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Biological properties and production of bacteriocins-like-inhibitory substances by *Lactobacillus* spp. strains from human vagina.

BLIS production by *Lactobacillus* strains

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ABSTRACT

Aims: The aim of this study was to characterize *Lactobacillus* strains for their biological properties and amensalistic activities against genital and non-genital pathogens.

Methods and Results: For the purpose, some special characteristics (H_2O_2, biofilm and antimicrobial substances production) as well as safety properties of 112 lactobacilli were evaluated.

All the strains had good amensalistic characteristics, in particular cell-free supernatants (CFS) of ten strains showed antibacterial activity against bacteria, as well as *Candida* spp. Moreover, these ten strains were excellent biofilm producers.

Conclusions: These results provide evidences for the possible use as probiotics for vaginal co-therapy in cases of dysbiosis.

Significance and Impact of Study: Nowadays, the problem of antibiotic resistance is constantly increasing, even though resources and energy are invested in order to increase knowledge on the mechanisms of action. Bacteriocins have a rapid mechanism of action, act at extremely low concentrations, are generally sensitive to proteases and they usually have a narrow killing spectrum: these characteristics reduce the possibility of the bacterium to develop resistance to it. Our study is focused on the feasibility of a high production of antimicrobial substances and their characterization in order to be exploited as a therapeutic alterative or in co-therapy with antibiotics in case of vaginal dysbiosis.

Keywords: *Lactobacillus*, biofilm, probiotics, antimicrobials, bacteriocins, biomolecules

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INTRODUCTION

The vagina is a habitat in which, under normal conditions, there is a balance between different bacterial species in a commensal symbiosis with the host. Lactobacilli account for 50-90% of the vaginal microaerobic microbiota in most healthy women (Beamer et al., 2017) and they are present in concentrations of 10^7-10^8 cfu g^{-1} of vaginal fluid, where glycogen is considered the maximum source of fermentable carbohydrates although the presence of the enzyme alfa-amylase is necessary to break down glycogen into a form usable by lactobacilli strains which cannot directly metabolize glycogen (Miller et al., 2016, Fuochi et al., 2017b). Any woman differ from another for many reasons: variables can be included hygiene, sexual activity, number of sexual partners, diet, use of antibiotics, age, and many other issues relating to socio-economic status (Marrazzo et al., 2002, Yamamoto et al., 2009, Martinez-Pena et al., 2013, Pendharkar et al., 2013). *L. crispatus, L. gasseri,* and *L. jensenii* were identified as predominant species in non-cultural analysis of samples, taken from healthy, sexually active women, while *L. rhamnosus, L. paracasei, L. fermentum, L. plantarum, L. vaginalis,* and *L. iners* have occasionally been identified (Antonio et al., 1999, Falsen et al., 1999, Vasquez et al., 2002).

In cases of vaginal diseases, the amount of lactobacilli decreases or it remains predominant but the bacterial composition changes (Kuebrich, 2004, Vitali et al., 2007). The most common vaginal disorders are linked to bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), and aerobic vaginitis (AV) (Tempera et al., 2009, Tempera and Furneri, 2010).

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BV occurs when *Lactobacillus* spp., are replaced by anaerobic bacteria, such as *Gardenella vaginalis, Mobiluncus curtisii,* and/or *Mycoplasma hominis*: 20–30 % of women of reproductive age with sexually transmitted infections suffer from BV, and the percentage is higher in high-risk populations (Bautista et al., 2016). AV differs from BV for several reasons: at the microbiological level, there is an increase of Gram-positive cocci, such as *Streptococcus agalactiae* and Gram-negative, in particular *E. coli*. The lactobacilli component of the microbiota decreases until it disappears completely (Donders et al., 2002, Kuebrich, 2004). From a clinical point of view, the infection is characterized by the presence of toxic leukocytes and parabasal cells, yellow secretion, foul odor (KOH negative test), elevated vaginal pH, redness, itching, burning and different degrees of dyspareunia (Donders et al., 2002, Kuebrich, 2004, Donders, 2007). One of the most important antibiotic used in case of AV is the fluoroquinolone ciprofloxacin, that's because it maintains stable the "good" vaginal microbiota in contempt of pathogens in cases of antibiotic therapies (Tempera et al., 2006, Tempera et al., 2009, Tempera and Furneri, 2010).

VVC is an infection caused by strains of *Candida* genus: more than 20% of healthy, asymptomatic and not-pregnant women during a visit showed a vaginal tract colonized by *Candida* ssp. and *C. albicans* is the most frequently isolated species (about 68.3%) (Bauters et al., 2002), followed by *Candida glabrata* (Achkar and Fries, 2010). The increase in the incidence of candidiasis is attributable to several factors: excessive use of antibiotics and oral contraceptives, as well as inadequate therapeutic treatments (Sobel, 1985).

In conclusion, in both aerobic vaginitis and vaginal candidiasis pathogenesis is closely related to the reduction of the component of vaginal lactobacilli. Lactobacilli are, in fact, predominant microorganisms in the vaginal environment of healthy women and play an

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important antimicrobial activity through the production of bacteriocins, hydrogen peroxide and lactic acid (Pascual et al., 2006, Kaewsrichan et al., 2006, Kubota et al., 2008, Tachedjian et al., 2017). Reducing the component of lactic acid bacteria makes the vaginal microbial more vulnerable to the onset of infections.

The main goal of the study has been the characterization of *Lactobacillus* strains in order to evaluate their amensalistic role and understanding the possible intervention in case of dysbiosis to restore the homeostasis of the vaginal microbiota.

For this aim, we focused our attention on the characterization of lactobacilli, previously recorded as vaginal isolates. Therefore, we have examined strains belonging to the collection of the Laboratory of Applied Microbiology: one hundred twelve strains were studied to assess special characteristics such as production of hydrogen peroxide, biofilm formation, and ability to produce antimicrobial substances. Moreover, the strains were tested for their safety: haemolytic activity and gelatin hydrolysis were done, and finally, cytotoxicity on human TR146 cells was evaluated.

MATERIALS AND METHODS

Bacterial Strains and Susceptibility Testing

In this study, seven references strains (*L. crispatus* ATCC33820 , *L. delbrueckii* ATCC9649, *L. fermentum* ATCC14931, *L. gasseri* ATCC33323, *L. jensenii* ATCC25258, *L. rhamnosus* ATCC7469, *L. salivarius* ATCC11741) and 112 strains of *Lactobacillus* spp (available in Laboratory of Applied Microbiology, Department of Biomedical and Biotechnological Sciences, Università degli Studi di Catania, Italy) were used (Table S1).

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Preliminary methods to isolate clinical strains are available in supporting information file.

Determination of the profile of susceptibility/resistance to antibiotics was evaluated to test the safety: broth dilution method was performed in agreement by CLSI M100 (CLSI, 2017b).

Molecular Identification

Overnight grown cultures of vaginal isolates strains were used for DNA extraction. RTP Bacterial DNA Mini Kit (Stratec Biomedical AG) was used for the purpose. Moreover, the samples obtained were visualized by electrophoresis (70V) in agarose gel 1.5% as previously described (Fuochi et al., 2015). Subsequently, molecular identification was carried out by 16S rDNA PCR/RFLP and, multiplex-PCRs techniques (Fuochi et al., 2015, Fuochi, 2016).

More details about identification at spp. level are available in supporting information file.

Evaluation of Special Characteristics

Detection of H_2O_2 production

MRS agar medium with horseradish peroxidase and TMB (3,3',5,5'-tetramethylbenzidine) was used for the evaluation and quantification of the production of hydrogen peroxide by isolates of *Lactobacillus* strains as previously described (Mareike, 2011, Fuochi, 2016). In brief, MRS agar medium, peroxidase (Sigma P6782) (A) and TMB (Sigma T2885) (B) solutions were prepared. After autoclaving, MRS agar medium was cooled to 50°C, and then A and B solutions were added. The plates were filled and stored in the dark at 4°C before use. The production was evaluated applying 0.5 McFarland bacterial inoculum to the plates and then, they were incubated at 37°C for 48h under microaerobic

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conditions, and finally, at room temperature for 24h in the dark. White colonies were classified as negative, instead colonies from brown to dark blue were classified as positive.

Preparation of lactobacilli cell-free supernatants (CFS)

The antimicrobial substances produced by *Lactobacillus* strains were obtained as following described: strains were grown at 37°C in MRS broth with 20% glycerol for 72h (Fuochi et al., 2017c). Then, cells were separated by centrifugation at 8,000 rpm for 25 min at 4°C and CFS were collected after filtration at 0.22 μm. Moreover, CFS were adjusted at 6.8 pH with NaOH 1 mol l^{-1} (Toba, 1991).

Evaluation of antimicrobial activity

Inhibitory activity was performed against certain Gram positive and Gram negative organisms: *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603 following the CLSI document (CLSI, 2017a, CLSI, 2017b) for the microdilution broth method.

Minimum Inhibitory Concentration (MIC) values (expressed as % v/v) were determined : MIC value corresponded to the lowest dilution of supernatant that did not result in any visible growth of the microorganism compared with the growth in the control well (Furneri et al., 2012, CLSI, 2017b, Ronsisvalle et al., 2017). All experiments were repeated six times.

Moreover, activity against *Candida* strains was determined by microplate assay following the CLSI document M27-A3 (CLSI, 2017a). A suspension of each *Candida* strain was prepared in sterile saline (NaCl 0.85% w/v). The suspensions were adjusted to $1.0x10^6$

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CFU/mL and then they were diluted as to obtain a concentration of $1.0x10^4$ CFU/mL in RPMI 1640. A mixture of 100 µl *Candida* suspensions in RPMI (final concentration $0.5x10^3$ CFU/mL) and 100 µl of CFS tested were added to each well of a sterile 96-well microplate (Thermo Scientific™ Sterilin™). MRS (20% glycerol) broth was used as a positive control. The plates were incubated at 35°C aerobically, and the growth of *Candida* was recorded as OD at 490 nm using a microplate reader (Gen5 Microplate Reader, BioTek Instruments). MIC values ranges were determined after 24 and 48h of incubation (Fuochi et al., 2017a).

Effect of enzymes and heat treatments on CFSs

Treatment with proteinase K 30 U mg^{-1} and trypsin 10 U mg^{-1} (Sigma-Aldrich) at a final concentration of 1 mg ml-1 was performed on each CFS with antimicrobial activity. Briefly, CFS were incubated for 2 hours at 37°C and then, residual activity was determined (Corsetti et al., 2004).

Moreover, to determine the thermal stability, each CFS were heated at 60°C, 80°C, and 100°C for 10 min using a water bath and finally, by sterilization at 121°C for 15 min. Then, residual activity was determined after cooled (Alajekwu and Ike, 2017).

Biofilm production

The assay for *in vitro* biofilm formation was performed in polystyrene 96 wells microplates, making $1.5x10^5$ CFU/mL inoculum of overnight broth culture (Roccasalva, 2008, Carmo et al., 2016). After aerobic incubation for 18-24h at 37°C the absorbance at 600nm (OD600) (BioRad Microplate Reader Model 680) was recorded and the culture broth was removed. Plates were washed with PBS for 3 times in order to remove any planktonic cells freely suspended. The cells were therefore heat fixed 1 hour at 60°C and then stained 10

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min with 200µl of crystal violet. Repeated washings with water removed the excess of dye and then, crystal violet residues were dissolved in methanol/acetic acid solution (10%/7.5%). A second read at 570nm (OD570) was recorded using BioRad Microplate Reader Model 680. The biofilm index was calculated using the following formula OD570/OD600 * 0.4. The cut-off value was considered OD 0.061 (Kubota et al., 2008).

Evaluation of Safety

Haemolytic activity

Haemolytic activity was evaluated streaking onto blood agar plates (5% horse blood, bioMérieux) an overnight suspension for each strain. All plates were incubated at 37°C under microaerobic conditions for 48 h, then the plates were examined for signs of haemolysis. *Streptococcus pyogenes* ATCC 19615 (β-haemolytic strain) and *S. mutans* ATCC 25175 (α-haemolytic strain) were used as positive controls (Ruoff, 2003, Hütt et al., 2016).

Gelatin Hydrolysis

Nutrient gelatin plate method was performed as described by dela Cruz & Torres (dela Cruz and Torres, 2012). Overnight grown cultures of lactobacilli strains were streaked onto culture plates pre-filled with nutrient gelatin (4 g l^{-1} peptone, 1 g l^{-1} yeast extract, 12 g l^{-1} gelatin, and 15 g l^{-1} agar). All plates were incubated at 35°C under microaerobic conditions for 24 h. Gelatin hydrolysis was indicated by clear zones around gelatinase-positive colonies. *Bacillus subtilis* ATTC 23857 was used as positive control.

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Citotoxicity

Cell cultures

The human TR146 cell line, an in vitro model of human buccal epithelium, was provided by Sigma-Aldrich (Milan, Italy) and cultured in HAMS F12 containing 10% fetal calf serum, 2 mmol l^{-1} glutamine, 100 U/ml penicillin and 100 μg ml^{-1} streptomycin at 37°C in a humidified, 95% air/5% CO_2 atmosphere. The medium was changed every 2–3 d. One day before the experiment, cells were trypsinized, counted, and plated in 96 wells-plates (20.000 cells/well). Experimental TR146 cells were treated or not (untreated controls) with CFS After 24 hours each sample was tested for the viability test.

Cell viability assay

The effect of CFS on cell viability was evaluated with a test based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases of metabolically active cells. The optical density at λ=550 nm was measured using a microplate reader.

Statistical Analysis

Data from cell viability assay were analyzed by one-way ANOVA with correction for multiple comparison by Bonferroni. All the analysis were considered significant with p value <0.05. All results and graphs were generated using GraphPad® Prism ver. 6 software.

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RESULTS

Molecular Identification and Susceptibility Testing

The identification of the species of *Lactobacillus* isolates (Figure S1) was conducted by the use of two different methods based on the analysis of the bacterial 16S rDNA (Tables S1-S2) (Song et al., 2000, Randazzo et al., 2004).

Seven species were identified: *L. crispatus, L. gasseri, L. rhamnosus, L. delbrueckii, L. fermentum, L. jensenii, L. salivarius*. The results show (Table 1) that the most species present in the vaginal tract is *L. crispatus*, followed by *L. gasseri* (28.60%) and *L. rhamnosus* (10.70%).

Table 2 shows MIC values to erythromycin (ERY), tetracycline (TET), clindamycin (DA), chloramphenicol (CAF), ciprofloxacin (CIP), gentamicin (GEN) and ampicillin (AMP): all strains of *Lactobacillus* tested were resistant to CIP, contrariwise they were susceptible to all other antibiotics.

Evaluation of Special Characteristics

Detection of H_2O_2 production

Seven *Lactobacillus* type strains, and 98 wild strains were grown on MRS medium with horseradish peroxide and TMB (Table 3). Unfortunately, 10 strains of *L. gasseri*, and 4 of *L. rhamnosus* were not grown on this medium. Forty-six strains did not accumulate hydrogen peroxide (19 of *L. crispatus*, 1 of *L. fermentum*, 11 of *L. gasseri*, 4 of *L. jensenii*, 8 of *L. rhamnosus* and 3 of *L. salivarius*). Fifty-one strains were exhibited a weakly positive reaction

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(29 of *L. crispatus*, 8 of *L. delbrueckii*, 3 of *L. fermentum*, 11 of *L. gasseri*). Finally, one strain of *L. salivarius* exhibited a strong positive accumulation of hydrogen peroxide.

Evaluation of antimicrobial activity

Above all strains tested only ten CFS showed antibacterial activity with MIC values in range between 50-12.5% v/v: two strains of *L. crispatus*, three *L. gasseri*, one *L. fermentum*, two *L. rhamnosus*, one *L. jensenii* and finally, one strain of *L. salivarius.*

These strains showed good activity both against Gram positive and Gram negative pathogens (Table 4). The supernatant produced by ML1 *L. salivarius* showed the best activity: 12.5% against *E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumoniae*. Instead, the supernatant produced by AD4 *L. rhamnosus* strains was not active against *E. faecalis*, *S. aureus* and *K. pneumoniae*, and its activity was showed only against *E. coli* and *P. aeruginosa* (50% v/v).

The antifungal activity of ten CFS was evaluated using the microdilution assay as described by CLSI (CLSI, 2017a). The activity was evaluated against two type strains of *C. albicans* and eight clinical strains (six *Candida albicans*, one *Candida glabrata*, and one *Candida tropicalis*).

The range of concentrations (% v/v) tested was 25% - 0.025%, for a total of eleven dilutions. As shown in the Table 5, after 24h incubation all isolates of *Candida* were susceptible to CFS of *Lactobacillus* strains equally. Moreover, the minimum inhibitory concentrations (MICs) at 48h was 0.19% for all strains tested (Table 5).

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Effect of enzymes and heat treatments on CFSs

To verify the protein nature of the ten CFS with antimicrobial activity, enzymatic and heat treatments were performed. After treatments, antimicrobial activity was determined against *E. coli* and *S. aureus*, selected as reference strains for Gram negative and Gram positive, respectively.

CFS were inactivated by Proteinase K and Trypsin. Moreover, seven CFS were resistant at 60°C and 80°C but they were inactivated at 100°C and after sterilization, while CFS from ML1 *L. salivarius* and SA2 *L. gasseri* were resistant until 100°C and inactivated only after sterilization at 121°C (Table 6). Finally, SA4 *L. gasseri* remained active after heat treatment at 100°C against *S. aureus* but it was not active after the same treatment against *E. coli*.

The inactivation by enzymes indicated the proteinaceous nature of CFS: therefore they belong to the category of BLIS because their complete characterization for amino acid sequences has not been done yet.

Biofilm production

Biofilm mass values were recorded after overnight growth at 37°C. The strains were divided by means of BI (Biofilm index): non-producers (OD <0.061), weak producers (0.061 <OD <0.120), medium-sized producers (0.121 <OD <0.300) and strong producers (OD> 0.300) (Roccasalva, 2008). Figure 1 shows Biofilm index of ten BLIS producing *Lactobacillus* strains grown in three different medium broth: MRS, MRS 2% glycerol and MRS 2% inulin.

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All strains tested were biofilm producers but they showed a strong biofilm production when grown in MRS containing 2% w/v inulin ($p < 0.004$).

Evaluation of Safety: Haemolytic activity, Gelatin Hydrolysis, Cytotoxicity

No one of lactobacilli under examination caused lysis on blood agar plates. Moreover, we achieved the same results for gelatin hydrolysis test on nutrient agar and for the cytotoxicity test.

In particular, CFS produced by VV1, ML4, SA2, SA1, AD4 and ML1 at concentrations equal to 50% and 25% v/v were tested on TR146 cell line. CFS had not significant effects in this model of human buccal epithelium (Figure 2).

DISCUSSION

As previously described (Fuochi et al., 2017b), the genus *Lactobacillus* plays a major role in women vagina, exerting its beneficial functions by H_2O_2 production, biofilm formation, lactic acid and bacteriocins production; indeed vaginal microbiota is involved in the homeostasis of mammal female's physiological conditions, avoiding vaginal infections such as aerobic vaginitis, bacterial vaginosis, and candidiasis (Vitali et al., 2007, Tempera and Furneri, 2010, Heczko et al., 2015, Jasarevic et al., 2015, Tachedjian et al., 2017).

On 112 strains tested the following seven species were identified *L. crispatus*, *L. gasseri*, *L. rhamnosus*, *L. delbrueckii*, *L. fermentum*, *L. jensenii*, *L. salivarius*. The most common strain was *L. crispatus* with 42.8% of strains isolated, followed by *L. gasseri* (28.6%) and *L. rhamnosus* (10.7%). The taxonomic classification of the isolated lactobacilli confirms the

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reported dominance of *L. crispatus* and *L. gasseri* in a healthy vagina (Antonio et al., 2005, Martin et al., 2008). *L. crispatus* and *L. gasseri* produce various compounds that are potent inhibitors of BV-associated bacterial species, and for this reason, women who are these species in vagina are less likely to develop BV (Abdelmaksoud et al., 2016). *L. fermentum* and *L. gasseri* are species very resistant to low pH and excellent biofilm producers (Fuochi et al., 2015, Fuochi et al., 2017c), important characteristics to inhibit most responsible bacteria for aerobic vaginitis and *Candida* spp for vulvovaginal candidiasis. Moreover, *L. gasseri* has already shown in past the best survival rate to bile salts (Fuochi et al., 2015), one of the most important characteristic to survive in the body when *Lactobacillus* strains are in taken by oral administration.

In this study, strains were tested for their susceptibility to various antibiotics because this kind of profile is necessary to assess the safety of the strains regarding the nowadays problem about antibiotic-resistance: the selection of strains for use as feed additives or probiotic should be oriented towards the least resistant organism (FEEDAP, 2012). The strains isolated were resistant only to ciprofloxacin, perfectly according to microbiological cut-off values defined by major standing committees (FEEDAP, 2012, Testing, 2018). Resistance to fluoroquinolones is important in case of vaginal infections; therefore, this class of antibiotics should be used in therapy to not affect the *Lactobacillus* component of the vaginal microbiota (Tempera et al., 2009).

Moreover, as widely discussed above, it is well known that strains of the genus *Lactobacillus,* and more generally LAB (Lactic Acid Bacteria), are able to produce by-products of lactic acid metabolism with intrinsic antibacterial activity (Tachedjian et al., 2017). These metabolites are not the only substances with antimicrobial activity; in fact, these

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strains are able to produce compounds as bacteriocins, which mostly show an antimicrobial spectrum closely related to the productive strain (Nissen-Meyer and Nes, 1997, Nissen-Meyer et al., 2010, Kristiansen et al., 2016).

Although the production of these substances requires a significant metabolic effort, on the other hand, within polymicrobial community such as the human microbiota, different strains must compete with the same resources, so this capacity represents a benefit at the population level (Dykes, 1995, Riley and Wertz, 2002).

Despite the chemical composition and the mechanism of action of the substances contained in the supernatants tested in this study is still to be defined, the tests carried out in this paper demonstrate both their biological activity and their antimicrobial spectrum against the human pathogens such as *E. coli, S. aureus, E. faecalis, P. aeruginosa, K. pneumoniae*.

Beyond their biological activity, a fascinating feature of these substances is their diversity and complexity. In fact same bacterial species isolated from different habitats (human, animal or plant) are able to produce substances with different chemical structures and biological properties.

No exception are the seven bacterial species tested in this study (*L. fermentum, L. crispatus, L. gasseri, L. jensenii, L. rhamnosus, L. salivarius*), some of which are already known for the production of substances with antimicrobial activity (Zhu et al., 2000, Kaewsrichan et al., 2006, Pascual et al., 2008, Dimitrijevic et al., 2009, Riaz et al., 2010, Nakamura et al., 2013, Jeong and Moon, 2015).

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It is interesting to notice that the same species can produce molecules with different spectrum of action (Gram positive, Gram negative and yeast) and different chemical composition.

In 2004, Gardner *et al*. (Gardner and West, 2004) tried to explain this high heterogeneity in the production of bacteriocins identifying two critical factors such as relatedness and competition scale. The relatedness measures the association between genotypes of cells killed by bacteriocins and cells that produce bacteriocins. Instead, the competition is, for instance, the substrate in limited quantity for which they compete.

In this context, the biofilm production would seem to be consistent with the evolution of bacteriocins production. In fact, although the conditions and the physical processes that favor the production of these molecules *in vivo* are not yet fully known, some authors (Bucci et al., 2011) have tried to correlate the role of microbial biofilm in the evolution of production of bacteriocins through analytical and computational approaches.

Looking our results, we think that these strains tested, in particular BLIS-BI producers, have brilliant probiotic characteristics: in this perspective the identification of BLIS, by H-NMR could help to understand the mechanisms of defence against pathogens, with final goal of being able to take advantage for clinical applications in vaginal diseases.

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Some results of this paper are part of a PhD thesis deposited at http://dspace.unict.it (Archivia): http://hdl.handle.net/10761/3878

SUPPORTING INFORMATION

Supporting information file describes in detail material and methods about the protocols followed for the isolation and identification of *Lactobacillus* strains. These methods have not been described in detail in the paper because they were done in the past, and they were not been the purpose of this work.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Table 1. Molecular identification of 112 strains of vaginal lattobacilli.

Species Identified	Number of strains isolated	Percentage %
L. crispatus	48	42.8
L. delbrueckii	8	7.1
L. fermentum	4	3.6
L. gasseri	32	28.6
L. jensenii	4	3.6
L. rhamnosus	12	10.7
L. salivarius	4	3.6

Table 2. MIC values (µg/mL) for erythromycin (ERY), tetracycline (TET), clindamycin (DA), chloramphenicol (CAF), ciprofloxacin (CIP), gentamicin (GEN), ampicillin (AMP).

Species	MIC values (µg mL^{-1})						
	ERY	TET	DA	CAF	CIP	GEN	AMP
L. crispatus	0.12	0.25	0.12	0.12	>16	2	≤0.03
L. delbrueckii	0.12	0.25	0.12	0.12	>16	2	≤0.03
L. fermentum	0.12	0.25	0.12	0.12	>16	2	≤0.03
L. gasseri	0.12	0.25	0.12	0.12	>16	1	≤0.03
L. jensenii	0.12	0.25	0.12	0.12	>16	0.5	≤0.03
L. rhamnosus	0.25	0.5	0.25	0.12	>16	1	≤0.03
L. salivarius	0.25	0.5	0.25	0.12	>16	0.5	≤0.03

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Table 3. Hydrogen peroxide production of *Lactobacillus* type strain and *Lactobacillus* spp. clinical isolates.

Species Identified	**H_2O_2 production by isolated strains**	**H_2O_2 production by ATCC strains**
L. crispatus	19 strains - 29 strains +	ATCC 33820 ++
L. delbrueckii	8 strains+	ATCC 9649 +
L. fermentum	1 strain - 3 strains +	ATCC 14931 ++
L. gasseri	10 strains *NG* 11 strains - 11 strains +	ATCC 33323 -
L. jensenii	4 strains -	ATCC 25258 -
L. rhamnosus	4 strains NG 8 strains -	ATCC 7469 -
L. salivarius	3 strains - 1 strain +++	ATCC 11741 ++
NG: not grown; -negative; +: slightly positive; ++:moderate; +++:strong		

Table 4. MIC values of CFS produced by vaginal lactobacilli against pathogens.

Strains	**MIC values (% v/v)**				
	Escherichia coli ATCC 25922	*Enterococcus faecalis* ATCC 29212	*Staphylococcus aureus* ATCC 25923	*Pseudomonas aeruginosa* ATCC 27853	*Klebsiella pneumoniae* ATCC 700603
VV1 *L. crispatus*	25	50	50	25	25
RB2 *L. crispatus*	50	NA	50	25	50
ML4 *L. fermentum*	25	50	25	12.5	25
SA2 *L. gasseri*	25	25	25	12.5	25
SL4 *L. gasseri*	50	50	50	25	50
SA4 *L. gasseri*	25	25	25	12.5	25
SA1 *L. jensenii*	25	50	25	25	25
AD4 *L. rhamnosus*	50	NA	NA	50	NA
AD2 *L. rhamnosus*	25	25	25	12.5	25
ML1 *L. salivarius*	12.5	25	12.5	12.5	12.5

NA: not active

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Table 5. MIC values of CFS produced by vaginal lactobacilli against *Candida* strains.

Strains	MIC % v/v	
	24h	48h
Candida albicans ATCC90028	0.048	0.19
Candida albicans ATCC10231	0.048	0.19
Candida albicans (1) clinical isolate	0.048	0.19
Candida albicans (6) clinical isolate	0.048	0.19
Candida albicans (10) clinical isolate	0.048	0.19
Candida albicans (C2) clinical isolate	0.048	0.19
Candida albicans (C4) clinical isolate	0.048	0.19
Candida albicans (C8) clinical isolate	0.048	0.19
Candida glabrata (14) clinical isolate	0.048	0.19
Candida tropicalis (21) clinical isolate	0.048	0.19

number in brackets is the reference number of the clinical isolate

Table 6. Effect of enzymes and heat treatment on CFS against *E. coli* ‡ and *S. aureus* □

Strains	Treatment									
	Enzymes		Temperature (°C)							
	Proteinase K‡,□	Trypsin‡,□	60‡	80‡	100‡	121‡	60□	80□	100□	121□
VV1 *L. crispatus*	NA	NA	+	+	NA	NA	+	+	NA	NA
RB2 *L. crispatus*	NA	NA	+	+	NA	NA	+	+	NA	NA
ML4 *L. fermentum*	NA	NA	+	+	NA	NA	+	+	NA	NA
SA2 *L. gasseri*	NA	NA	+	+	+	NA	+	+	+	NA
SL4 *L. gasseri*	NA	NA	+	+	NA	NA	+	+	NA	NA
SA4 *L. gasseri*	NA	NA	+	+	NA	NA	+	+	+	NA
SA1 *L. jensenii*	NA	NA	+	+	NA	NA	+	+	NA	NA
AD4 *L. rhamnosus*	NA	NA	+	+	NA	NA	+	+	NA	NA
AD2 *L. rhamnosus*	NA	NA	+	+	NA	NA	+	+	NA	NA
ML1 *L. salivarius*	NA	NA	+	+	+	NA	+	+	+	NA

NA: not active; +: activity

‡:Treatment against *E. coli*

□: Treatment against *S. aureus*

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Legend:

Fig 1. Biofilm formation by ten *Lactobacillus* strains recorded after overnight growth in 2 different medium at 37°C.

Legend symbols:

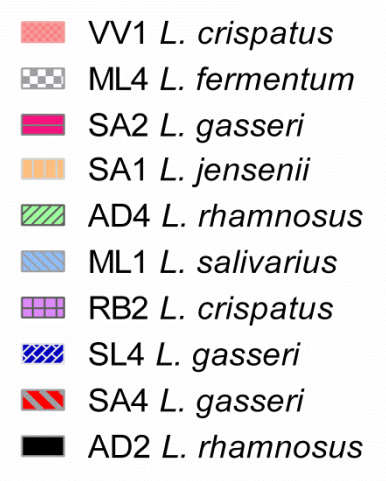

Fig 2. MTT assay on TR146 cell line, *in vitro* model of human buccal epithelium, after treatment with CFS produced by vaginal lactobacilli VV1, ML4, SA2, SA1, AD4 and ML1 at concentrations equal to 50% and 25% v/v versus untreated cells. Bars represent the mean with SD of six independent experiments. p value <0.05

SUPPLEMENTARY MATERIALS

Fig S1. Vaginal smears examined under the microscope after Gram staining (Fuochi, 2016).

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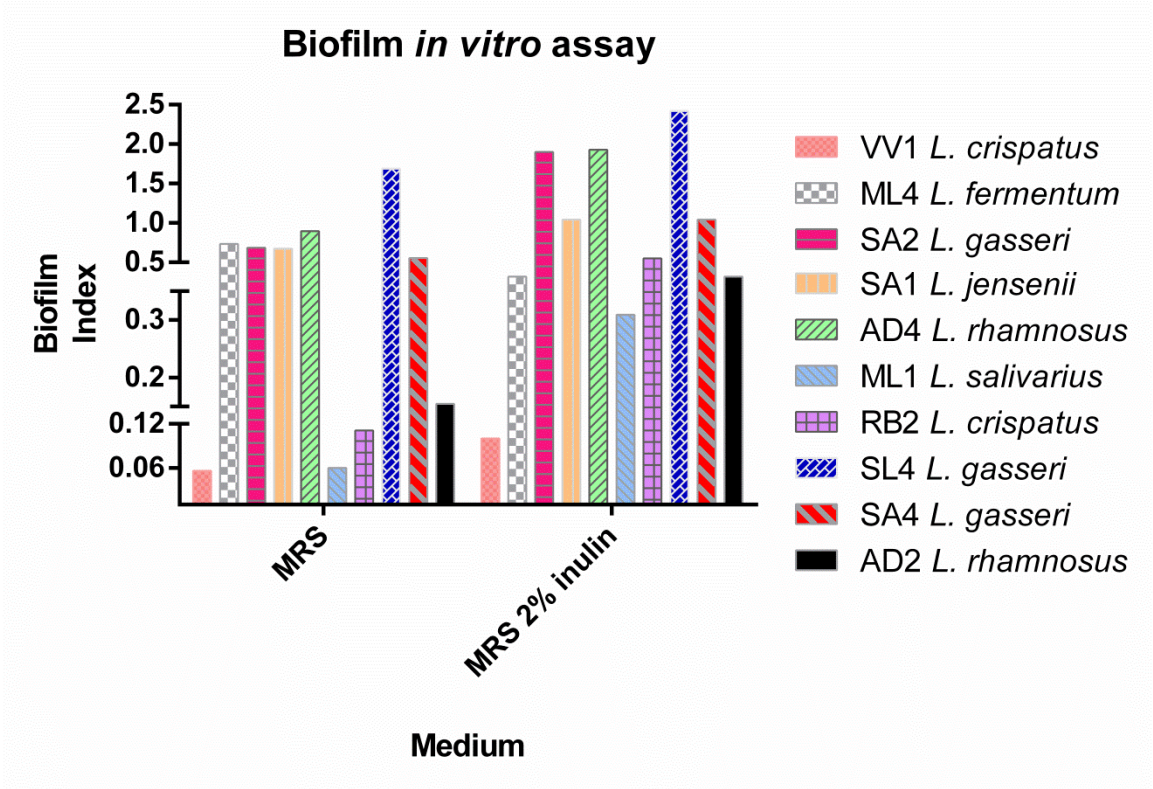

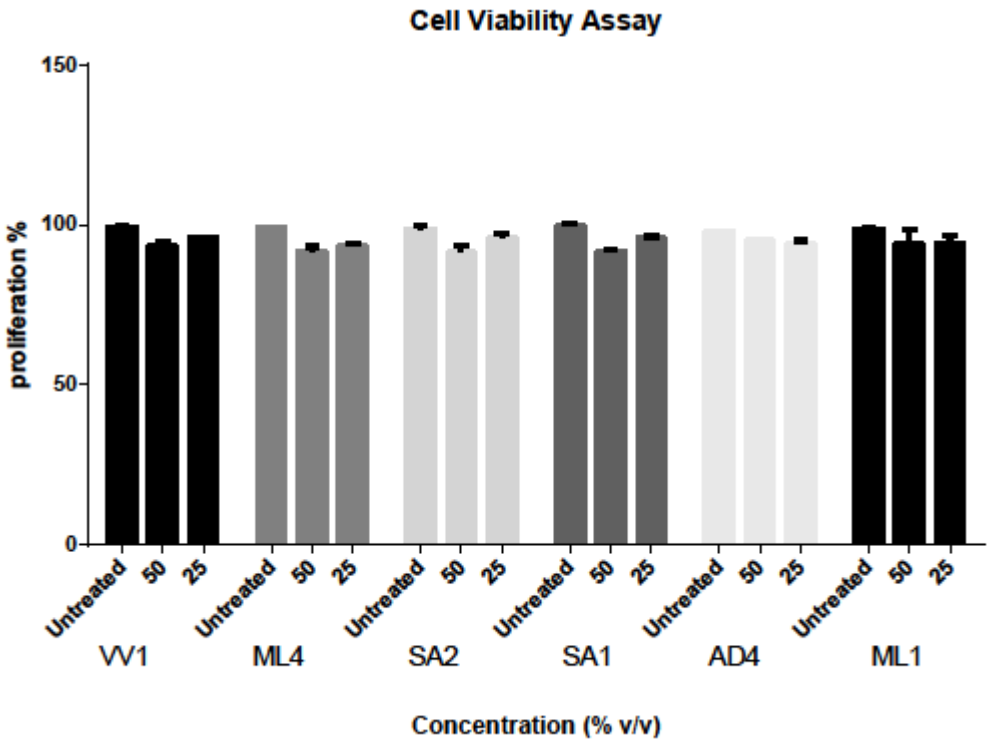

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