J. Gen. Appl. Microbiol., **59**, 425–436 (2013)

Full Paper

Antimicrobial and anti-adhesive properties of biosurfactant produced by lactobacilli isolates, biofilm formation and aggregation ability

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(Received December 10, 2012; Accepted August 16, 2013)

This study aimed to investigate the antimicrobial and anti-adhesive properties of biosurfactant extracted from ten lactobacilli species isolated from Egyptian dairy products. The produced biosurfactants showed distinct antimicrobial and anti-adhesive activities against several pathogenic microorganisms. Furthermore, lactobacilli isolates were studied for biofilm formation and lactic acid production in different growth media. All lactobacilli isolates produced biofilm on polystyrene surface in all media tested to different degrees. *L. acidophilus* showed the highest biofilm formation in Rogosa medium. However, the highest lactic acid production was recorded by *L. brevis* (39.63 g/L), followed by *L. reuteri* (33.32 g/L) in MRS medium. Evaluation based on in vitro studies including auto-aggregation and co-aggregation with three pathogenic bacterial strains was further analyzed. All lactobacilli isolates tested were able to auto-aggregate (ranging from 51.12% to 78.17% assessed at 5 h of incubation). The lactobacilli isolates co-aggregate with the tested bacterial strains to different degrees; among them *L. delbrueckii* showed the highest scores of co-aggregation with *Candida albicans* ATC70014, reaching 59.37%. The aggregation ability exhibited by the isolated lactobacilli, together with the antimicrobial and anti-adhesive properties observed for their biosurfactants, opens future prospects for their use against microorganisms responsible for diseases and infections and as effective probiotic strains.

Key Words——anti-adhesive, antimicrobial, auto-aggregation, biofilm, biosurfactant, co-aggregation, lactobacilli

Introduction

Lactic acid bacteria (LAB) comprise a wide range of genera and include a considerable number of species. These bacteria are the major component of the starters used in fermentation, especially for dairy products. *Lactobacillus* is one of the most important genera of LAB (Coeuret et al., 2003). Different bacterial species belonging to the genus *Lactobacillus* are part of the human and animal commensal intestinal flora (Vaughan et al., 2005). It is reported that lactobacilli isolated from dairy products have shown a long history of safe use (WHO/FAO, 2001). They are used widely as starter cultures in the food industry, e.g. fermented milk or meat products, alcoholic beverages, sourdough and silage (Carr et al., 2002). Lactic acid production by lactobacilli that are used by the food industry has been studied extensively (Kyla-Nikkila et al., 2000).

In the last years, there has been an increasing recognition of the role of lactobacilli in the maintenance of the homeostasis within dynamic ecosystems such as

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the gastrointestinal and urogenital tracts, and in the prevention of colonization and infection caused by pathogenic microorganisms (Boris and Barbés, 2000). Some lactobacilli play this protective role by producing compounds such as hydrogen peroxide, lactic acid, bacteriocins and biosurfactants, which inhibit the growth of potential pathogens (Pascual et al., 2008). Furthermore, lactobacilli interfere with pathogens by competitive exclusion from receptors present on the surface of the epithelial cells and by co-aggregation with them, which contributes to the creation of a barrier that prevents colonization by pathogens (Schachtsiek et al., 2004). Previous studies indicate that auto-aggregation of probiotic strains is necessary for adherence to epithelial cells and mucosal surfaces, and this increases their colonization in environments with short residence times, such as the oral cavity and the urogenital tract (Voltan et al., 2007).

A biofilm is a thin layer of microorganisms that adhere to the surface of an organic or inorganic structure, together with their secreted polymers. Biofilms are the predominant phenotype of nearly all bacteria in their natural habitat, whether pathogenic or environmental. Indeed, bacteria in a biofilm environment can be up to 1,000 times more resistant to antibiotics than the same bacteria circulating in a planktonic state (McCarthy, 2001). Co-aggregation and co-adhesion are the two forms of physical interactions that are thought to play an important role in biofilm formation (Bradshaw et al., 1996).

The aim of the present study was to investigate the antimicrobial and anti-adhesion properties of the biosurfactant extracted from lactobacilli isolates against several pathogenic microorganisms. Microbial adhesions to solvents and lactic acid production as well as biofilm formation were also investigated. Moreover, the study was also extended to study the auto-aggregation and co-aggregation abilities of the isolated lactobacilli species.

Materials and Methods

Bacterial strains and culture conditions. The lactobacilli species were isolated from 24 samples of traditional Egyptian dairy products collected from the Cairo markets as described by Rushdy and Gomaa (2013). Bacterial isolates that were gram-positive and catalase-negative rods were selected for further identification by the API 50CHL kit system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions (Ghanbari et al., 2009) at the microbial culture collection center (MIRCIN), Faculty of Agriculture, Ain Shams University. The isolates were identified by using the API WEB software version 5.0 from bioMérieux and Bergey's Manual of Determinative Bacteriology (Buchanan and Gibson, 1974) for comparison of assimilation and/or fermentation pattern. All identified isolates were kept at −70°C in De Man-Rogosa-Sharpe broth (MRS) (De Man et al., 1960) with glycerol (30% v/v). They were activated and grown in the same medium.

For antimicrobial and anti-adhesive assays, the following microorganisms kindly provided by the Fermentation Biotechnology and Applied Microbiology (FERM-BAM) Centre, Al-Azhar University, Cairo, Egypt were used: *Bacillus subtilis* 1020 (originally ATCC 6633), *Bacillus subtilis* 1250 (originally NCTC 10400), *Bacillus cereus* 1283 (originally ATCC 14579) , *Staphylococcus aureus* 1351 (originally NCTC 7447), *Pseudomonas aeruginosa* 1259 (originally ATCC 10145), *Escherichia coli* 1319 (originally NCTC 10418), *Salmonella typhi* 1350 (originally NCIMB 9331), *Proteus vulgaris* 1227 (originally ATCC 27973), *Serratia marcescens* 1247 (originally ATCC 25179) and *Candida albicans* 22 CBS 5703 (originally ATCC 70014). On the other hand, *Bacillus* sp. 2BSG-PDA-16 and *Bacillus* sp. DV2-37 were isolated from crude oil polluted water samples collected from a mangrove region on the western coast of the Red Sea, Egypt, and identified by 16S rRNA sequencing method as described by Gomaa (2013). All these bacterial strains were cultured in Luria-Bertani (LB) medium at 37°C, while *Candida albicans* ATCC 70014 was cultivated on Yeast Nitrogen Base agar (YNB) (Sigma-Aldrich, St. Louis, MO).

Surface activity. To select the isolates showing the highest surface activity, bacteria were cultivated in 100 ml flasks containing 20 ml MRS broth at 30°C under static conditions for 48 h. One milliliter of each culture broth was centrifuged at 10,000 $\times g$ for 10 min and the supernatants filter sterilized. Surface activity was measured by an oil spreading assay (Morikawa et al., 2000) by using 20 μl of crude oil previously deposited onto the surface of 20 ml of distilled water in a Petri dish (90 mm in diameter) to form a thin membrane. Twenty microliters of each bacterial supernatant was gently put onto the center of the oil membrane. Diameters of clearly formed oil displaced circles were measured.

Biosurfactant production. For biosurfactant production, a seed culture was prepared by transferring a single colony of the lactobacilli isolate from MRS agar culture into 20 ml of modified MRS broth without Tween 80 and incubating it overnight at 30°C under static conditions. Thereafter, the 20 ml was inoculated in 1 L of modified MRS broth in a 5 L flask and incubated again for 72 h at 30°C and 150 rpm. The broth culture was then centrifuged at 10,000 × *g* for 30 min and the supernatant was collected. To exclude the possibility that the biosurfactant was adherent to the bacterial cell wall, bacteria separated from the supernatant were washed three times and re-suspended in 100 ml of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). The bacteria were left at room temperature for 2 h with gentle stirring for biosurfactant release (Rodrigues et al., 2006b). Subsequently, the bacteria were removed by centrifugation and the remaining supernatant was collected, filtered (0.22 μm) and dialyzed against demineralized water at 4°C (molar mass range 6,000–8,000 kDa).

For the biosurfactant extraction, the supernatant was acidified to pH 2 with 6 M HCl, stored overnight at 4°C and extracted three times with chloroform/methanol (2 : 1). The organic fraction was evaporated to dryness under vacuum conditions, and acetone was added to recover the raw biosurfactant. The acetone was evaporated and the biosurfactant was collected and dissolved in 10% dimethyl sulfoxide (DMSO) at a concentration of 10 mg/L.

Antimicrobial assays. The antimicrobial activity of the isolated crude biosurfactant against several microbial strains was determined by agar well diffusion assay (AWDA) as described by Ennahar et al. (1999). One milliliter of the pathogenic strains (with approximately 10^5 CFU /ml) was incorporated into soft agar (1%, v/v) plates of Luria-Bertani (LB) medium containing (g/L): tryptone, 10; yeast extract, 5; and sodium chloride, 10 (Sambrook et al., 2001). The crude biosurfactants extracted from lactobacilli isolates were pipetted into holes drilled into the agar. Controls were maintained with DMSO only. The plates were incubated aerobically for 24 h at 37°C, and then observed for clear inhibition zones around the well. The test for each crude biosurfactant against each pathogenic strain was carried out three times with duplicates each time.

Anti-adhesive activity. The anti-adhesive activity of the crude biosurfactant extracted from lactobacilli isolates against several microbial strains was quantified according to the procedure described by Heinemann et al. (2000). Briefly, the wells of a sterile 96-well flat-bottomed polystyrene microtiter plate were filled with 200 μl of the crude biosurfactant. The plate was incubated for 18 h at 4°C and subsequently washed twice with PBS. Control wells contained PBS buffer only. An aliquot of 200 μl of a washed bacterial suspension (10^8 CFU / ml) was added and incubated in the wells for 24 h at 37°C. Unattached microorganisms were removed by washing the wells three times with PBS. The adherent microorganisms were fixed with 200 μl of 99% methanol per well, and after 15 min the plates were emptied and left to dry. Then the plates were stained for 5 min with 200 μl of 2% crystal violet used for Gram staining per well. Excess stain was rinsed out by placing the plate under running tap water. Subsequently, the plates were air dried, the dye bound to the adherent microorganisms was resolubilized with 200 μl of 33% (v/v) glacial acetic acid per well and the optical density readings of each well were measured at 595 nm. The microbial inhibition percentages for each microorganism were calculated as follows:

$$\% \text{ Microbial inhibition} = [1-(A_1-A_0)] \times 100$$

where A_1 represents the absorbance of the well with a biosurfactant and A_0 the absorbance of the control well.

Microbial adhesion to solvents. Microbial adhesion to solvents was measured according to the method of Bellon-Fontaine et al. (1996). Bacteria were harvested in the stationary phase by centrifugation at 10,000 × *g* for 30 min, washed twice, and re-suspended in 0.1 mol/L KNO_3 (pH 6.2) to approximately 10^8 CFU/ml. The absorbance of the cell suspension was measured at 600 nm (A_0). One milliliter of solvent was added to 3 ml of cell suspension. Three different solvents were tested in this study, xylene, chloroform and ethyl acetate. After 10 min of pre-incubation at room temperature, the two-phase system was mixed by vortexing for 2 min. The aqueous phase was removed after 20 min of incubation at room temperature, and its absorbance at 600 nm (A_1) was measured. The percentage of bacterial adhesion to solvent was calculated as $(1-A_1/A_0) \times 100$.

Biofilm formation and quantification. Biofilm production by pure cultures of lactobacilli was quantified in polystyrene microtiter plates (Stepanovic et al., 2004). Briefly, 96-well flat-bottomed polystyrene microtiter plate wells were filled with 200 μl of MRS. For biofilm forma-

tion by individual isolates of lactobacilli, 20 ml of each of the overnight grown cultures (OD adjusted to McFarland No. 0.5 as a standard solution, 10^8 CFU/ml) was added to each well. The experiment was performed in triplicate with appropriate medium control (MRS alone without culture). The plates were incubated at 37°C for 24 h. Unattached microorganisms were removed by washing the wells thrice with sterile phosphate-buffered saline (PBS).The adherent microorganisms were fixed with 200 µl of methanol (99% purity) per well, and after 15 min, the plates were emptied and left to dry. Then the plates were stained for 5 min with 200 µl of 2% crystal violet, shaking the plates three times to help the colorant to get the bottom of the well. Excess stain was rinsed out by placing the plate under running tap water. Subsequently, the plates were air dried, the dye bound to the adherent microorganisms was resolubilized with 200 µl of 33% (v⁄v) glacial acetic acid per well, and the absorbance of each well was measured at 595 nm. All steps were carried out at room temperature. The capability of lactobacilli isolates to produce biofilm in different media was tested by using Rogosa broth (g/L) (sodium acetate 15, tryptone 10, dextrose 10, monopotassium phosphate 6, yeast extract 5, sucrose 5, arabionose 5, ammonium citrate 2, sorbitan monooleate 1, magnesium sulfate 0.57, manganase sulfate 0.12 and ferrous sulfate 0.03), LAPT (g/L) (peptone 15, tryptone 10, yeast extract 10, glucose 10 and Tween 80 1 ml) and MRS media. Tests were done in triplicate on three separate occasions and the results averaged. The data obtained were used to classify the strains as high producers (OD higher than 0.500), producers (OD between 0.500 and 0.100) or poor producers (OD lower than 0.100) (Maldonado et al., 2007).

Production of lactic acid. Lactic acid production was quantified in grams per liter, by acid-base titration, according to Edema and Sanni (2008).

Auto-aggregation and co-aggregation assays. Auto-aggregation of bacterial isolates was evaluated by the method of Del Re et al. (2000) with certain modifications. The tested bacteria were grown at 37°C for 24 h on MRS medium. After centrifugation at 10,000 $\times g$ for 30 min, cells were washed twice and re-suspended in sterile phosphate buffered saline solution (pH 7.2) to give a viable concentration of about 10^7–10^8 CFU/ml.

Four milliliters of the cell suspension was mixed for 10 s to determine auto-aggregation during 5 h of incubation at room temperature. The upper suspension was used in each hour by transferring 0.1 ml to another 3.9 ml of phosphate buffer solution, and the optical density at 600 nm was measured. Percent auto-aggregation was calculated by the formula: $1- (A_t /A_0) \times 100$, where A_t represents the absorbance at time t = 1, 2, 3, 4 or 5, and A_0 the absorbance at $t = 0$.

The S-layer proteins of lactobacilli isolates were removed by extraction with 5 mol /L LiCl (30 min at 37°C). Subsequently, bacterial cell suspensions were washed twice and resuspended in PBS for aggregation assays. For determination of co-aggregation with *Staphylococcus aureus* 1351 (originally NCTC 7447), *Proteus vulgaris* 1227 (originally ATCC 27973) and *Candida albicans* 22 CBS 5703 (originally ATCC 70014), the cell suspension was prepared similarly to the auto-aggregation assay. Two milliliter aliquots of the cell suspensions were mixed together by vortexing for 10 s. About 4 ml of individual cell suspension was set aside as a control at the same time. The absorbance at 600 nm was measured after mixing and incubation at room temperature for 5 h. The quantitative co-aggregation rate of paired isolates was calculated using the equation: % Co-aggregation = $OD_{Tot} - OD_S /OD_{Tot} \times 100$, where the OD_{Tot} value refers to the initial OD, taken immediately after the relevant strains were paired; and OD_S refers to the OD of the supernatant, after the mixture was centrifuged after 5 h (Malik et al., 2003).

Statistical analysis. Results are presented as mean value ± standard deviation (SD). The Microsoft Excel 2003 and SAS 9.1.3 statistical program were used for data analysis.

Results and Discussion

Identification of lactobacilli isolates

Twenty-three lactobacilli species were isolated from 24 dairy samples of traditional Egyptian dairy products on MRS medium. Ten of them were selected for further assays on the basis of their surface activity (data not shown). They were tentatively identified with an API 50CHL kit system, and are summarized in Table 1. These isolates need further study for their final identification, but the species names shown in Table 1 were used in the present study.

Antimicrobial activity of biosurfactant

In the present study, a total of ten biosurfactant preparations extracted from lactobacilli isolates were found to produce inhibition zones against the tested

Table 1. Identification of lactobacilli isolates using API 50CHL kit system.

Code	API 50CHL identification	(% similarity)[a]
L02	*Lactobacillus paracasei*	(99%)
L07	*Lactobacillus plantarum*	(99%)
L10	*Lactobacillus delbrueckii*	(98%)
L12	*Lactobacillus acidophilus*	(99%)
L16	*Lactobacillus casei*	(97%)
L18	*Lactobacillus fermentum*	(99%)
L20	*Lactobacillus rhamnosus*	(98%)
L22	*Lactobacillus* spp.	(87%)
L24	*Lactobacillus reuteri*	(99%)
L32	*Lactobacillus brevis*	(99%)

[a]The percentages following the scientific names of strains represent the similarities from the computer-aided database of the Apiweb TM API 50 CH V5.1 software.

strains except *Bacillus cereus* 1283 (originally ATCC 14579), *Escherichia coli* 1319 (originally NCTC 10418) and *Serratia marcescens* 1247 (originally ATCC 25179). The average of their inhibition zones ranged from 1.8 to 12.9 mm (Table 2). The biosurfactant derived from *L. fermentum* exhibited the highest inhibition to the growth of *Candida albicans* 22 CBS 5703 (originally ATCC 70014) based on the size of inhibition zone having reached 12.9 mm. In this sense, the antimicrobial activity of the crude biosurfactant extracted from lactobacilli isolates was similar to that obtained with the biosurfactants isolated from *L. paracasei* ssp. *paracasei* A20 against *C. albicans*, *Staph. aureus* and *Staph. Epidermidis* (Gudina et al., 2010 a).

Santos et al. (2009) reported that lactobacilli, as probiotic agents, are believed to interfere with pathogens by different mechanisms. One of their mechanisms is biosurfactant production. Biosurfactants, a structurally diverse group of surface active molecules synthesized by microorganisms, have attracted attention in recent years. They have several advantages over synthetic surfactants, such as low toxicity, inherent good biodegradability and ecological acceptability. Biosurfactants include unique amphipathic properties derived from their complex structures, which include a hydrophilic moiety and a hydrophobic portion (Vater et al., 2002). They include glycolipids, lipopeptides, polysaccharide-protein complexes, protein-like substances, lipopolysaccharides, phospholipids, fatty acids and neutral lipids (Van Hamme et al., 2006). Therefore, it is reasonable to expect diverse properties and physiological functions of biosurfactants including enhanc-

Table 2. The antimicrobial activity of biosurfactant derived from lactobacilli isolates against pathogenic microorganisms.

Biosurfactant-producing lactobacilli isolates	Mean diameter of inhibition zone (mm)											
	Bacillus subtilis ATCC 6633	*Bacillus subtilis* NCTC-10400	*Bacillus* sp. BSG-PDA-16	*Bacillus* sp. DV2-37	*Bacillus cereus* ATCC 14579	*Staphylococcus aureus* NCTC-7447	*Pseudomonas aeruginosa* ATCC-10145	*Escherichia coli* NCTC-10418	*Salmonella typhi* NCIMB-9331	*Proteus vulgaris* ATCC 27973	*Serratia marcescens* ATCC 25179	*Candida albicans* ATCC 70014
L. paracasei	6.2 ± 0.05	7.5 ± 0.04	5.7 ± 0.00	6.4 ± 0.04	—	4.0 ± 0.02	—	—	11.7 ± 0.06	11.9 ± 0.28	—	7.6 ± 0.00
L. plantarum	12.3 ± 0.00	5.6 ± 0.03	3.0 ± 0.01	—	—	4.2 ± 0.11	4.2 ± 0.11	—	7.6 ± 0.14	11.0 ± 0.0	—	5.1 ± 0.15
L. delbrueckii	7.8 ± 0.16	—	5.9 ± 0.02	—	—	9.9 ± 0.12	—	—	—	4.5 ± 0.18	—	8.5 ± 0.09
L. acidophilus	5.7 ± 0.04	3.9 ± 0.00	5.5 ± 0.05	4.8 ± 0.01	—	7.8 ± 0.01	—	—	—	12.5 ± 0.02	—	7.4 ± 0.15
L. casei	—	—	1.8 ± 0.03	—	—	6.3 ± 0.13	—	—	—	5.0 ± 0.01	—	10.0 ± 0.01
L. fermentum	—	—	—	—	—	5.0 ± 0.02	—	—	—	5.5 ± 0.02	—	12.9 ± 0.04
L. rhamnosus	6.6 ± 0.07	—	5.0 ± 0.06	—	—	5.2 ± 0.07	—	—	—	5.7 ± 0.09	—	5.6 ± 0.34
L. spp.	11.0 ± 0.09	6.1 ± 0.04	10.4 ± 0.04	6.0 ± 0.00	—	3.8 ± 0.05	—	—	2.1 ± 0.05	11.2 ± 0.13	—	10.4 ± 0.06
L. reuteri	10.3 ± 0.06	5.4 ± 0.02	6.9 ± 0.03	5.3 ± 0.01	—	10.2 ± 0.03	—	—	—	6.6 ± 0.07	—	10.3 ± 0.39
L. brevis	5.1 ± 0.01	5.5 ± 0.02	7.3 ± 0.01	6.8 ± 0.02	—	10.0 ± 0.00	—	—	—	4.7 ± 0.11	—	6.2 ± 0.00

Each value is the mean of three repeat experiments with a duplicate each ± standard deviations of values obtained from triplicate experiments.
Note: — = no inhibition.

ing the solubility of hydrophobic/water-insoluble compounds (which facilitates their uptake into the cell), heavy metal binding, bacterial pathogenesis, cell adhesion and aggregation, quorum sensing and biofilm formation (Singh and Cameotra, 2004).

Anti-adhesion activity of biosurfactant

The results cited in Table 3 show that the produced biosurfactants exhibited a considerable anti-adhesive activity against three bacterial strains tested. The highest anti-adhesive activity was recorded for *L. fermentum* biosurfactant against *Candida albicans* 22 CBS 5703 (originally ATCC 70014) (84.69%). On the other hand, the lowest activity was recorded for *L. delbrueckii* biosurfactant against *Proteus vulgaris* 1227 (originally ATCC 27973) (9.66%). Comparatively, Gudina et al. (2010b) reported that biosurfactants produced by *L. acidophilus* and *L. paracasei* ssp*. paracasei A20* showed lower anti-adhesive activities against *C. albicans* strains (adhesion reduction of about 25% and 50%, respectively).

Another valuable application of biosurfactants is their use as anti-adhesive agents against pathogens. Adsorption of a biosurfactant to a substratum surface modifies its hydrophobicity, interfering in the microbial adhesion and desorption processes. Many lactobacilli have been found to produce biosurfactants which tend to accumulate at the liquid-air interface such as *L. lactis*, *L. acidophilus*, *L. fermentum*, *L. casei*, and *L. rhamnosus* strains (Rodriguez et al*.*, 2006a). These biosurfactants not only prevent adhesion of bacteria to the surfaces but also induce detachment of already adherent cells. Involvement of biosurfactants in microbial adhesion and desorption has been widely described, and constitutes an effective strategy to reduce microbial adhesion and combat colonization by pathogenic microorganisms, not only in the biomedical field, but also in other areas, such as the food industry (Falagas and Makris, 2009).

Adhesion to solvents

With respect to microbial adhesion to solvents, three different solvents were tested in this study: xylene, which is an apolar solvent; chloroform, a monopolar and acidic solvent; and ethyl acetate, a monopolar and basic solvent. Results presented in Fig. 1 show that the highest percentages of adhesion for all lactobacilli isolates were recorded with xylene, demonstrating the hydrophobic cell surface of these isolates. The highest hydrophobicity values (73.31 and 73.10%) were calculated for *L. casei* and *L.* spp., respectively. Many previous studies on the physicochemistry of microbial cell surfaces have shown that the presence of (glyco-) proteinaceous material at the cell surface results in higher hydrophobicity (Pelletier et al., 1997). The values obtained with the other two solvents, chloroform and ethyl acetate, were regarded as a measure of electron donor (basic) and electron acceptor (acidic) characteristics of bacteria, respectively (Bellon-Fontaine et al., 1996). All isolates showed stronger affinity for chloroform, which is an acidic solvent and electron acceptor, than for ethyl acetate, which is a basic solvent and electron donor.

Biofilm formation

In the present study, a microtiter plate format assay was used to assay biofilm formation. Three different growth media were tested: MRS, Rogosa and LAPT.

Table 3. Microbial inhibition percentages obtained from the anti-adhesion assays with the crude biosurfactant from lactobacilli isolates. Results are expressed as means ± standard deviations of values obtained from triplicate experiments

Biosurfactant-producing lactobacilli isolates	*Staphylococcus aureus* NCTC 7447	*Proteus vulgaris* ATCC 27973	*Candida albicans* ATCC 70014
L. paracasei	18.00 ± 2.3	62.41 ± 2.5	45.16 ± 1.2
L. plantarum	19.73 ± 1.4	55.25 ± 3.7	12.09 ± 2.6
L. delbrueckii	47.36 ± 1.1	9.66 ± 4.6	47.59 ± 1.6
L. acidophilus	30.92 ± 1.2	75.17 ± 2.9	44.35 ± 1.9
L. casei	30.26 ± 1.6	10.11 ± 2.1	59.67 ± 1.2
L. fermentum	28.94 ± 2.2	10.34 ± 2.8	84.69 ± 1.1
L. rhamnosus	29.60 ± 1.3	30.34 ± 1.6	16.12 ± 1.0
L. spp.	12.32 ± 1.9	71.03 ± 1.0	74.19 ± 1.3
L. reuteri	60.52 ± 2.0	38.62 ± 0.0	64.51 ± 1.6
L. brevis	52.21 ± 1.7	19.31 ± 1.4	21.77 ± 1.0

The isolates showed comparable rates in the biofilm assay using different media. It was reported that increases in nutrient concentration increased biofilm formation (Rochex and Lebeault, 2007) and our study corroborates the same where biofilm formation is less pronounced in LAPT medium than in Rogosa and MRS media. However, under all conditions tested, *L. acidophilus* showed by far the best biofilm formation properties on polystyrene (Fig. 2).

Biofilms are defined as communities of microorganisms that are encased in a self-synthesized extracellular polymeric matrix (EPS) and grow attached to a biotic or abiotic surface (Gotz, 2002). The substances (EPS) are mainly made up polysaccharides, but in addition proteins and nucleic acids, lipids, mineral ions and various cellular debris have been recorded (Sutherland, 2001). Biofilm formation was thought to be one of the microbial survival strategies because it provides microorganisms with important advantages including (i) increased access to nutrients; (ii) protection against toxins and antibiotics; (iii) maintenance of extracellular enzyme activities and (iv) shelter from predation (Dang and Lovell, 2000). Whole cell hydrophobicity, auto-aggregation, and co-aggregation are important for colonization and biofilm development in flowing environments (Rickard et al., 2004).

Lactic acid production

The results for quantification of lactic acid production by the lactobacilli isolates varied according to the

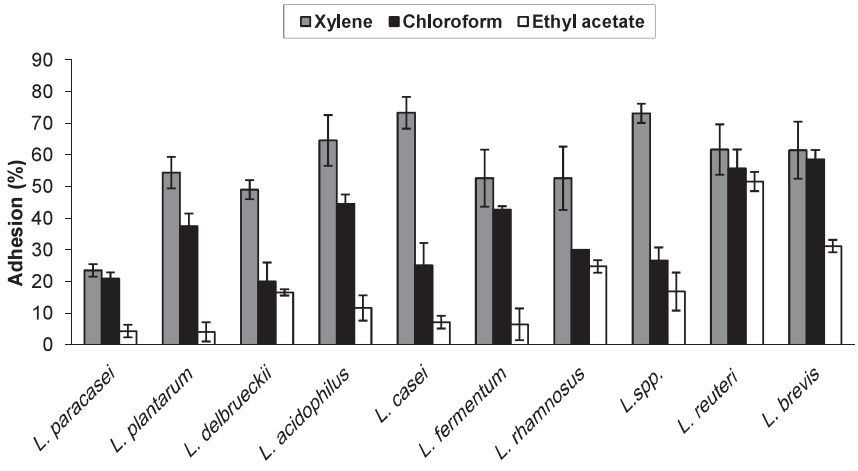

Fig. 1. Adhesion of lactobacilli isolates to xylene, chloroform and ethyl acetate.
Results are averages of triplicate experiments and error bars represent the standard deviation values.

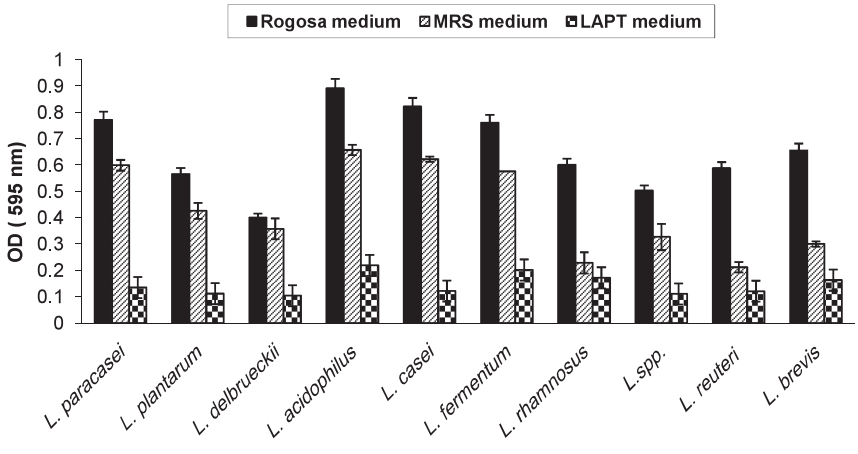

Fig. 2. Biofilm formation of lactobacilli isolates using the microtiter plate assay after incubation at 37°C for 24 h in different growth media.
The error bars represent standard deviations of three replicated assays.

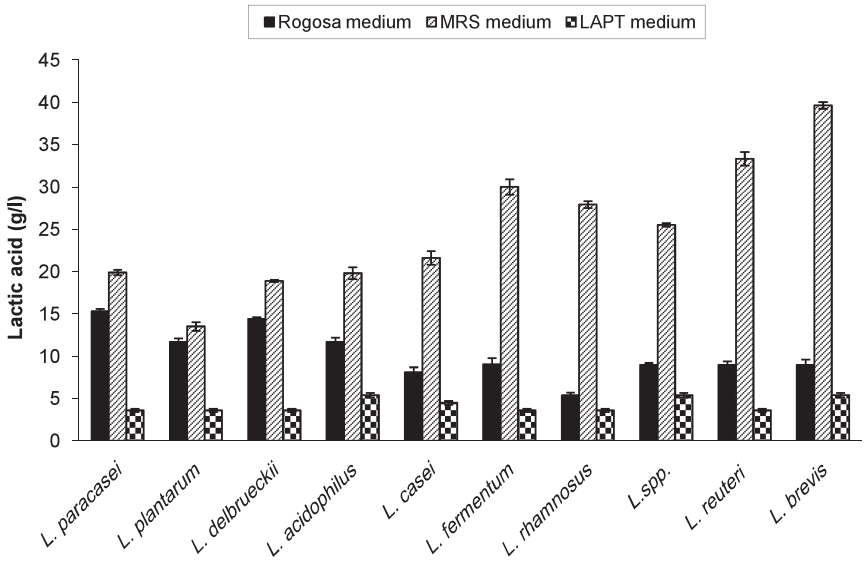

Fig. 3. Lactic acid production expressed in g/L by lactobacilli isolates under different growth media.

Results are averages of triplicate experiments and error bars represent the standard deviation values.

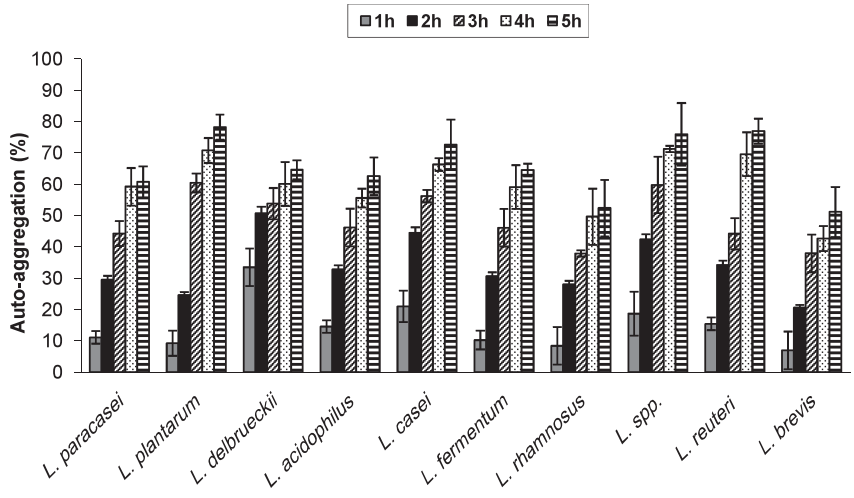

Fig. 4. Auto-aggregation kinetics of lactobacilli isolates cells re-suspended in PBS (pH 7.2) evaluated on MRS broth.

Error bars represent standard deviations of the mean values of results from three replicated experiments.

growth medium used (Fig. 3). In general, the levels of lactic acid production by lactobacilli isolates were higher in MRS broth than in Rogosa or LAPT broth. The highest amount of lactic acid was produced by *L. brevis* followed by *L. reuteri* then *L. fermentum* reaching 39.63, 33.32 and 30.00 g/L, respectively.

The ability of the lactobacilli isolates to produce toxic metabolites such as lactic acid has been suggested as being responsible for their ability to inhibit other bacteria (Juven et al., 1992). Makras et al. (2006) also reported that the antibacterial activity of *L. acidophilus* IBB 801, *L. amylovorus* DCE 471, *L. casei* and *L. rhamnosus* GG was solely due to the production of lactic acid.

Auto-aggregation

Results illustrated in Fig. 4 show that all lactobacilli isolates tested in the present study exhibited some de-

gree of auto-aggregation at all time points tested and the highest degree of auto-aggregation was observed after 5 h of incubation. In the beginning, the percentage of auto-aggregation ranged between 6.99–33.5%, and then continually increased every hour. After 5 h, the auto-aggregation registered a high percentage of 51.12–78.17%. The results presented in this study were comparable with the levels reported by Todorov et al. (2008) for other lactobacilli: *L. pentosus* ST712BZ (67%) and *L. paracasei* ST284BZ (99%). Among some probiotic strains, auto-aggregation appears to be the first step for adhesion to intestinal epithelial cells. Moreover, bacteria with co-aggregation abilities may form a barrier preventing colonization by pathogenic microorganisms (Del Re et al., 2000). The proteinaceous nature of some surface components has been demonstrated and surface layer (S-layer) proteins detected in some lactobacilli isolates may be involved in adherence (Mukai and Arihara, 1994). Determination of these properties on bacterial strains before and after the re-

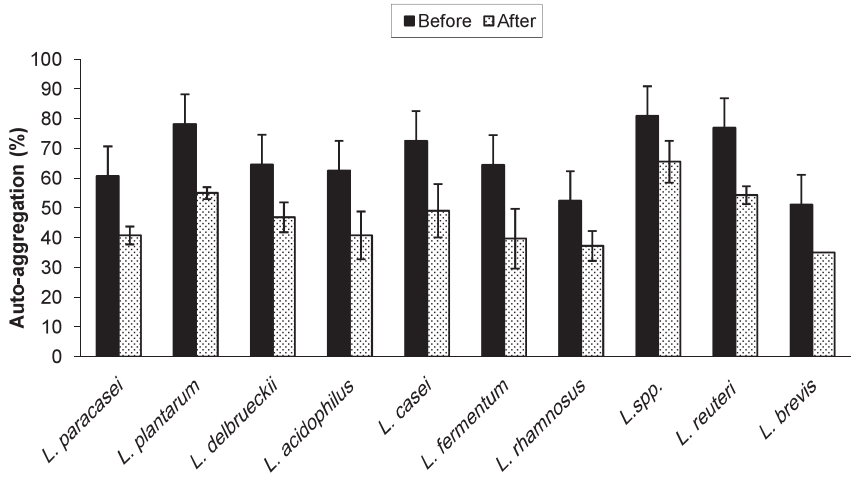

Fig. 5. Comparison of the auto-aggregation ability of lactobacilli isolates before and after the treatment with 5 mol /L LiCl.

Error bars represent standard deviations of the mean values of results from three replicated experiments.

Table 4. Co-aggregation values recorded for lactobacilli isolates with *Staphylococcus aureus* NCTC 7447, *Proteus vulgaris* ATCC 27973 and *Candida albicans* ATCC 70014 after 5 h incubation at room temperature in PBS (pH 7.2).

Lactobacilli isolates	Co-aggregation indices (%)		
	Staphylococcus aureus NCTC 7447	*Proteus vulgaris* ATCC 27973	*Candida albicans* ATCC 70014
L. paracasei	33.23 ± 1.6	26.51 ± 2.1	35.48 ± 3.1
L. plantarum	36.92 ± 1.4	45.76 ± 0.0	29.24 ± 2.0
L. delbrueckii	48.88 ± 0.1	45.40 ± 2.2	59.37 ± 1.4
L. acidophilus	22.10 ± 1.6	22.05 ± 1.8	38.65 ± 2.6
L. casei	23.98 ± 2.6	30.42 ± 0.5	35.27 ± 2.1
L. fermentum	40.40 ± 2.0	37.35 ± 0.0	50.24 ± 1.4
L. rhamnosus	36.63 ± 4.6	22.19 ± 0.5	27.55 ± 1.8
L. spp.	26.37 ± 2.3	29.86 ± 2.8	27.92 ± 1.2
L. reuteri	39.63 ± 0.5	23.62 ± 2.2	32.96 ± 1.0
L. brevis	40.43 ± 0.8	26.61 ± 1.8	45.22 ± 1.0

Results are expressed as means ± standard deviations of values obtained from triplicate experiments.

moval of S-layers may confirm the role of such proteins in the interaction (Golowczyc et al., 2009). The results illustrated in Fig. 5 show the reduction of auto-aggregation after removing S-layer proteins from the cell surface. Similarly, Kos et al. (2003) reported the removal of the S-layer by treatment with 5 mol/L LiCl reduces the auto-aggregation ability of *L. acidophilus* M92.

Co-aggregation

Generally, all of the tested lactobacilli isolates in this study exhibited high auto-aggregation and moderate co-aggregation (Table 4). Similarly, *L. acidophilus* M92 showed a high score in auto-aggregation but a lower score in co-aggregation with pathogens (Del Re et al., 2000). Co-aggregation between *L. acidophilus* and *Proteus vulgaris* 1227 (originally ATCC 27973) showed the lowest inhibition percentage (22.05%). On the other hand, the co-aggregation percentage of *L. delbrueckii* with the *Staphylococcus aureus* 1351 (originally NCTC 7447) and *Candida albicans* 22 CBS 5703 (originally ATCC 70014) showed the highest inhibition percentage, reaching 48.88% and 59.37%, respectively. Quorum sensing signaling molecules could explain the high co-aggregation indices observed with *L. delbrueckii* (Jacobs and Chenia, 2011). Bacterial co-aggregation is defined as cell-to-cell adherence of different bacterial species or strains (Shen et al., 2005). Co-aggregation between lactobacilli and pathogenic microorganisms contributes to the creation of a barrier that prevents their adhesion to the epithelia and subsequent access to the tissues, constituting an important host defense mechanism against infections in the urogenital and gastrointestinal tracts. Thus, food-associated lactobacilli that co-aggregate numerous pathogens are of special interest with regard to potential applications.

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