion of bile salts through multidrug resistance proteins (Price *et al.*, 2006), or high bile salt hydrolase activity (Noriega *et al.*, 2006). These findings indicate that bile promotes in bacteria a very complex response, involving several cellular mechanisms.

The isolation of bile salt-resistant mutants in *Bifidobacterium* showed that bile resistance is correlated with multiple factors, including different surface properties, and changes in the enzymatic profiles and protein-expression patterns (Gueimonde *et al.*, 2005; Sánchez *et al.*, 2005; Noriega *et al.*, 2006). This prompted the present study, in which the authors aimed to characterize the effect of bile on the surface

of *Bifidobacterium*, and the influence of the acquisition of a bile-resistant phenotype in this effect.

Material and methods

Microorganisms and growth conditions

The bacterial strains used in this study were *B. animalis* ssp. *lactis* IPLA 4549 and its ox gall-resistant derivative *B. animalis* ssp. *lactis* 4549dOx (Ruas-Madiedo *et al.*, 2005). The mutant has a stable resistance phenotype, and probably harbours several mutations since it displays remarkable physiological changes compared with the wild-type (WT) strain (Gueimonde *et al.*, 2005; Ruas-Madiedo *et al.*, 2005; Noriega *et al.*, 2006). Strains were cultured on Man, Rogosa & Sharpe (MRS) agar (BD Diagnostic Systems, Sparks, MD) supplemented with 0.05% L-cysteine (MRSC) (w/v) (Sigma, St. Louis, MO). Single colonies were picked and cultured in liquid MRSC medium in an anaerobic chamber (Mac500; Down Whitley Scientific, West Yorkshire, UK) under a 10% H_2, 10% CO_2, and 80% N_2 atmosphere at 37 °C. MRSC broth was inoculated at 1% with fresh overnight cultures and incubated anaerobically. When necessary, bile salts (ox gall, Oxoid Limited, Hampshire, UK) were added to a final concentration of 3.0 g L^{-1}.

Analysis of membrane lateral diffusion

To determine the lateral diffusion of fatty acid chains in the membrane, measurements of intermolecular pyrene excimerization were carried out according to Aricha *et al.* (2004). Bacterial samples (two independent cultures for each condition) were fixed with formaldehyde (final concentration, 0.25%), washed twice with phosphate-buffered saline (PBS) containing 0.25% formaldehyde (pH 7.4), and incubated with constant mild stirring at 37 °C during 40 min with different concentrations of pyrene, ranging from 0.06 to 0.5 μM. With this low pyrene concentration, excimer formation is not detected in the absence of cells. The extent of incorporation of the probe was not significantly different for any of the strains/conditions used. An untreated portion of the sample without pyrene was incubated under the same conditions, and served as a scattering control. Measurements were carried out in duplicate for each sample at 37 °C with a Cary Eclipse spectrofluorometer (Varian, Palo Alto, CA) with excitation at 335 nm, emission at 374 nm (for the monomer) and 470 nm (for the excimer), and 5- and 5-nm slits, respectively.

Fatty acid composition and protein and phospholipid determinations

The fatty acid composition was determined by GC at the DSMZ external service (Braunschweig, Germany) using the Microbial Identification System (MIDI, Newark, DE; http://www.midi-inc.com). Two or three independent cultures were made for each condition. Cells were collected by centrifugation, washed twice with sterile deionized water and the pellets were frozen at − 80 °C. The fatty acids were converted to methyl esters and extracted in a four-step procedure according to the DSMZ protocols.

For the phospholipid and protein determinations in membrane fractions, inside-out membrane vesicles were obtained essentially as indicated in a previous work (Sánchez *et al.*, 2006) with minor modifications. Cells were grown in 1 L of MRSC, with or without added bile salts, and collected at late exponential phase. Two independent cultures for each condition were analysed. Cells were centrifuged at 2800 ***g*** at 4 °C for 15 min and washed twice with 50 mM HEPES-potassium buffer, pH 7.0. The pellet was resuspended in 20 mL of the same buffer supplemented with 10 mM $MgSO_4$, and treated with 5 mg mL^{-1} lysozyme and 25 U mL^{-1} mutanolysin. The suspension was incubated at 37 °C for 4 h with constant stirring. Cells were broken by passage through a cell disruptor (Constant Systems, Northants, UK) and the suspension was incubated for 20 min at 30 °C with 100 μg mL^{-1} DNase A (Sigma). Unbroken cells and cell debris were removed by two centrifugation steps at 13 000 ***g*** for 20 min at 4 °C. Membrane vesicles were centrifuged at 200 000 ***g*** for 30 min at 4 °C, resuspended in 50 mM HEPES-potassium buffer pH 7.0, frozen in liquid nitrogen and stored at − 80 °C until use.

Total membrane protein was assayed according to Lowry *et al.* (1951) in the presence of 0.5% sodium dodecyl sulphate, using bovine serum albumin as the standard. The concentration of phospholipids in the membrane fraction was determined by the measurement of the inorganic phosphate released after perchloric acid oxidation (Rouser *et al.*, 1970). Glass tubes were washed with perchloric acid and dried before their use. Samples (400 μL) were added to the tubes and then dried at 180 °C. Perchloric acid (300 μL) was added and the preparations were heated for 30 min at 180 °C, using perchloric acid-washed glass marbles on top of the tubes. Amounts of 1.4 mL of deionized water, 200 μL of 2.5% ammonium molibdate (w/v) and 200 μL of 10% ascorbic acid (w/v) were sequentially added, shaking vigorously between each step. Tubes were incubated for 20 min at 60 °C and the absorbance of the samples was measured at 797 nm. Quantification was carried out by comparisons with a series of inorganic phosphate standards.

Transmission electron microscopy (TEM)

Samples for TEM were obtained from 10 mL of cells grown in the absence or presence of bile. Two independent cultures were analysed for each condition. Cells were collected by low-spin centrifugation and the pellet was resuspended with

a PBS solution containing 2% glutaraldehide and 1% paraformaldehide. Cells were fixed during 3 h at room temperature. Subsequently, cells were treated with osmium tetroxide (1%) for 90 min at 4 °C and dehydrated in gradually increasing concentrations of acetone, and embedded in Epon-812. The sections were stained with uranile acetate and lead citrate and examined in an electron microscope (Leica EM UC6) in the Electron Microscopy Service from the CNB (Centro Nacional de Biotecnología, CSIC, Madrid).

Results and discussion

The adaptation to bile of *Bifidobacterium* is the result of a sum of complex phenomena directed to maintain the optimal viability of the cell under stress conditions (Garrigues *et al.*, 2005; Gueimonde *et al.*, 2005; Ruas-Madiedo *et al.*, 2005; Noriega *et al.*, 2006; Sánchez *et al.*, 2006). In this respect, the cell surface is the first physical barrier of defence against environmental cytotoxic compounds. The deleterious action of lipophilic compounds is due to their membrane accumulation, resulting in alteration of its structure and function. In particular, bile salts have been related to the ability to dissipate membrane potential, rendering the bacterial membrane permeable to protons and causing cell death (Kurdi *et al.*, 2006). Therefore, bacteria able to survive under bile challenge exhibit adaptation changes in their membrane lipids to compensate for the membrane potential dissipation effect of these compounds. Thus, a shift in the membrane characteristics could have a direct influence on bile tolerance. The authors took the opportunity of using a strain previously isolated and partially characterized by them obtained by progressive adaptation to increasing concentrations of ox bile, *B. animalis* ssp. *lactis* 4549dOx, which had acquired a stable bile-resistance phenotype and the capacity to resist high bile concentrations (Sánchez *et al.*, 2006). Then, to know the cell surface response to bile of *B. animalis* and the influence of the acquisition of the bile-resistant phenotype in that response, the fatty acid composition and the membrane lateral diffusion of the WT and the mutant strains with and without bile were compared. In order to perform in similar physiological conditions the different analyses of this work, the cells were always grown until the late exponential phase of growth ($OD_{600\,nm}$ *c.* 5.0 for the WT and 3.0 for the mutant, and between 0.8 and 1.0 for both strains in the presence of bile). Under these conditions, the pH values of the cultures in the absence of bile were 4.7 for the WT and 4.9 for the mutant, and 5.4 and 5.5 for the WT and the mutant in the presence of bile, respectively. These data show that the acidic pH, especially in the absence of bile, could partially influence the physiological response of the cells. In this way, previous results from the authors' group demonstrated that the bile resistance phenotype of *B. animalis* ssp. *lactis* 4549dOx was lost when grown in buffered MRSC medium, suggesting that the bile resistance level reached by the mutant, but not by the WT, depended on the external pH (Noriega *et al.*, 2004). Taking these results into account, all the experimental work in batch cultures was carried out under uncontrolled pH conditions.

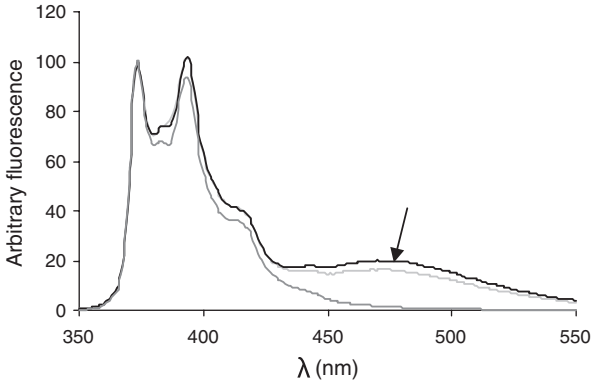

Fig. 1. Fluorescence emission spectra of 2 μM pyrene (excitation at 335 nm) normalized by the intensity of the band at 374 nm in buffer (dark grey) and in cell suspensions of *Bifidobacterium animalis* 4549dOx grown in the absence (black) or presence (light grey) of bile. The arrow indicates de excimerization peak.

Lateral diffusion of pyrene was examined, which is directly related to the membrane viscosity and the lateral diffusion of the fatty acyl chains (Aricha *et al.*, 2004). This fluorescent probe, when excited by light, can form a complex with an identical unexcited probe molecule. Such a complex is called an excimer and the intermolecular excimerization of pyrene is recognized by the production of a new fluorescent band at a longer wavelength (470 nm) than the usual emission wavelength of the monomer (374 nm) (Fig. 1). The rate of excimer formation depends on pyrene concentration, excimer lifetime and pyrene excimerization coefficient (K_a), the latter being diffusion controlled (Vanounou *et al.*, 2003). The excimer-to-monomer ratio increases linearly with the pyrene concentration and the probe's lateral diffusion can be derived from the slope of the curve (Aricha *et al.*, 2004). *Bifidobacterium* cells labelled with pyrene displayed a fluorescence spectrum characteristic for pyrene derivatives in a hydrophobic environment. Initial experiments confirmed that the probe was almost completely embedded (more than 90%) into the bifidobacterial membrane (data not shown). Figure 1 shows the emission spectra of pyrene in the membrane and the influence of bile on the excimer formation. A significant decrease in the pyrene excimerization coefficient, 28.6% ($P < 0.05$), was measured in the bile-resistant mutant compared with the WT. Similarly, the presence of bile in the growth medium promoted a decrease of K_a of 28.6% and 24% in the WT and the mutant, respectively ($P < 0.05$ in both strains) (Table 1).

Table 1. Pyrene excimerization coefficient (K_a) and protein/phospholipid ratios (mean ± SD) in *Bifidobacterium animalis* IPLA 4549 and its bile resistant mutant *B. animalis* 4549dOx grown in the presence (+bile) or absence (−bile) of ox gall

	B. animalis IPLA 4549			*B. animalis* 4549dOx		
	−bile	+bile	% decrease	−bile	+bile	% decrease
K_a	0.35 ± 0.02	0.25 ± 0.01	28.6	0.25 ± 0.02	0.19 ± 0.04	24.0
Protein/phospholipid ratio	7.94 ± 1.19	ND		4.24 ± 0.33	2.36 ± 0.57	44.3

ND, not determined.

Table 2. Fatty acid composition (% of total fatty acids; mean ± SD) of *Bifidobacterium animalis* IPLA 4549 and its bile resistant mutant *B. animalis* 4549dOx grown in the presence (+bile) or absence (−bile) of ox gall

	B. animalis IPLA 4549		*B. animalis* 4549dOx	
Fatty acids	−bile	+bile	−bile	+bile
9 : 0	–	0.14 ± 0.03	–	0.19 ± 0.01
10 : 0	0.27 ± 0.02	–	0.34 ± 0.11	0.07 ± 0.01
12 : 0	0.63 ± 0.06	0.14 ± 0.06	2.27 ± 1.02	0.19 ± 0.01
13 : 0	–	0.11 ± 0.05	–	–
14 : 0*	3.09 ± 0.02	2.36 ± 1.04	6.60 ± 2.44	1.8 ± 0.25
15 : 0*	–	1.09 ± 0.18	–	0.99 ± 0.10
16 : 0*	17.29 ± 1.99	17.45 ± 1.52	20.29 ± 1.80	16.42 ± 0.63
16 : 1w7	0.89 ± 0.04	0.19 ± 0.03	2.17 ± 0.61	0.29 ± 0.02
16 : 1w9	0.25 ± 0.01	0.92 ± 0.07	0.29 ± 0.07	1.34 ± 0.02
16 : 1w11	–	–	–	0.47 ± 0.03
17 : 0	–	0.44 ± 0.18	–	0.48 ± 0.02
18 : 0	4.04 ± 0.55	4.51 ± 2.51	4.04 ± 1.06	4.60 ± 0.32
18 : 0 12OH	3.02 ± 0.26	12.51 ± 3.15	–	–
18 : 1w9*	49.26 ± 2.59	43.79 ± 0.03	39.71 ± 0.66	54.06 ± 0.62
18 : 1w11*	–	0.35 ± 0.01	–	0.49 ± 0.01
18 : 2w9,12	–	3.65 ± 0.29	–	4.65 ± 0.08
19 : 0cyc*	11.11 ± 1.76	0.57 ± 0.20	12.31 ± 0.95	0.57 ± 0.03
Total BCFA (iso+anteiso 15+16+17)	–	1.97 ± 0.50	–	1.54 ± 0.07
Not identified	10.19 ± 1.33	9.85 ± 1.37	11.99 ± 3.38	11.90 ± 0.03
Total DMA	10.60 ± 0.30	2.96 ± 1.29	12.93 ± 0.48	4.25 ± 0.12
Total SFA	28.58 ± 2.16	38.82 ± 0.22	36.90 ± 3.56	24.76 ± 0.66
Total USFA	61.99 ± 0.89	49.61 ± 0.45	54.75 ± 2.42	61.93 ± 0.58
USFA/SFA	2.18 ± 0.19	1.28 ± 0.00	1.49 ± 0.16	2.50 ± 0.09

*Fatty acids also including DMA forms.

–, not detected.

USFA, unsaturated fatty acids; SFA, saturated fatty acids; BCFA, branched chain fatty acids; DMA, dimethyl acetal fatty acids.

Because pyrene excimer lifetime does not show variations during the time of analysis, the decrease in K_a was due to reduced lateral diffusion of the probe, probably indicating increased membrane viscosity in the bile-resistant mutant compared with the WT, and in both strains in the presence of bile. The significance of these alterations in the biophysical characteristics of the membrane could be related to enable better control of some functional aspects, such as transporters and signal-transduction systems (Barenholz *et al.*, 1996). Furthermore, the regulation of membrane fluidity through fatty acid alterations is a way for the bacteria to respond to environmental challenge (Denich *et al.*, 2003).

A preliminary thin-layer chromatography analysis of polar lipids in *B. animalis* IPLA 4549 and *B. animalis* 4549dOx indicated important qualitative changes in the lipid composition of their membranes (data not shown). This encouraged the study of fatty acid profiles of both strains grown in the absence and presence of bile. Two major fatty acids (accounting for over 50% of the total fatty acids) were found, corresponding to 16 : 0 and 18 : 1w9c (Table 2). The most significant change observed in the mutant with respect to the WT was a large increase of saturated fatty acids (SFA), and a decrease of the unsaturated ones (USFA), and this was reflected in a ratio USFA : SFA of 2.18 for the WT and 1.49 for the mutant (Table 2). This is in agreement with

the pyrene excimerization rates, since a higher proportion of SFA is usually related to a decreased mobility of membrane probes, generally reflected in decreasing the numbers of double bonds in acyl chains or increasing their length (Beney & Gervais, 2001; Loffhagen *et al.*, 2004).

On the other hand, different environmental factors have been shown to influence the fatty acid composition of bacteria (Quivey *et al.*, 2000; Guerzoni *et al.*, 2001; Fozo *et al.*, 2004). In this case, the unusual 10-hydroxy-octanodecanoic acid (18 : 0 12OH) was only detected in the WT, and its percentage increased more than four times in the presence of bile. In *Lactobacillus acidophilus*, an increase in hydroxy fatty acids has been reported when grown at suboptimal temperatures (Fernández-Murga *et al.*, 2001). These molecules seem to play a role in cell membrane permeability. In the authors' strains, WT and mutant, bile promoted a decrease of cyclic fatty acids. A similar reduction was reported for *Lactobacillus reuteri*, attributed to the inhibition exerted by bile on some fatty-acid-metabolizing enzymes (Taranto *et al.*, 2003). In contrast, bile induced the synthesis of branched-chain 15 : 0, 16 : 0 and 17 : 0 fatty acids (Table 2) in WT and mutant strains. A higher quantity of branched-chain fatty acids has been previously detected under alkaline conditions in *Listeria monocytogenes* (Giotis *et al.*, 2007). These fatty acids, typical of Gram-positive bacteria (Hopkins & MacFarlane, 2000), have physicochemical characteristics very similar to USFA. Thus, its presence could contribute to the modification of the viscosity of the membrane when cells were grown in the presence of bile. In this respect, Goitis and colleagues suggest that the balance between the different branched-chain fatty acids could be critical in pH adaptation. However, the influence of bile on the ratios USFA/SFA was different in both strains. Whereas bile induced an increase in the ratio in the mutant, a decrease was detected in the WT. Related to this, it is worthwhile to point out that a much lower protein to phospholipid ratio in the cell membranes of the mutant strain was detected in the presence of bile (44.3% of reduction), compared with the conditions in which bile was absent (Table 1). Membrane proteins represent mechanical barriers for lipids. The presence of proteins decreases the diffusion coefficient of lipids in biological membranes, in a way that is related to the protein to lipid ratio (Engelke *et al.*, 1996). Thus, the relative decrease of protein in the membrane of the mutant in the presence of bile is not in agreement with the decrease of K_a detected in the mutant under the same conditions. In relation to this, it must be pointed out that only phospholipids (and not the total lipid fraction) are considered in the ratio; therefore the influence of the other lipids is going unnoticed in the analysis. In addition, pyrene lateral diffusion is directly related to the lateral diffusion of fatty acid chains in the membrane, but other factors also affect the physical state and the viscosity of the membrane, such as (1) rotational diffusion of the fatty acyl chains inside the bilayer and (2) transbilayer lipid diffusion ('flip-flop') (Aricha *et al.*, 2004). Furthermore, possible secondary effects, such as changes in the activities of membrane-embedded enzymes as a result of altered membrane characteristics, should be taken into account.

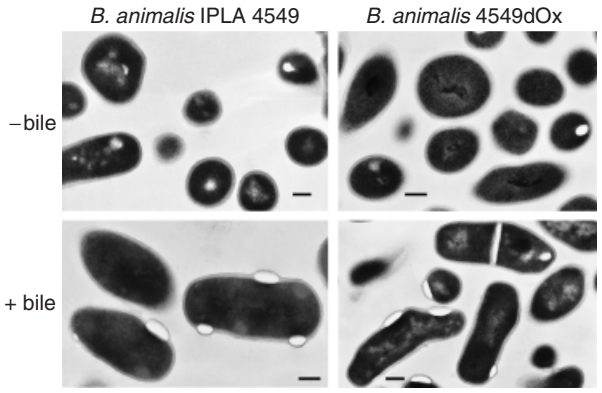

Fig. 2. Transmission electron microscopy of *Bifidobacterium animalis* IPLA 4549 and *B. animalis* 4549dOx cells grown in the absence (−bile) or in the presence (+bile) of ox gall. The bar corresponds to 250 nm.

Unfortunately, the protein to phospholipid ratio could not be calculated for the WT since bile interferes with the colorimetric reaction of phospholipid determination, and the membranes of the WT accumulated bile to a much higher extent than the membranes of the mutant. This suggests that the WT can embed bile salts into its membrane, and the mutant does it in a much lower proportion. This fact also indicates that the membrane of the mutant could be more impermeable to bile than the membrane of the WT, partially explaining the higher bile tolerance of *B. animalis* 4549dOx.

Morphological changes also occurred as a consequence of bile action. In previous studies, it was observed, by optical microscopy, that bile strongly influences the cell shape in *Bifidobacterium* (Margolles *et al.*, 2003). In this work, it was analysed by TEM the influence that the adaptation or the exposure to bile has in *B. animalis* cells. Remarkably, the most significant changes were apparent when cells, either WT or mutant, were grown in the presence of bile (Fig. 2). In the absence of bile, clearly defined cell walls and cell membranes were detected. In contrast, bile-treated cells displayed marked changes on the cell surface and vesicle-like structures became apparent. These structures retained an electron-dense surrounding cover, and an interior space, which was transparent to the electrons. In this respect, a similar result has been reported for *Lactobacillus reuteri* as a reponse to the bile salt deoxycholate (Taranto *et al.*, 2006). Recently, McBroom & Kuehn (2007) demonstrated that *Escherichia coli* alleviates stress by producing membrane vesicles for removal of toxic missfolded proteins, and they

suggest this vesiculation phenotype as a general envelope stress response mechanism in Gram-negative bacteria. The data of this paper could indicate a similar behaviour in *B. animalis*. This surface response under stress conditions has not been described before in *Bifidobacteria*, and further research is needed to characterize this phenomenon. In short, bile has the capacity to modulate the lateral diffusion of *B. animalis* membranes and their fatty acid composition, and to change the ratio between membrane proteins and phospholipids. Long-term exposure to bile, represented in this work by the bile-adapted strain, also induced stable changes in the membrane features, which are maintained even in the absence of bile, likely contributing to the stable bile-resistance phenotype of *B. animalis* 4549dOx.

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References

Araya M, Morelli L, Reid G, Sanders ME & Stanton C (2002) Guidelines for the evaluation of probiotics in foods. FAO/WHO Report. On line (http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf).

Aricha B, Fishov I, Cohen Z *et al.* (2004) Differences in membrane fluidity and fatty acid composition between phenotypic variants of *Streptococcus pneumoniae*. *J Bacteriol* **186**: 4638–4644.

Barenholz Y, Cohen T, Haas E & Ottolenghi M (1996) Lateral organization of pyrene-labeled lipids in bilayers as determined from the deviation from equilibrium between pyrene monomers and excimers. *J Biol Chem* **271**: 3085–3090.

Begley M, Gahan CG & Hill C (2005) The interaction between bacteria and bile. *FEMS Microbiol Rev* **29**: 625–651.

Beney L & Gervais P (2001) Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses. *Appl Microbiol Biotechnol* **57**: 34–42.

Bron PA, Marco M, Hoffer SM, Van Mullekom E, de Vos WM & Kleerebezem M (2004) Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract. *J Bacteriol* **186**: 7829–7835.

Denich TJ, Beaudette LA, Lee H & Trevors JT (2003) Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. *J Microbiol Methods* **52**: 149–182.

Engelke M, Bojarski P, Diehl HA & Kubicki A (1996) Protein-dependent reduction of the pyrene excimer formation in membranes. *J Membr Biol* **153**: 117–123.

Fernández-Murga ML, Font de Valdez G & Disalvo EA (2001) Effect of lipid composition on the stability of cellular membranes during freeze–thawing of *Lactobacillus acidophilus* grown at different temperatures. *Arch Biochem Biophys* **388**: 179–184.

Fozo EM, Kajfasz JK & Quivey RG Jr (2004) Low pH-induced membrane fatty acid alterations in oral bacteria. *FEMS Microbiol Lett* **238**: 291–295.

Garrigues C, Stuer-Lauridsen B & Johansen E (2005) Characterization of *Bifidobacterium animalis* subsp. *lactis* BB-12 and other probiotic bacteria using genomics, transcriptomics and proteomics. *Aust J Dairy Technol* **60**: 84–92.

Giotis ES, McDowell DA, Blair IS & Wilkinson BJ (2007) Role of branched-chain fatty acids in pH stress tolerance in *Listeria monocytogenes*. *Appl Environ Microbiol* **73**: 997–1001.

Gómez-Zavaglia A, Kociubinski G, Pérez P, Disalvo E & De Antoni G (2002) Effect of bile on the lipid composition and surface properties of bifidobacteria. *J Appl Microbiol* **93**: 794–799.

Gueimonde M, Noriega L, Margolles A, de los Reyes-Gavilán CG & Salminen S (2005) Ability of *Bifidobacterium* strains with acquired resistance to bile to adhere to human intestinal mucus. *Int J Food Microbiol* **101**: 341–346.

Guerzoni ME, Lanciotti R & Cocconcelli PS (2001) Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*. *Microbiology* **147**: 2255–2264.

Harmsen HJ, Wildeboer-Veloo AC, Raangs GC *et al.* (2000) Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr* **30**: 61–67.

Hopkins MJ & MacFarlane GT (2000) Evaluation of 16s rRNA and cellular fatty acid profiles as markers of human intestinal bacterial growth in the chemostat. *J Appl Microbiol* **89**: 668–677.

Kurdi P, Kawanishi K, Mizutani K & Yokota A (2006) Mechanism of growth inhibition by free bile acids in *Lactobacilli* and *Bifidobacteria*. *J Bacteriol* **188**: 1979–1986.

Loffhagen N, Hartig C & Babel W (2004) *Pseudomonas putida* NCTC 10936 balances membrane fluidity in response to physical and chemical stress by changing the saturation degree and the *trans*/*cis* ratio of fatty acids. *Biosci Biotechnol Biochem* **68**: 317–323.

Lowry OH, Rosebrough NJ, Farr AL & Randall R (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275.

Margolles A, García L, Sánchez B, Gueimonde M & de Los Reyes-Gavilán CG (2003) Characterisation of a *Bifidobacterium* strain with acquired resistance to cholate – a preliminary study. *Int J Food Microbiol* **82**: 191–198.

Masco L, Huys G, De Brandt E, Temmerman R & Swings J (2005) Culture-dependent and culture-independent qualitative analysis of probiotic products claimed to contain *Bifidobacteria*. *Int J Food Microbiol* **102**: 221–230.

McBroom AJ & Kuehn MJ (2007) Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. *Mol Microbiol* **63**: 545–558.

Noriega L, Gueimonde M, Sánchez B, Margolles A & de los Reyes-Gavilán CG (2004) Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low pH and cross-resistance to bile salts in *Bifidobacterium*. *Int J Food Microbiol* **94**: 79–86.

Noriega L, Cuevas I, Margolles A & de los Reyes-Gavilán CG (2006) Deconjugation and bile salts hydrolase activity by *Bifidobacterium* strains with acquired resistance to bile. *Int Dairy J* **16**: 850–855.

Price CE, Reid SJ, Driessen AJ & Abratt VR (2006) The *Bifidobacterium longum* NCIMB 702259T *ctr* gene codes for a novel cholate transporter. *Appl Environ Microbiol* **72**: 923–926.

Quivey RG Jr, Faustoferri R, Monahan K & Marquis R (2000) Shifts in membrane fatty acid profiles associated with acid adaptation of *Streptococcus mutans*. *FEMS Microbiol Lett* **189**: 89–92.

Rouser G, Fkeischer S & Yamamoto A (1970) Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5**: 494–496.

Ruas-Madiedo P, Hernández-Barranco A, Margolles A & de los Reyes-Gavilán CG (2005) A bile salt-resistant derivative of *Bifidobacterium animalis* has an altered fermentation pattern when grown on glucose and maltose. *Appl Environ Microbiol* **71**: 6564–6570.

Salminen SJ, Gueimonde M & Isolauri E (2005) Probiotics that modify disease risk. *J Nutr* **135**: 1294–1298.

Sánchez B, Champomier-Vergès M-C, Anglade P *et al.* (2005) Proteomic analysis of global changes in protein expression during bile salt exposure of *Bifidobacterium longum* NCIMB 8809. *J Bacteriol* **187**: 5799–5808.

Sánchez B, de los Reyes-Gavilán CG & Margolles A (2006) The F_1F_0-ATPase of *Bifidobacterium animalis* is involved in bile tolerance. *Environ Microbiol* **8**: 1825–1833.

Taranto MP, Fernández-Murga ML, Lorca G & de Valdez GF (2003) Bile salts and cholesterol induce changes in the lipid cell membrane of *Lactobacillus reuteri*. *J Appl Microbiol* **95**: 86–91.

Taranto MP, Pérez-Martínez G & Font de Valdez G (2006) Effect of bile acid on the cell membrane functionality of lactic acid bacteria for oral administration. *Res Microbiol* **157**: 720–725.

Vanounou S, Parola AH & Fishov I (2003) Phosphatidylethanolamine and phosphatidylglycerol are segregated into different domains in bacterial membrane. A study with pyrene-labelled phospholipids. *Mol Microbiol* **49**: 1067–1079.