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L. Plantarum Supernatant Affects *P. Mirabilis* And *C. Albicans* Biofilm Formation.

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ABSTRACT: **Biofilm-forming bacterium represents a major medical problem because of the decreased susceptibility of bacteria, within the biofilm, to host defenses and antibiotic treatments. In particular, *Proteus mirabilis* and *Candida albicans* biofilm play an important role in various wound and urinary tract infections. The aim of this study was to evaluate the ability of different culture supernatants derived from *Lactobacillus* spp. to contrast *P. mirabilis* and *C. albicans* biofilm formation. We demonstrated that supernatants derived from *L. plantarum* markedly contrast *P. mirabilis* and *C. albicans* biofilm formation. The supernatants did not lost their activity if collected at 24, 48 or 72 hours. In addition, *L. plantarum* supernatant reduced the vitality of *P. mirabilis* and *C. albicans* sessile cells. The results here presented might suggest the ability of molecules released by *L. plantarum* to act both on preformed biofilm and on microrganisms released by dispersed biofilm, avoiding distant colonization.**

***Keywords**: Antimicrobial agents, biofilm, Proteus mirabilis, Candida albicans, Lactobacillus plantarum, urinary tract infections*

I. INTRODUCTION

The microbial flora of the vagina in healthy women is a natural ecosystem in which pathogenic and non-pathogenic microorganisms are balanced [1]. Lactobacilli are dominant in this habitat, at 107 to 108 CFU/g of vaginal fluid in healthy premenopausal women. Among them, those belonging to the *Lactobacillus acidophilus* group and *L. fermentum* are most frequently isolated, although others, such as *L. plantarum*, *L. brevis*, *L. jensenii*, *L. casei*, *L. delbrueckii*, and *L. salivarius*, are isolated as well [2,3]. Lactobacilli play a major role in maintaining the urogenital health by preventing the overgrowth and invasion of pathogenic bacteria by a combination of competitive exclusion, competition for nutrients and antimicrobial substances production such as, hydrogen peroxide, organic acids, bacteriocins and biosurfactants [4]. In particular, the production of lactic acid maintains a low pH in the vagina, thus preventing the overgrowth of pathogens [5].

Bacteria of the genus Proteus are widely distributed in the environment and the intestine of many mammals, including humans [6]. Their particular resistance to antimicrobial compounds enables them to survive in the hospital environment. In addition, they have the ability to form biofilm, an attached structure with microbial cells and populations embedded in a polysaccharide layer. The biofilm facilitates survival, enables better adaptation to the conditions of the external environment and more effective use of nutrition [7]. *Proteus mirabilis* is the causative agent of a variety of opportunistic nosocomial infections, in particular it is associated with urinary tract infections (UTI) in patients undergoing urinary catheterization. The artificial surface of the implants facilitates adhesion of bacteria, which can adhere to the catheter forming biofilm. This can justify the large number of the catheter-associated urinary tract infections.

Candida spp. are unusual causes of UTI in healthy individuals, but common in the hospital setting or among patients with predisposing diseases. In addition, urinary catheterization and bacterial UTIs caused by Enterobacteriaceae may facilitate the initial step in the process [8]. *Candida spp*. employ a repertoire of virulence factors, including phenotypic switching, dimorphism, biofilm, and hydrolytic enzymes, to colonize and then invade the urinary tract [8]. Biofilm is critical to candidal growth on biomedical devices, such as urinary catheters that are composed of latex coated with silicone elastomer. Both of these compounds have been shown to support more biofilm production than either polyvinylchloride or polyurethane [9]. Biofilms of *Candida albicans* formed in vitro on catheter material consist of matrix-enclosed microcolonies of yeasts and hyphae, arranged in a bilayer structure. The biofilms are resistant to a range of antifungal agents currently in clinical use, including amphotericin B and fluconazole, and there appear to be multiple resistance mechanisms. However, mixed biofilms containing *Candida* and bacterial species suggest that extensive and striking interactions occur between the prokaryotic and eukaryotic cells in these adherent populations.

Since urinary tract infections are frequently associated with a decrease in the vaginal lactobacilli population, a high vaginal pH, and an increase in Enterobacteriaceae colonization, it has been suggested that lactobacilli play a protective role against both urinary tract infections and bacterial vaginosis [1].

The aim of this study was to evaluate the effect of different culture supernatants derived from Lactobacillus

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spp. on *Proteus mirabilis* and *Candida albicans* biofilm formation.

II. MATERIAL AND METHODS

A. Bacterial isolation and culture conditions

The six *Lactobacillus* strains used in this study were originally isolated from a vaginal swab of healthy post-pubertal and pre-menopausal woman, after informed consent. The sample was pre-cultured anaerobically in MRS broth for 24h at 37°C. From these cultures a streak was made, using a sterile cotton swab, on LAMVAB to allow the selective growth of *Lactobacillus* spp [10]. The plates were incubated under microaerophilic conditions at 37°C for 48 hours and then sub-cultured for an additional 24 hours in MRS broth (OXOID). Purified cultures were maintained at -80 °C in the same medium containing 15% (w/v) glycerol (Sigma Chemie GmbH, Deisenhofen, Germany).

P. mirabilis was isolated from the urine of an adult woman with urinary-catheter-associated bacteriuria, after informed consent [11]. Identification of pure colonies were done according to microscopic examination and API 20E test kit (bio-Merieux, Marcy-l'Etoile, France). This strain was stored at -80°C in Luria-Bertani (LB) broth supplemented with 15% (w/v) glycerol, grown aerobically at 37 ºC, with shaking.

C. albicans was obtained from clinical sample of an healthy patient, after informed consent. Two methods were used to identify the strain: API ID 32 C™ (bio-Merieux, Marcy-l'Etoile, France) and Vitek Yeast Biochemical Card™ (bio-Merieux, Marcy-l'Etoile, France). *C. albicans* was routinely grown in Sabouraud dextrose agar (DifcoTM; Becton-Dickinson, Sparks, MD), enriched with yeast extract, at 35°C for 48 h. The strain was stored at -80°C in broth supplemented with 15% (w/v) glycerol.

B. Supernatant Collection

Lactobacillus spp cultures were performed and supernatants were collected at 24, 48 and 72 hours. In these samples, OD600nm was determined. Cells were separated from the medium by centrifugation at 4000 x g, 4°C for 15 min; the cell pellet was discarded and the supernatant was stored at −20°C until further processing. The supernatants were filter-sterilized using 0.22 µm microfilters (Millipore Syringe Filter, Merck, Darmstadt, Germany). The cell-free supernatant was adjusted to pH 6.5-7.0 using 10 N NaOH.

C. Identification of Lactobacilli by Two-Step Multiplex PCR Assays

Lactobacilli analyzed in this study were first grouped by a multiplex PCR (designated multiplex PCR-G) and then identified to the species level by four multiplex PCR assays (named multiplex PCR II-1, multiplex PCR II-2, multiplex PCR III and multiplex PCR IV), as reported by Song et al. [12]. Primers used for the grouping and species identification are given in Table 1. Total DNA samples were extracted from pure cultures of *Lactobacillus spp*. by using QIAamp DNA Mini Kit according to manufacturer's protocol (QIAGEN, Milan Italy). One □l of DNA was amplified in a reaction mixture containing 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 10 µM dNTP, 10 µM primer mix comprising one portion of each primer (Ldel-7, LU-1, LU-3, LU-5 and Lac-2 for multiplex PCR-G; LU-5, L par-4 and RhaII for multiplex PCR-III; Lfer-4, Lpla-3, Lpla-2, L reu-1, Lreu-4, Lsal-1, Lsal-2 and Lfer-3 for multiplex PCR- IV) and 2.5 U of Taq DNA polymerase (Roche Diagnostics, Milan, Italy), in a final volume of 25 µl. PCR was carried out for 35 cycles. Each cycle consisted of 95°C for 20 s for denaturation; annealing and extension was performed for 2 min at 55°C for multiplex PCR-G, 62°C for multiplex PCR III and 60°C for multiplex PCR IV. A cycle of 72°C for 5 min was added to the final extension. Amplicons were analyzed by electrophoresis on 2% agarose gel followed by ethidium bromide staining.

D. Biofilm Formation and Effect of Lactobacilli Culture Supernatant on P.mirabilis Biofilm

Biofilm formation was assayed by measuring the ability of cells to adhere to sterile 96-well polystyrene flat-bottom microtiter plate (BD Falcon, Mississauga, Ontario Canada) [13]. Twenty □l of *P. mirabilis* growth overnight in LB broth and 80 □l of MRS broth were added in FBS coated microtiter well, to allow biofilm formation, and incubated at 37°C for 16 h. After incubation, the wells were rinsed with phosphate-buffered saline (PBS) and air dried at room temperature for 45 min. Two hundred microliters of crystal violet (1%) solution was added to each well, and the dishes were incubated for 30 min. Then, the wells were washed four times with distilled water and immediately discolored with 200 µl of 95% ethanol. Elapsed 45 minutes after the last procedure, 100 µl of discolored solution was transferred to a well of a new plate and the crystal violet measured at 570 nm in an ELISA reader (MICROPLATES Reader, Biorad. Milan, Italy).

To assess the activity of *Lactobacillus spp*. supernatants against preformed *P. mirabilis* biofilm, following 16 h of adhesion and biofilm formation, planktonic cells were removed from the wells and the plate was rinsed with 100 µl of phosphate buffered saline (PBS). Cells adhered to the polystyrene microtiter plate were treated with 100 µl of *L. paracasei*, *L. rhamnosus*, *L. reuteri*, *L. salivarius*, *L. plantarum* or *L. fermentum* supernatant, respectively, and the plate was further incubated for 16 h at 37°C. Non-treated cells were incubated with 20 µl of LB and 80 □l of MRS broth, which served as a negative control.

To assess the ability of *L. plantarum* supernatants to prevent *Proteus* biofilm formation, 100 µl of supernatant was added to polystyrene microtiter plate and incubated overnight at 4°C. The supernatant was aspirated and the plates were washed once in sterile PBS. The 96-well microtiter plates were then seeded with *P. mirabilis* for biofilm formation, as above described, and the plate was further incubated for 16 h at 37°C. Non-treated cells were pre-incubated with 20 µl of LB and 80 □l of MRS broth, which served as a negative control.

To assess the ability of *Lactobacillus plantarum* supernatants to inhibit *Proteus* biofilm formation, 20 □l of *P. mirabilis* growth overnight in LB broth was added to 80 µl of supernatant in polystyrene microtiter plate and incubated 16 h at 37°C. Twenty µl of *P. mirabilis* in LB and

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80 µl of MRS broth served as a negative control. At the end of each experiments crystal violet-staining was performed to assess biofilm formation. The amount of biofilm formed was measured subtracting the absorbance from the absorbance values of the control wells. The percentage of biofilm reduction was calculated using the following formula: [(Ac-At)/Ac] x 100, were Ac is the OD_{570} for control well and At is OD_{570} for biofilm in presence of supernatants.

E. Biofilm Formation and Effect of Lactobacilli Culture Supernatant on C.albicans Biofilm

One hundred µl of *C. albicans* cells (1.0 × 106 cells/ml in BHI broth) were introduced into 96 wells. The plates were incubated for 24 h at 37°C [14]. Biofilm developed in each well was washed twice with 200 µl of distilled water and then dried for 45 min. In each well 200 µl of 0.4% crystal violet were added for 45 min. After this procedure the wells were washed four times with distilled water, discolored with 200 µl of 95% ethanol, and the crystal violet measured as above reported.

To assess the activity of *Lactobacillus spp*. supernatants against preformed *C. albicans* biofilm, following 24 h of adhesion and biofilm formation, planktonic cells were removed from the wells and the plate was rinsed with 100 µl of phosphate buffered saline (PBS). Cells adhered to the polystyrene microtiter plate were treated with 100 µl of *L. plantarum* supernatant, and the plate was further incubated for 24 h at 35°C. Non-treated cells were incubated with 100 µl of BHI broth, which served as a negative control.

To assess the ability of *L. plantarum* supernatants to prevent *C. albicans* biofilm formation, 100 µl of supernatant was added to polystyrene microtiter plate and incubated overnight at 4°C. The supernatant was aspirated and the plates were washed once in sterile PBS. The 96-well microtiter plates were then seeded with *C. albicans* for biofilm formation, as above described, and the plate was further incubated for 24 h at 35°C. Non-treated cells were pre-incubated with 100 µl of BHI broth, which served as a negative control.

To assess the ability of *L. plantarum* supernatants to inhibit *C. albicans* biofilm formation, 100 µl of *C. albicans* (1.0 × 106 cells/mL in BHI broth) was added to 100 µl of L. plantarum supernatant in polystyrene microtiter plate and incubated 24 h at 35°C. One hundred µl of *C. albicans* in BHI broth and 100 µl of MRS served as a negative control. At the end of each experiments crystal violet-staining was performed to assess biofilm formation. The amount of biofilm formed was measured as above reported.

F. Assay for the Evaluation of Biofilm Vitality

For the evaluation of biofilm vitality after exposure to *L. plantarum* supernatant, 100 µl of planktonic cells were diluted (1:10) in water and plated on agar plates. After overnight incubation the colonies were counted to calculate the CFU/well. Well surfaces were vigorously scraped with a sterile tip, and re-suspended in a 100 µl of sterile water, as previously reported [15]. As performed for planktonic cells, serial dilutions were plated and incubated 24 or 48 h to calculate the CFU/well for *P. mirabilis* and *C. albicans*, respectively. The tests were performed in three independent experiments, each in triplicate.

G. Statistical Analysis

Each experiment was performed at least three times. The results are expressed as mean ± standard deviation (SD). Student's t test was used to determine statistical differences between the means, and $p<0.05$ was considered a significant difference.

III. RESULTS

A. Isolation of Vaginal Lactobacillus

Lactobacilli isolated from vaginal swabs were able to grow on LAMVAB. Growth was determined by the appearance of colonies over 1 mm in diameter and a yellow discoloration of the medium, due to acid production. Most colonies were green or blue, due to the uptake of bromocresol green into the cells (Fig.1) .

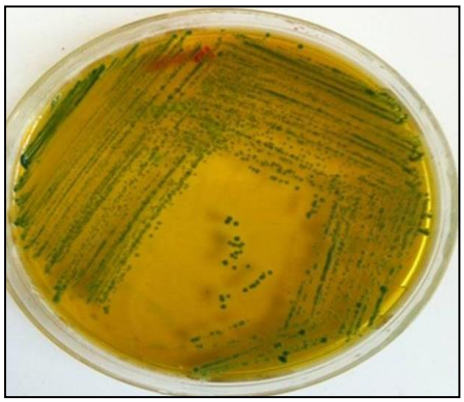

Figure 1: Lactobacilli isolated from vaginal swabs grown on LAMVAB plates.

B. Multiplex PCR for Grouping of Lactobacilli

For a correct identification of the *Lactobacillus spp*. we performed a two-step multiplex PCR assays, as previously reported [12], comprising the first multiplex PCR for grouping of *lactobacilli* followed by a multiplex PCR assay(s) for each group to identify at the species level. As expected, multiplex PCR-G generated two major amplicons of 400 and 350 bp (Fig.2). Based on the amplicon size reported for multiplex PCR-G we divided our strains in group III (amplicon of 400 bp) and IV (amplicon of 350 bp), respectively. Successively, the second multiplex PCR assays for identification of *lactobacilli* at the species level was performed. As reported in figure 2 *L. paracasei* (312 bp) and *L. rhamnosus* (132 bp) were identified at the species level by using the primers specific for the group III, whereas, *L. reuteri* (303 bp), *L. salivarius* (411 bp), *L. plantarum* (248 bp) and *L. fermentum* (192 bp) were

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identified at the species level by using the primers specific for the group IV.

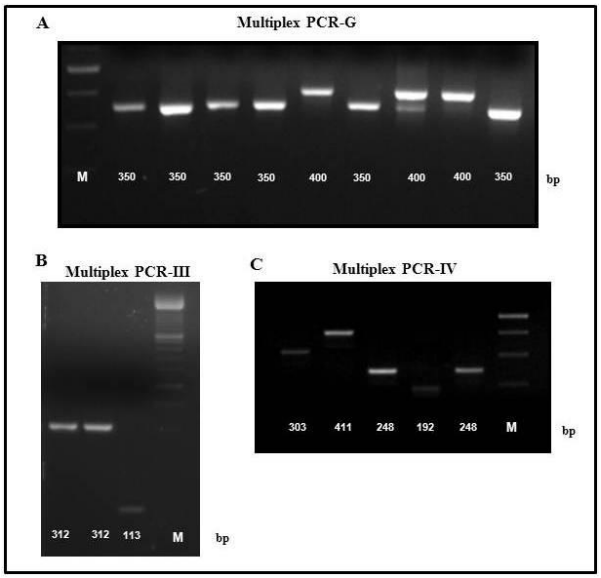

Figure 2: Agarose gel electrophoresis of PCR products from multiplex PCR-G (A) and multiplex PCR assays at the species level (B and C). M, 100 bp ladder (Roche diagnostics). Bp: base pairs (PCR products for each group of lactobacilli).

C. *Antibiofilm activities on P. mirabilis*

In order to test the efficacy of *Lactobacillus spp* supernatants on preformed biofilm, *P. mirabilis* was allowed to grown on 96-well microtiter plates for 24 h and, successively, incubated with 100 µl of supernatants derived from different species of *Lactobacillus*, collected at 24 h of culture. As shown in figure 3 the supernatants derived from *L. plantarum* and *L. rhamnosus* significantly reduced the adhesion of *P. mirabilis* to polystyrene well (percent of reduction was about 61% and 32%, respectively).

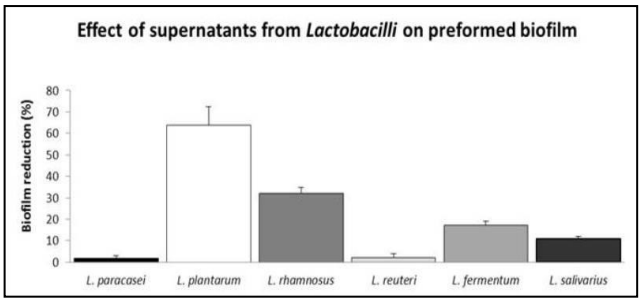

Figure 3: Effects of *Lactobacilli* supernatants collected at 24 h on *P. mirabilis* preformed biofilm. The results are expressed as percentage of biofilm reduction compared to respective control wells. The data shown are representative of three different experiments ± SD

The same effect was not obtained with the supernatants derived from *L. paracasei, L. salivarius, L. fermentum* and *L. reuteri.* To test if a longer culture time might increase the release of molecules by *Lactobacilli spp*. possessing the anti-biofilm properties, *Lactobacilli* supernatants were also collected at 48 and 72 hours. As shown in figure 4, *L. plantarum* supernatant, collected at 48 and 72 h, was still able to strongly reduce the adhesion of *P. mirabilis* whereas the supernatant derived from *L. paracasei, L. salivarius, L. fermentum* and *L. reuteri*, collected at the same time of culture, did not display anti-biofilm properties (data not shown).

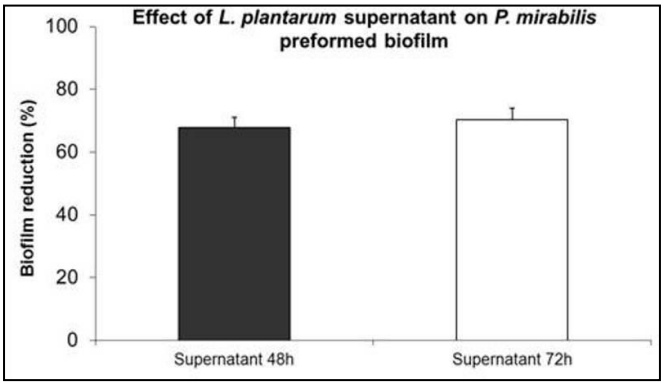

Figure 4: Effects of *L. plantarum* supernatants collected at 48 and 72 h on *P. mirabilis* preformed biofilm. The results are expressed as percentage of biofilm reduction compared to respective control wells. The data shown are representative of three different experiments ± SD.

Since the best effect was observed when *L. plantarum* culture supernatant was used, we were prompted to further investigate if this supernatant was able to prevent or inhibit the biofilm formation, too. Furthermore, as it is known that UTIs caused by *Enterobacteriaceae* may favor *Candida spp*. infections [8] we investigated the effect of the supernatants on *C. albicans* biofilm, too. First of all we analyzed the effect of *L. plantarum* supernatant, collected at 24, 48 or 72 hours, on preformed *C. albicans* biofilm. As shown in figure 5 *L. plantarum* supernatant was able to strongly reduce the adhesion of *C. albicans* to polystyrene well.

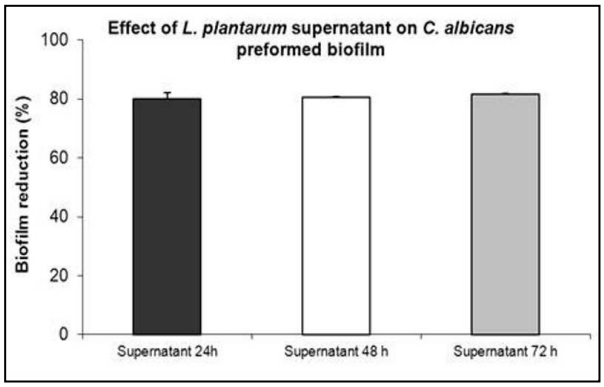

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Figure 5: Effects of *L. plantarum* supernatants collected at 24, 48 and 72 h on *C. albicans* preformed biofilm. The results are expressed as percentage of biofilm reduction compared to respective control wells. The data shown are representative of three different experiments ± SD.

Successively, we investigated the effect of *L. plantarum* supernatants on the prevention or inhibition of biofilm formation. As shown in figure 6 and 7, respectively, *L. plantarum* supernatants, collected at 24, 48 and 72 h, were both able to prevent and inhibit *P. mirabilis* and *C. albicans* biofilm formation, after 24 h of incubation at 37°C. However, the supernatants displayed their best effect on *P. mirabilis* biofilm growth; in fact, a strong biofilm inhibition (65, 75 and 78 %, respectively) was observed when the supernatants collected at all the time (24, 48 and 72 h) were used (Fig. 7A). On *C. albicans* the effects were slighter with a percentage of biofilm inhibition of about 14, 28 and 38 %, respectively at 24, 48 and 72 h (Fig. 7B).

D. Evaluation of Biofilm Vitality

Biofilm-related infections are problematic since cells embedded in biofilms are resistant to host immune response and antimicrobial chemotherapy. By considering the interesting results until now obtained we were encouraged to analyze if *L. plantarum* supernatant had the ability to kill the cells embedded in *P. mirabilis* and *C. albicans* biofilm. Biofilm vitality was assessed by counting the CFU/well. As shown in figure 8, *L. plantarum* supernatants collected at 24, 48 and 72 hours were able to reduce the vitality of planktonic and sessile cells of both microrganisms, but the effect was visible in a stronger extent on *P. mirabilis* cells.

Figure 6: Effects of *L. plantarum* supernatants collected at 24, 48 and 72 h on *P. mirabilis* (A) and *C. albicans* (B) biofilm formation. The results are expressed as percentage of biofilm reduction compared to respective control wells. The data shown are representative of three different experiments ± SD

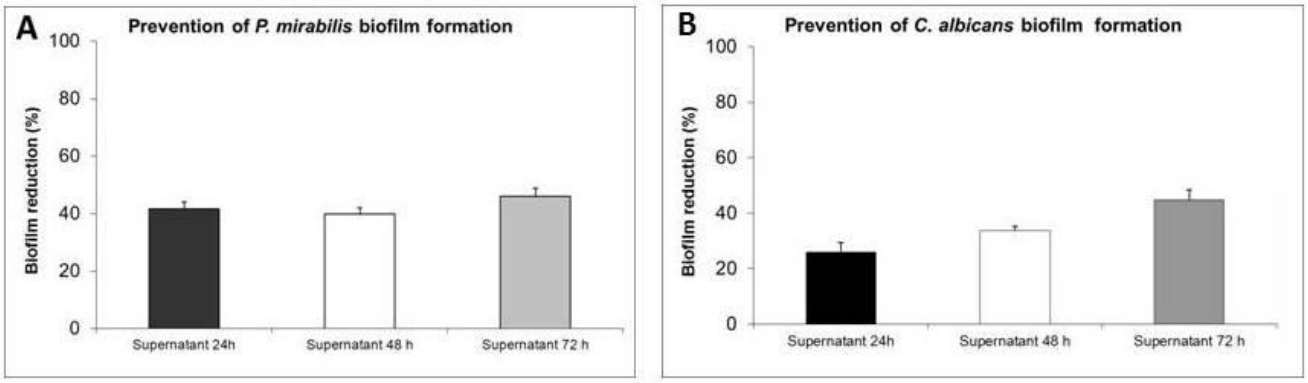

Figure 7: Effects of *L. plantarum* supernatants collected at 24, 48 and 72 h on *P. mirabilis* (A) and *C. albicans* (B) biofilm inhibition. The results are expressed as percentage of biofilm reduction compared to respective control wells. The data shown are representative of three different experiments ± SD.

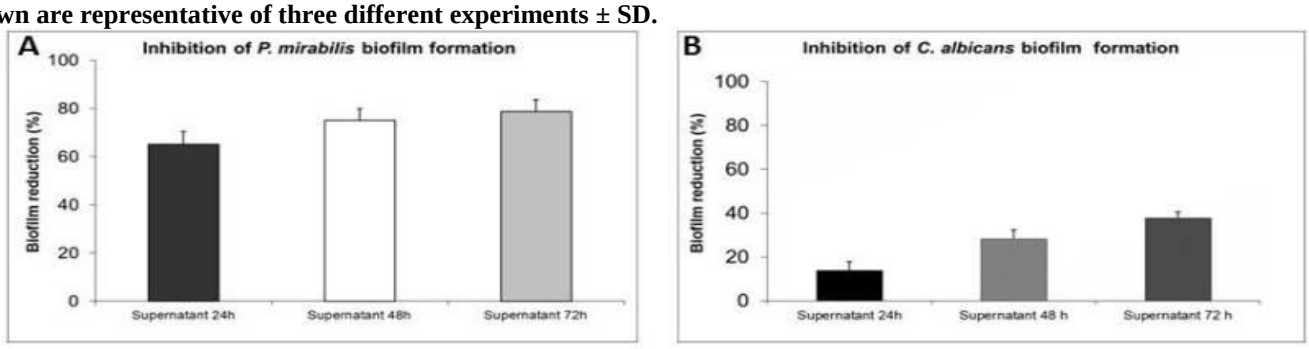

Figure 8: Effects of *L. plantarum* supernatants collected at 24, 48 and 72 h on viability of planctonic (A) and sessile (B) *P. mirabilis* cells, and on planctonic (C) and sessile (D) *C. albicans* cells. The results are expressed as percentage of biofilm reduction compared to respective control wells. The data shown are representative of three different experiments ± SD.

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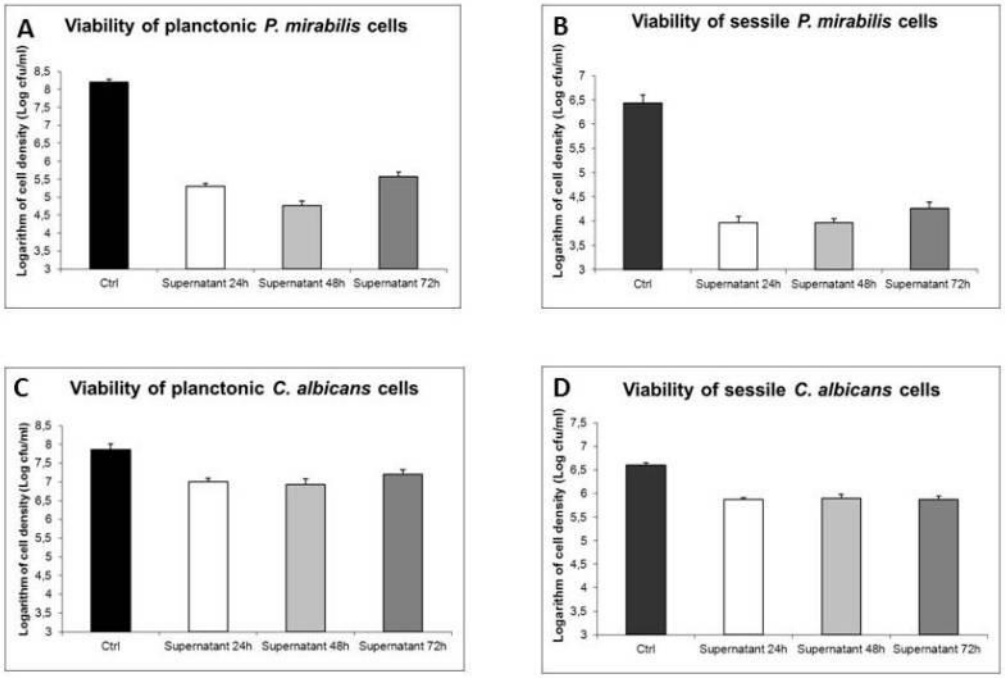

IV. DISCUSSION

There exists a global threat for the existing antimicrobial agents due to the widespread emergence of microbial drug resistance. Thus the discovery of a novel antimicrobial agents to contrast the growth of antibiotic-resistant organism represent a great challenge for the researchers.

UTI causative bacteria have changed their susceptibility to antimicrobial agents after decades of exclusive use. This has favored resistance emergence among the most common uro-pathogens like *E. coli*, *P. mirabilis*, *P. aeruginosa* or *P. stuartii* [16]. *P. mirabilis*, a common cause of complicated UTI that are frequently difficult to treat, has emerged as a serious public health problem and some strains have developed mutations involving resistance to different drugs [17]. Concomitantly, *Candida spp*. complicate the treatment of UTI among patients with predisposing diseases. Although antibiotic therapy generally reverses the symptoms caused by planktonic cells, microrganisms within a biofilm can display up to 1000-fold increased resistance to antibiotic or biocide treatment [7]. For this reason biofilm infections typically show recurring symptoms, even after cycles of antibiotic therapy. In this context, identification of natural compounds which can limit formation of bacterial biofilms represent an important task for the researchers working in this field. Here we demonstrated that supernatants derived from *L. plantarum* markedly reduce the formation of *P. mirabilis* and *C. albicans* biofilm. In addition, the supernatants derived from *L. rhamnosus*, *L. paracasei*, *L. salivarius*, *L. fermentum* and *L. reuteri* were also tested, showing a reduced or scarce effect on *P. mirabilis* pre-formed biofilm. The supernatants did not lost their activity if collected at 24, 48 or 72 hours, indicating that the molecule/s active were accumulated in the culture medium and not degraded in the time. From a medical standpoint, molecules that inhibit the formation of a bacterial biofilm are of great interest and could be used as a prophylactic measure; however, molecules that disperse pre-formed biofilms could be as well of importance to treat established biofilm infections in combination to traditional antibiotic therapy. One of the mechanism of biofilm resistance to antibiotics is the inability of the antibiotic to penetrate the full depth of the biofilm, as the polymeric matrix of a biofilm is known to retard the diffusion of antibiotics [18]. The slow growth rates of biofilm communities have also been posited as a factor in increased resistance to eradication [18]. Microrganisms embedded deep within the biofilm matrix grow more slowly due to lack of nutrients and oxygen. Cells with reduced metabolic activity are thus more recalcitrant to antimicrobial therapies as almost all antibiotics mainly target biosynthetic processes, occurring in actively growing bacteria [19]. In addition, the main problem associated to biofilm infections is the dissemination of biofilm cells into the systemic circulation. In order to colonize new surfaces and to prevent density-mediated starvation within the mature biofilm, the cells detach and disseminate [20]. This may result in bloodstream infection, depending on the host immune system and bioburden of cells released. Single cells released by shedding are susceptible to antibiotics and can be controlled by antimicrobial therapy and/or the host's immune system. However, those released in clumps retain antibiotic resistance and may embolize at a distant anatomic site to develop metastatic infections such as endocarditis or osteomyelitis [21]. Worthy of note are our results showing that *L. plantarum* supernatants displayed their effect also on pre-formed biofilm. The effect is not only on planktonic cells but also on sessile one, as showed by biofilm vitality assay, highlighting the ability of the supernatant to reduce the vitality of sessile cells embedded in the biopolymer produced by microorganisms. However, the supernatant

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elicited a stronger effect on *P. mirabilis* biofilm compared with *C. albicans*. This might depend on different cell surface components interacting with the molecules active in the supernatants.

V. CONCLUSIONS

In summary, the results here presented might suggest the ability of the molecules released by *L. plantarum* to act both on preformed biofilm and on microrganisms released by dispersed biofilm, avoiding distant colonization. The strength of this study rely in the ability of *L. plantarum* supernatant to contrast the growth and biofilm formation of both *P. mirabilis* and *C. albicans*, thus resulting useful in the treatment of pathologies due to mixed biofilms. Further studies are, however, necessary to characterize the class of molecules responsible of such effects, paving the way to the development of new pharmaceutical for the treatment of UTIs.

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