Lactobacilli and its Metabolites as Potential Probiotics against *Gardnerella vaginalis*

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1. General remarks

Bacterial vaginosis (BV) is the most prevalent vaginal disorder in women of childbearing age, posing some significant health risks, such as abnormal pregnancy, pelvic inflammatory disease and an increased risk of sexual transmitted infections, including HIV [1]. It is characterized by a disruption in the normal balance of bacteria in the vagina with a reduction on lactobacilli number and an overgrowth of anaerobes [2]. Despite several species have been associated with this condition [3], several studies point out for the higher virulence potential of *Gardnerella vaginalis* [4, 5, 6] and therefore it has been proposed as the main etiological candidate of BV.

Current BV treatment is based in antibiotic therapy leading to an increased resistance of BV anaerobes and to a severe reduction of healthy lactobacilli strains in the vaginal epithelium [7, 8, 9]. Therefore, a more appropriate treatment is required, aiming to decrease *G. vaginalis* and also to promote the lactobacilli re-colonization in BV patients [10].

Previous studies have shown the potential of lactobacilli in preventing vaginal colonization by pathogens [11, 12] through different mechanisms, which may include auto-aggregation, co-aggregation with pathogenic microorganisms, adhesion to epithelial cells and/or by the production of some metabolites (such as lactic acid, hydrogen peroxide, bacteriocins, bacteriocin-like proteins and biosurfactants) [13]. Thus, the probiotic properties of lactobacilli should be explore in order to evaluate their potential as a more effective treatment for BV.

2. Materials and methods

2.1 Lactobacilli isolation from vaginal exudate samples

Vaginal exudate samples were obtained from volunteer women after informed consent as approved by the Minho University Institutional Review Board (SECVS 003-2013). Samples obtained were used for lactobacilli isolation. All colonies obtained in Columbia Blood Agar (CBA) after a 72h incubation at 37ºC and under anaerobic conditions, were tested for catalase and oxidase presence and analysed by microscopy after Gram-staining. Colonies composed by rod shape cells, with positive reaction for Gram-staining and negative reaction for catalase and oxidase tests, were selected for further testing. In order to confirm the genus of the isolated bacteria, a PCR reaction was performed using primers previously design for specific detection of lactobacilli species [15].. A total of 51 lactobacilli strains were thus obtained.

2.2 Screening for probiotic activity of lactobacilli against *G. vaginalis*

The 51 vaginal lactobacilli isolated and 35 lactobacilli strains from culture collections were used to evaluate their effect on growth of 9 *G. vaginalis* strains.Lactobacilli potential to affect *G. vaginalis* growth was screened by an agar sopt test [12], in order to select potential probiotic candidates. . Briefly, lactobacilli strains were cultured for 48 h to 72 h at 37ºC in sBHI broth (supplemented Brain Heart Infusion) or MRS broth, under a 5-10% CO_2 atmosphere (according to each strain's requirements) [16]. Subsequently, cells were harvested, washed once with water and resuspended in PBS pH7.0 with agitation. After 2 hours, cells were removed by centrifugation and the PBS extract (which may contain intracellular biosurfactants) were saved. The medium (supernatant) was also saved in order to evaluate the possible presence of other metabolites such as lactic acid, bacteriocins, bacteriocins-like molecules and extracellular biosurfactants. Fifty microliters of both medium and PBS extract was spotted onto the surface of the CBA plate, previously spread with a suspension of *G. vaginalis*, and then incubated at 37ºC at 10% CO2 atmosphere. After 48h the *G. vaginalis* growth inhibition around the spot was evaluated.The assays were performed in triplicate using independent cultures.

2.3 Evaluation of the probiotic activity of lactobacilli extracellular metabolites against *G. vaginalis*

The evaluation of the probiotic activity of the lactobacilli supernatants against *G. vaginalis* was based on the microdilution method performed in 96-well culture plates, as described by Gudiña et al. [17], with some modifications. Briefly, 3ul of an overnight G. vaginalis culture, previously adjusted to an absorbance of 0.100-0.200 at 620 nm, were added to 200 µL of sBHI medium containing 40 to 80% (V/V) of supernatant. Positive controls did not contain supernatant. A non-probiotic strain was used as negative control. The 96-well microplates were incubated for 48 h at 37 °C under anaerobic conditions. Afterwards, the optical density at 620 nm of each well was recorded using a microplate reader and the results analysed

2.4 Lactobacilli's active metabolites identification

The antimicrobial activity of supernatants that were found to be active against *G. vaginalis* was accessed and characterized, according to Pascual et al. [12], with some modifications. Briefly, pH of the medium, which could be perturbed by the presence of lactic acid, was measured after each lactobacilli incubation period. To evaluate the possible effect oflactic acidon *G. vaginalis* growth, pH was adjusted to neutral and tested as described in section 2.2. To evaluate the possible effect of hydrogen peroxide, 1 ml of filtered supernatant (0.45 µm) was incubatedwith 5mg/ml catalase in 50 mM KH_2PO_4 incubated at room temperature for 1 h and then tested as described in section 2.2. Regarding the presence of bacteriocins and/or bacteriocin-like proteins , filtered supernatant (0.45 µm) was incubated with 0.3 mg/mltrypsin in (conc buffer pH) and protease (final concentration of 0.3 mg/ml each), separately.for 1 h at 37ºC,and then used to inoculate CBA plates previously grown with *G. vaginalis*Finally, the presence/absence of inhibition halos was evaluated.

3. Results and discussion

3.1 Screening of lactobacilli

The initial experimental step of our work consisted in a qualitative analysis of the lactobacilli's effect on *G. vaginalis growth*, by evaluating the presenceof inhibition halos around the drop of supernatant. Inhibition was scored positive when a clear or haemolytic zone around the spot of each supernatant tested was noticeable. The results are represented in Fig.1 and Fig.2.

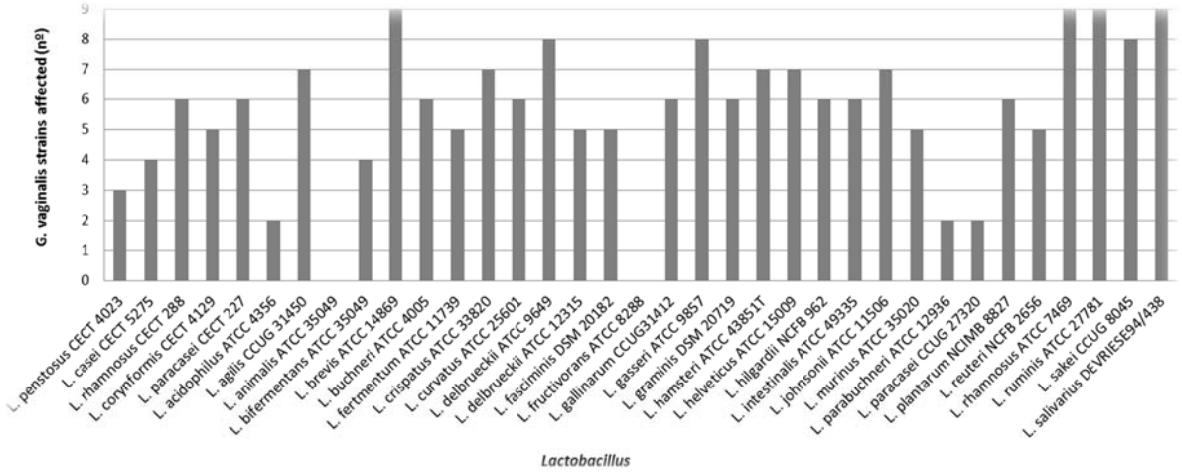

Fig.1 Screening of probiotic activities from culture collection lactobacilli species against *G. vaginalis* strains.

To validate our assays and select the more interesting lactobacilli to be used in the following step of our work, we focused on the ones with the widest spectrum of action.

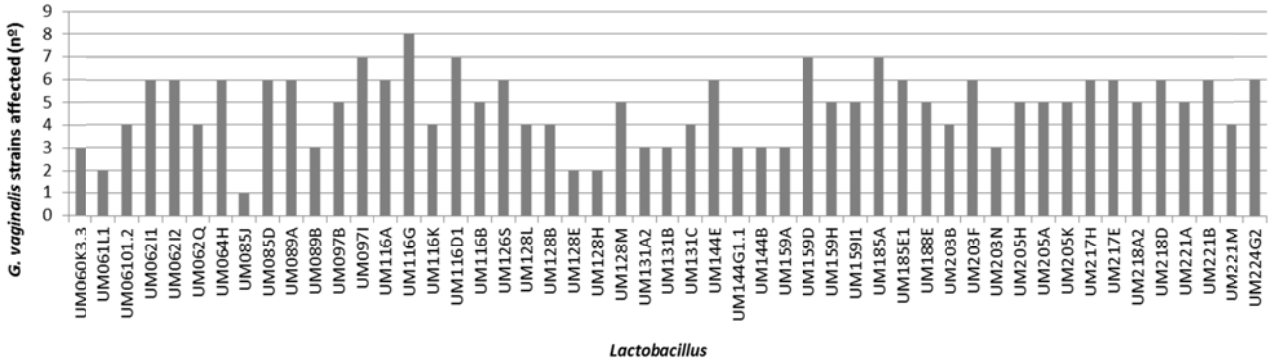

Fig.2 Screening of probiotic activities from vaginal isolated lactobacilli species against *G. vaginalis* strains.

All lactobacilli strains tested were apparently able to inhibit the growth of *G. vaginalis* strains. However, in the culture collection group, 2 lactobacilli strains did not show any activity against *G. vaginalis* and 4 lactobacilli strains were able to inhibit all the *G. vaginalis* strains tested. A possible explanation for the absence of activity from the previous 2 lactobacilli strains could be due to the low concentration of the metabolite(s) produced, i.e. not enough to inhibit *G. vaginalis* growth. Regarding the vaginal isolates, all lactobacilli strains were able to inhibit the growth of *G. vaginalis*. However, their spectrum of action was usually lower when compared to the culture collection lactobacilli strains. Taking into account these results, we selected 10 lactobacilli strains to proceed with the antimicrobial activity assays.

3.2 Evaluation of the probiotic activity of lactobacilli supernatants against *G. vaginalis*

None of the supernatants revealed any significant probiotic activity in the microdilution test assays. However, all the 10 strains with 80% (V/V) in the culture medium were simultaneously capable to inhibit all *G. vaginalis* strains used, as shown in Fig.3.

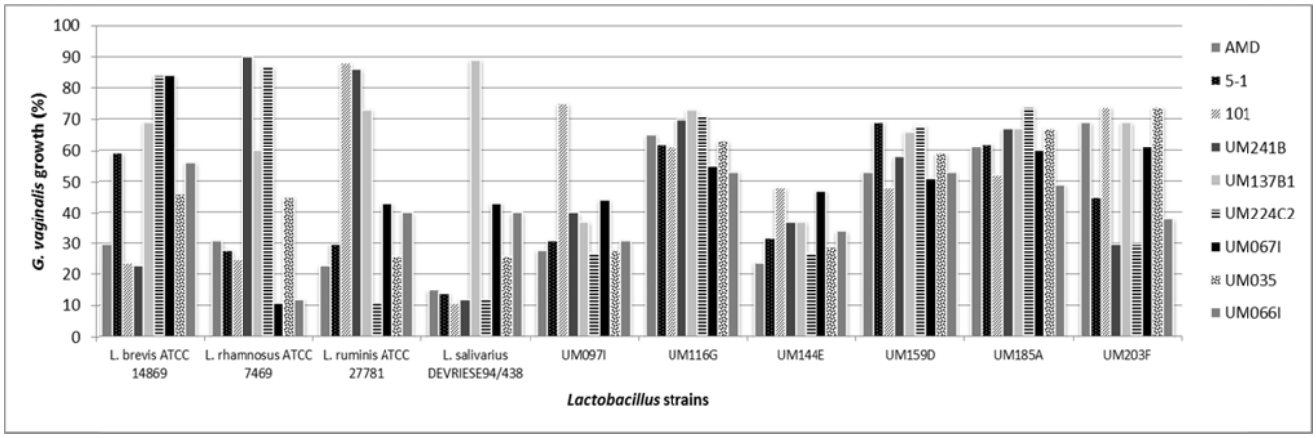

Fig.3 *G. vaginalis* strains growth in presence of lactobactilli supernatants, as comparing to the control.

According to Fig.3, it can be seen that in general lactobacilli strains from culture collections present a more pronounced inhibition of *G. vaginalis* growth comparing to the vaginal isolates, thus confirming our previous qualitative observations. Again, this may be due to the metabolites concentrations in the cell-free supernatants, since the lactobacilli from culture collections grew much more than the vaginal isolates. Therefore, it can be expected that whatever metabolite is responsible for inhibiting *G. vaginalis* growth, it was produced at higher concentrations, along with the higher growth rates, thus promoting a greater growth inhibition. Additionally, the *L. salivarius* DEVRIESE94/438 strain was able to inhibit almost all *G. vaginalis* strains tested to values below 50% of growth,and in some cases, to below 90%, except for UM137B1,. Similar results were obtained for UM097I and UM144E isolates, although they were less effective against each *G. vaginalis* strain.

3.3 Identification of the metabolites responsible for G. vaginalis growth inhibition.

Based on the previous results, it was crucial to determine the active compound responsible for such inhibition. It is important to notice that the PBS extract's superficial tensions were measured to evaluate the presence of intracellular biosurfactants [18]. None of the tested PBS extracts showed a significant reduction of the superficial tension, thus the presence of intracellular biosurfactants was excluded as the possible active agent responsible for *G. vaginalis* growth inhibition. Similar results were obtained for the extracellular biosurfactants. Six lactobacilli strains were used to evaluate the presence of lactic acid, hydrogen peroxide and/or bacteriocins (table 1).

Table 1. Metabolites produced by the selected lactobacilli strains and responsible for G. vaginalis growth inhibition.

		Lactobacilli strains					
		L. brevis ATCC 14869	UM159D	*L. rhamnosus* ATCC 7469	UM203F	UM116D1	*L. salivarius* DEVRIESE 94/438
G. vaginalis strains	AMD	LA			BC	HP	LA
	5-1	LA		LA			LA
	101			LA	HP	HP	
	UM241B	LA		LA			
	UM137B1					BC	
	UM224C2	LA	LA		BC	HP	BC
	UM067I			LA	HP, LA	LA	LA
	UM035	LA		LA		HP	
	UM066I	LA		LA			HP, BC

LA – Lactic acid; HP – Hydrogen peroxide; BC - Bacteriocins

The effect of lactic acid against *G. vaginalis* growth was the most prevalent one among all the metabolites evaluated, thus suggesting that *G. vaginalis* is more sensitive to acidic pH. For some *G. vaginalis* strains, bacteriocins or hydrogen peroxide appeared to be involved in the antimicrobial activity of the lactobacilli. Also, for 2 *G. vaginalis* strains, a synergetic effect between two metabolites seemed to be the cause of growth inhibition. It is important to note that for *L. salivarius* DEVRIESE94/438 strain, lactic acid was responsible for a reduction of almost 90% in AMD and 5-1 strains, and a bacteriocin showed a similar effect on the UM224C2 strain.

4. Conclusions

Our results suggest the existence of lactobacilli strains with a relevant probiotic activity against *G. vaginalis* strains, demonstrating their potential clinical significance. In adition, we observed the production of 3 distinct types of metabolites, being lactic acid the most effective against *G. vaginalis*. Moreover, an up-and-coming candidate, *L. salivarius* DEVRIESE94/438, showed an excellent ability to inhibit *G. vaginalis* growth. Although further work must be conducted in order to fully characterize the active agents produced by lactobacilli, we believe that these results are promising and may contribute for a new and more effective BV treatment than the currently used therapies.

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