

VAGINAL LACTOBACILLI AS POTENTIAL PROBIOTICS AGAINST *Candida* spp.

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ABSTRACT

Urogenital infections affect millions of people every year worldwide. The treatment of these diseases usually requires the use of antimicrobial agents, and more recently, the use of probiotic lactic acid bacteria (LAB) cultures for the management of vaginal infections has been extensively studied. In this work, 11 vaginal lactobacilli isolates, previously obtained from healthy patients, were studied to screen microorganisms with probiotic properties against *Candida* spp. The LAB were tested for their ability of auto-aggregation, co-aggregation with *C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis*, adhesion to Caco-2 epithelial cells and production of lactic acid and hydrogen peroxide (H₂O₂). All lactobacilli isolates tested were able to auto-aggregate (ranging from 25.3% to 75.4% assessed at 4 hours of incubation) and to co-aggregate with the four *Candida* species into different degrees; among them *L. crispatus* showed the highest scores of co-aggregation. The highest amount of lactic acid was produced by *L. salivarius* (13.9 g/l), followed by *L. johnsonii* (6.5 g/l), *L. acidophilus* (5.5 g/l), and *L. jensenii* (5.4 g/l). All isolates produced H₂O₂, but the highest levels (3 - 10 mg/l) were observed for *L. acidophilus*, *L. crispatus*, *L. gasseri*, *L. johnsonii*, and *L. vaginalis*. Only *L. agilis*, *L. jensenii*, *L. johnsonii* and *L. ruminus* were able to adhere to epithelial Caco-2 cells. Among the isolates evaluated, *L. agilis*, *L. jensenii*, *L. johnsonii*, and *L. ruminus* exhibited simultaneously several desirable properties as potential probiotic strains justifying future studies to evaluate their technological properties in different pharmaceutical preparations for human use.

Key words: *Lactobacillus* spp., probiotic, *Candida* spp.

INTRODUCTION

In women of childbearing age, the vaginal ecosystem is dominated by *Lactobacillus* spp. (41). These microorganisms can prevent the colonization of the urogenital tract by several pathogens and they are important for women's reproductive and general healthy (17, 24, 40, 42).

Lactobacilli modulate the vaginal microbiota by different mechanisms such as: (i) auto-aggregation, (ii) production of lactic acid, hydrogen peroxide, bacteriocins, and biosurfactants, (iii) co-aggregation with pathogenic microorganisms, and (iv) adhesion to epithelial cells (17, 29, 37).

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Bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC) are the most prevalent vaginal infections worldwide (30). BV is responsible for up to 50% of all the cases of vaginal infections and it is characterized by a significant reduction in lactobacilli population, and increase in facultative aerobic and anaerobic pathogens (10, 16).

VVC affects up to 75% of women at least once in their lives and despite pruritus and vaginal discharge are usual complaints associated with this disease neither is specific to the infection (34). The majority of cases of VVC (ca. 90%) caused by *Candida albicans* are treated with oral or topical antifungal agents, with increasing reports on episodes of VVC due to non-*albicans* species (27, 28). There is an overgrowing concern about the spread use of over-the-counter preparations (such as topical azole agents) which may contribute for the selection of non-*albicans* resistant strains that are normally more difficult to be eradicated (23, 35).

Probiotics are defined as live microorganisms which when administered in adequate quantity confer health benefits to the host and lactobacilli of human origin are potential probiotics against urogenital tract infections (11, 25). Some clinical studies showed positive results for the use of *L. fermentum* RC-14 and *L. rhamnosus* GR-1 to treat patients with BV by oral intake and intravaginal administration (1, 2). Also, a recent clinical trial showed that oral administration of capsules containing *L. fermentum* RC-14 and *L. rhamnosus* GR-1 was effective as adjuvant in the treatment of patients diagnosed with VVC (20).

Probiotics do not show collateral effects usually seen for traditional antibacterial and antifungal agents because they act by several mechanisms, which minimize punctual mutations involved in the emergence of antimicrobial resistant pathogens. The technology necessary to produce probiotic agents does not appear to be complex, and this can stimulate their production at reasonable costs. This scenario certainly encourages more researches to be undertaken to select and test new strains with probiotic properties.

The aim of the present work was to evaluate the ability of *Lactobacillus* spp., previously isolated from the vaginal

microbiota of healthy Brazilian patients, as potential probiotics against *Candida* species.

MATERIALS AND METHODS

Strains

A total of 11 vaginal *Lactobacillus* spp. were previously isolated from a group of 64 healthy Brazilian women (21) and the use of the strains for this study was approved by local Ethic Review Board (250/CEP-CSE-FMRP-USP). The isolates studied were *L. acidophilus*, *L. agilis*, *L. coleohominis*, *L. crispatus*, *L. fermentum*, *L. gasseri*, *L. jensenii*, *L. johnsonii*, *L. salivarius*, *L. ruminus* and *L. vaginalis*. Additionally, for the study of adhesion to epithelial cells, *L. bulgaricus* and *L. rhamnosus* GG were employed as negative and positive controls, respectively. The bacterial strains were kept at -70°C in MRS broth (de Man, Rogosa and Sharpe – Oxoid, UK) added of 20 % (v/v) of glycerol.

A total of four *Candida* spp. strains were used in this study, to know: *C. albicans* ATCC 18804, *C. tropicalis* ATCC 750, *C. krusei* ATCC 20298 and *C. glabrata* ATCC 2001. The yeast strains were kept in SDA (Sabouraud-dextrose agar – Oxoid, UK) at room temperature.

Auto-aggregation studies

Lactobacillus spp. was grown overnight at 37°C in MRS broth (1.0%, v/v), centrifuged at 6,000g for 15min (Fanem, mod. 208 N, Brazil) and cell pellets were resuspended in phosphate buffered saline (PBS) to obtain an optical density (O.D.) of 0.6 at 600nm (UVmini-1240, Shimadzu, Japan). Auto-aggregation inversely correlated with O.D. and it was monitored every 1h for up to 4h of incubation (13, 25). Gram staining was used to visualize the aggregates under oil immersion microscopy with 100 times magnification (CX-31 – Olympus, Japan).

Co-aggregation studies

Culture plates of 24 wells containing round glass slides were added of: i) 500µL of an overnight culture of

Lactobacillus spp. grown at 37°C in MRS broth and ii) 500µL of an overnight culture of *Candida* spp. grown at 37°C in BHI broth (Brain-Heart Infusion – Oxoid, UK). Plates were incubated at 37°C for 4h in an orbital shaker at 100 rpm (CT-712, Cientec, Brazil) and co-aggregation was determined by Gram staining of the round glass slides and observation under oil immersion microscopy (CX-31 – Olympus, Japan). Scoring was done according to Reid *et al.* (31).

Production of lactic acid

Homofermentative metabolism was verified by absence of production of gas from glucose (33) and lactic acid production was quantified in grams per liter, by acid-base titration, according to Edema and Sanni (9).

Production of hydrogen peroxide

Determination of hydrogen peroxide (H₂O₂) production by *Lactobacillus* isolates was performed according to Wilks *et al.* (41) with modifications. Briefly, lactobacilli were grown in MRS broth (Oxoid, UK) for 24h at 37°C and 100µL-aliquots of the broths were seeded on MRS agar plates (Oxoid, UK) and incubated for 48h at 37°C, under anaerobic atmosphere. Selected colonies were put in contact with strips containing peroxidase (Merckoquant Peroxide Test - Merck, Germany). Different tones of blue products were visually compared, with a scale provided by the manufacturer. Results were expressed in ranges of H₂O₂ production according to Wilks *et al.* (41).

Adhesion to the epithelial cells

Adhesion to intestinal epithelial Caco-2 cells (ATCC 7348406) was evaluated according to Duprè *et al.* (8). Briefly, Caco-2 cells were cultivated at 37°C under 5% CO₂ in RPMI medium (Gibco, USA) supplemented with 10% of fetal bovine serum and 100U/ml of streptomycin and penicillin (Sigma, USA). When confluent growth was achieved, adhered cells were trypsinized, transferred to 24-well plates containing round glass slides and re-incubated. After 24 hours, the RPMI medium with antibiotics was removed and replaced by RPMI supplemented with 2% of fetal bovine serum. Bacterial cultures were previously grown overnight at 37°C in MRS broth,

diluted in RPMI containing 2% of fetal bovine serum and added to each well (ca. 10⁶ bacteria) containing the Caco-2 cells and incubation was done at 37°C for 3 hours. After that, slides were washed, fixed, stained with May–Grunwald–Giemsa and analyzed under oil immersion microscopy with 100 times magnification (CX-31 – Olympus, Japan).

The number of bacteria adhered to Caco-2 cells was obtained by scoring adhesion to 100 random eukaryotic cells, using criteria proposed by Del Re *et al.* (7) as non-adhesive (<5 bacteria/100 cells), adhesive (6-40 bacteria/100 cells) and strongly adhesive (>40 bacteria/100 cells). *L. bulgaricus* and *L. rhamnosus* GG were used as negative and positive controls, respectively.

RESULTS AND DISCUSSION

All lactobacilli isolates tested in the present study exhibited some degree of auto-aggregation at all time-points tested, but the highest degree of auto-aggregation was observed after 4h of incubation at 37°C for *L. jensenii*, followed by *L. ruminus* and *L. acidophilus* (Figure 1). Figure 1 also illustrates auto-aggregation observed for *L. acidophilus* in two time-points. The ability of auto-aggregation of vaginal lactobacilli is an intrinsic characteristic and may substantially increase the colonization of environments with short residence times (25). According to Juarez-Tomás *et al.* (13) the ability of auto-aggregation is higher in acid environments where probiotic lactobacilli are more adapted to survive and represents the first step towards the formation of biofilms by lactobacilli strains, which helps to inhibit the overgrowth and proliferation of pathogenic microorganisms (14, 37).

The co-aggregation scores obtained after 4h of incubation at 37°C for *Lactobacillus* spp. and *Candida* spp. are shown in Table 1 and illustrated in Figure 2. *L. crispatus* showed macroscopically visible clumps when evaluated with all four *Candida* strains. The co-aggregation can create a microenvironment around the pathogen with a higher concentration of inhibitory substances and it can also block the dissemination of pathogens to tissue receptors (22, 29).

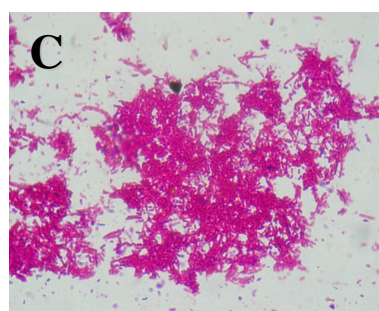
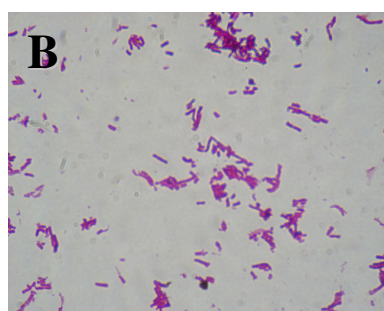
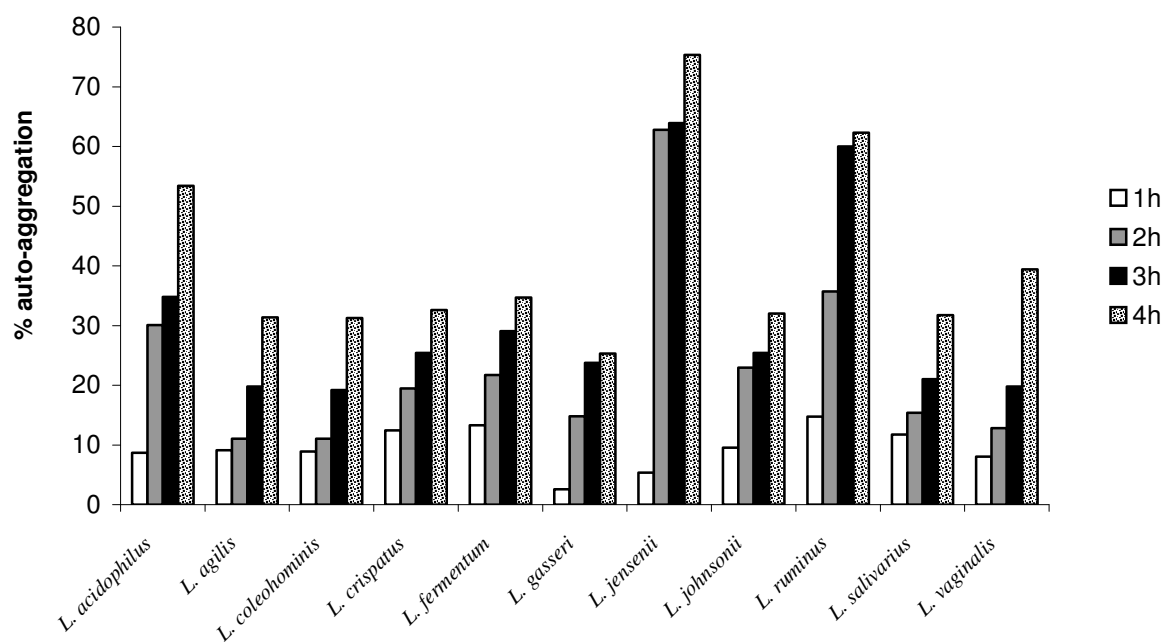


Figure 1. Auto-aggregation of *Lactobacillus* spp. evaluated in a 4-hour study (A) and microphotographies (magnification of 1000x) showing specifically auto-aggregation of *L. acidophilus* assessed after 1h (B) and 4h (C) of incubation at 37°C.

Table 1. Co-aggregation scores obtained with *Lactobacillus* spp. and *Candida* spp. at 4h of incubation at 37°C, according classification of Reid *et al.* (31)*

	<i>C. albicans</i>	<i>C. glabrata</i>	<i>C. krusei</i>	<i>C. tropicalis</i>
<i>L. acidophilus</i>	3	3	2	3
<i>L. agilis</i>	1	3	2	3
<i>L. coleohominus</i>	3	3	4	4
<i>L. crispatus</i>	4	4	4	4
<i>L. fermentum</i>	4	4	3	3
<i>L. gasseri</i>	1	1	3	3
<i>L. jenssenii</i>	1	3	3	2
<i>L. johnsonii</i>	3	4	3	2
<i>L. ruminus</i>	4	3	2	3
<i>L. salivarius</i>	3	1	4	2
<i>L. vaginalis</i>	3	4	2	4

*No aggregation (0); small aggregates with small visible clusters of bacteria (1); aggregates with larger numbers of bacteria (2); macroscopically visible clumps with larger groups of bacteria settled in the center of the well (3) and macroscopically visible clumps (4).

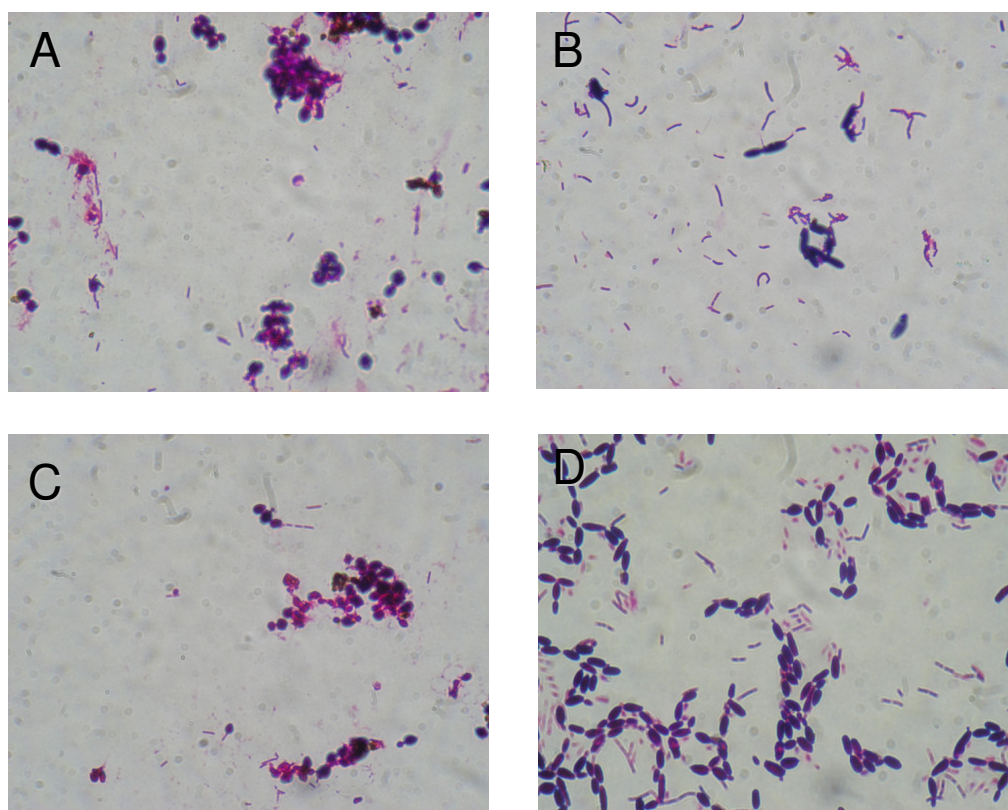


Figure 2. Microphotographies (magnification of 1000x) showing co-aggregation between *C. albicans* x *L. gasseri* (A), *C. krusei* x *L. vaginalis* (B), *C. albicans* x *L. johnsonii* (C) and *C. albicans* x *L. ruminus* (D) assessed after 4h of incubation at 37°C, and respectively scored as: small aggregates with small visible clusters of bacteria (1); aggregates with larger numbers of bacteria (2); macroscopically visible clumps with larger groups of bacteria settled in the center of the well (3) and macroscopically visible clumps (4) according to Reid *et al.* (31).

The results for quantification of lactic acid produced by the lactobacilli isolates are summarized in Table 2. The highest amount of lactic acid was produced by *L. salivarius*, followed by *L. johnsonii*, *L. acidophilus* and *L. jensenii*. The production of organic acids helps to keep the vaginal pH below 4.5 and creates a hostile environment for the growth and survival of pathogenic microorganisms (3). In a study conducted by Valore *et al.* (40) with vaginal exudates samples obtained from healthy patients, a higher antimicrobial activity was verified for samples with the highest levels of lactic acid.

Hydrogen peroxide is another antagonistic compound produced by lactobacilli and its production is normally assessed by using qualitative methods, such as incorporation of the peroxide in agar medium and revelation by addition of

tetramethylbenzidine (19, 24). However, quantitative results may help to better understand the role of H₂O₂ in healthy and infected vaginal environments (38, 41). In this study, despite H₂O₂ production by all isolates, the highest levels were observed for *L. acidophilus*, *L. crispatus*, *L. gasseri*, *L. johnsonii*, and *L. vaginalis* (Table 3).

In vaginal exudates, H₂O₂ is converted to reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and hydroxyl free radicals that are highly toxic against several microorganisms (15). Besides that, lactobacilli keep a high oxidation potential in the vaginal environment, which inhibits multiplication of strictly anaerobic bacteria (3). The absence of hydrogen peroxide-producing lactobacilli has been related to a higher risk of BV, recurrent urinary tract infection by *E. coli*

and increased susceptibility to the infection by Human Immunodeficiency Virus (HIV-1) (31, 38).

Table 2. Lactic acid production by vaginal *Lactobacillus* spp. isolates, expressed in g/l

Species	Lactic acid (g/l)*
<i>L. acidophilus</i>	5.52
<i>L. agilis</i>	1.72
<i>L. coleohominis</i>	0.77
<i>L. crispatus</i>	1.32
<i>L. fermentum</i>	1.22
<i>L. gasseri</i>	1.35
<i>L. jensenii</i>	5.42
<i>L. johnsonii</i>	6.50
<i>L. ruminus</i>	1.22
<i>L. salivarius</i>	13.95
<i>L. vaginalis</i>	1.72

*According to Edema and Sanni (9)

Table 3. Semi-quantification of H₂O₂ production by vaginal *Lactobacillus* spp. isolates obtained from healthy patients

Production of H ₂ O ₂ (mg/l)*	
1-3	3-10
<i>L. agilis</i>	<i>L. acidophilus</i>
<i>L. coleohominis</i>	<i>L. crispatus</i>
<i>L. fermentum</i>	<i>L. gasseri</i>
<i>L. jensenii</i>	<i>L. johnsonii</i>
<i>L. ruminus</i>	<i>L. vaginalis</i>
<i>L. salivarius</i>	

*Production of H₂O₂ was scored according to Wilks *et al.* (41) as: negative, 1-3, 3-10, 10-30, 30-100 mg/l of H₂O₂.

is the first step towards the formation of a barrier by lactobacilli that will prevent colonization by pathogenic microorganisms. In our study lactobacilli isolates were tested for adhesion to epithelial tissue, using Caco-2 cell, which presents a confluent growth when cultivated *in vitro* and shows characteristics of mature enterocytes, making it an excellent model for studying adherence of microorganisms (6, 12). Among all lactobacilli isolates tested, only *L. agilis*, *L. jensenii*, *L. johnsonii* and *L. ruminus* were able to adhere to epithelial Caco-2 cells (Table 4 and Figure 3) indicating adhesion is specific for each bacterial strain, as verified also by Chauvière *et al.* (6).

Some vaginal *Lactobacillus* species are capable of synthesizing antimicrobial peptides known as bacteriocins (5). In our study, we have also evaluated bacteriocin production of all lactobacilli isolates against *Candida* strains by agar antagonism method, but no inhibitory activity was detected (data not shown). Osset *et al.* (26) studied the production of bacteriocin by several LAB isolates against *C. albicans* and *C. glabrata* and they did not observe inhibition zones when agar plate method was used. However, in broth cultures those authors verified that some lactobacilli were inhibitory against *C. albicans* to some extent. In our experiments, highest inhibitory activity against *C. albicans* assessed in BHI broth was obtained for both *L. jensenii* and *L. johnsonii* isolates (data not shown).

Ström *et al.* (36) observed that several antifungal compounds, such as cyclic dipeptides, pyroglutamic acid and lactones were produced by *Lactobacillus* isolates and played an important role against *Candida* spp. Thus further investigation is required to clarify the nature of the inhibitory substance produced by lactobacilli isolates against the yeast in our study.

In conclusion, our results indicated that among the 11 vaginal lactobacilli isolates tested, *L. agilis*, *L. jensenii*, *L. johnsonii*, and *L. ruminus* exhibited desirable properties as potential probiotic strains including the ability to adhere to epithelial mucosa, to auto-aggregate, co-aggregate with *Candida* species, and to produce both lactic acid and H₂O₂. Also, future studies are encouraged to assess technological

Atassi *et al.* (4) affirmed that adhesion to epithelial tissue

properties of those microorganisms for clinical use, including determination of their viability and stability in pharmaceutical preparations such as capsules resistant to gastrointestinal tract

for oral intake and ovules/capsules for intravaginal administration.

Table 4. Classification of adhesion of vaginal isolates of *Lactobacillus* spp. to Caco-2 cells

Non-adhesive* (<5 bacteria/100 Caco-2 cells)	Adhesive* (6-40 bacteria/100 Caco-2 cells)
<i>L. acidophilus</i>	<i>L. agilis</i>
<i>L. colehominis</i>	<i>L. jensenii</i>
<i>L. crispatus</i>	<i>L. johnsonii</i>
<i>L. fermentum</i>	<i>L. ruminus</i>
<i>L. gasseri</i>	<i>L. rhamnosus</i> [‡]
<i>L. salivarius</i>	
<i>L. vaginalis</i>	
<i>L. bulgaricus</i> [‡]	

*Adhesion was classified according to Del Re *et al.* (7). [‡]*L. bulgaricus* and *L. rhamnosus* GG were used as negative and positive controls, respectively.

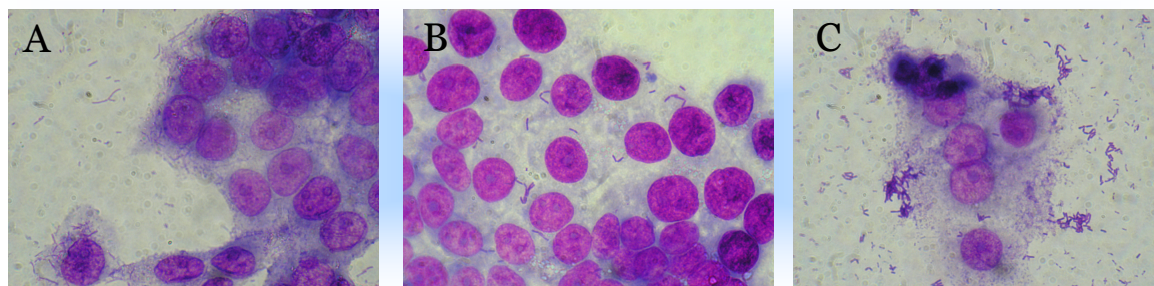


Figure 3. Microphotographies (magnification of 1000x) showing adhesion of *L. rhamnosus* (A), *L. bulgaricus* (B), and *L. agilis* (C) to the epithelial Caco-2 cell line.

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