In vitro evaluation on HeLa cells of protective mechanisms of probiotic lactobacilli against Candida clinical isolates

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Keywords
Candida infection, HeLa cell culture, inhibition mechanisms, lactobacilli, probiotics.

Abstract
Aims: To characterize in vitro the ability of human Lactobacillus strains to inhibit the adhesion, to displace and to compete with clinically isolated Candida strains.

Methods and Results: Three types of assays were performed to determine the inhibitory effect of Lactobacillus plantarum 319, Lactobacillus rhamnosus IMC 501, Lactobacillus paracasei IMC 502 and a specific probiotic combination (SYNBIO) on adhesion of Candida pathogens to HeLa cells: blockage by exclusion (lactobacilli and HeLa followed by pathogens), competition (lactobacilli, HeLa and pathogens together) and displacement (pathogens and HeLa followed by the addition of lactobacilli). Bacterial adhesion to HeLa was quantified by microscopy after May-Grünwald/Giemsa stain. The inhibition results highlight a significant ($P < 0.05$) competition of the considered probiotics against all the Candida strains. The results suggest that the probiotic strains used in this study could prevent colonization of the urogenital tract by relevant pathogens such as Candida strains through barrier and interference mechanisms (mainly displacement and competition), but the degree of inhibition of adhesion was bacterial strain-dependent.

Conclusions: The results support the potential of these Lactobacillus probiotic strains as anti-infective agents in the vagina and encourage further studies about their capacity to prevent and manage urogenital tract infections in females.

Significance and Impact of the Study: To optimize the defensive properties of the vaginal microbiota, improving the health of many women by probiotic intervention.

Introduction
It has long been understood that interactions between microbes and the host vagina have major implications for the well-being of the female. The microbial species that inhabit the vaginal tract play an important role in the maintenance of health and prevention of infection. Under physiological conditions, the vagina primarily harbours lactobacilli which ideally confer, in mutualism with the vaginal epithelium, colonization resistance to other micro-organisms, thereby preventing ascending or systemic infection. The most common Lactobacillus species are Lact. iners, Lact. crispatus, Lact. gasseri and Lact. jensenii, followed by Lact. acidophilus, Lact. fermentum, Lact. plantarum, Lact. brevis, Lact. casei, Lact. vaginalis, Lact. delbrueckii, Lact. salivarius, Lact. reuteri and Lact. rhamnosus (Vásquez et al. 2002; Anukam et al. 2005). All the factors such as hormonal changes (particularly...
oestrogen), vaginal pH and glycogen content can affect the colonization of the lactobacilli in the vagina (Burton and Reid 2002). Menstrual cycle can also cause hormonal changes.

In women, the depletion of vaginal lactobacilli leads to an overgrowth of diverse aerobic, anaerobic and microaerophilic species. The long-term efficiency of antimicrobial treatment is often limited by relapse, most likely due to an inability to re-establish the normal Lactobacillus-dominated vaginal flora.

There have been several attempts to restore the normal vaginal flora by re-colonizing with lactobacilli (Cribby et al. 2008; Borges et al. 2014). However, the correct choice of Lactobacillus strains has not always been used.

In this regard, probiotics provide a health benefit to the host and are promoted as alternatives for the treatment and prevention of infectious diseases and other conditions. A recent important area of probiotic research is in the management of vaginally acquired infections (Borges et al. 2014). Several Lactobacillus species produce compounds that kill or inhibit the growth of vaginally acquired pathogens (Kaewsrichan et al. 2006). Other lactobacilli reduce the adherence of pathogens to urogenital epithelial cells in culture (Osset et al. 2001).

Probiotics have been documented to be beneficial in curing vaginal infections as well as reducing its recurrence and have been administered both orally and vaginally (Senok 2009). Mechanisms through which probiotics play a role in bacterial vaginitis treatment include: (i) occupation of specific adhesion sites at the epithelial surface of the urinary tract, (ii) maintenance of a low pH and production of antimicrobial substances like acids, hydrogen peroxide and bacteriocins, (iii) degradation of polyamines and (iv) the production of surfactants with anti-adhesive properties (Goldin and Gorbach 2008).

Recent advances are concentrated on the characterization and development of probiotics formulation to reduce, inhibit or, in same case, cure genital infection, especially vaginal yeast infection. The problem is correlated with the fact that fungi have emerged as major causes of human disease, especially among the immune-compromised and hospitalized people (McNeil et al. 2001; Eggimann et al. 2003; Fridkin 2005). Numerous factors have contributed to the increase in fungal infections, such as mucosal or cutaneous barrier disruption, defects in the number and function of neutrophils or in cell-mediated immunity, metabolic dysfunction and extremes of age, but one of the most important factor is the increase in the use of broad-spectrum antibiotics (Pfaller and Diekema 2007). The last factor is also responsible for the increase of the antibiotics resistance of fungi and hence, for the problem related to the treatment of vaginal yeast infection (Walsh et al. 2004). In fact, today, it is not only difficult to cure this disease but also to reduce the recurrence of disease in time, without considering the enormous economic weight that the diagnosis and treatment of the pathology have on the public health care. So, alternative therapeutic agents need to be sought, and it has been suggested that the administration of lactobacilli can restore ecological balance in the vagina by controlling the infectivity of pathogenic microbes (Mailander-Sanchez et al. 2011).

In a previous study (Verdenelli et al. 2014), we investigated how a collection of wild-type lactobacilli isolated from human intestinal tract of older people adhere to vaginal human cancerogenic cells (HeLa) and exert a protective activity against Candida strains.

Some of these lactobacilli demonstrated promising properties such as adhesion, coaggregation abilities, antimicrobial activity against pathogenic Candida strains, technological characteristics that make them good candidates for the realization of formulations suitable for vaginal health.

The purpose of this study was to assess the capacity of previously selected Lactobacillus strains, already proved to adhere to vaginal epithelium, to remove or inhibit the adhesion of Candida species from vaginal cells with the aim of demonstrating the ability of these bacteria to reduce pathogen infections and compete for the same environment.

Materials and methods

Microbial strains, cell line and growth condition

The Lactobacillus strains used in this study (Table 1) were isolated from older participants of Italy during an EU project named Crownalife (Silvi et al. 2003). Lactobacillus rhamnosus IMC 501® and Lactobacillus paracasei IMC 502® were characterized as probiotics in previous studies (Verdenelli et al. 2009, 2011). Lactobacillus plantarum 319 was characterized as probiotic (Coman et al. 2014a,b) and the deposit in DSMZ collection is currently ongoing. All the strains have been shown to adhere to HeLa cells (Verdenelli et al. 2014). Candida strains (Table 1) were clinically isolated from human vagina and supplied by Istituto Superiore di Sanità in Rome. Lactobacilli were grown in MRS (de Man, Rogosa, Sharpe) broth (Oxoid, Basingstoke, UK), whereas Candida strains were grown in Sabouraud (SAB) dextrose medium (Oxoid). All the strains were grown aerobically at 37°C. The HeLa cell line was grown in Dulbecco’s Minimal Essential Medium (DMEM; PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% foetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic/antimycotic. Cells were cultured at 37°C in a humidified (95%) atmosphere 5%
CO₂ and subcultured twice per week and the medium changed every 3 days.

Adhesion test

Three types of assays were performed to study the ability of lactobacilli to block the adherence of Candida strains to HeLa cells: blockage by exclusion, by displacement or by competition.

Exclusion test

To assess the capacity of lactobacilli strains to influence the adherence of pathogens to vaginal human cells, interference experiments were performed against Candida clinical isolates. Adhesion reaction was performed using 6-well culture plate containing a sterile coverslip in each well. Each well was then added with 2 ml of HeLa cell suspension at a concentration of 4 × 10⁵ cells ml⁻¹ and incubated in a 5% CO₂ atmosphere at 37°C. After 48 h, when the cells were grown to approx. 60% confluence, they were washed twice with PBS and inoculated with 1 ml of lactobacilli suspension at a concentration of 1.5 × 10⁸ cells ml⁻¹. The plates were then incubated for 1 h at 37°C in a 5% CO₂ atmosphere to allow the adhesion of bacteria on cells. After 1 h, the cells were washed twice with PBS to remove all nonadhering bacteria and again inoculated, but in this case with Candida strains in the same experimental condition (1.5 × 10⁸ cells ml⁻¹). At the end, the cells were washed, stained with the May-Grünwald/Giemsa stain and analysed under the microscope (100×; Leitz Laborlux 12 microscope, Ernst Leitz, Wetzlar GmbH, Germany). Further, each HeLa cell was scored for the presence and number of bacteria and Candida strains attached, and a number of 100 cells was checked for micro-organism adhesion.

Displacement test

The displacement test evaluates the ability of the Lactobacillus strains to displace the already-adhered pathogens, and occurs with the same protocols of the exclusion test; but in this case, the sequence of the inoculation was reversed, which implies that the HeLa cells were inoculated first with Candida strains and then with lactobacilli.

Competition test

Competitive exclusion of the pathogens by tested probiotics was determined as described previously, but in this case, lactobacilli and Candida were added together to HeLa cells incubated for 1 h at 37°C in a 5% CO₂ atmosphere.

The results of the three conditions (i.e. exclusion, displacement and competition) were expressed as the mean number of Candida cells per HeLa cell and compared with the number obtained in the adhesion without lactobacilli (control value). The control values were taken as 100% of adhesion and the inhibition of pathogens adherence was calculated by subtracting each adhesion percentage from their corresponding value.

Data analysis, calculations and statistical analysis

The results of exclusion, displacement and competition assay are expressed as the average of three independent experiments. Significant differences between mean values were determined by Tukey’s test after one-way analysis of variance ANOVA (one-way ANOVA) using GraphPad Prism® 5.1 program (GraphPad Software, San Diego, CA, USA). A P-value <0.05 was considered statistically significant.

Results

The three lactobacilli (319, IMC 501®, IMC 502®) and the SYNBIO® (IMC 501®–IMC 502® combination) (Synbiotec Srl, Italy) were able to exclude, compete with and displace all the Candida strains tested. The effect was dependent on the each probiotic strain and the pathogen assayed (Table 2, Fig. 1). The Lactobacillus strains tested showed a broad range of pathogen exclusion. The adhesion of Candida albicans ATCC 14503 and C. albicans ISS1 was significantly reduced by all strains. No significant differences were observed in the exclusion of Candida glabrata ISS3 and Candida krusei ISS4 by all strains. The adhesion of all the pathogens tested were significantly reduced by competition by all the lactobacilli ranging from 24.9 to 89.7% depending on the pathogen and the Lactobacillus strain. Degree of displacement of adhered Candida strains by all lactobacilli was significant.

Table 1 Lactobacillus and Candida strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus plantarum</td>
<td>Intestinal isolate</td>
</tr>
<tr>
<td>Lactobacillus rhamnosus IMC 501®</td>
<td>Intestinal isolate</td>
</tr>
<tr>
<td>Lactobacillus paracasei IMC 502®</td>
<td>Intestinal isolate</td>
</tr>
<tr>
<td>SYNBIO®*</td>
<td></td>
</tr>
<tr>
<td>*Combination 1 : 1 of Lact. rhamnosus IMC 501® and Lact. paracasei IMC 502®</td>
<td></td>
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</tbody>
</table>

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Table 2 Percentage of adherence inhibition† of different strains of Candida to HeLa cells by Lactobacillus plantarum 319, Lactobacillus rhamnosus IMC 501®, Lactobacillus paracasei IMC 502® and SYNBIΟ® under conditions of exclusion, competition and displacement

<table>
<thead>
<tr>
<th>Strain</th>
<th>C. albicans ATCC 14503</th>
<th>C. albicans ISS1</th>
<th>C. albicans ISS2</th>
<th>C. albicans ISS7</th>
<th>C. glabrata ISS3</th>
<th>C. krusei ISS4</th>
<th>C. parapsilosis ISS5</th>
<th>C. tropicalis ISS6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exclusion</td>
<td></td>
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</tr>
<tr>
<td>Lact. plantarum 319</td>
<td>95.5 ± 0.4*</td>
<td>78.4 ± 5.4*</td>
<td>49.2 ± 0.2*</td>
<td>715.6 ± 1.3*</td>
<td>27.3 ± 3.5*</td>
<td>0.1 ± 20.9</td>
<td>38.9 ± 4.9*</td>
<td>50.6 ± 1.3*</td>
</tr>
<tr>
<td>Lact. rhamnosus IMC 501®</td>
<td>69.6 ± 3.1*</td>
<td>37.6 ± 1.5*</td>
<td>20.2 ± 0.6</td>
<td>235.0 ± 0.4*</td>
<td>-80.0 ± 24.2</td>
<td>-44.2 ± 4.2</td>
<td>27.3 ± 9.3</td>
<td>5.15 ± 2.2</td>
</tr>
<tr>
<td>Lact. paracasei IMC 502®</td>
<td>69.2 ± 0.4*</td>
<td>59.6 ± 0.9*</td>
<td>29.1 ± 2.2</td>
<td>-200 ± 2.3</td>
<td>19.2 ± 8.2</td>
<td>-18.1 ± 18.5</td>
<td>11.0 ± 9.0</td>
<td>29.5 ± 3.7*</td>
</tr>
<tr>
<td>SYNBIΟ®</td>
<td>51.8 ± 3.2*</td>
<td>55.8 ± 5.2*</td>
<td>-13.7 ± 17.3</td>
<td>40.4 ± 11.5*</td>
<td>17.1 ± 11.3</td>
<td>14.7 ± 55.4</td>
<td>39.9 ± 11.0*</td>
<td>38.8 ± 5.1*</td>
</tr>
<tr>
<td>Displacement</td>
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<td></td>
</tr>
<tr>
<td>Lact. plantarum 319</td>
<td>42.1 ± 21.7</td>
<td>41.3 ± 1.6*</td>
<td>80.6 ± 0.2*</td>
<td>91.8 ± 0.04*</td>
<td>93.0 ± 8.1*</td>
<td>84.9 ± 6.4*</td>
<td>85.4 ± 4.9*</td>
<td>51.7 ± 6.5*</td>
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<tr>
<td>Lact. rhamnosus IMC 501®</td>
<td>38.0 ± 28.2</td>
<td>62.9 ± 2.6*</td>
<td>55.3 ± 3.1*</td>
<td>75.1 ± 0.1*</td>
<td>77.9 ± 4.2*</td>
<td>85.2 ± 0.2*</td>
<td>76.3 ± 0.4*</td>
<td>38.3 ± 9.4*</td>
</tr>
<tr>
<td>Lact. paracasei IMC 502®</td>
<td>21.8 ± 24</td>
<td>49.7 ± 1.6*</td>
<td>87.9 ± 0.7*</td>
<td>80.6 ± 8.6*</td>
<td>79.8 ± 0.5*</td>
<td>-2.9 ± 9.3</td>
<td>48.2 ± 4.9*</td>
<td>19.7 ± 6.9</td>
</tr>
<tr>
<td>SYNBIΟ®</td>
<td>5.8 ± 24</td>
<td>31.9 ± 5.3*</td>
<td>74.3 ± 0.1*</td>
<td>44.7 ± 10.9*</td>
<td>91.6 ± 4.8*</td>
<td>-21.3 ± 10.2</td>
<td>51.4 ± 1.3*</td>
<td>22 ± 15.8</td>
</tr>
<tr>
<td>Competition</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lact. plantarum 319</td>
<td>60.9 ± 3.3*</td>
<td>65.5 ± 3.3*</td>
<td>75.2 ± 1.3*</td>
<td>89.3 ± 13.1*</td>
<td>86.4 ± 1.2*</td>
<td>57.0 ± 10.2</td>
<td>76.3 ± 3.5*</td>
<td>66.4 ± 8.0*</td>
</tr>
<tr>
<td>Lact. rhamnosus IMC 501®</td>
<td>44.7 ± 2.3*</td>
<td>64.3 ± 0.1*</td>
<td>62.1 ± 3.3*</td>
<td>76.8 ± 1.3*</td>
<td>67.6 ± 2.2*</td>
<td>76.3 ± 2.0*</td>
<td>29.4 ± 15.0*</td>
<td>54.5 ± 4.0*</td>
</tr>
<tr>
<td>Lact. paracasei IMC 502®</td>
<td>58.7 ± 4.3*</td>
<td>42.7 ± 7.3*</td>
<td>61.1 ± 2.3*</td>
<td>74.2 ± 1.8*</td>
<td>67.4 ± 0.5*</td>
<td>69.3 ± 1.1*</td>
<td>62.4 ± 1.6*</td>
<td>89.7 ± 4.5*</td>
</tr>
<tr>
<td>SYNBIΟ®</td>
<td>24.9 ± 11.2*</td>
<td>55.5 ± 5.5*</td>
<td>78.3 ± 1.1*</td>
<td>63.0 ± 0.3*</td>
<td>86.9 ± 0.5*</td>
<td>76.7 ± 4.7*</td>
<td>77.6 ± 3.5*</td>
<td>81.4 ± 6.4*</td>
</tr>
</tbody>
</table>

*P < 0.05 by Tukey’s test/one-way ANOVA.
†Adhesion of Candida strains to HeLa was quantified in the absence (control value, 100%) and presence of lactobacilli under the three conditions tested. The inhibition percentages were calculated by subtracting each adhesion percentage from their corresponding control value.
for *C. albicans* ISS1, ISS2, ISS7, *C. glabrata* ISS3 and *Candida parapsilosis* ISS5. No displacement was observed with *C. albicans* ATCC 14503, in particular, *Lact. plantarum* 319 and SYNBIO/C226 showed the highest inhibition percentage against *Candida* strains in the three types of assays. It is interesting to note that in several cases, the SYNBIO® combination enhance the inhibition ability of the single strains *Lact. rhamnosus* IMC 501® and *Lact. paracasei* IMC 502®. This was demonstrated for *C. krusei* ISS4, *C. parapsilosis* ISS5, *Candida tropicalis* ISS6 and *C. albicans* ISS7 in the exclusion test, for *C. albicans* ISS2, *C. glabrata* ISS3, *C. krusei* ISS4 and *C. parapsilosis* ISS5 in the competition test and for *C. glabrata* ISS3 in displacement test. In general, the results shown in Table 2 demonstrated that the exclusion profiles for *Candida* strains by lactobacilli were very different from those of competition and displacement. Degrees of exclusion were generally much lower than the degree of inhibition achieved by competition and displacement. This is true for both strains of *C. albicans* and for non-albicans strains. Figure 1 shows the effect of the tested *Lactobacillus* strains on the attachment of the different *Candida* strains to HeLa cells under the conditions of exclusion, displacement and competition.

The *Lactobacillus* strains tested were able to exclude, compete with and displace the *Candida* strains to different degrees (Fig. 2). The main mechanisms of action that determined a significant reduction in *Candida* were displacement and competition, in particular against non-albicans strains. *Lactobacillus plantarum* 319 was also able to significantly reduce the attachment of *Candida* strains, except for *C. krusei* ISS4, with the exclusion mechanisms.

**Discussion**

The rationale for replenishing the vagina with non-pathogenic organisms, such as probiotic lactobacilli, has been recently reviewed (Homayouni et al. 2014). The effectiveness of treatment is certainly related to the ability of lactobacilli to adhere to the vaginal mucosa and exert an antipathogenic activity. The capacity to adhere to human epithelial cells, as well as the antagonism towards pathogen establishment is also considered as crucial by the FAO/WHO for the *in vitro* evaluation of potential probiotic candidates (FAO/WHO, 2006). The *Lactobacillus* strains used in this study have previously shown adhesive properties (Verdenelli et al. 2014) and antagonistic effects against urogenital pathogens (Coman et al. 2014a,b). In the present study, we assessed their capability of interfering with the adhesion of vaginal pathogens, such as *Candida* strains in a model human cell line. In the healthy urogenital tract of adult females, it is supposed that the
Effect of probiotics on *Candida* adhesion

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indigenous lactobacilli exclude the colonization of pathogenic bacteria by occupying or masking (by steric hindrance) their potential binding sites in the mucosa (Chan et al. 1984; Spencer and Chesson 1994). However, in a depleted lactobacilli environment, such as an infected urogenital tract, it should be expected that exogenous probiotic lactobacilli have the capacity to compete for the same receptors and displace previously attached pathogens (Reid 2000). Therefore, we investigated the blocking of C. albicans pathogen adherence by lactobacilli, under three possible interactive mechanisms: exclusion by adhered Lactobacillus strains, displacement of adhered pathogens and competition for receptor sites (inhibition test). In the exclusion assays, we found enormous differences among the Lactobacillus strains in their ability to block the adherence of Candida strains to HeLa cells. Moreover, the same Lactobacillus displayed different blocking capacity according to the Candida strain studied. Lactobacillus plantarum 319 showed the greatest blocking activity by exclusion mechanism against six of eight Candida strains tested including all the C. albicans strains which are the most frequent agents causing vaginal infection (Hani et al. 2015). No strain was able to significantly inhibit by exclusion mechanism, the adhesion of C. glabrata and C. krusei strains, which are non-albicans Candida (NAC) species that cause 35 ± 65% of all candidaemias in the general patient population (Krcmery and Barnes 2002). All the lactobacilli tested demonstrated the ability to strongly reduce the adherence of invading yeast cells in the competition assay. The degree of competition is determined by the affinity of adhesins on the respective bacterial surfaces for the specific receptors that they are competing for; or their relative positions in the case of steric hindrance (Lee et al. 2003). Moreover, the ability to significantly inhibit the attachment of pathogens by displacement indicates that affinity of lactobacilli for the specific receptors is higher than that of the pathogenic strain tested, as shown by Kaewsrichan et al. (2006). SYNBIOS® combination (Lact. rhamnosus IMC 501® and Lact. paracasei IMC 502®) was able to significantly reduce the attachment of C. glabrata to HeLa cells by competition and displacement at a higher percentage than the two strains taken individually. This specific synergy of the two strains, already highlighted in other studies on adhesion (Verdenelli et al. 2009, 2014), may be of interest in situations in which the individual strains are not able to counter specific pathogens. This is the case of C. parapsilosis ISS5: neither Lact. rhamnosus IMC 501® nor Lact. paracasei IMC 502® were able to significantly inhibit the attachment to HeLa while SYNBIOS® did so. The results of this work are even more interesting because clinically isolated Candida strains were tested, some of which are among other resistant strains to imidazole antifungals.

It is important to emphasize that a balanced vaginal microflora results in an unfavourable environment to the attraction of pathogenic bacteria. This implies that lactobacilli with a significant ability to block pathogens with exclusion mechanism are certainly valid to maintain vaginal health. By contrast, in patients with recurrent vaginal infections, lactobacilli with high capacity to compete and/ or displace previously attacked pathogens could definitely result of greater impact. This study has shown that the same strain of Lactobacillus can exert various activities of inhibition in relation to the pathogen tested. This implies and reinforces the concept and the importance of proper probiotics characterization. Since probiotic properties are strain specifics, it would be interesting to use more than one probiotic strain in the same vaginal product. Our study demonstrated that Lact. plantarum 319 and SYNBIOS® have the most significant (P < 0.05) interference activity on vaginal pathogens using different mechanisms of action. The use of a combination of these probiotic strains in a vaginal formulation might be effective not only as prevention in healthy women but also in the reduction of vaginal colonization by potential pathogenic bacteria and yeasts.

Conflict of Interest

No conflict of interest declared.

References


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