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ORIGINAL ARTICLE

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

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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 ($P < 0{\cdot}05$) 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. jenesenii,* 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%

Table 1 *Lactobacillus* and *Candida* strains used in this study

Strain	Origin
Lactobacillus plantarum 319	Intestinal isolate
Lactobacillus rhamnosus IMC 501®	Intestinal isolate
Lactobacillus paracasei IMC 502®	Intestinal isolate
SYNBIO®*	
Candida albicans ATCC 14053	Culture collection
Candida albicans ISS1	Clinical isolate
Candida albicans ISS2	Clinical isolate
Candida albicans ISS7	Clinical isolate
Candida glabrata ISS3	Clinical isolate
Candida krusei ISS4	Clinical isolate
Candida parapsilosis ISS5	Clinical isolate
Candida tropicalis ISS6	Clinical isolate

*Combination 1 : 1 of *Lact. rhamnosus* IMC 501® and *Lact. paracasei* IMC 502®.

CO_2 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 4×10^5 cells ml^{-1} and incubated in a 5% CO_2 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 $1{\cdot}5 \times 10^9$ cells ml^{-1}. The plates were then incubated for 1 h at 37°C in a 5% CO_2 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 ($1{\cdot}5 \times 10^9$ 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% CO_2 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 $<0{\cdot}05$ 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 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 SYNBIO® under conditions of exclusion, competition and displacement

	% of inhibition							
Strain	*C. albicans* ATCC 14503	*C. albicans* ISS1	*C. albicans* ISS2	*C. albicans* ISS7	*C. glabrata* ISS3	*C. krusei* ISS4	*C. parapsilosis* ISS5	*C. tropicalis* ISS6
Exclusion								
Lact. plantarum 319	95·5 ± 0·4*	78·4 ± 5·4*	49·2 ± 0·2*	71·5 ± 1·3*	27·3 ± 3·5	0·1 ± 20·9	38·9 ± 4·9*	50·6 ± 1·3*
Lact. rhamnosus IMC 501®	69·6 ± 3·1*	37·6 ± 1·5*	20·2 ± 0·6	23·5 ± 0·4*	−80·0 ± 24·2	−44·2 ± 4·2	27·3 ± 9·3	5·15 ± 2·2
Lact. paracasei IMC 502®	69·2 ± 0·4*	59·6 ± 0·9*	29·1 ± 2·2	−2·0 ± 2·3	19·2 ± 8·2	−18·1 ± 18·5	11·0 ± 9·0	29·5 ± 3·7*
SYNBIO®	51·8 ± 3·2*	55·8 ± 5·2*	−13·7 ± 17·3	40·4 ± 11·5*	17·1 ± 11·3	14·7 ± 55·4	39·9 ± 11·0*	38·8 ± 5·1*
Displacement								
Lact. plantarum 319	42·1 ± 21·7	41·3 ± 1·6*	80·6 ± 0·2*	91·8 ± 0·04*	93·0 ± 8·1*	84·9 ± 6·4*	85·4 ± 4·9*	51·7 ± 6·5*
Lact. rhamnosus IMC 501®	38·0 ± 28·2	62·9 ± 2·6*	55·3 ± 3·1*	75·1 ± 0·1*	77·9 ± 4·2*	85·2 ± 0·2*	76·3 ± 0·4*	38·3 ± 9·4*
Lact. paracasei IMC 502®	21·8 ± 2·4	49·7 ± 1·6*	87·9 ± 0·7*	80·6 ± 8·6*	79·8 ± 0·5*	−2·9 ± 9·3	48·2 ± 4·9*	19·7 ± 6·9
SYNBIO®	5·8 ± 2·4	31·9 ± 5·3*	74·3 ± 0·1*	44·7 ± 10·9*	91·6 ± 4·8*	−21·3 ± 10·2	51·4 ± 1·3*	2·2 ± 15·8
Competition								
Lact. plantarum 319	60·9 ± 3·3*	65·5 ± 3·3*	75·2 ± 1·3*	89·3 ± 13·1*	86·4 ± 1·2*	57 ± 10·2*	76·3 ± 3·5*	66·4 ± 8·0*
Lact. rhamnosus IMC 501®	44·7 ± 2·3*	64·3 ± 0·1*	62·1 ± 3·3*	76·8 ± 1·3*	67·6 ± 2·2*	76·3 ± 2·0*	29·4 ± 15·0*	54·5 ± 4·0*
Lact. paracasei IMC 502®	58·7 ± 4·3*	42·7 ± 7·3*	61·1 ± 2·3*	74·2 ± 1·8*	67·4 ± 0·5*	69·3 ± 1·1*	62·4 ± 1·6*	89·7 ± 4·5*
SYNBIO®	24·9 ± 11·2*	55·5 ± 5·5*	78·3 ± 1·1*	63·0 ± 0·3*	86·9 ± 0·5*	76·7 ± 4·7*	77·6 ± 3·5*	81·4 ± 6·4*

*$P < 0{\cdot}05$ 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.

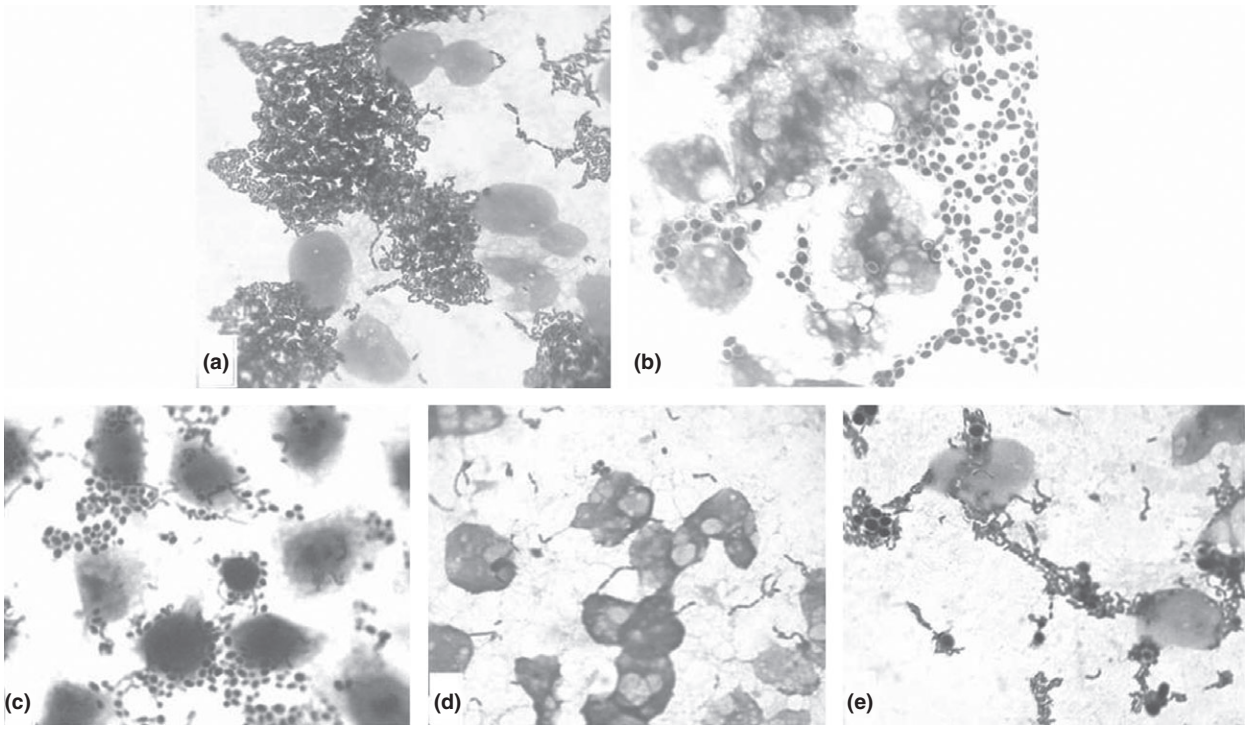

Figure 1 Adherence of *Lactobacillus plantarum* 319 (a) and *Candida glabrata* ISS3 (b) to vaginal epithelial cells (HeLa cells) as observed by light microscope after May-Grunwald/Giemsa stain under condition of exclusion (c) displacement (d) and competition (e).

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® 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 antiphatogenic 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

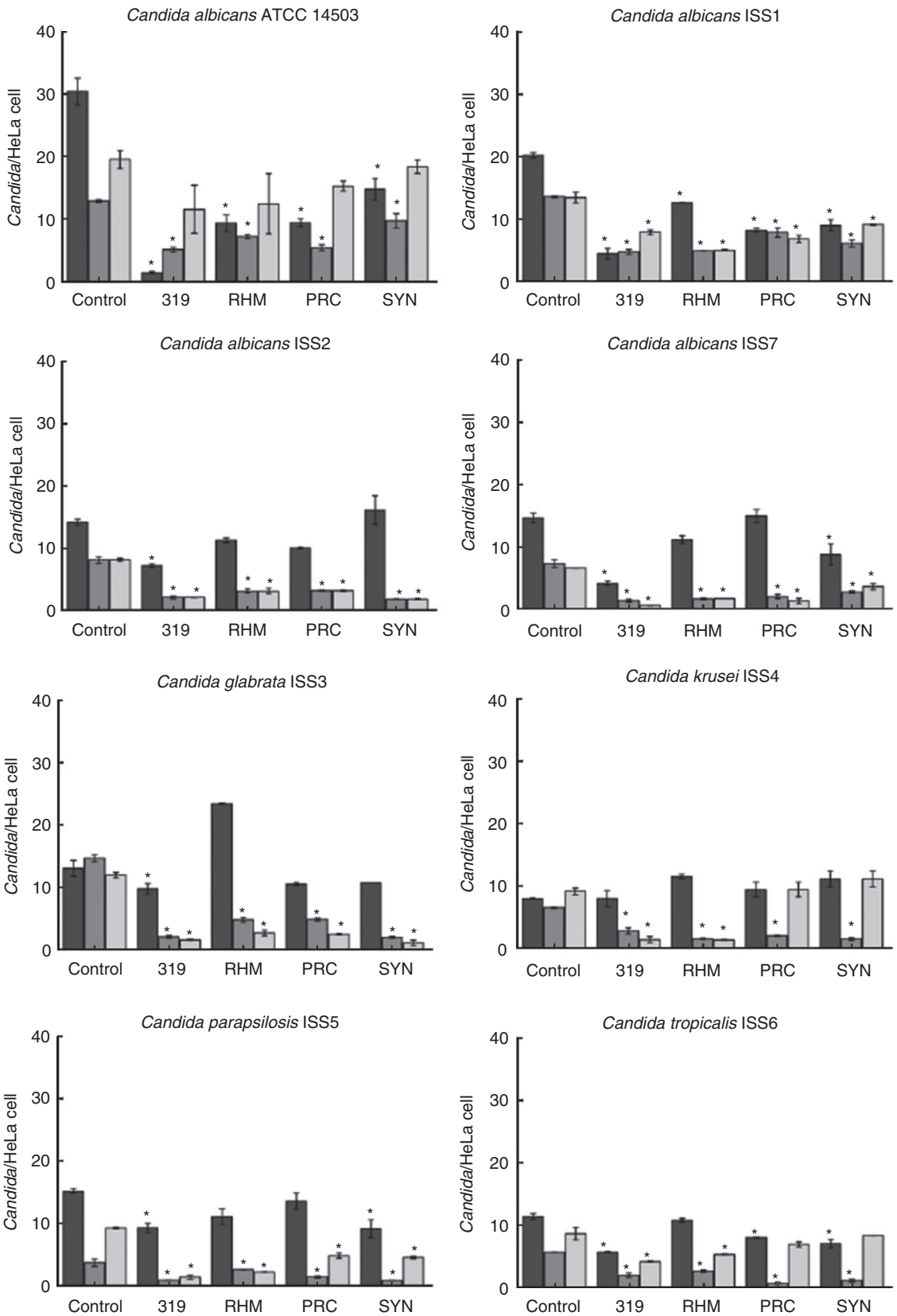
Candida albicans ATCC 14503
Candida albicans ISS1
Candida albicans ISS2
Candida albicans ISS7
Candida glabrata ISS3
Candida krusei ISS4
Candida parapsilosis ISS5
Candida tropicalis ISS6
Candida/HeLa cell
0
10
20
30
40
Control
319
RHM
PRC
SYN

Figure 2 Inhibition of adhesion to HeLa cells of different *Candida* strains by the probiotic lactobacilli tested under the condition of ■ exclusion, ■ displacement and ■ competition. *Candida* adhesion to HeLa cells was quantified in 100 consecutive cells by microscopy (×100) after May-Grünwald/Giemsa stain. Results are expressed as *Candida*/HeLa cell and the data presented are the mean ± SD of three independent assays. CP, positive control; 319, *Lactobacillus plantarum* 319; RHM, *Lactobacillus rhamnosus* IMC 501®; PRC, *Lactobacillus paracasei* IMC 502®; SYN, SYNBIO®. *Significantly different from the control ($P < 0{\cdot}05$) by Tukey's test/one-way ANOVA.

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 blockage of *Candida* 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 stearic 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). SYNBIO® 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 SYNBIO® 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 SYNBIO® have the most significant ($P < 0{\cdot}05$) 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.

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