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MORPHOMETRIC PARAMETERS, ZETA POTENTIAL AND GROWTH RATE OF *Lactobacillus casei* Shirota BY EFFECT OF DIFFERENT BILE SALTS

PARÁMETROS MORFOMÉTRICOS, POTENCIAL ZETA Y TASA DE CRECIMIENTO DE *Lactobacillus casei* Shirota POR EFECTO DE DIFERENTES SALES BILIARES

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Abstract

Effects of primary and secondary bile salts, conjugated to glycine [glycocholic (GCA) and glycodeoxycholic acid (GDCA)], taurine [taurocholic acid (TCA), taurodeoxycholic acid (TDCA)] on morphometry and growth rate of *Lactobacillus casei* Shirota were studied. Images obtained by scanning electron microscopy showed modification ($p < 0.05$) in cell minor axis and aspect ratio. The former was modified by GCA while the second by all the bile salts used. The growth rate (μ) values were: 1.95, 0.66, 0.32, 1.89 and 1.86 h^{-1} in MRS medium without salts and with GCA, GDCA, TCA and TDCA, respectively. Estimated doubling time was: 0.35, 0.36 and 0.37 h for MRS medium without salts, and with TCA and TDCA, respectively. For GCA and GDCA, a decrease in growth was observed from 4 h and 2 h of incubation, respectively. Bile salt hydrolase activity was determined qualitatively and quantitatively. *L. casei* Shirota showed activity on taurine-conjugated bile salts. The zeta potential was determined by using the same conditions as those for quantification of bile salt hydrolase activity and different potentials for each dispersion system were found; the zeta potential values were related to the metabolism of *L. casei* Shirota.

Keywords: *Lactobacillus casei* Shirota, growth rate, bile salts, morphometric parameters, bile salt hydrolase activity, zeta potential.

Resumen

Se estudió el efecto de sales biliares primarias y secundarias, conjugadas a glicina [ácido glicocólico (GCA), ácido glicodeoxicólico (GDCA)] y taurina [ácido taurocólico (TCA), ácido taurodeoxicólico (TDCA)] sobre los parámetros morfométricos y la tasa de crecimiento de *Lactobacillus casei* Shirota. Imágenes obtenidas por microscopia electrónica de barrido mostraron modificación ($p < 0.05$) del eje menor y la relación de aspecto celular. El primero fue modificado por la sal primaria GCA y el segundo por todas las sales. La tasa especifica de crecimiento (μ) fue: 1.95, 0.66, 0.32, 1.89 y 1.86 h^{-1} en medio MRS sin sales, con GCA, GDCA, TCA y TDCA, respectivamente. El tiempo de duplicación estimado fue: 0.35, 0.36 y 0.37 h para medio MRS sin sales, TCA y TDCA, respectivamente. Para GCA y GDCA, el crecimiento disminuyó desde las 4 y 2 h de incubación, respectivamente. La actividad sal biliar hidrolasa fue determinada cualitativa y cuantitativamente. *L. casei* Shirota mostró actividad sobre sales conjugadas a taurina. Se determinó el potencial zeta para *L. casei* Shirota baja las mismas condiciones que para la cuantificación de la actividad sal biliar hidrolasa y se encontraron diferentes valores para cada sistema de dispersión asociados al metabolismo de la bacteria.

Palabras clave: *Lactobacillus casei* Shirota, tasa de crecimiento, sales biliares, parámetros morfométricos, actividad sal biliar hidrolasa, potencial zeta.

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1 Introduction

Addition of bile salts to culture media to study the tolerance of probiotics to gastrointestinal tract conditions is a common practice (Begley, Gahan and Hill, 2005) and the numerous physicochemical changes and possible perturbations to microbial communities within this tract have not been adequately studied (Sooresh *et al*., 2012). Cell membrane integrity determines ion distribution at both sides of the cell membrane and consequently surface potential (i.e. the charge distribution at the bacterial surface) is related to the conditions at which bacteria grow. Thus, the surface potential (in the form of zeta potential) of individual cells can be considered as an index of the metabolic state of the bacteria (Gomez Zavaglia *et al*., 2003) and in particular, the presence of bile salts in culture media affects the bacterial surface properties of microorganisms (Schar-Zammaretti *et al*., 2005).

In recent years, image analysis methods have been applied for quantitative evaluation of morphology and texture of biological objects by extracting information from images captured with different acquisition systems. The extracted information (morphological parameters) is useful for translating the biological systems' complexity into numeric data in order to improve the understanding of structure-function relationships of such systems, for instance food and micoorganisms, and to characterise their morphological features such as shape and roughness (Chanona-Pérez *et al*., 2008). A correlation of the changes in the zeta potential with the observed changes in the cell surface by electron microscopy would suggest that surface potential measurements are suitable to be used for detecting surface changes in bacteria, assuming that zeta potential is the difference in electrical potential between the slip plane around the surface of the bacterium and the bulk-surrounding medium, i.e. it is a measure of the net distribution of electrical charge on the surface of the bacterium (Fernandez Murga, Font de Valdez and Disalvo, 2000).

A number of foods containing probiotic microorganisms have been widely promoted given the health benefits they can offer to consumers (Granato *et al*., 2010; Villa-García *et al*., 2013). Probiotics are defined as "live microorganisms, which upon ingestion of a certain amount exert health benefits on the host beyond inherent basic nutrition" (Ejtahed *et al*., 2011; González-Olivares *et al*., 2011; Rodríguez-Huezo *et al*., 2011). Most of the studies on probiotics correspond to genera Lactobacillus and Bifidobacterium (Patel *et al*, 2010). In particular, Lactobacillus casei Shirota has been widely used as a probiotic (Dunne *et al*., 1999; Coeuret, Gueguen and Vernoux, 2004; Ohashi, Umesaki and Ushida, 2004; Cogan *et al*., 2007; Figueroa-González *et al*., 2010; Guo *et al*., 2011). The preservation of probiotic viability represents a challenge during production and storage of functional foods, where a considerable amount of cell-disruptive agents exist due to the influence of manufacturing parameters upon cell metabolism including, temperature, diffusion, processing time and mechanical pressure, among others. Several obstacles are further encountered during gastro-intestinal (GI) transit (pH, enzymes, bile salts), all of which limit probiotic survival and functionality for the conveyance of health benefits (Doherty *et al*., 2011; Klaenhammer and Kullen, 1999). Hence, information about morphological and growth rate parameters when *Lactobacillus casei* Shirota is grown in media containing bile acids is of relevance.

Bile acids are synthesised from cholesterol and conjugated to either glycine or taurine in the liver. Then, they pass into the intestine, where the amino acid may be hydrolysed from the conjugated bile acid by means of enzymes (Moser and Savage, 2001) produced by gastrointestinal microorganisms such as *Bacteroides*, *Clostridium*, *Enterococcus*, *Bifidobacterium*, and *Lactobacillus*. Particularly in the case of *Lactobacillus*, some authors have reported bile salt hydrolase (BSH) activity in different subspecies of *Lactobacillus casei*; however, others have reported the opposite (Gilliland, Staley and Bush, 1984; Brashears, Gilliland and Buck, 1998; Corzo and Gilliland, 1999 a, b; Tanaka *et al*., 1999; Liong and Shah, 2005; Kim *et al*., 2008; Patel *et al*., 2010).

Bile salt hydrolase is an enzyme (EC 3.5.1.24) which catalyses the hydrolysis of conjugated bile salts in the amide bond on the C-24 position of the steroid. In a host, after deconjugation, bile salts are precipitated and excreted via the faeces (Shimada, Bricknell, and Finegold, 1969). Cholesterol, the precursor of bile salts, is used to replace the amount of bile salts lost and this diminishes serum cholesterol (De Smet, De Boever, and Verstraete, 1998; Begley, Hill and Gahan, 2006).

In vitro, BSH activity is determined by qualitative and quantitative methods. In the former, a plate assay is often carried out; however, many times, available non-consumed bile salts may produce false negative results. The latter may consist of measurement of conjugated bile acid by HPLC, quantification of free

bile acids by spectrophotometry, or measurement of the amount of taurine and glycine released after hydrolysis from the conjugated bile salts by using a colorimetric assay. These methods are reliable but complicated and time-consuming (Guo *et al*., 2011).

The aim of this work was to study the effect of different bile salts on cell morphology and growth rate as well as the relation of zeta potential with the bile salt hydrolase activity of *Lactobacillus casei* Shirota.

2 Material and methods

2.1 Microorganisms

Lactobacillus casei Shirota, isolated from a commercial product (Yakult ®), was used in this study and biochemical characteristics such as Gram staining and catalase activity were evaluated. The spread of the microorganism was performed by using MRS agar and incubating at 37 °C for 24 h. Biomass was transferred to MRS broth and incubated at 37 °C for 24 h in order to prepare enough experimental stock. Subsequently, the broth containing the biomass was centrifuged at 29286 g for 30 minutes at 4 °C under sterile conditions. The cell pellet was suspended in 10 mL of MRS broth with 50% glycerol (Schillinger, Holzapfel Guigas and Heinrich, 2005). *E. coli* ATCC 160211was used as negative control in all experiments.

2.2 Bile salts effect on the growth rate of L. casei Shirota

Growth rate determinations were done in triplicate by using MRS broth without salts and 4 MRS media individually added with 0.5% w/v of the following bile salts: glycocholic, glycodeoxycholic, taurocholic and taurodeoxycholic acid (Sigma-Aldrich, USA). The initial inoculum consisted of *L. casei* Shirota in MRS broth at 37 °C with a concentration of 3.9×10^4 CFU/mL which corresponded to 0.05 absorbance at 600 nm. After inoculation, the culture media were incubated at 37 °C in a New Brunswick Scientific shaker G25 (140 rpm, 1 inch, 2.54 cm of circular orbit) for 72 h. Growth was measured to each broth at 0, 2, 4, 6, 8, 12, 24, 48, and 72 h by plate count (Paw *et al*., 1994). Specific growth rate (μ) expressed in h^{-1} and duplication time expressed in h, were estimated by using Eqs. 1 and 2.

Specific growth rate (μ):

$$\mu = \frac{\ln N_t - \ln N_0}{t - t_0} \tag{1}$$

N_t: number of bacteria at any time.
N_0: number of bacteria at start.
t: time.
t_0: initial time.

Duplication time was estimated (T_d) as follows:

$$T_d = \frac{\ln_2}{\mu} \tag{2}$$

2.3 Determination of the morphology of L. casei Shirota by scanning electron microscopy

Lactobacillus casei Shirota was grown in MRS broth during 8 h (initial) and 72 h (no-salts) as well as in MRS broth individually added with 0.5% w/v of the following bile salts: glycocholic, glycodeoxycholic, taurocholic and taurodeoxycholic acids during 72 h. After incubation at 37 °C, 1 mL of each broth was used for examination by scanning electron microscopy (SEM) JEOL JSM-5800 (JEOL USA Inc.). Each sample was prepared by washing three times with phosphate buffer 0.1 M pH 7.2 at room temperature and fixed with glutaraldehyde 2.5% and 1% osmium tetroxide (0.1 M) for 1 hour at room temperature and sequentially dehydrated by using ethanol at 30, 40, 50, 60, 70, 80 and 90 %. To ensure complete ethanol saturation, samples were then immersed in fresh 100% ethanol three times for ten minutes and were critical-point dried by using an EMS 850 Critical Point Dryer (Electron Microscopy Science, USA.). Samples were finally coated with gold, by using a Denton Vacuum Desk-2 (Denton Vaccum, USA) and examined by SEM.

2.4 Image analysis

The SEM micrographs obtained at 5000x (720 × 480 pixels resolution) were processed by digital image analysis (DIA) in order to measure the length of cell major (shortest distance between the rounded edges of the cell projected area) and minor (shortest distance between the flat edges of the cell projected area) axes, which are perpendicular to each other, and the aspect ratio (major axis/minor axis) of the cells at the initial stage and after each treatment. The software used was ImageJ 1.48g (National Institutes of Health, USA). By using the scale provided by the SEM, the scale for measurements was calibrated at 26 pixel/μm; thus, the measuring tools of the software package were directly used to determine the parameters mentioned above. Approximately, 20 cells from each micrograph were randomly selected to extract their morphometric data.

2.5 Zeta potential determination

L. casei Shirota was inoculated (1×10^9 CFU/mL) in type 1 water cm during 8 h (initial) and 72 h (no-salts) and in the same water individually added with the following bile salts at concentration of 0.5% w/v, glycocholic, glycodeoxycholic, taurocholic and taurodeoxycholic acids and incubated during 72 h. After incubation at 37 °C, pH and zeta potential were determined by means of a zeta potential analyser (Zeta Plus, Brookhaven Instruments Corporation, USA). Each test was considered as a different dispersion system.

2.6 Bile salt hydrolase activity

2.6.1 Qualitative test in Petri dish

MRS agar Petri dishes were prepared by adding individually 0.5% w/v from each of the following bile salts: glycocholic, glycodeoxycholic, taurocholic and taurodeoxycholic acid, and 0.37 g/L $CaCl_2$. *Lactobacillus casei* Shirota was spread on the medium by using the streaking technique and incubated aerobically at 37 °C for 72 h. The test was considered positive when a precipitate of bile acid was formed around the colony (opaque halo) (Lim, Kim and Lee, 2004).

2.6.2 Qualitative test in thin layer chromatography (TLC)

In order to assess BSH activity by TLC, MRS agar individually added with 0.5% w/v of glycocholic, glycodeoxycholic, taurocholic and taurodeoxycholic acids and 0.37 g/L $CaCl_2$ was prepared. Each plate was inoculated with a sample of the microorganism by the streaking technique and incubated aerobically at 37 °C for 72 h. After that, a colony was taken from the plate together with all the precipitate caused by the presumptive BSH activity and dissolved in 1 mL of HCl 0.01 N at 45 °C. An aliquot of 1 mL of ethyl acetate was added, stirred vigorously and allowed to stand until phase separation was observed. A sample of 1 μL from the organic phase (ethyl acetate) was taken and revealed on a (20×20) cm^2 silica gel 60 plate (Merck, Darmstadt, Germany).

The mobile phase consisted of isoamyl acetate, propionic acid, n-propanol and water (40:30:20:10). Standards at 0.5% w/v (cholic and deoxycholic acid) were dissolved in ethyl acetate and analysed by using the same procedure. When the solvent front was 1 to 2 cm from the top edge of the plate, the plates were dried at room temperature. After that, the plates were dipped in *p*-anisaldehyde and heated to 110 °C for 5 min (Šušković, Matosic, and Besendorfer, 2000; Guo *et al*., 2011).

2.6.3 Quantification of bile salt hydrolase activity

The method described by Tanaka *et al*. (1999) was adapted to quantify BSH activity. *L. casei* Shirota was inoculated at a concentration of 1×10^8 CFU/mL in type 1 water containing 0.5% w/v of each of the following acids: glycocholic, glycodeoxycholic, taurocholic or taurodeoxycholic and incubated at 37° C for 72 h. After incubation, a 1 mL aliquot was centrifuged for 5 min at 29286 g at 4 °C and 50 μL of the supernatant were mixed with 50 μL of sodium phosphate buffer (0.1 M, pH 6.0) and 100 μL of a solution of each bile salt at 0.5 % w/v.

With the purpose of protecting the enzyme against oxidation, 10 μL of dithiothreitol (DTT) 10 mM were added and after incubation at 37°C for 30 min, an aliquot of 100 μL of trichloroacetic acid was added to the system in order to stop the enzyme activity. Subsequently, the mixture was centrifuged during 5 min at 29286 g, at 4 °C and 50 μL of the supernatant were mixed with 50 μL of distilled water and 1.9 mL of ninhydrin reagent [0.5 mL of 1% w/v ninhydrin in citrate buffer 0.5 M (pH 5.5), 1.2 mL glycerol, 0.2 mL of buffer solution 0.5 M citrate, pH 5.5]. The mixture was boiled for 14 min, cooled for 3 min by using an ice bath and the absorbance at 570 nm was, finally, determined. Previously, a standard curve was prepared for each assay using glycine or taurine and 10 μL of DTT. The unit of BSH activity was defined as the amount of enzyme catalysing the release of 1 mmol of amino acids per 1 mL of substrate per minute and expressed per unit gram of protein. Protein concentration was determined by the Bradford method by using the Bio-Rad Protein kit (Bio-Rad, USA, CA); bovine serum albumin was used as a standard for all tests (Tanaka *et al*., 1999).

2.7 Statistical analysis

ANOVA tests were performed (SigmaPlot 12.5, Systat Software, USA) with the aim of determining the statistical significance of the morphometric parameters measured by DIA. Student-Newman-Keuls test was used for comparison of means. The significance level was set at $2\alpha = 0.05$.

3 Results and discussion

3.1 Bile salts effect on L. casei Shirota growth

Figure 1 shows the negative effect of primary and secondary bile salts conjugated to glycine and taurine on bacterium rate growth. The growth in MRS medium without salts is shown in Figure 1a, the growth rate obtained was $\mu = 1.95\ h^{-1}$. The addition of glycocholic and glicodeoxicholic acids decreased the initial rate to $0.66\ h^{-1}$ (Fig. 1(b)) and $0.32\ h^{-1}$ (Fig. 1c), respectively. The growth rate obtained for taurine conjugates were similar to the media without salts, the obtained rates were, $1.89\ h^{-1}$ and $1.95\ h^{-1}$ for taurocholic (Fig. 1d) and taurodeoxycholic acids (Fig. 1e), respectively. While duplication time was estimated as 0.35, 0.36 and 0.37 h, for no-salts, taurocholic and taurodeoxycholic acids, respectively, it was not possible to estimate this parameter for glycocholic and glycodeoxycholic acids because a decrease in the growth rate was observed from 4 and 2 h of incubation (Fig. 1b and c). The major function of bile *in vivo* is to act as an emulsifier of lipids (De Smet, De Boever, and Verstraete, 1998). Therefore, bile has an antimicrobial action, which primarily disrupts cell membranes. Several factors determine the exact outcome of bile effects on cell membranes such as alterations in charge, hydrophobicity and lipid fluidity. First, the concentration of bile is of major importance; the type and structure of bile imposing the stress as well as cell membrane architecture and composition are factors that play a key role in bile resistance (Begley, Gahan and Hill, 2005).

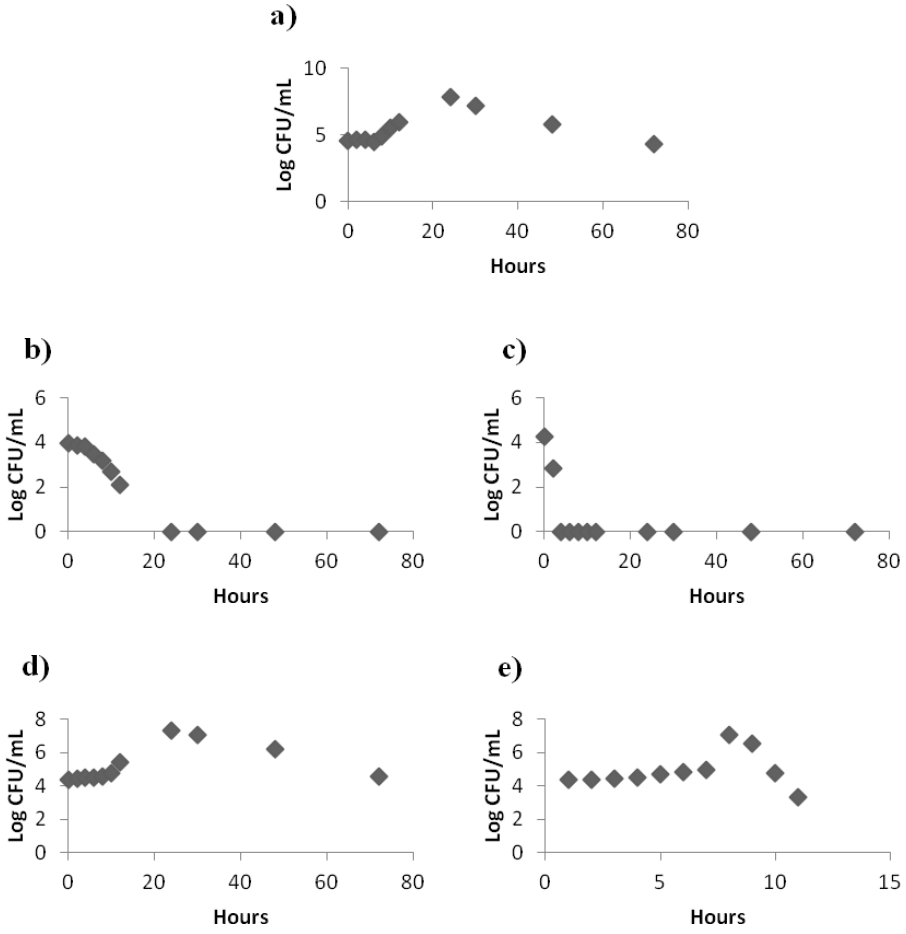

Fig. 1. Effect of bile salts on the growth of L. casei Shirota. a) No-salts ($\mu = 1.95\ h^{-1}$), b) glycocholic acid ($\mu = 0.66\ h^{-1}$), c) glycodeoxycholic acid ($\mu = 0.32\ h^{-1}$), d) taurocholic acid ($\mu = 1.89\ h^{-1}$), and e) taurodeoxycholic acid ($\mu = 1.86\ h^{-1}$).

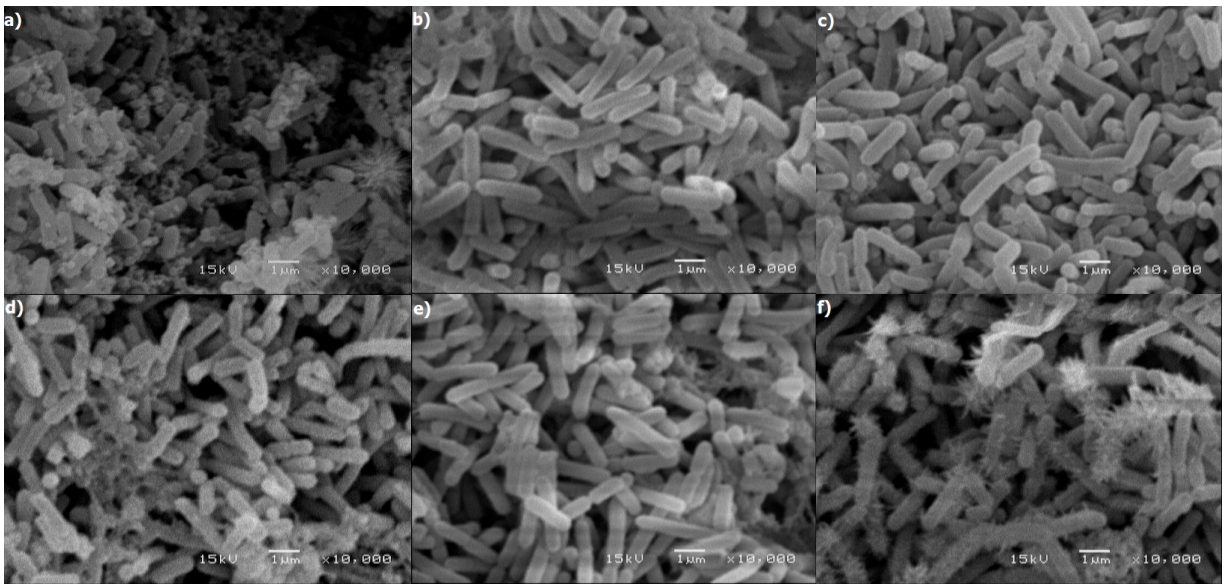

Fig. 2. SEM micrographs of *L. casei* Shirota. a) Initial (8 h), b) No-salts (72 h), c) glycocholic acid (72 h), d) glycodeoxycholic acid (72 h), e) taurocholic acid (72 h), f) taurodeoxycholic acid (72 h).

According to some authors, the survival capacity of probiotics with no BSH activity in intestine may result altered (Begley, Hill and Gahan, 2006). In the present study, it was confirmed that different types of bile salts (primary or secondary, conjugated to glycine or taurine) differently affect the growth rate of *L. casei* Shirota. Regarding the effect of glycocholic and glycodeoxycholic acids on *L. casei* Shirota growth rate, this might be related to the lack of glycine-conjugated bile acid hydrolase activity (Fig. 4).

3.2 Digital image analysis

SEM micrographs of *L. casei* Shirota are shown in Figure 2. Image analysis revealed few changes of morphometric parameters influenced by primary and secondary bile salts conjugated to glycine or taurine. The results for aspect ratio of the cells are shown in Figure 3. While the variation in the length of cell minor axis was significant ($p < 0.05$) between cells grown in glycocholic acid (0.43 μm) (primary bile salt), as compared to all others (Initial: 0.49; No-salts: 0.51; GDC: 0.49; TC: 0.49; TDC: 0.53 μm), there was no significant difference in the length of the major axis (Initial: 1.83; No-salts: 1.56; GC: 1.44; GDC: 1.63; TC: 1.57; TDC: 1.69 μm). Cell aspect ratio was the morphological parameter in which more differences were observed (Fig. 3). Between initial and no-salts samples there were significant differences ($p < 0.05$), which might be attributed to incubation time. Nevertheless, between no-salts and glycocholic, glycodeoxycholic, taurocholic and taurodeoxycholic acids, there were significant ($p < 0.05$) differences that are probably related to the presence and type (primary or secondary, conjugated to glycine or taurine) of each bile salt, to the effect of each bile salt on the growth rate and also might be related to the BSH activity, since cell aspect ratio was different between each type

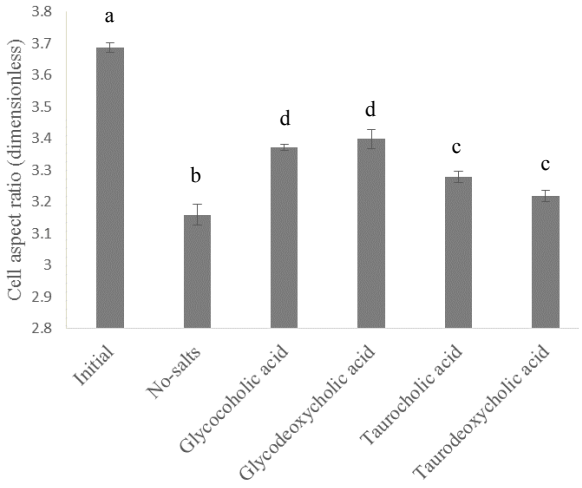

Fig. 3. Results of cell aspect ratio of *Lactobacillus casei* Shirota obtained by digital image analysis at the initial stage (8 h) and after 72 h of treatment with bile salts. Different letters on each bar mean significant statistical difference.

of bile salt used. Changes in cell aspect ratio can be read as modifications in cell shape, as observed in the SEM micrographs; these modifications might be associated also to the growth rate and the stress experienced by the lipolytic effect of bile salts. In the case of taurine conjugates (primary and secondary), the cell aspect ratio of *L. casei* Shirota was higher, as well the BSH activity showed the same behaviour; hence, the structural conformation adopted by *L. casei* Shirota and the hydrolase activity might have the aim of protecting the cell structure.

3.3 Zeta potential determination

In this work it was measured the zeta potential of a *L. casei* Shirota in the presence of different bile salts: glycocholic, glycodeoxycholic, taurocholic and taurodeoxycholic acids. Zeta potential for each dispersion system is summarised in Table 1. After 72 h of incubation each dispersion system showed different pH. However, it is important to highlight that each dispersion system showed a characteristic zeta potential. As stated by some authors, the surface properties of microorganisms depend on the culturing conditions, the metabolic state of the bacteria and the composition of the media including the addition of components such as bile salts (Schär-Zammaretti *et al*., 2005). Thus, the presence of bile salts in growth media in the present work induced the production of some hydrolases by *L. casei* Shirota, which could have modified the surface properties of the microorganism. Therefore, the measurement of this potential might be used as an indicator of the bacterium metabolic state when they are subjected to any particular growth condition (Gomez Zavaglia *et al*., 2003), in the case of the present work, to the presence of different bile salts. Values of zeta potential of each dispersion system might be related to different bile salt hydrolase activity for each type of bile salt (primary or secondary, conjugated to glycine or taurine); nonetheless, a systematic study on this matter is required to verify if all changes in zeta potential are due to the metabolic activity.

3.4 Bile salt hydrolase activity

3.4.1 Qualitative test in Petri dish

BSH activity observed in Petri dish is shown in Table 2. *L. casei* Shirota showed BSH activity only on primary and secondary taurine-conjugated salts.

Table 1. Zeta Potential of *L. casei* Shirota in different bile salts at 72 h

	Zeta Potential (mV)	pH
Initial (8h)	-3.11	3.63
No-salts	-2.64	3.59
Glycocholic acid	13.09	4.73
Glycodeoxycholic acid	7.77	4.19
Taurocholic acid	-12.39	3.41
Taurodeoxycholic acid	6.54	3.39

This is in agreement with Schillinger, Holzapfel Guigas and Heinrich, (2005) who studied the activity of *Lactobacillus* isolated from the commercial product Yakult® and other eight different *Lactobacillus paracasei* (BFE 675 , BFE 697, BFE 715, BFE 723, BFE 732, BFE 736, BFE 742, BFE 743) isolated from several fermented foods [drinkable yogurt (Actimel), yogurt (ABC), Chr.Hansen (*L. casei* 01), yogurt (Yogosan), yogurt (Biogarde plus), yogurt (BI'AC), drinkable yogurt (Procult) and yogurt (Ib Hoch 3)]. The latter did not show taurodeoxycholic acid BSH activity by using an assay similar to the one used in the present work. Some authors have reported that the capability to hydrolyse taurine-conjugates bile salts is not related to the *L. casei* species (Dashkevicz and Feighner, 1989). These authors tested various *L. casei* ATCC 4646, ATCC 4961, ATCC 393, ATCC 4224, ATCC 4007 and ATCC 4940 and did not found activity in BSH Petri dish assay for primary (taurochenodeoxycholic acid) and secondary (taurodeoxycholic acid) bile salts. The results were confirmed by Dashkevicz and Feighner (1989) when doing a chemical assay by using radioisotope C^{14}. Moreover, the same authors suggested that this activity is present in other species of *Lactobacillus*, such as *L. acidophilus*, *L. salivarius* and *L. fermentum*. This assumption was made after evaluating seven different strains of *L. casei*, eighteen different strains of *L. acidophilus*, ten different strains of *L. salivarius* and fourteen different strains of *L. fermentum* (Dashkevicz and Feighner, 1989). While significantly, the most abundant bile acids in human bile are cholic and chenodeoxycholic, all bile acids are conjugated inside the body with either glycine (glycol-conjugated) or taurine (tauro-conjugated) (Patel *et al*., 2010; Begley *et al*., 2005); furthermore, experiments focusing on the resistance of lactobacilli are made against conjugated bile salts (De Smet *et al*., 1995).

Table 2. Bile salt hydrolase activity of *L. casei* Shirota by qualitative methods

	Activity by Petri assay				Activiy by TLC			
Strain	GC	GDC	TC	TDC	GC	GDC	TC	TDC
L. casei Shirota	-	-	+	+	-	-	+	+
E. coli ATCC 160211	-	-	-	-	-	-	-	-

GC: glycocholic acid; GDC: glycodeoxycholic acid; TC: taurocholic acid; TDC: taurodeoxycholic acid. Symbol + indicates presence of bile salt hydrolase activity. Symbol - indicates absence of bile salt hydrolase activity.

3.4.2 Qualitative test in thin layer chromatography (TLC)

The BSH activity results obtained in TLC are shown in Table 2. Through TLC technique it was confirmed that *L. casei* Shirota had BSH activity for primary and secondary bile salts associated to taurine. In both salts, though the associated amino acid is the same, there are differences in their steroidal chains at position 7α. Another microorganism, *Lactobacillus buchneri* JCM1069, has not BSH activity when taurocholic acid (primary bile salt) is used as a substrate, but this microorganism has the ability of hydrolysing taurine-conjugates secondary bile salts (taurodeoxycholic acid) (Patel *et al*., 2010). Moreover, the ratio of glyco-conjugates to taurine-conjugates bile salts in human bile is usually 3:1. *In vitro* experiments have determined that the glyco-conjugates bile salts are more toxic than taurine-conjugates bile salts (Begley, Gahan Hill, 2006). However, the ratio between both bile salts depends on the type of population and feeding; for example, the ratio may be 0.1:1, as in the case of people with diets rich in taurine-containing foods such as meat and seafood (Begley, Hill and Gahan, 2005).

The findings obtained by both qualitative methods were consistent, since in Petri dishes with the culture medium used, as by thin layer chromatography, it was found that *L. casei* Shirota only hydrolysed taurocholic and taurodeoxycholic acids.

3.4.3 BSH activity

The results of quantifying the BSH activity from *L. casei* Shirota are shown in Figure 4. These results agree with those obtained in the two qualitative tests. BSH activity obtained for taurocholic acid was 1046 U/g of protein and for taurodeoxycholic acid it was 265 U/g of protein. The latter represents only 25% of the activity on taurocholic acid. BSH activity of one hundred different strains of *Lactobacillus* was determined by Lundeen and Savage (1990) who found that one strain expressed two hydrolases, which were named A and B. A hydrolase showed an activity of 32.8 and 49.4 mmol/min per g of protein for taurocholic and taurodeoxycholic acid, respectively. B hydrolase showed an activity of 41.3 and 72.7 mmol/min per g of protein for the same salts. Other authors reported BSH activity of *Lactobacillus reuteri* for taurocholic and taurodeoxycholic acids of 14 and 26 (mmol of amino acid liberated/min per g of protein), respectively (Taranto, Sesma and Font de Valdez, 1999). In another study, it was reported the BSH activity of five strains of *Bifidobacteria* when taurine- and glycine-conjugated salts were used as a substrate. The specific enzyme activity had a variation within 0.83 to 1.37 U/mL (a unit was defined as the amount of enzyme that released 1 mol of amino acid from the substrate

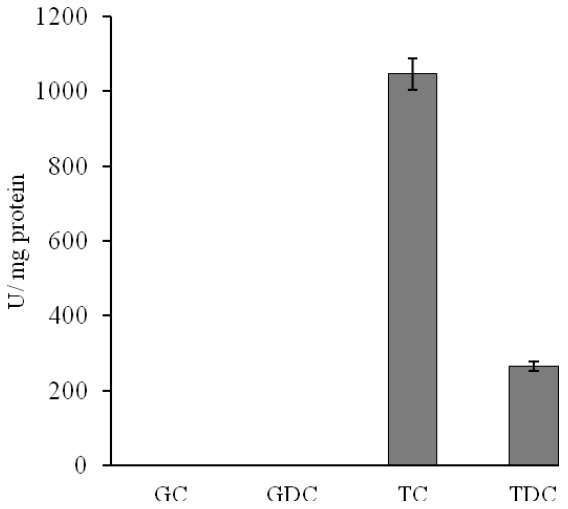

Fig. 4. Quantitative determination of bile salt hydrolase activity from L. casei Shirota. GC: glycocholic acid; GDC: glycodeoxycholic acid; TC: taurocholic acid; TDC: taurodeoxycholic acid.

per minute) (Liong and Shah, 2005). By comparing the results obtained in the present work with those previously mentioned, the BSH activity from *L. casei* Shirota was higher when either taurocholic or taurodeoxycholic acids was used. This is the first report in which BSH activity on taurine-conjugates is reported in *L. casei* Shirota by both qualitative and quantitative analyses.

As mentioned in the results of zeta potential, there might be a relationship between the presence of bile salts, the induced bile salt hydrolase activity and zeta potential since each bile salt under study induced different bile salt hydrolase activity and also a different zeta potential in *L. casei* Shirota, which means a modification of the surface properties of this microorganism.

Conclusions

Primary and secondary bile salts, conjugated to glycine or taurine, affected in different way the growth rate and the morphometric parameters of *L. casei* Shirota. Glyco-conjugates had a negative effect on the growth rate (μ). Additionally, glyco-conjugates modified the length of cell minor axis, and both glyco- and tauro- conjugates modified the cell aspect ratio. Zeta potential seems to be related to the bile salt hydrolase activity. It is also important to note that when comparing the qualitative and quantitative methods used for determining the BSH activity, *L. casei* Shirota exhibited activity on primary taurine-conjugated salts. There are not previous reports on the hydrolase activity of bile salts and the change of zeta potential, growth rate and morphometric parameters in *L. casei* Shirota by the different bile salts used, nevertheless, more research work is needed.

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