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Lactobacilli and lactoferrin: Biotherapeutic effects for vaginal health

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ABSTRACT

Protective human vaginal microbiota is characterized by several microbial strains, mainly members of *Lactobacillus* genus. During vaginal infections, the balance among these strains is altered producing opportunistic or pathogenic bacteria overgrowth. Antibiotic treatments often select for resistant organism or lead to adverse effects on beneficial microbiota. In this study the effect of a combination of *L. acidophilus* GLA-14 and *L. rhamnosus* HN001 with lactoferrin, a glycoprotein of the innate immune system exerting antimicrobial, immunomodulatory, and prebiotic actions, has been investigated. Results obtained using a human cervix *in vitro* model showed that supernatants from both probiotics exert a beneficial effect on cervix cells and that both strains were able to grown in biofilm and exhibited aggregation and adherence properties to biotic or abiotic surfaces. These properties are modulated and even increased by lactoferrin demonstrating the beneficial activity of this biotherapeutic agent combination to prevent and manage urogenital tract infections in women.

1. Introduction

The human vagina is colonized by various microorganisms, the vaginal microbiota, which can have a significant impact on the health of women, their partners, and their neonates (Srinivasan & Fredricks, 2008). The normal vaginal microbiota is dominated by *Lactobacillus* spp. that are believed to promote a healthy vaginal ecosystem by producing antimicrobial compounds such as lactic acid, hydrogen peroxide, and bacteriocins thereby excluding pathogens from this niche (Aroutcheva, Simoes, & Faro, 2001). The *Lactobacillus* spp. capability of adhering to vaginal cells and mucus, thus competing with pathogen adherence and multiplication, further helps to preserve the healthy vaginal status (Boris, Suárez, Vázquez & Barbés, 1998). Moreover, it has also been reported that *Lactobacillus* colonization of gastric mucosa aids to accelerate ulcer healing (Elliott, Buret, McKnight, Miller, & Wallace, 1998).

Protective vaginal Lactobacilli are active against both aerobic and anaerobic microorganisms such as *Escherichia* spp. and *Gardnerella vaginalis* (McGroarty & Reid, 1988; McLean & McGroarty, 1996; Bertuccini, Russo, Iosi, & Superti, 2017). Consequently, changes in the normal vaginal microbiota are associated to diseases such as bacterial vaginosis (BV) and aerobic vaginitis (AV), in which the protective Lactobacilli are replaced by pathogenic anaerobic or aerobic microorganisms, respectively (Donders et al., 2002; Donders, 2010). BV is one of the most frequent causes of vaginal discharge (Sumati & Saritha, 2009). Vulvar irritation due to epithelial cell damage is often present. Conversely, typical AV markers are an increased inflammatory response or prominent signs of epithelial atrophy or both. The latter condition, if severe, is also called desquamative inflammatory vaginitis (Sobel, Reichman, Misra, & Yoo, 2011; Donders, Ruban, & Bellen, 2015). With the aging of the population, atrophic vaginitis and desquamative inflammatory vaginitis, both associated with hypoestrogenism, are frequently seen in women with persistent vaginitis (Nyirjesy, 2014).

Both BV and AV require a combined oral or local treatment with any of the following that may yield the best results: antibiotic (infectious component), steroids (inflammatory component), and/or estrogens (atrophy component). Complementary alternative treatments are also of common use, particularly in young women with disruptive symptoms and under severe stress conditions (Nyirjesy et al., 2011). Interest by the community in nutraceutical based treatments has also increased and, in this view, lactoferrin (Lf), a safe, natural component of most exocrine biological fluids, including vaginal secretions, deserves attention as a possible therapeutic agent. Lf is a ~80 kDa iron binding multifunctional glycoprotein constituting one of the major antimicrobial components of the innate immune system (González-Chávez, Arévalo-Gallegos & Rascón-Cruz, 2009). Biological properties reported for Lf include antimicrobial activity against a wide range of pathogenic bacteria, fungi, protozoa and viruses, as well as anti inflammatory,

Abbreviations: BV, bacterial vaginosis; AV, aerobic vaginitis; bLf, bovine lactoferrin; SEM, scanning electron microscopy

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antitumor and immunomodulatory activities (González-Chávez, Arévalo-Gallegos & Rascón-Cruz, 2009). As other milk glycoconjugates, Lf functions as soluble receptor mimetic that inhibits pathogen binding to the mucosal cell surface. Despite Lf broad spectrum of antimicrobial and immunomodulatory activities mentioned above, relatively little is known about Lf capacity to modulate vaginal microbiota growth and biofilm formation capability, as well as their interactions (or interactions of their products) with target human cells.

Concerning the possible influence of lactoferrin on vaginal microbiota, it has been demonstrated that the administration of bovine lactoferrin (bLf) in a woman with a refractory vaginitis resulted in a *Lactobacillus* predominant vaginal flora (Otsuki et al., 2014).

Based on the above rationale and the mentioned observation we have here addressed the ability of selected Lactobacilli, *Lactobacillus acidophilus* GLA-14 and *Lactobacillus rhamnosus* HN001, alone or in combination, and in the presence or absence of bLf, to adhere to and promote the healthy status of cervical epithelial cells.

2. Materials and methods

2.1. Lactobacillus strains

Lactobacillus strains used in the current *in vitro* study were: *Lactobacillus acidophilus* GLA-14 (BCCM/LMG Bacteria Collection, LMG S 29159) and *Lactobacillus rhamnosus* HN001 (American Tissue Culture Collection, ATCC Number: SD5675). Both strains were stored in milk yeast extract (13% nonfat milk, 1% yeast extract, Sigma Aldrich, Milan, Italy, MYE) at −80 °C. Before the experiments, each strain was transferred from the frozen stock culture to De Man Rogosa Sharpe broth (MRS broth; Sigma Aldrich, Milan, Italy) incubated for 48 h or 24 h at 37 °C, for *L. acidophilus* GLA-14 and *L. rhamnosus* HN001, respectively, under non agitated aerobic conditions.

2.2. Chemicals

Milk derivative bovine lactoferrin (bLf) was obtained from Morinaga (Milk Industry Co., Ltd., Japan).

2.3. Cells

HeLa epithelial cells (American Tissue Culture Collection, ATCC CCL-2™), derived from a biopsy of a cervical tumor, were cultured in Dulbecco's Modified Eagle Medium (DMEM, High Glucose, Euroclone, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, HyClone, European Union) and 2 mM/L glutamine (Euroclone, Milan, Italy) at 37 °C and in 5% CO_2.

2.4. Analysis of sensitivity of L. acidophilus GLA-14 and L. rhamnosus HN001 to bLf

Lactobacillus acidophilus GLA-14 and *Lactobacillus rhamnosus* HN001 were grown in MRS broth (Sigma Aldrich, Milan, Italy) at 37 °C in microaerophilic conditions. After 18 h incubation, bacteria were diluted in MRS broth (Sigma Aldrich, Milan, Italy) at the final concentration of 5×10^5 Colony Forming Units (CFU)/ml. Serial dilutions of bLf (from 0.5 to 4 mg/ml) were incubated at 37 °C with probiotic cells. After 24 h incubation, the growth of Lactobacilli was evaluated by measuring the optical density at a wavelength of 590 nm.

2.5. Effect of probiotic supernatants on cell viability

The supernatant of cultures of probiotic strains used in this study (hereafter referred to as probiotic supernatants) was obtained from 24 h liquid cultures in MRS broth (Sigma Aldrich, Milan, Italy). The supernatant used was in all cases referred to a probiotic cell density of 10^8 CFU/ml. After culture centrifugation (10 min, 1000 × *g*), the supernatants were harvested and sterilized by membrane filtration (0.2 µm). The final cell free supernatants (CFSs) were stored at −20 °C until assayed without or with pH adjustment to values near neutrality using 1 M NaOH. CFSs were diluted in DMEM at the final concentration of 6% and incubated with the HeLa cells for 24 and 48 h at 37 °C in a 5% CO_2 atmosphere. After incubation, cell viability was measured by neutral red assay.

2.6. L. acidophilus GLA-14 and L. rhamnosus HN001 biofilm assay (Crystal Violet assay)

For biofilm analysis, samples were processed using a quantitative adherence assay as previously described (Terraf, Juárez Tomás, Nader-Macías, & Silva, 2012) with slight modifications. Overnight cultures of Lactobacilli were diluted 1:10 in 96 well polystyrene microtitre plates in MRS or Tryptone Soy Broth (TSB, Sigma Aldrich, Milan, Italy) medium, in presence of bLf (1 or 4 mg/ml). After 48 h incubation at 37 °C, biofilm was measured by discarding the medium and gently rinsing the wells with Phosphate Buffered Saline (PBS, pH 7.2) (four times), oven drying at 60 °C for 1 h, then staining the dried extracellular matrix with 1% Hucker Crystal Violet (Sigma Aldrich, Milan, Italy) in H_2O (100 µl per well). After 5 min of incubation at room temperature, microplates were rinsed four times with water and dried on blotting paper. The dye was solubilized with ethyl alcohol 95% (200 µl per well) and transferred in a new 96 well plate; absorbance at 590 nm was determined using a microtitre plate reader (PerkinElmer, Monza, Italy). For each experiment, background staining was corrected by subtracting the crystal violet bound to uninoculated controls. Each experiment was performed at least in triplicate.

2.7. Ultrastructure of L. acidophilus GLA-14 and L. rhamnosus HN001 biofilm by scanning electron microscopy

For this assay, the support used to test the biofilm was modified (glass coverslips instead of polystyrene microplates). The assay was performed as described previously by us (Ammendolia et al., 2010) with slight modifications. Biofilms were grown and formed on 12 mm glass coverslips in 12 well polystyrene tissue culture plates (BD, Falcon, BioScientifica S.r.l., Rome, Italy) containing 1 ml of each medium (in presence or absence of 4 mg/ml bLf) under static aerobic conditions for 72 h at 37 °C. The broth was then removed, the biofilms were carefully rinsed with saline, and samples were fixed and processed for scanning electron microscopy (SEM).

2.8. Interactions between Lactobacilli and HeLa cells and biofilm formation

Adhesion assay was performed by utilizing *Lactobacillus acidophilus* GLA-14 and *Lactobacillus rhamnosus* HN001, alone or in combination, in presence or in absence of 1 mg/ml bLf. For this assay, HeLa cells were grown at 37 °C and 5% CO_2 on 12 mm glass coverslips in 24 well tissue culture plates (BD, Falcon, BioScientifica S.r.l., Rome, Italy) until they reached a density of 2×10^5 cells/well (≈90% confluence). Semi confluent cell monolayers were washed twice with PBS and 1 ml suspension of Lactobacilli at a concentration of 2×10^7 bacteria/ml in DMEM was added. After 4 h incubation at 37 °C, HeLa cells were washed three times with PBS to remove unbound bacteria and processed for SEM.

2.9. Adhesion to abiotic surface

Lactobacillus strains were tested for their capacity to aggregate and adhere to abiotic surfaces. Briefly, 1 ml of *Lactobacillus acidophilus* GLA-14 (10^9 CFU/ml) was mixed with 1 ml of *Lactobacillus rhamnosus* HN001 (10^9 CFU/ml) in PBS on a vortex mixer for at least 10 s and then incubated on 12 mm glass coverslips in 12 well tissue culture tray (BD, Falcon, BioScientifica S.r.l., Rome, Italy) for 24 h at 37 °C, under