Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Phenotypic surface properties (aggregation, adhesion and biofilm formation) and presence of related genes in beneficial vaginal *lactobacilli*

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Keywords
adhesion, auto-aggregation, biofilm, pili, sortases, vaginal lactobacilli.

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2014/0842: received 7 May 2014, revised 2 August 2014 and accepted 30 August 2014

doi:10.1111/jam.12642

Abstract

Aims: To evaluate the phenotypic expression of auto-aggregation, adhesion to mucin and biofilm formation of lactobacilli isolated from human vagina and the presence of related genes.

Methods and Results: Seven different strains of three *Lactobacillus* species (*Lactobacillus gasseri*, *Lactobacillus rhamnosus* and *Lactobacillus reuteri*) were evaluated. The auto-aggregation property was determined by spectrophotometric assay and flow cytometry. Adhesion and biofilm formation were assayed by crystal violet staining. The presence of the genes encoding sortases, pilin subunits and surface proteins was evaluated by polymerase chain reactions. The two *Lact. reuteri* strains assayed showed high auto-aggregation, adhesion to mucin and biofilm formation ability. In these strains, the genes encoding three adhesion proteins were identified. In *Lact. rhamnosus* CRL (Centro de Referencia para Lactobacilos Culture Collection) 1332, pilus-encoding genes were detected. In all *Lact. rhamnosus* strains assayed, two genes encoding for other surface proteins related to adhesion and biofilm formation were detected.

Conclusions: The vaginal lactobacilli assayed exhibited phenotypic and genetic characteristics that were specific for each strain.

Significance and Impact of the Study: This is the first study on auto-aggregation, adhesion and biofilm formation of vaginal *Lactobacillus* strains by phenotypic and genetic assays.

Introduction

The human microbiome of the vaginal tract of healthy women is dominated by different *Lactobacillus* species (Human Microbiome Project Consortium 2012). The health-promoting effects of probiotic strains could be related to their persistence in a specific ecological niche and to their adhesion to mucosal surfaces (Younes *et al.* 2012). Different *Lactobacillus* strains used and proposed as vaginal probiotics such as *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 express antimicrobial activity and different surface properties believed to help in reducing pathogen colonization in the urogenital tract (Martinez *et al.* 2009; Younes *et al.* 2012).

The molecular mechanisms involved in the adhesion and colonization of Gram-positive bacteria are well documented in different pathogens (Cossart and Jonquières 2000; Sillanpää *et al.* 2010; Sheen *et al.* 2011). In several Gram-positive bacteria, surface proteins with a conserved C-terminal LPXTG motif (e.g. surface Ig-binding *Staphylococcus aureus* protein A), a C-terminal hydrophobic region and a positively charged tail have been shown to play a role in virulence (Cossart and Jonquières 2000).

In Gram-positive pathogens, the presence of pili and their relation with the adhesion and virulence was demonstrated (Sillanpää *et al.* 2010; Sheen *et al.* 2011; Hendrickx *et al.* 2012). Pili are proteinaceous appendages of 1–10 nm in diameter protruding 2–3 μm outside of

bacterial cells (Kang *et al.* 2007). Gram-positive bacteria pili are formed by 2 or 3 LPXTG proteins termed pilins that are covalently polymerized by transpeptidation reactions catalysed by pilus-specific class C sortases (SrtC) and anchored to wall by class A sortases (SrtA) (Ton-That *et al.* 2000). Two types of pilins have been described; one of them forms the pilus shaft, and the other are ancillary pilins that are at the tip, at the base or spread along the pilus shaft (Kang *et al.* 2007).

Pili are known to be involved in adhesion and biofilm formation (Sillanpää *et al.* 2010). Biofilms are differentiated bacterial communities encased in a protective and adhesive matrix (Costerton *et al.* 1995). Sillanpää *et al.* (2010) demonstrated that pili (codified by the $ebpABC_{fm}$ operon) of *Enterococcus faecium* TX82 are relevant for their ability to form biofilm and cause infection in an ascending urinary tract infection model. Serine-rich repeat proteins and PilA (pilin A protein) in *Streptococcus agalactiae* (a urogenital opportunistic pathogen) contribute to adherence to vaginal and cervical epithelial cells and to vaginal colonization in mice (Sheen *et al.* 2011). *Bacillus cereus* (a bacterium that causes food poisoning) can elaborate pili on its cell surface using a sortase-mediated mechanism (Hendrickx *et al.* 2012).

There is little information available on the presence and role of sortases, LPXTG proteins or pili in beneficial micro-organisms (Kankainen *et al.* 2009; Dieye *et al.* 2010; Oxaran *et al.* 2012). Sortase C (involved in pilus assembly) and three pilin subunits (SpaA, SpaB and SpaC) have been described in *Lactobacillus rhamnosus* GG (Kankainen *et al.* 2009). SpaA forms a pilus shaft, whereas SpaB is observed at regular intervals and SpaC is a pilus tip. Oxaran *et al.* (2012) showed that when pili were present in genetically modified *Lactococcus lactis* IL1403 cells, auto-aggregation (i.e. formation of multicellular clumps between micro-organisms of the same strain) and formation of thick biofilm were induced. On the other hand, only one study was reported on the importance of sortase A-dependent proteins in the high auto-aggregating capability, adhesion to vaginal cells and biofilm formation of a vaginal *Lactobacillus* strain (*Lactobacillus plantarum* CMPG5300) (Malik *et al.* 2013).

The ability to form biofilm of vaginal *Lactobacillus rhamnosus* CRL 1332, *Lactobacillus reuteri* CRL 1324, but not of *Lactobacillus gasseri* CRL 1263, was previously demonstrated in culture media without Tween 80 (Leccese Terraf *et al.* 2012). In this work, the objectives were to assess the phenotypic expression of different surface properties (auto-aggregation, biofilm formation and mucin adhesion) in several vaginal *Lactobacillus* (VL) strains and to determine the presence of genes related to these characteristics.

Materials and methods

Micro-organisms and growth conditions

Seven *Lactobacillus* strains of three different species originally isolated from human vagina in Tucumán, Argentina and previously preselected for their beneficial properties were used (Table 1). In this study, *Lact. reuteri* CRL (from the CERELA Culture Collection) 1327, *Lact. rhamnosus* CRL 1508 and *Lact. rhamnosus* CRL 1511 were genetically identified by ribosomal 16S-DNA sequencing according to the technique described by Kullen *et al.* (2000).

Micro-organisms were stored in milk yeast extract [% (w/v): 13 nonfat milk, 0·5 yeast extract and 1 glucose] at −70°C. Before the experiments, each strain was transferred from the frozen stock culture to MRS (De Man-Rogosa-Sharpe) broth (De Man *et al.* 1960), incubated for 24 h at 37°C under nonagitated aerobic conditions and subcultured in the same medium and conditions at 37°C for 12 h. From the second subculture, a third subculture was performed in MRS and without Tween 80 MRS and incubated at 37°C for 14 h under nonagitated aerobic conditions.

Auto-aggregation ability

A 7 × 2 × 8 experimental design was applied to determine the effects of the following factors on the auto-aggregation ability of VL: VL strain (seven strains), cell suspension initial concentration [initial optical density at

Table 1 Vaginal *Lactobacillus* strains evaluated in this work

Vaginal *Lactobacillus* strain	Relevant beneficial properties
*Lact. gasseri** CRL 1263	Inhibition of urogenital pathogens (Juárez Tomás *et al.* 2011)
Lact. gasseri‡ CRL 1294	Auto-aggregating ability (Ocaña and Nader-Macías 2002; Juárez Tomás *et al.* 2005a)
*Lact. reuteri** CRL 1324	Biofilm formation (Leccese Terraf *et al.* 2012)
Lact. reuteri† CRL 1327	Inhibition of urogenital pathogens (Ocaña *et al.* 1999)
*Lact. rhamnosus** CRL 1332	Biofilm formation (Leccese Terraf *et al.* 2012)
Lact. rhamnosus† CRL 1508	Production of H_2O_2 (Juárez Tomás *et al.* 2005b)
Lact. rhamnosus† CRL 1511	Production of H_2O_2 (Juárez Tomás *et al.* 2005b)

CRL: Centro de Referencia para Lactobacilos Culture Collection.
The strains were genetically identified by ribosomal 16S-DNA sequencing: *Juárez Tomás *et al.* 2011; †In the present study; ‡Unpublished result.

540 nm ($OD_{540\ nm}$) adjusted to 0·6 and 1·5] and time (eight times). The extent of auto-aggregation was assessed by spectrophotometry and flow cytometry, detailed as follows.

Auto-aggregation by spectrophotometric assay

The technique described by Ocaña and Nader-Macías (2002), with modifications, was applied. Briefly, the third VL subcultures (grown in MRS_{-T} for 14 h) were centrifuged (6000 ***g***, 15 min), washed with saline (S) [0·85% (w/v) NaCl] and resuspended in S to an $OD_{540\ nm}$ of 0·6 and 1·5. Variation of $OD_{540\ nm}$ of cellular suspensions was monitored every 1 h for 4 h, without agitation of these suspensions during spectrophotometric determinations.

The percentage of auto-aggregation was calculated by the following expression:

$$\text{Auto-aggregation}\,(\%) = [1 - (OD_{final}/OD_{initial})] \times 100$$

where $OD_{initial}$ is the OD at initial time ($t = 0$) of auto-aggregation assay, and OD_{final} is the OD at each time after the beginning of the assay (in minutes).

Also, the cell sediment from suspensions was collected at the end of the assay (4 h) and observed by optical microscopy (1000x).

Auto-aggregation by flow cytometry

Auto-aggregation ability was also assessed according to the technique described by Mackenzie *et al.* (2010), with modifications. The cellular suspensions of $OD_{540\ nm}$ equal to 0·6 and 1·5 were prepared as described above. Cell aggregation of S-washed cells was quantified by measuring forward scatter (FSC) and side scatter (SSC) signals detected by photodiode and photomultiplier tube (PMT) sensors, respectively. Data were acquired on a FACS Calibur flow cytometer (BD Bioscience, San Diego, CA, USA) and analysed using FCS Express 4 Flow Cytometer (De Novo Software, Glendale, CA, USA). During data analysis, gates for events with high FSC and high SSC were used to determine the percentage of aggregating cells. The controls were cell clumps homogenized into a single cell suspension using sonication (2 min at power 30).

Biofilm formation by VL strains

In a 7 × 2 experimental design, biofilm formation was evaluated considering the following factors: VL strain (seven strains) and Tween 80 concentration in MRS broth (0 and 0·1%). *Lact. rhamnosus* CRL 1332 and *Lact. reuteri* CRL 1324 were used as positive controls, and *Lact. gasseri* CRL 1263 as negative control of biofilm formation (Leccese Terraf *et al.* 2012).

For this assay, the third subculture of each VL strain was performed in both MRS and MRS_{-T}. The inoculum was prepared with bacterial pellets from the third subculture washed and resuspended in saline up to an optical density at 540 nm ($OD_{540\ nm}$) of 1·5. The microplate assay was carried out according to the technique previously described (Leccese Terraf *et al.* 2012), using 30% acetic acid as crystal violet eluent. Each strain was assayed in at least three independent experiments, each with four replicates. Additionally, a sterile culture medium was always included as negative control.

Ultrastructure of VL biofilms by scanning electron microscopy (SEM)

The ultrastructure of biofilm formed by *Lact. reuteri* CRL 1327, *Lact. rhamnosus* CRL 1508 and *Lact. rhamnosus* CRL 1511 was assessed by SEM according to the technique described by Leccese Terraf *et al.* (2012).

Adhesion and biofilm formation on mucin

Adhesion and biofilm formation of VL on immobilized mucin (type III, Sigma Chemicals Co., St Louis, MO) were assayed in 96-well Polysorp microplates (Nunc Immuno plates, Nunc, Roskilde, Denmark) according to the technique described by McMillan *et al.* (2013), with modifications. Plates were covered with 300 μl of 0·1 mg ml^{-1} mucin in PBS and incubated for 30 min at 37°C and then at 4°C overnight. Wells were washed with PBS and blocked with PBS-1% bovine serum albumin (BSA) for 2 h at room temperature; then wells were washed and allowed to dry.

For the adhesion assay, 100 μl of a cell suspension of each strain in saline ($OD_{540\ nm}$ adjusted to 1·5, from the third subculture grown for 14 h in MRS_{-T}) was added to each well, and plates were incubated for 2 h at 37°C. For the biofilm formation assay, 200 μl of the $OD_{540\ nm}$ adjusted to 1·5 cell suspension of each strain was inoculated into 5 ml of MRS_{-T}; 200 μl were added to microplates and the plates were incubated 72 h at 37°C. After removing nonadhered cells by washes with 100 μl (in the adhesion assay) or 200 μl (in the biofilm formation assay) of PBS, adhered cells were detected by staining with 0·1% crystal violet (for 30 min). After washing, the plates were dried and the stain was released with 30% acetic acid (100 μl per well), and the $OD_{570\ nm}$ was determined in a plate reader as previously described (Leccese Terraf *et al.* 2012). PBS alone was included in all experiments to account for any mucin binding by PBS, and their absorbance values were subtracted from the values of wells inoculated with VL cell suspensions. Experiments were carried out in triplicate three times with each strain.

Detection of genes related to sortases, pili and surface proteins in VL

To study the presence of genes involved in aggregation, adhesion and biofilm formation phenotypes of some *Lactobacillus* species, different gene-specific primers were designed (Table S1). DNA extraction was carried out from pure cultures of VL grown in MRS broth for 16 h at 37°C following the method described by Pospiech and Neumann (1995). Polymerase chain reactions (PCR) were performed in a final volume of 50 μl containing 10 μl of 5× PCR buffer, 100 μmol l^{-1} deoxynucleotides, 1 μmol l^{-1} primer, 1·5 mmol l^{-1} $MgCl_2$, 1·25 units of Taq polymerase and 10 ng μl^{-1} of DNA. The amplification program was as follows: one cycle of 95°C for 5 min, 30 repetitions of 95°C for 1 min, 52°C for 1 min and 72°C for 2 min, extension at 72°C for 10 min. PCR products were separated by electrophoresis on 1·5% agarose gels in 1× TAE buffer at 90 V. Bands of PCR products were cut from the agarose gel and purified with a High Pure PCR Purification kit (Roche, Bassel, Switzerland), following the manufacturer's instructions. Sequencing reactions were carried out in an automatic DNA sequencer (Applied Biosystems model 3130, Foster City, CA, USA). The sequences were assembled and analysed using MEGA 5 software (Molecular Evolutionary Genetics Analysis version 5, free software, Hachioji, Tokyo, Japan) and subsequently compared with those in the GenBank using the BLASTN tool (NCBI).

Statistical analysis

An analysis of variance using the general linear model (ANOVA-GLM) was applied to determine: (i) the effects of the VL strain, initial concentration of the cell suspension and time on the auto-aggregation extent, considering as a response of interest the auto-aggregation percentages obtained by the spectrophotometric method; (ii) the effects of the VL strain and of the initial concentration of the cell suspension on the auto-aggregation extent, considering as a response of interest the auto-aggregation percentages obtained by flow cytometry; (iii) the effects of the VL strain and Tween 80 concentration on biofilm formation in the assays of biofilm formation on polystyrene microplates; (iv) the effects of the VL strain on adhesion and biofilm formation on immobilized mucin. In all analyses, the significant differences between mean values of the response of interest for each treatment were determined by Tukey's test. A P value < 0·05 was considered as statistically significant.

Pearson's correlations were estimated to determine the relationship between the auto-aggregation values determined by OD measurements and by flow cytometry at different inoculum concentrations (bacterial suspensions adjusted to $OD_{540\ nm}$ of 0·6 and 1·5). The statistical significances of Pearson's correlations were calculated and a P value < 0·05 was considered as statistically significant. All statistical analyses were performed using MINITAB 16 Statistical Software (Minitab Inc., State College, PA, USA).

Results

Auto-aggregation ability

The auto-aggregation capability of VL was evaluated from bacteria grown in MRS_{-T} broth. ANOVA indicated that auto-aggregation percentages were significantly affected by the strain and time length of the spectrophotometric assay ($P < 0·05$). However, the initial concentration of the cell suspension was not significant. Consequently, only the aggregation kinetics of VL with high cell suspension initial concentration ($OD_{540\ nm} = 1·5$) are shown (Fig. 1a). The auto-aggregation kinetics depended on the VL strain (statistically significant strain-time interaction).

Lactobacillus reuteri CRL 1324 and *Lact. reuteri* CRL 1327 displayed the highest percentages of auto-aggregation (86 and 80%, respectively, at the end of assay) (Fig. 1a). Maximal auto-aggregation percentages of *Lact. rhamnosus* (CRL 1332, CRL 1508 and CRL 1511) and *Lact. gasseri* (CRL 1263 and CRL 1294) strains were similar.

Figure 1b shows a cell sediment and an upper clear solution when *Lact. reuteri* CRL 1324 and *Lact. reuteri* CRL 1327 suspensions were kept without shaking for 4 h. In contrast, most of the other strains showed a relatively homogenous suspension with no differentiation of the upper layer. In addition, cell aggregates of *Lact. reuteri* strains and *Lact. rhamnosus* CRL 1332 were observed by optical microscopy. In contrast, single cells of *Lact. rhamnsosus* CRL 1508 and CRL 1511 and *Lact. gasseri* CRL 1263 and CRL 1294 were also observed. As examples, Fig. 1c shows the microscopic cellular patterns of CRL 1324, CRL 1332 and CRL 1508.

Flow cytometry analysis revealed that the aggregation levels of the cell suspensions of *Lact. reuteri* CRL 1324 (9·14% at an $OD_{540\ nm} = 1·5$, and 8·18% at an $OD_{540\ nm} = 0·6$) and *Lact. reuteri* CRL 1327 (4·55% at an $OD_{540\ nm} = 1·5$, and 2·55% at an $OD_{540\ nm} = 0·6$) were significantly higher than those of *Lact. rhamnosus* and *Lact. gasseri* strains (Fig. 2). Moreover, *Lact. gasseri* CRL 1294 and *Lact. rhamnosus* CRL 1332 and CRL 1511 showed slightly higher auto-aggregation percentages than CRL 1263 and CRL 1508. When estimating the relationships between the auto-aggregation percentages from

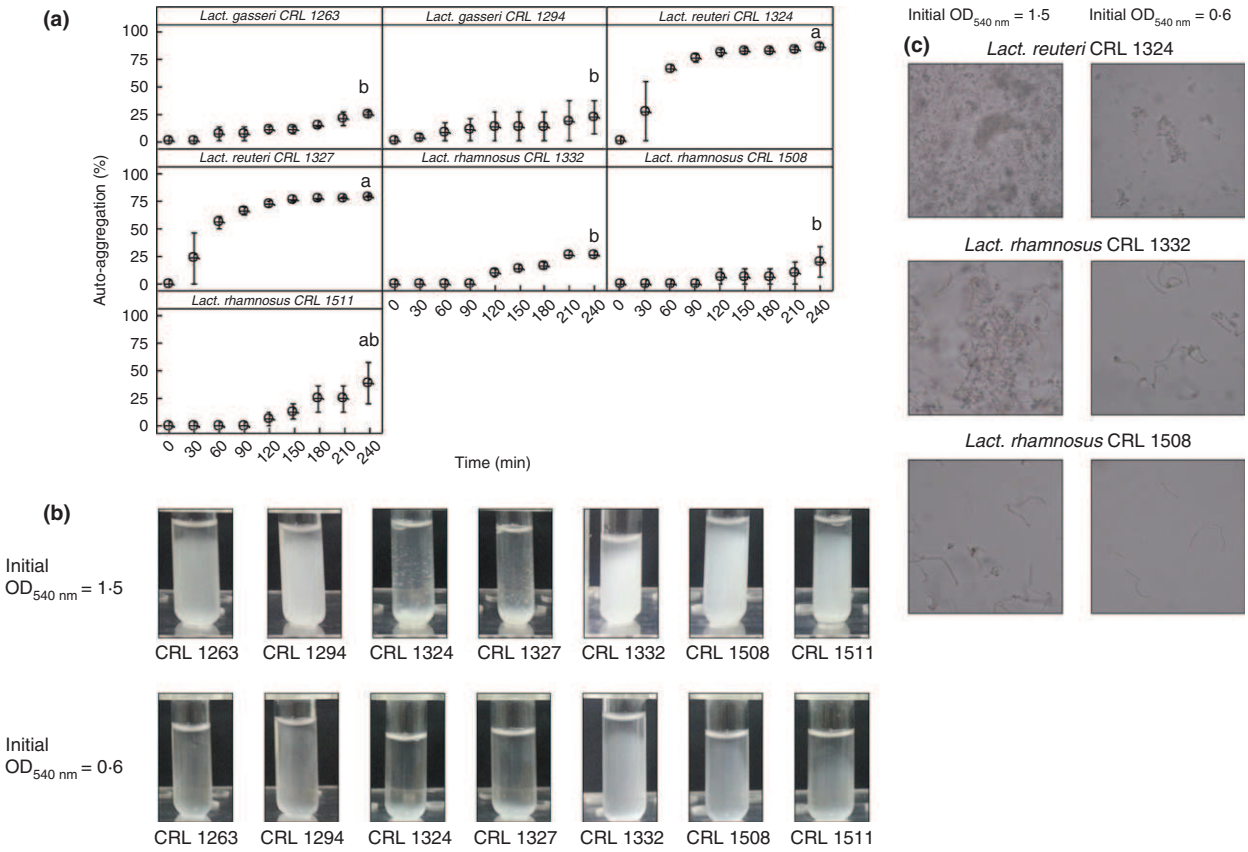

Figure 1 Auto-aggregation of saline-washed cells of different vaginal *Lactobacillus* (VL) strains. (a) Auto-aggregation monitored spectrophotometrically and expressed as auto-aggregation percentage. The data are plotted as average values of aggregation percentages of cell suspensions adjusted to an initial $OD_{540\ nm}$ = 1·5. Statistically significant differences ($P < 0{\cdot}05$) in the aggregation percentages at 240 min are indicated by different letters. (b) Macroscopic observation of VL auto-aggregation from cell suspensions adjusted to initial $OD_{540\ nm}$ = 0·6 and 1·5, after 240 min of assay. (c) Optical microscopy images of *Lact. reuteri* CRL 1324 and *Lact. rhamnosus* CRL 1332 cell clumps, and *Lact. rhamnosus* CRL 1508 single cells.

$OD_{540\ nm}$ and flow cytometry results, significant positive correlations (Pearson's correlation value = 0·706, $P = 0{\cdot}005$) were obtained.

Biofilm formation

Lactobacillus rhamnosus CRL 1332 and *Lact. reuteri* CRL 1324 were able to form biofilm in medium without Tween 80 as evidenced in a previous work (Leccese Terraf *et al.* 2012). Consequently, these VL strains were used as positive controls in this study. ANOVA results indicated that the VL strain and Tween 80 concentration factors exerted statistically significant effects on biofilm formation in polystyrene microplates (Fig. 3a). The VL strain–Tween 80 concentration interaction was also statistically significant, indicating that the absence of Tween 80 favoured biofilm formation only in some VL strains.

Lactobacillus reuteri CRL 1327 was able to produce a higher biofilm in MRS_{-T} ($OD_{570\ nm}$ mean value = 3·1) than *Lact. rhamnosus* strains. $OD_{570\ nm}$ values of *Lact. reuteri* CRL 1327 were similar to those of *Lact. reuteri* CRL 1324 ($OD_{570\ nm}$ mean value = 3·0) used as a positive biofilm control (Fig. 3a). *Lactobacillus rhamnosus* CRL 1508 and CRL 1511 were able to produce detectable biofilm in MRS_{-T} ($OD_{570\ nm}$ mean value = 0·3 for both strains). The $OD_{570\ nm}$ values of these strains were significantly lower than those of *Lact. rhamnosus* CRL 1332 ($OD_{570\ nm}$ mean value = 2·2). None of the strains was able to form biofilm in standard MRS broth. *Lactobacillus gasseri* CRL 1294 did not form detectable biofilm in any of the conditions assayed (similar to *Lact. gasseri* CRL 1263 negative control).

Biofilm formation was also analysed by SEM in *Lact. reuteri* CRL 1327, *Lact. rhamnosus* CRL 1508 and CRL 1511 using glass coverslips incubated in MRS_{-T} broth. Figure 3b, c, d show that the morphological characteristics of the biofilms were different. The biofilm produced by *Lact. reuteri* CRL 1327 was homo-

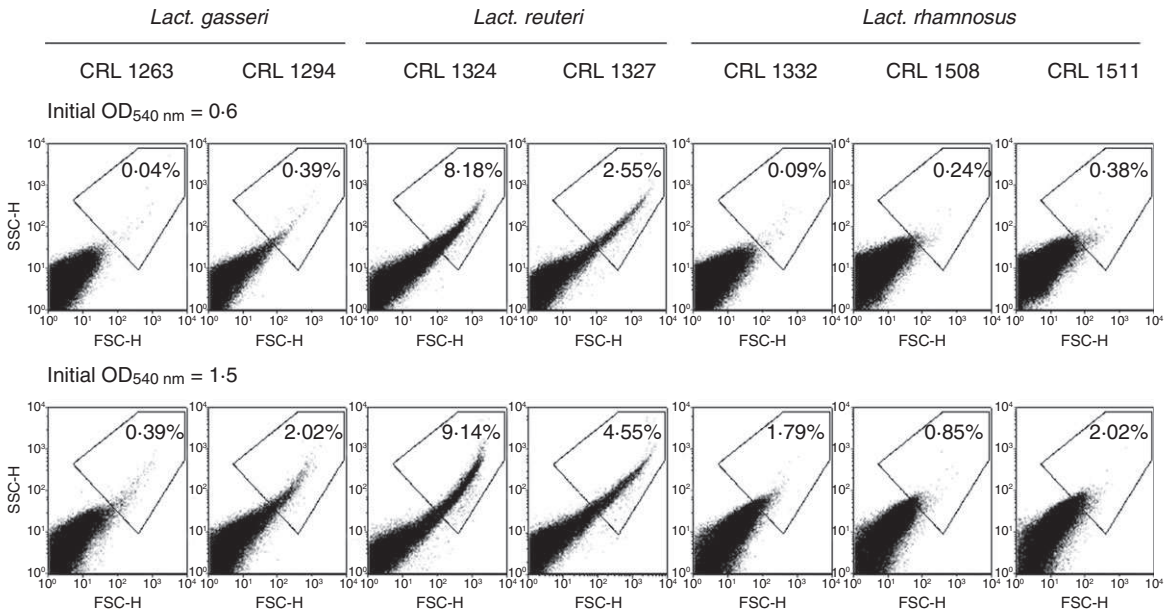

Figure 2 Auto-aggregation of vaginal *Lactobacillus* strains analysed by flow cytometry. Saline-washed bacterial cells were used to prepare the cell suspensions adjusted to initial $OD_{540\ nm}$ = 0·6 and 1·5. Cell aggregation was quantified by determining the percentage of events with high SSC and FSC. These cell clumps consisted of multiple cells and could be homogenized into a single cell suspension using sonication (data not shown).

geneous, covering the whole surface, with abundant extracellular material (Fig. 3b). However, few cell clusters were observed for *Lact. rhamnosus* CRL 1508 and *Lact. rhamnosus* CRL 1511 biofilms (Fig. 3c,d).

Adhesion and biofilm formation on mucin

When assaying VL adhesion to immobilized mucin in microplates, significant differences were observed between the adhesion ability of the various VL strains (Fig. 4). Binding to mucin was significantly higher in *Lact. reuteri* CRL 1324 and *Lact. reuteri* CRL 1327 ($OD_{570\ nm}$ mean value = 3·47 ± 0·10) than in the other strains. In this assay, no significant differences in mucin binding between *Lact. rhamnosus* CRL 1332, CRL 1508 and CRL 1511 were observed ($OD_{570\ nm}$ mean value = 1·72 ± 0·07). *Lactobacillus gasseri* CRL 1263 and CRL 1294 were able to bind to immobilized mucin ($OD_{570\ nm}$ mean value = 0·85 ± 0·12), but the adhesion ability of CRL 1294 was significantly lower than the one obtained in most of the other strains.

When evaluating biofilm formation by VL on immobilized mucin, the strain effect (from ANOVA) was also statistically significant (Fig. 4). Biofilm formation by *Lact. rhamnosus* CRL 1332 ($OD_{570\ nm}$ mean values = 1·6) was similar to *Lact. reuteri* CRL 1324 and CRL 1327, and it was significantly higher than that of the other *Lact. rhamnosus* strains ($OD_{570\ nm}$ mean value = 0·35) and *Lact. gasseri* strains ($OD_{570\ nm}$ mean value = 0·15).

Detection of genes related to sortase enzymes and pili in VL

StrA genes encoding for sortase A enzyme were identified in *Lact. reuteri* CRL 1324 and CRL 1327 as well as in *Lact. gasseri* CRL 1263 and CRL 1294 (Table 2). In the case of *Lact. rhamnosus*, *srtA*, *srtC1* and *srtC2* genes encoding for three different classes of sortases were screened. *Lactobacillus rhamnosus* CRL 1332, CRL 1508 and CRL 1511 were positive for *srtA* and *strC2*. However, the *strC1* gene encoding for the sortase C1 enzyme was only identified in CRL 1332 under the studied conditions (Table 2).

Furthermore, the presence of genes encoding for different pilin subunits, previously reported in the *Lact. rhamnosus* species, was evaluated in this study (Table 2). In *Lact. rhamnosus* CRL 1332, genes encoding for SpaA, SpaB and SpaC subunits were amplified. Also, *spaD*, *spaE* and *spaF* genes were evidenced in this strain. With respect to CRL 1508 and CRL 1511, only *spaD* and *spaF* genes were detected in the assayed conditions.

Presence of genes related to surface proteins involved in adhesion and biofilm formation

Four genes related to adhesion proteins were studied in *Lact. reuteri* CRL 1324 and CRL 1327 (Table 2). In both strains, genes encoding for a mucus adhesion promoting protein (MapA), a mucus-binding protein (MubI) and a cell and mucus-binding protein (CmbA) were identified.

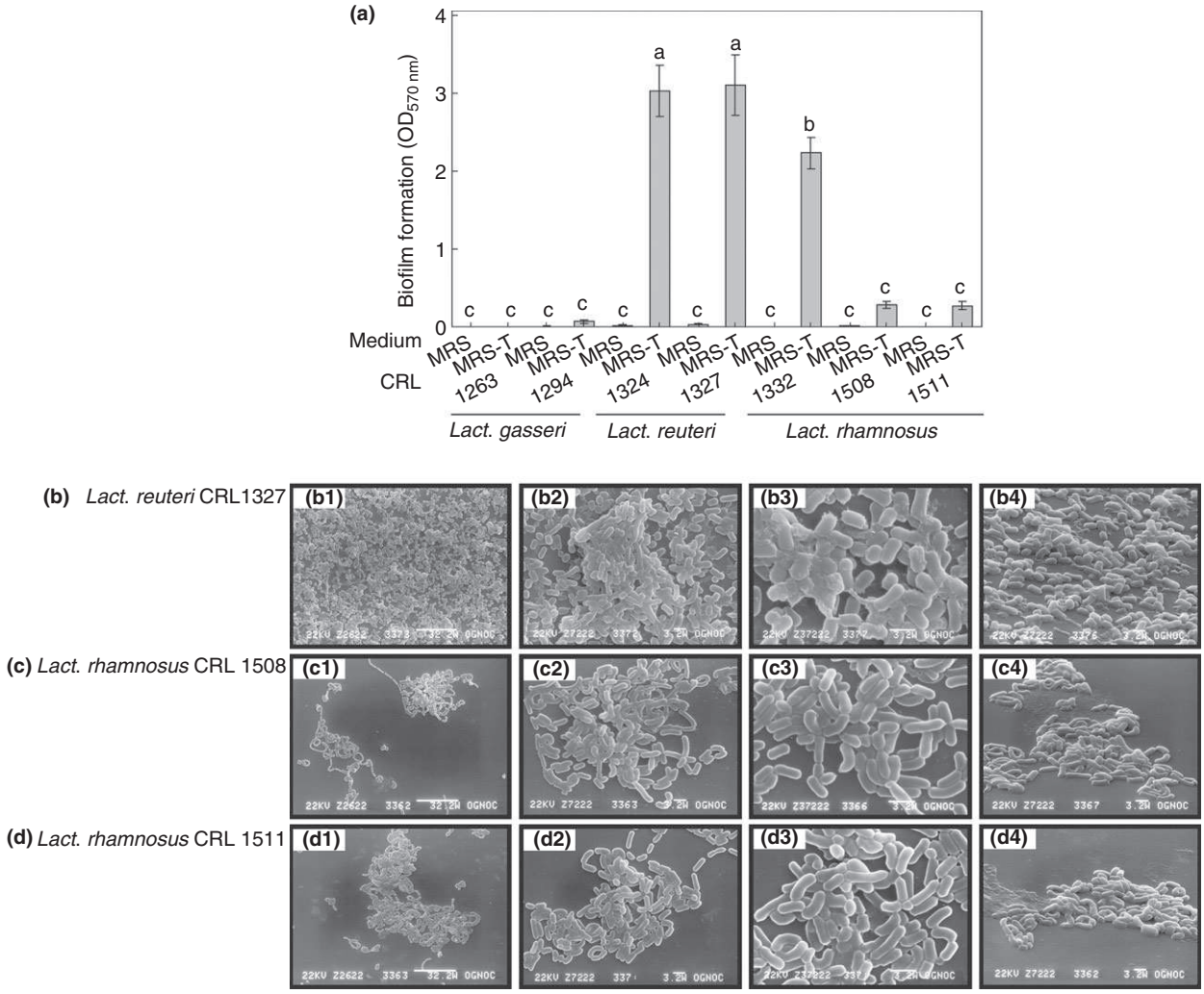

Figure 3 Biofilm formation of seven vaginal *Lactobacillus* (VL) strains. (a) Biofilm formed on polystyrene microplates after VL growth in MRS and MRS without Tween 80 (MRS-T). The data express the mean $OD_{570\ nm}$ values ± standard error of acetic acid-solubilized crystal violet-stained cultures from microplates. Different letters indicate significant differences ($P < 0{\cdot}05$) in biofilm formation between the different conditions assayed for the seven strains according to Tukey's test. Scanning electron microscopy images of biofilm formed by *Lact. reuteri* CRL 1327 (b), *Lact. rhamnosus* CRL 1508 (c) and *Lact. rhamnosus* CRL 1511 (d) in MRS_{-T}. Biofilm cells at 2400× (b-1, c-1, d-1), at 7200× (b-2, c-2, d-2), at 15000× (b-3, c-3, d-3) and biofilm cells at 7200× an angle of 45° (b-4, c-4, d-4).

However, no genes encoding for a mucus-binding protein (MubII) were detected in the *Lact. reuteri* strains assayed.

For *Lact. rhamnosus* strains, genes related to two adhesion factors were identified. The presence of *mabA* (modulator of adhesion and biofilm formation) gene was demonstrated in *Lact. rhamnosus* CRL 1332, CRL 1508 and CRL 1511. Also, a gene encoding for the mucus-binding factor (MBF) was evidenced in the three *Lact. rhamnosus* strains. However, the *welE* gene (encoding for a glycosyltransferase enzyme for the synthesis of a galactose-rich polysaccharide) was not detected in any of the strains by the technique applied.

The presence of the specific genes evaluated in VL strains was confirmed by sequencing the amplified products, which showed 100% identity with the available sequences in the GenBank (with the exception of the *spaF* gene, which showed 95-100% identity).

Summary of the main phenotypic and genetic features determined in VL

The expression of individual surface properties (aggregation, adhesion and biofilm formation) and the presence of related genes in seven VL strains are summarized in (Table 2). *Lactobacillus reuteri* CRL 1324 and CRL

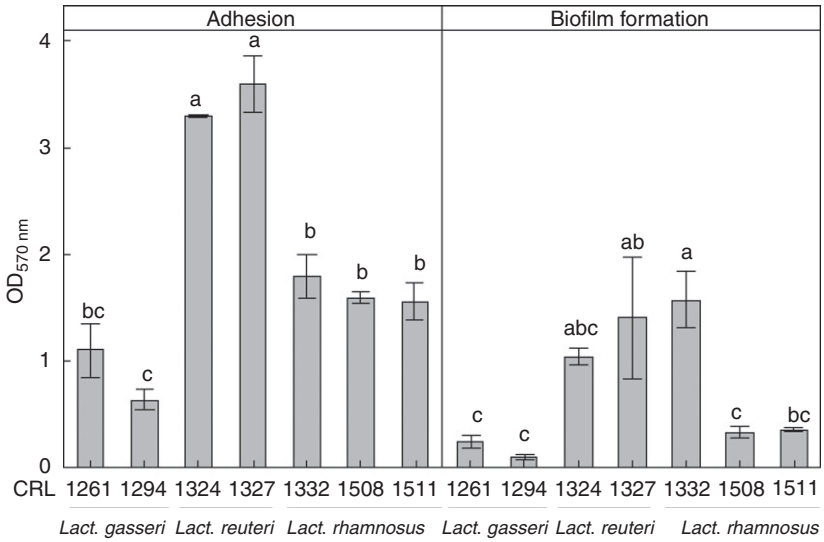

Figure 4 Adhesion and biofilm formation of vaginal *Lactobacillus* on immobilized mucin. The data express the mean $OD_{570\ nm}$ values ± standard error of acetic acid-solubilized crystal violet-stained cultures from microplates. Different letters indicate significant differences ($P < 0{\cdot}05$) in the adhesion or biofilm formation between the different strains according to Tukey's test.

1327 and *Lact. rhamnosus* CRL 1332 express interesting surface properties and contain genes encoding for relevant proteins related to these characteristics. These strains were selected for further studies as candidate micro-organisms for inclusion in vaginal probiotics.

Discussion

The phenomena related to the surface properties of beneficial vaginal lactobacilli such as aggregation, adhesion and biofilm formation capabilities could be involved in their subsequent permanence and colonization and in the beneficial effects on the host (Younes *et al.* 2012).

The phenotypic expression of bacterial surface characteristics can be evaluated by different methodologies. In this work, two different approaches were used to assay auto-aggregation ability in VL cells: a spectrophotometric assay complemented with a flow cytometry study. Through the conventional spectrophotometric assay, the microbial aggregation was followed during the length of the assay. By flow cytometry, information on the size and complexity of cell aggregates was obtained. A significant positive correlation was obtained between the results of the two techniques; therefore, complementary data could be obtained. *Lactobacillus reuteri* strains showed the highest percentages of auto-aggregation compared with *Lact. rhamnosus* and *Lact. gasseri* strains. In a similar way, Mackenzie *et al.* (2010) reported that auto-aggregation is a strain-dependent multifactorial process, because the aggregation levels of PBS-washed cells of *Lact. reuteri* ATCC 53608 and ATCC 55739 were significantly higher than those of *Lact. reuteri* 1063N and DSM 20016T.

The auto-aggregation percentages of *Lact. gasseri* CRL 1294 determined in previous works using VL cell suspensions in PBS were higher than the values obtained in this work using bacterial suspensions in saline (Juárez Tomás *et al.* 2005a). Opposite results were observed for *Lact. reuteri* CRL 1324 using PBS (Juárez Tomás *et al.* 2005b; De Gregorio *et al.* 2014), which can be supported by the fact that the aggregation phenomenon is affected by ionic strength (Mackenzie *et al.* 2010). Mackenzie *et al.* (2010) demonstrated that *Lact. reuteri* DSM 20016^T (a human isolate) exhibited high aggregation during growth in MRS broth, but cell aggregates were lower when diluted in PBS. In contrast, *Lact. rhamnosus* and *Listeria monocytogenes* showed the highest auto-aggregation abilities in PBS (29·4% and 24·7%, respectively) than in trypticase soy broth (17·5 and 5·2%, respectively) (Woo and Ahn 2013).

The vaginal epithelium is covered by a protective layer of mucus, which is mainly composed of mucins (Dasari *et al.* 2007). In this study, most of the VL strains studied were able to adhere and form biofilm on mucin. The highest mucin adhesion ability was observed for *Lact. reuteri* strains while high biofilm formation capability was evidenced for *Lact. rhamnosus* CRL 1332 and *Lact. reuteri* strains, indicating that adhesion and biofilm formation are strain-dependent. Mackenzie *et al.* (2010) demonstrated that the binding properties of lactobacilli to host intestinal mucus are strain-specific because *Lact. reuteri* ATCC 53608 (a pig isolate) and *Lact. reuteri*

Table 2 Individual surface properties and the presence of related genes in vaginal *Lactobacillus* (VL) strains

	VL strains						
	Lact. gasseri		*Lact. reuteri*		*Lact. rhamnosus*		
Phenotypic expression of surface properties	CRL 1263	CRL 1294	CRL 1324	CRL 1327	CRL1332	CRL1508	CRL1511
Auto-aggregation							
(%)* from $OD_{540\ nm}$	24·04	21·66	86·33	79·00	26·66	19·99	39·33
(%)* from FC	0·39	2·02	9·14	4·55	1·79	0·85	2·02
Cell clumps†	−	−	+	+	+	−	−
Biofilm formation on polystyrene							
$OD_{570\ nm}$‡	0·0	0·1	3·0	3·1	2·2	0·3	0·3
Biofilm formation on mucin							
$OD_{570\ nm}$‡	0·2	0·1	1·0	1·4	1·6	0·3	0·4
Adhesion to mucin*							
$OD_{570\ nm}$‡	1·1	0·6	3·3	3·6	1·8	1·6	1·6
Presence of genes related to surface properties§							
Enzyme sortases							
srtA (sortase A)	+	+	+	+	+	+	+
srtC1 (sortase C1)					+	−	−
srtC2 (sortase C2)					+	+	+
Pilins							
spaA (pilin A)					+	−	−
spaB (pilin B)					+	−	−
spaC (pilin C)					+	−	−
spaD (pilin D)					+	+	+
spaE (pilin E)					+	−	−
spaF (pilin F)					+	+	+
Surface proteins							
mabA (modulator of adhesion and biofilm formation)					+	+	+
MBF (mucus-binding factor)					+	+	+
mapA (mucus adhesion promoting protein)			+	+			
mubI (mucus-binding proteins)			+	+			
mubII (mucus-binding proteins)			−	−			
cmbA (mucus- binding protein A)			+	+			
welE (glycosyltransferase enzyme)					−	−	−

*The data represent the mean values of auto-aggregation percentages from optical density values at 540 nm ($OD_{540\ nm}$, at the end of spectrophotometric assay) or from flow cytometry (FC) of cell suspensions in saline adjusted to an initial $OD_{540\ nm}$ = 1·5.
†Optical microscopy: +, presence of cell aggregates; −, absence of cell aggregates (single cells).
‡The data represent the mean optical density at 570 nm ($OD_{570\ nm}$) values of acetic acid-solubilized, crystal violet-stained cultures from microplates, in assays of biofilm formation on polystyrene microplates and of adhesion and biofilm formation on immobilized mucin.
§Genes related to surface properties: +, presence of gene and −, gene not detected under the assayed conditions.

ATCC 55739 (a rat isolate) showed higher binding to mouse mucus than other 23 *Lact. reuteri* strains.

The surface of Gram-positive organisms consists in a cell wall made of peptidoglycan grafted with proteins, teichoic acids, lipoteichoic acids and polysaccharides (Sengupta *et al.* 2013). Sortase A (SrtA, a transpeptidase) can anchor different cell surface proteins that contain the sorting signal LPXTG motif, which could facilitate cell adhesion (Spirig *et al.* 2011; Remus *et al.* 2013). SrtA has been identified in the genomes of different *Lactobacillus* species (Call and Klaenhammer 2013). In the present study, the presence of the *srtA* gene was evidenced for the first time in *Lact. rhamnosus*, *Lact. reuteri* and *Lact. gasseri* strains isolated from human vagina. Further studies will be performed to determine whether the presence of SrtA favours the expression of some surface properties that promote intimate associations of VL with the host.

With respect to the *Lact. gasseri* species, the available literature has only reported the presence of genes encoding for a high number of putative mucus-binding proteins (Azcarate-Peril *et al.* 2008; Kleerebezem *et al.* 2010). To the best of our knowledge, their functional role has not yet been demonstrated; therefore, the genes related to putative mucus-binding proteins of *Lact. gasseri* were not studied in this work.

Lactobacillus rhamnosus GG, the most studied probiotic organism (isolated from healthy human gut microbiota), contains two putative clusters of pilus-encoding genes: the *spaCBA* cluster [genes for spaA, spaB, spaC (pilins) and srtC1 (sortase C1)] and the *spaFED* cluster [genes for spaD, spaE, spaF (pilins) and srtC2 (sortase C2)] (Kankainen *et al.* 2009). SpaCBA pili of *Lact. rhamnosus* GG have an important role in its strong adhesive interaction with the Caco-2 cell intestinal epithelial cell line and with biofilm formation (Lebeer *et al.* 2012). Two pilin subunits codified in the *spaCBA* cluster, SpaB and SpaC, and only a single pilin subunit (SpaF) of the *spaFED* cluster are potentially responsible for the adhesion of *Lact. rhamnosus* GG to mucus (Kankainen *et al.* 2009; von Ossowski *et al.* 2010). In the present study, the presence of pilus-related genes in vaginal *Lact. rhamnosus* strains was evaluated. Only in *Lact. rhamnosus* CRL 1332 were the complete *spaCBA* and *spaFDE* clusters identified. However, in *Lact. rhamnosus* CRL 1508 and CRL 1511, only a few genes involved in pili formation were detected.

In addition to pili, other cell surface proteins in *Lact. rhamnosus* strains such as MabA (modulator of adhesion and biofilm) and MBF (mucus-binding factor) have been described in relation to their adhesion properties (Vélez *et al.* 2010; von Ossowski *et al.* 2011). In this work, *MabA* and *MBF* genes were detected in three vaginal *Lact. rhamnosus* strains assayed, which can explain the ability of these strains to adhere and form biofilm on mucin. MabA adhesion protein has been described in *Lact. rhamnosus* GG; a knockout mutant strain in this protein showed a lower biofilm formation capability and a lower adhesion to intestinal epithelial cells and tissues of the murine gastrointestinal tract (Vélez *et al.* 2010). On the other hand, von Ossowski *et al.* (2011) showed that MBF, a LPXTG like protein, was distributed throughout the cell surface and participated in the adhesive interaction between *Lact. rhamnosus* GG and mucus.

The adhesion ability of beneficial micro-organisms can be also related to bacterial surface polysaccharides (Lebeer *et al.* 2009). We were not able to identify *welE* gene [encoding the priming glycosyltransferase involved in the biosynthesis of a long, galactose-rich exopolysaccharide (EPS)] in vaginal *Lact. rhamnosus* CRL 1332, CRL 1508 and CRL 1511. This gene was reported in *Lact. rhamnosus* GG (Lebeer *et al.* 2009). The deprivation of EPS molecules resulted in an increased adherence and biofilm formation capability of *Lact. rhamnosus* GG, possibly because of a lower shielding of adhesins such as fimbria-like structures (Lebeer *et al.* 2009, 2012).

In *Lact. reuteri* strains, several adhesion proteins have been described. CnBP, which binds to collagen type-I, was the first surface protein involved in adhesion described in *Lact. reuteri* NCIB 11951 (Aleljung *et al.* 1994; Roos *et al.* 1996). In addition, MapA (mucus adhesion promoting protein) is other protein involved in the adhesion phenomenon of *Lact. reuteri* strains (Rojas *et al.* 2002). This protein binds to both mucus (Rojas *et al.* 2002) and Caco-2 cells (Miyoshi *et al.* 2006). CnBP and MapA are considered homologues in the light of their similarity at the amino acid level (94%).

Mucus-binding proteins (MUBs) have been associated with mechanisms of adherence of lactobacilli to the host in *Lact. reuteri* strains (Roos and Jonsson 2002; Mackenzie *et al.* 2010). These proteins were described in *Lact. reuteri* ATCC 53608 and *Lact. reuteri* ATCC 55739 that showed the highest cell aggregation and binding to mouse and pig gastric mucin, compared with other *Lact. reuteri* strains. However, *Lact. reuteri* 1063N that produces a truncated MUB showed low aggregation and mucus-binding capacities (Mackenzie *et al.* 2010). This adhesion protein presents a high genetic variability among strains. Then, we used two different sequences, MubI of *Lact. reuteri* I5007 and MubII of *Lact. reuteri* ATCC 53608, to design primers in order to identify MUB protein in VL strains.

Recently, Jensen *et al.* (2014) described that a putative sortase-dependent protein encoded by the *hmpref0563_10633* gene in *Lact. reuteri* ATCC PTA 6475 plays a significant role in the ability to adhere to cells and mucus. Also, the authors proposed that this new adhesion protein should be referred as cell and mucus-binding protein A (CmbA). In this study, the genes encoding for three adhesion proteins (MubI, MapA and CmbA) in vaginal *Lact. reuteri* CRL 1324 and CRL 1327 were identified. We were not able to demonstrate the presence of *mubII* gene in the strains evaluated.

It is of fundamental importance to know the surface properties of commensal and potential probiotic bacteria, to predict their adhesion ability and permanence in the hosts, but the knowledge of the bacterial molecular mechanisms mediating adhesion to the vaginal mucosa is still limited. This is the first study on the beneficial surface properties of different vaginal *Lactobacillus* strains through phenotypic and genetic assays. The VL strains assayed exhibited *in vitro* surface characteristics that were specific for each strain and that could be associated with the presence of some genes. However, additional molecular studies are necessary to evaluate the potential correlation between phenotypic and genetic features in vaginal lactobacilli. We chose three strains (*Lact. reuteri* CRL 1324 and CRL 1327 and *Lact. rhamnosus* CRL 1332) for further *in vitro* and *in vivo* studies, to go deeper in the knowledge of the mechanisms for the establishment and beneficial effects of lactobacilli in the vaginal niche.

Acknowledgements

This paper was supported by CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina) (PIP 744) and ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica) (PICT 2012-1187). *Lactobacillus gasseri* CRL 1294 was licensed to Probiotical-Anidral for industrial production. None of the authors have a conflict of interest for the publication of the manuscript.

Conflict of Interest

No conflict of interest declared.

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