Characterization of the adherence properties of human Lactobacilli strains to be used as vaginal probiotics

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Abstract

In the present work, the adhesion of 43 human lactobacilli isolates to mucin has been studied. The most adherent strains were selected, and their capacities to adhere to three epithelial cell lines were studied. All intestinal strains and one vaginal isolate adhered to HT-29 cells. The latter was the most adherent to Caco-2 cells, although two of the intestinal isolates were also highly adherent. Moreover, five of the eight strains strongly adhered to HeLa cells. The binding of an Actinomyces neuii clinical isolate to HeLa cells was enhanced by two of the lactobacilli and by their secreted proteins, while those of another two strains almost abolished it. None of the strains were able to interfere with the adhesion of Candida albicans to HeLa cells. The components of the extracellular proteome of all strains were identified by MALDI-TOF/MS. Among them, a collagen-binding A precursor and aggregation-promoting factor–like proteins are suggested to participate on adhesion to Caco-2 and HeLa cells, respectively. In this way, several proteins with LysM domains might explain the ability of some bacterial supernatants to block A. neuii adhesion to HeLa cell cultures. Finally, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) could explain the good adhesion of some strains to mucin.

Introduction

The balance between the different microorganisms inhabiting the human vagina is important for the maintenance of its homeostasis, affecting directly the health status of the woman. Among the resident microorganisms, the Lactobacillus isolates represent at least 70% of the bacteria sampled (Redondo-López et al., 1990; Martín et al., 2008b) being the most dominant L. crispatus, L. jensenii, and L. gasseri and in less extent L. salivarius, L. vaginalis, and L. iners (Boyd et al., 2005; Martin et al., 2008a, b). Because of their relative abundance, lactobacilli have been proposed as probiotics to be used against the establishment and overgrowth of pathogenic microorganisms in the vagina. These benefits would be exerted by two different mechanisms: (i) competition for attachment sites on epithelial cells and pathogen co-aggregation and (ii) production of antimicrobial compounds (Lepargneur & Rousseau, 2002). The first leads to formation of a biofilm that prevents the colonization by undesirable microorganisms (Antonio et al., 2005). In addition, some lactobacilli can co-aggregate with these potential pathogens, such as Escherichia coli, Candida albicans, and Gardnerella vaginalis, which may help in the clearance of the pathogen (Boris et al., 1998; Osset et al., 2001). The antimicrobials are mainly organic acids produced from the fermentation of sugars, which leads to the typical low pH of the vagina. This low pH is able to inhibit the growth of most pathogens (Boskey et al., 2001).

Probiotics are defined as ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’ (FAO/WHO, 2006). Use of lactobacilli as probiotic agents in the human genitourinary tract has a long history of safe use, which dates from 1915 (Newman, 1915). Among the physiological traits that are desirable for potential probiotic lactobacilli, adhesion to...
epithelial surfaces is of paramount importance. It is well known that, in healthy women, the cervix produces mucus that is mainly composed of mucin, among other components (Moghissi et al., 1960) acting as a protective barrier for the uterus and the vagina (Wang & Lee, 2002). A good adhesion to mucus is thus a desirable characteristic, which may increase the residence time of probiotic lactobacilli, as happens with intestinal Lactobacillus strains (McGrady et al., 1995; Perea Vélez et al., 2007). The quick turnover of the vaginal mucosa makes adhesion a crucial feature for the establishment and colonization of probiotic lactobacilli; thus, it is necessary to characterize the bacterial adhesion an efficient in vitro model (Van den Abbeele et al., 2009).

In the present study, the adhesion abilities of 32 vaginal and 11 intestinal Lactobacillus strains to mucin have been characterized. Among them, eight strains were selected because of their good probiotic characteristic, which may increase the residence time of probiotic lactobacilli; thus, it is necessary to characterize the bacterial adhesion an efficient in vitro model (Van den Abbeele et al., 2009).

Materials and methods

Bacterial strains, cell lines, and growth conditions

The Lactobacillus strains used in this study were isolated from the vagina of fertile women or had an intestinal origin and were selected because of their good probiotic properties (Martin et al., 2008a, unpublished data). Actinomyces neuii R1 was isolated from a vaginal swab of a woman with vulvovaginitis, whereas C. albicans CECT 1392, Lactobacillus rhamnosus GG (ATCC 53103), and Lactobacillus plantarum 299V (DSM 9843) were obtained from the Colección Española de Cultivos Tipo, the American Type Culture Collection and the German Collection of Microorganisms and Cell Cultures, respectively.

Lactobacilli were grown in MRS broth (Difco, Detroit), whereas C. albicans and A. neuii were grown in BHI broth (Oxoid, Cambridge, UK) supplemented with 1% (w/v) yeast extract (Difco), 0.1% (w/v) maltose (VWR, Haasrode, Belgium), 0.1% (w/v) glucose (VWR), and 1% (v/v) defibrinated horse blood (Eurobio, Les Ulis, France) (supplemented BHI, S-BHI). When appropriated, 1.5% (w/v) agar (Difco) was added to the liquid medium. Actinomyces neuii, C. albicans, and agar cultures of the lactobacilli were incubated in anaerobic jars using the AnaeroGen Compact system (Oxoid). All the strains were grown at 37 °C.

The Caco-2 (HTB-37) (LGC-Standards, Molsheim, France) cell line was routinely grown in Eagle’s minimal essential medium (EMEM) (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). HeLa (ATCC CCL-2) and HT-29 (HTB-38) (LGC-Standards) cell lines were grown in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) (Sigma-Aldrich). Both culture broths were supplemented with 10% (w/v) heat-inactivated fetal bovine serum (GibcoBRL, Eragny, France) and with penicillin G/streptomycin (5000 IU mL⁻¹, 5000 µg mL⁻¹) (Sigma-Aldrich). Cultures were incubated in 25 cm² tissue culture flasks (Nunc, Roskilde, Denmark) at 37 °C in a 5% (v/v) CO₂ atmosphere until confluence. For adhesion assays, 2500 cells per well were seeded in 12-well culture plates (Nunc) and cultivated, with a daily change of the culture medium until confluence (Tallon et al., 2007).

Adhesion assays

Adhesion to porcine gastric mucin (Porcine gastric mucin, type III, Sigma-Aldrich), Caco-2, HT-29, and HeLa monolayers of the lactobacilli and the pathogenic bacteria was tested following the procedure described by Tallon and co-workers (Tallon et al., 2007), using around 10⁶ CFUs (as determined by plate count) for the adhesion to mucin and 50 bacteria per eukaryotic cell for the adhesion to epithelial cell tests. Overnight bacterial cultures, in the early stationary phase of growth, and confluent eukaryotic cell cultures were used in all cases. Assays were performed at least in triplicate, and the data are expressed as the mean ± SD.

Protein binding assay

Binding assays were performed using the surface proteins extracted from 50 mL of culture or secreted proteins extracted from 20 mL of culture and mucin as coated matrix on 96-well plates as described before (Sánchez et al., 2009). Proteins were resolved by SDS-PAGE and then visualized by standard silver staining. Proteins able to bind mucin were identified by its relative electrophoretic mobility with respect to the surface proteins profiles.

Interference with pathogen adhesion

The effect of the eight Lactobacillus strains on the adhesion of C. albicans CECT 1392 and A. neuii R1 to HeLa cells was performed as described earlier, using a probiotic/pathogen ratio of 10 : 1. After incubation with the cell line monolayers and five PBS washes, aliquots of the cultures or their dilutions were transferred to plates containing
S-BHI with 20 μg mL⁻¹ penicillin G (selective for *C. albicans*) or 16 μg mL⁻¹ erythromycin (Sigma-Aldrich) (selective for *A. neuii*). The susceptibility of all *Lactobacillus* strains to both antibiotics was confirmed prior to the adhesion assays.

To check the effect of extracellular proteins on the adhesion of the two pathogens to HeLa cells, 250 or 500 μg of crude extracellular protein preparations was added to each well and incubated at 37 °C for 1 h prior to the adhesion assays, which were performed using 50 microorganisms per eukaryotic cell, as already described. At least three independent assays were performed, and the results were expressed as the mean ± SD.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism Software version 5.00 for Windows (San Diego, CA). The groups were compared using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison post hoc analysis. A *P*-value of < 0.05 was considered significant.

**Results**

**Adhesion to mucin of the *Lactobacillus* strains**

Adhesion of 43 human lactobacilli, isolated from the gastrointestinal tract or from vagina, to mucin was first characterized (Supporting Information, Table S1). Of the 43 strains tested, 27 showed higher adhesion capabilities to mucin than *L. rhamnosus* GG being statistical significant for 10 of them (*P*-value < 0.05). In fact, the best performing strain, *L. plantarum* Li70, adhered 51 times more than *L. rhamnosus* GG. In the rest of the experiments, only the eight most adherent lactobacilli with different RAPD profile were selected (Table 1, Data S1). Strain Lv67 was also selected as a negative control.

**Bacterial adhesion to epithelial cell lines**

Adhesion was tested using two epithelial cell lines of intestinal origin (Caco-2 and HT-29) and the vaginal cell line HeLa (Fig. 1). *Lactobacillus casei* Li71, *L. gasseri* Lv19, and *L. plantarum* Li68 were the most adherent strains to HeLa cells. *Lactobacillus vaginalis* Lv67, *L. plantarum* Li68, and *L. casei* Li71 showed the best adhesion to Caco-2, and finally, *L. plantarum* Li68, *L. plantarum* Li69, *L. plantarum* Li70, *L. casei* Li71, and, to a lesser extent, *L. vaginalis* Lv67 were the most adherent to HT-29. All the adhesion values showed statistical differences (*P*-value < 0.05) comparing to each control in all the cell lines used.

**Table 1.** Adhesion to mucin, classification according to their RAPD profiles, and identification of the strains at the level species by partial sequencing of ribosomal DNA gene 16S. Data have been normalized using the adhesion values of *Lactobacillus casei* ssp. *rhamnosus* GG (ATTC 53103) because of its good adhesion capacities to this compound (Gueimonde et al., 2004), which was given the arbitrary value of 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>RAPD profile</th>
<th>Species</th>
<th>Adhesion (mean ± SD)</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li81</td>
<td>1a</td>
<td><em>Lactobacillus plantarum</em></td>
<td>23.32 ± 9.91*</td>
<td>Intestinal</td>
</tr>
<tr>
<td>Li82</td>
<td>1a</td>
<td><em>Lactobacillus plantarum</em></td>
<td>3.11 ± 1.61</td>
<td>Intestinal</td>
</tr>
<tr>
<td>Li83</td>
<td>1a</td>
<td><em>Lactobacillus plantarum</em></td>
<td>36.35 ± 15.17</td>
<td>Intestinal</td>
</tr>
<tr>
<td>Li84</td>
<td>1a</td>
<td><em>Lactobacillus plantarum</em></td>
<td>14.94 ± 12.28</td>
<td>Intestinal</td>
</tr>
<tr>
<td>Li85</td>
<td>1a</td>
<td><em>Lactobacillus plantarum</em></td>
<td>1.95 ± 1.02</td>
<td>Intestinal</td>
</tr>
<tr>
<td>Li86</td>
<td>1a</td>
<td><em>Lactobacillus plantarum</em></td>
<td>41.28 ± 27.98*</td>
<td>Intestinal</td>
</tr>
<tr>
<td>Li87</td>
<td>1a</td>
<td><em>Lactobacillus plantarum</em></td>
<td>14.46 ± 5.31*</td>
<td>Intestinal</td>
</tr>
<tr>
<td>Li69</td>
<td>1a</td>
<td><em>Lactobacillus plantarum</em></td>
<td>38.20 ± 2.17*</td>
<td>Intestinal</td>
</tr>
<tr>
<td>Li70</td>
<td>1b</td>
<td><em>Lactobacillus plantarum</em></td>
<td>51.81 ± 32.47*</td>
<td>Intestinal</td>
</tr>
<tr>
<td>Lv19</td>
<td>2</td>
<td><em>Lactobacillus gasseri</em></td>
<td>17.48 ± 8.01*</td>
<td>Vaginal</td>
</tr>
<tr>
<td>Li68</td>
<td>3</td>
<td><em>Lactobacillus plantarum</em></td>
<td>9.65 ± 1.24*</td>
<td>Intestinal</td>
</tr>
<tr>
<td>Lv72</td>
<td>4</td>
<td><em>Lactobacillus salivarius</em></td>
<td>4.49 ± 0.18*</td>
<td>Vaginal</td>
</tr>
<tr>
<td>Li71</td>
<td>5</td>
<td><em>Lactobacillus casei</em></td>
<td>17.44 ± 10.94*</td>
<td>Intestinal</td>
</tr>
<tr>
<td>Lv57</td>
<td>6</td>
<td><em>Lactobacillus jensenii</em></td>
<td>1.01 ± 0.08*</td>
<td>Vaginal</td>
</tr>
<tr>
<td>Lv67</td>
<td>7</td>
<td><em>Lactobacillus vaginalis</em></td>
<td>0.58 ± 0.67</td>
<td>Vaginal</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SD of at least three independent experiments.

*Strains more adherent than LGG (*P*-value < 0.05).

**Effect on pathogen adhesion**

The effect of the lactobacilli and their secreted proteins on the adhesion of the vaginal pathogens *C. albicans* and *A. neuii* to HeLa cells was then investigated (Fig. 2). Inhibition values were calculated as adherent bacteria per HeLa cell. *Lactobacillus gasseri* Lv19 and *L. plantarum* Li70 increased significantly the adhesion of *A. neuii* R1 to HeLa cells (*P*-value < 0.05 and 0.001, respectively), as well as their extracellular proteins (*P*-value < 0.001), although the proteins of Lv70 do not show statistical differences (Fig. 2a and b). Conversely, the proteins secreted by *L. plantarum* Li69 and *L. salivarius* Lv72 abrogated the adhesion of *A. neuii* to the same cell line (*P*-value < 0.05) (Fig. 2b). Regarding *C. albicans*, some *Lactobacillus* strains slightly enhanced the adhesion of the yeast (no significant differences) (Fig. 2c), while their secreted proteins did not have any effect (Fig. 2d).

**Identification of the *Lactobacillus* secreted and surface-associated proteins**

Crude preparations of the proteins secreted by the eight *Lactobacillus* strains in MRS broth (Fig. 3a) and their surface-associated proteins (Fig. 3b) were resolved by SDS-PAGE. The most intense bands were excised manually and identified by mass spectrometry (Tables S2 and S3).
Among the extracellular proteins detected, cell wall hydrolases, muramidases, peptidoglycan-binding polypeptides, and a precursor of the collagen-binding A protein were identified. In addition, some moonlighting proteins, such as glyceraldehyde 3-phosphate dehydrogenase, were also found. The bacterial lysis of the cultures was negligible, as can be deduced from the comparison of secreted protein/total protein profiles obtained by SDS-PAGE (Fig. 3c).

**Adhesion of extracellular proteins to mucin**

Analysis of the relative electrophoretic mobility of the proteins recovered after binding experiments suggested that the surface proteins ABC transporter periplasmic protein, ornithine carbamoyltransferase, and a high-affinity cystine-binding protein bound mucin (Fig. 4a). Also, the secreted GAPDH of *L. plantarum* Li69 and Li70 and that of *L. gasseri* Lv19 bound mucin, as it did muramidase and putative extracellular protein from *L. plantarum* Lv69 and Li70 (Fig. 4b).

**Discussion**

One of the tests considered as crucial by the FAO/WHO for the *in vitro* evaluation of potential probiotic candidates is their capacity to adhere to mucin and human epithelial cells, as well as their antagonism toward pathogen establishment (FAO/WHO, 2006). The eight most adherent *Lactobacillus* strains were selected, and their adhesion abilities to three cell lines, their capability of interfering with the adhesion of two vaginal pathogens to a model human cell line, and the identification of their extracellular proteins and their ability to bind mucin were established.

Presence of typical intestinal lactobacilli, such as *L. plantarum*, in vaginal environment has been reported previously and related to the decreased risk of bacterial vaginosis (Antonio *et al.*, 2005). Besides, the vaginal epithelium is also covered by a protective layer of mucus, which is mainly composed of mucins as the intestinal one, although no commercial vaginal mucin is available (Dasari *et al.*, 2007). In this context, mucins produced in the gastrointestinal and vaginal epithelium are very different. In the gut, MUC2 is mainly produced by goblet cells (McGuckin *et al.*, 2011), whereas in the vaginal epithelium, MUC1, MUC4, MUC5AC, MUC5B, or MUC6 is produced, depending on the location (Gipson *et al.*, 1997).

Regarding the adhesion experiments to human cell lines, the four intestinal isolates presented affinities to HT-29 cells in the order of the positive control *L. plantarum* 229V. Therefore, this is an especially valuable probiotic property that, join to their ability to resist bile salts and acid (data not shown), might allow the use of Lv67, Li68, and Li71 in restoration of the vaginal ecosystem through oral administration.
Binding of lactobacilli or their secreted compounds may either hinder colonization of the epithelium by potential pathogens, or create a barrier between them and the mucosal cells, thus excluding direct contact with the underlying epithelium. The lactobacilli strains were confronted to *C. albicans*, which is responsible for at least 85% of human candidiasis (Rein, 1997), and *A. neuii*, which is the second most frequent microorganism isolated in the Ison and Hay grade II and III vaginal microbiota represented by bacterial vaginosis-related organisms (Verhelst et al., 2005) and has been also associated with bacterial vaginosis in women with intrauterine devices (Chatwani & Amin-Hanjani, 1994). Four of the lactobacilli enhanced the adherence of *C. albicans* and *A. neuii* to HeLa cells, which contrasts with previous findings, where pathogen adhesion inhibition was reported (Boris et al., 1998; Osset et al., 2001). This fact suggests that this trait is strain specific. In fact, although the formation of a ternary complex pathogen–*Lactobacillus*–epithelial cell might enhance the antimicrobial effect of the lactic acid generated by this bacteria (Boris et al., 1997; Coudeyras et al., 2008), these ternary complexes could also enhance the pathogen adhesion as has been observed with *Lactobacillus acidophilus* and the adhesion of *C. albicans* to the contraceptive vaginal ring (Chassot et al., 2010).

Adhesion of *A. neuii* was very responsive to the addition of the extracellular proteins of the lactobacilli in a strain-dependent fashion. Five of them enhanced adsorption of the pathogen, thus reproducing the results obtained when whole bacterial cells were used. It is worth mentioning the extraordinary adhesion increment brought about by *L. gasseri* Lv19, which could be due to the secretion of an aggregation-promoting factor–like protein. In fact, it has already been described that these factors act as bridges between pathogen and human cells (Marcotte et al., 2004). This synergistic effect has also been described for some exopolysaccharides produced by several probiotic bacteria, including *L. rhamnusus* GG (Ruas-Madiedo et al., 2006).

Interestingly, the extracellular proteins of *L. plantarum* Li69 and of *L. salivarius* Lv72 markedly inhibited the adhesion of *A. neuii* to HeLa cells. Among the different proteins secreted by these strains, several contained LysM domains, such as two peptidoglycan-binding proteins of Lv72. The LysM domain has been proposed to be the attachment site of the autolysin AcmA of *Lactococcus lactis* to peptidoglycan (Steen et al., 2003). Recently, an extracellular chitin-binding protein from *L. plantarum*, containing this domain, has been shown to attach to the cell surface and to selective bind N-acetylglucosamine-containing polymers (Sánchez et al., 2010). Notably, the Lv19 extracellular proteome, which enhanced *A. neuii* adhesion, did not include any LysM-bearing polypeptides. It is thus conceivable that binding of the LysM-bearing proteins to the *A. neuii* surface might block the ligands...
that recognize the surface of the HeLa cells, as already shown for other proteins (Spurbeck & Arvidson, 2010). This fact points out Lv72 as another good probiotic candidate, due also to its moderate ability to bind HeLa cells. However, other bacterial skills such as hydrogen peroxide, bacteriocin and acid production, and resistance to antibiotics, low pH, and spermicidal compounds, among other properties, have to be taken into account to do the correct selection of a vaginal probiotic (Martín et al., 2008a, b).

Besides, nowadays, there is a tendency to use a combination of various strains to cover the whole range of characteristics required in a vaginal probiotic.

Surface and secreted protein extracts are important to detect potential mucin-binding proteins. Among the surface proteins, ornithine carbamoyltransferase (R16) and amino acid ABC transporter periplasmic protein and high-affinity cystine-binding protein (both in band R126) of L. vaginalis Lv67 bound mucin. High-affinity cystine-binding proteins are surface proteins that are frequently suggested to be putative adhesions. For instance, BspA, a cystine-binding protein of Lactobacillus fermentum BR11, has been described as a collagen-binding protein (Hung et al., 2005).

Among the secreted protein fraction, an extracellular form of GADPH was able to bind mucin. The presence of surface-associated GAPDH is well known in a huge variety of microorganisms (Sánchez et al., 2008). As a secreted form, GAPDH has been shown to be a plasminogen- and fibrinogen-binding protein in E. coli (Egea et al., 2007). Furthermore, Neisseria meningitidis GAPDH-deficient mutant showed a significant reduction in adhesion to human epithelial and endothelial cells compared to the wild-type and complemented mutant (Tunio et al., 2010). However, care should be taken in the interpretation of these results, because the only criteria applied for
identification have been the comparison between their electrophoretical mobility with respect to the surface protein profiles.

In conclusion, the ability to adhere to mucin and to the epithelial cell cultures seems to be strain specific although some association with origin has been found for HT-29 cells. Some of the strains analyzed have good capacities on the models tested being good candidates to be used as vaginal probiotics alone or with other lactobacilli. The data presented in this work also suggest that certain extracellular proteins produced by intestinal and vaginal lactobacilli could act as potential mediators in the molecular interaction with both epithelial cells and pathogens. Further research is needed to establish the precise molecular mechanism of action of these proteins using convenient genetically modified strains.

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References


Adherence properties of human Lactobacillus strains


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Additional material and methods and results section.
Table S1. Adhesion of the 43 isolates to mucin.
Table S2. MS and MS/MS data corresponding to the identification of extracellular proteins produced by the different Lactobacillus strains.
Table S3. MS and MS/MS data corresponding to the identification of surface proteins produced by the different Lactobacillus strains.

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