Antimicrobial, Antibiofilm and Immunomodulatory Activities of *Lactobacillus rhamnosus and Lactobacillus gasseri* against some Bacterial Pathogens

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Abstract: In this study, two *Lactobacillus* (LAB) strains namely, *Lactobacillus rhamnosus* EMCC 1105 (*L. rhamnosus*) and *Lactobacillus gasseri* EMCC 1930 (*L. gasseri)* were tested for their antagonistic activities against *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) which are known to be frequently implicated in biofilm infections. The acidic cell free culture supernatant (CFS) of 24 h and 48 h cultures of both LAB stains showed antimicrobial effects against the three pathogens in radial diffusion assay. These effects were abolished upon neutralization of CFS indicating that this effect was due to acids only. Both LAB strains could effectively inhibit the biofilm formation of the three test pathogens and largely replaced them on polystyrene surfaces as demonstrated by crystal violet staining, viable count and scanning electron microscopy. Both of the tested LAB strains could inhibit the protease productivity of *S. aureus* in 24 h and 48 h dual species-biofilms. The supernatant of 24 h-dual biofilms of *P. aeruginosa* with *L. gasseri* also showed a significantly lower protease activity compared to that of *P. aeruginosa* individual biofilm. Neither LAB strains affected phospholipase C production by the test pathogens when they co-exist during biofilm formation. The different preparations of LAB strains caused no significant change in the levels of gamma interferon expressed by peripheral blood mononuclear cells in response to stimulation by the test pathogens *in vitro*. In conclusion, *L. gasseri* and *L. rhamnosus* can be considered as promising tools for combating biofilm infections.

Keywords: Probiotics, *Lactobacillus rhamnosus*, *Lactobacillus gasseri*, Biofilm, Antibiofilm, Gamma interferon.

1. INTRODUCTION

The role of probiotics in achieving health benefits is being intensely studied nowadays in different fields, with many species of bacteria and yeasts being investigated for their potential for various applications. One of the most important genera employed in this field is lactic acid bacteria LAB [1].

LAB have being studied for their nutritional effects and their protective effects against cardiovascular diseases, autoimmune diseases and cancer. Yet, their most promising application seems to be due to their anti-infective potential. This is of special importance particularly that the prevalence of antibiotic resistance is rapidly rising worldwide and the development of new antibiotics is not keeping in pace [2]. This brings us to the cusp of a “post-antibiotic era” and constitutes an urge to find suitable alternatives for combating infectious diseases other than the traditionally used antimicrobial agents [3-5].

Lactobacilli (LAB) have shown promising effects in prevention and treatment of antibiotic associated diarrhea, traveler’s diarrhea and other forms of diarrhea [6]. They have also shown a possible effect in treatment and prophylaxis against vaginal, urinary tract, wound and skin infections [1, 7-10].

These effects may be attributed to different factors such as the production of metabolites of direct antimicrobial activity, competition with the pathogenic organisms for nutrients, interfering with them for their adherence sites on epithelial cells, suppressing their production of cytotoxins or they may exert their protective action via an immunomodulatory effect [11,12].

Another important role that is being investigated is their ability to interfere with biofilm formation which is known to be implicated in more than 80% of infections [13,14]. Bacteria in biofilms are characterized by extreme resistance to antimicrobial agents compared to their planktonic counterparts. This develops as a result of several mechanisms that act synergistically providing protection to the bacteria within the biofilm [15]. In addition, bacterial cells in biofilms can effectively evade the host immune system possibly via inhibition of engulfment of biofilm cells by phagocytes. This may be due to biofilm matrix polymers which are of low immunogenicity and that have the ability to mechanically shield the surface-exposed epitopes of

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bacterial cells in biofilms from being recognized by the host immune system [16].

LAB have been studied as a promising tool to prevent or disperse the biofilm formation of growing pathogenic bacteria [17]. However, the conflicting results obtained from various studies suggest that these effects are just strain specific and should not be extrapolated from one strain to another even among the same species [14]. This implies that more studies are still needed to clarify the efficacy and the underlying mechanisms of the different LAB probiotic candidates and to evaluate the reliability of the probiotic products presented to the public.

In this study, two *Lactobacillus* strains, *Lactobacillus rhamnosus* EMCC 1105 and *Lactobacillus gasseri* EMCC 1930 were evaluated for their antimicrobial, antibiofilm activities and immunomodulatory activities. They were tested against *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus,* three bacterial pathogens that are commonly implicated in biofilm related infections [13].

These two strains proved to have antimicrobial, antibiofilm as well as an inhibitory effect against the proteolytic activity of *P. aeruginosa*, *E. coli* and *S. aureus*, three bacterial pathogens commonly implicated in biofilm infections.

2. MATERIALS AND METHODS

2.1. Microorganisms and Culture Conditions

Two probiotics *Lactobacillus* strains were used in this study, *Lactobacillus rhamnosus* EMCC 1105 and *Lactobacillus gasseri* EMCC 1930. Both strains were purchased from Cairo Microbiological Resources Centre (MIRCEN), Ain Shams University, Cairo, Egypt. They were cultivated in deMan, Rogosa and Sharpe (MRS) broth (Difco Laboratories, Detroit, Michigan, USA) at 37°C for the specified time periods.

The test pathogens used were *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). *P. aeruginosa* and *S. aureus* were recovered from clinical specimens and are available at Microbiology and Immunology Department, Faculty of pharmacy, Ain Shams University, Cairo, Egypt. *E. coli* was a reference strain (*E. coli* NCTC 10959) that was kindly provided as a gift by Dr. Omneya Helmy, Department of Microbiology and Immunology, Faculty of pharmacy, Cairo University, Cairo, Egypt. All organisms were cultivated in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Michigan, USA) at 37°C for 18 h unless otherwise is indicated.

2.2. Determination of the Antimicrobial Activity of LAB Strains Against Tested Bacterial Pathogens

The antimicrobial activity of both cell free supernatant (CFS) and neutralized cell free supernatant (NCFS) of the tested LAB strains was determined according to a method described by Coconnier *et al*. using radial diffusion assay [18]. LAB were grown in MRS broth at 37°C. Supernatants of 24 h or 48 h cultures were then collected and filtered through 0.22-µM-pore-size cellulose membrane filters (CHMLAB, Barcelona, spain) either before or after being neutralized using 1N sodium hydroxide (NaOH) (El-Nasr Chemicals Co., Qalyubiah, Egypt) solution to get CFS or NCFS, respectively.

The tested pathogens were grown in BHI broth for 18 hours at 37°C. The culture was then centrifuged at 12000 rpm for 5 min. The pellets were subsequently washed once with then resuspended in phosphate buffered saline (PBS). A volume containing 10^6 colony forming units (CFU) was used to inoculate 10 mL of sterile warm Mueller-Hinton (MH) agar (Difco Laboratories, Detroit, Michigan, USA). The seeded MH agar was then poured over a solidified basal layer of plain MH agar in a 10-cm-diameter Petri dish. Wells were then punched into the agar and each was filled with 150 µL of either CFS or NCFS of the tested LAB strains. Plates were incubated overnight at 37°C. The diameter of the clear zone around each well if any was measured in mm.

2.3. Testing the Effect of LAB as Antibiofilm Agents

Interaction of LAB strains with biofilm formation of the tested pathogens was studied according to Zago *et al*. [19]. The biofilm formation of the test pathogens was assessed in single-species biofilms and in dual-species biofilms with the tested LAB strains.

Biofilms were evaluated by counting colony-forming units (CFU/mL), crystal violet (CV) staining and scanning electron microscopy (SEM). The effect of LAB on certain hydrolytic enzymes produced during biofilm formation of the test pathogens was also evaluated.

2.3.1. Biofilm Formation

Biofilm assays were carried out on 96-well flat bottom microtiter plates (CellStar®, Germany) except

for SEM, 6-well plates were used instead. Briefly, the tested organisms were cultured in BHI broth for pathogenic strains or MRS broth for LAB strains and incubated at 37 °C overnight. After incubation, 1 mL of the culture produced for each test organism was centrifuged at 12,000 rpm for 5 min. The pellets obtained for the different test organisms were washed once with PBS, resuspended in their corresponding fresh culture medium and standardized to contain 2×10^6 CFU/mL.

In the assays in which 96-well plates were used, wells of test pathogens in single-species biofilms contained 100 µL of the standardized suspension of the test organism prepared before and 100 µL of fresh MRS medium. Similarly, single-species biofilms of LAB contained 100 µL of the standardized suspension of the test LAB strain and 100 µL of fresh BHI medium. Those of dual-species biofilms contained 100 µL of the standardized suspension of each of the tested pathogen and the tested LAB strain. For SEM in which 6-well plates were used, wells of single-species biofilms contained 1 mL of the test pathogen suspension and 1 mL of fresh MRS medium. Those of dual-species biofilms contained 1 mL of suspensions of each of the test pathogen and the test LAB strain. Plates were incubated at 37°C for 24 or 48.

2.3.2. Quantification of Total Biofilm Biomass

Total biofilm biomass was measured using a crystal violet (CV) staining method as described by Zago *et al*. and O'Toole *et al*. with minor modifications [19,20]. Briefly, after 24 h and 48 h of biofilm formation, the medium was totally aspirated and the non-adherent cells were removed by washing the wells twice with 150 µL of PBS. The single-species biofilms of the test pathogens and their dual-species biofilms with LAB were then fixed by incubation with methanol (El-Nasr Chemicals Co, Qalyubiah, Egypt) (200 µL/ well) for 15 min at room temperature. After methanol removal, the culture plates were allowed to dry at room temperature followed by the addition of 1% CV solution (200 µL / well) and incubation for 5 min. after decantation of CV solution, the wells were gently washed twice with PBS (150 µL / well) followed by the addition of 33% acetic acid (El-Nasr Chemicals Co, Qalyubiah, Egypt) (200 µL / well) to release and dissolve the stain. The solution is then transferred to a new flat bottom microtiter plate to quantify absorbance in a microtiter plate reader (Chromate 4300, Awareness technology, Palm city, Florida, USA) at 570 nm.

2.3.3. Quantification of Biofilm Cells

The count of cells (colony forming units, CFU) in biofilms was determined by viable count technique. The wells were gently washed twice with PBS to remove loosely attached cells. Next, 100 µL of PBS were added to each well and the adhered biofilm was carefully scraped off the wells with a sterile pipette tip for 1 min. The content of each well was aspirated into an Eppendorf tube and vigorously vortexed to separate any possible aggregation among the cells. Serial dilutions in PBS were made and the count of the pathogenic bacterial strains was determined by plating replicate aliquots of the suspensions on appropriate selective medium (MacConkey agar for *P. aeruginosa* and *E. coli* and mannitol salt agar for *S. aureus.* Both culture media are products of Lab M, Topley house, England). The plates were incubated for 24 h at 37°C and the count of the resultant colonies was expressed as CFU/mL.

2.3.4. Quantification of Extracellular Hydrolytic Enzymes

The culture broth in wells of 24 h and 48 h single-species biofilms of test pathogens and of their dual-species biofilms with LAB was aspirated and centrifuged for 5 min at 5,000 rpm to get the CFS. Both protease and phospholipase C (PLC) activities were determined for the test pathogens in the resultant CFS.

2.3.4.1. Protease Assay

This was carried according to Zhang *et al.* with some modifications [21,22]. A 125 µL aliquot of 2 % azocasein solution (Sigma Aldrich Co., Taufkirchen, Germany) in phosphate buffer was incubated with 75 µL of the tested CFS at 37°C for 45 min. The reaction was stopped by adding 600 µL of 10 % trichloroacetic acid (OXFORD LAB CHEM, Maharashtra, India) for 10 min at room temperature. After that, the mixture was centrifuged for 5 min at 12,500 rpm in a centrifuge, and 600 µL of the supernatant obtained were transferred to a tube containing 500 µL of 1 M NaOH. The absorbance of the resulting solution was measured spectrophotometrically at 440 nm. A control experiment was carried out in a similar manner except that the test CFS was replaced with fresh culture medium. The proteolytic activity was determined using a calibration curve constructed by Hafez 2005[23] and expressed in units/ml.

2.3.4.2. Phospholipase C Assay

This was carried out using a chromogenic assay described by Elleboudy *et al*. in 96-well microtiter

plates [24]. Each well contained 90 µL of p-nitrophenylphosphorylcholine (NPPC) reagent of the following composition: 250 mM Tris (hydroxymethyl)-aminomethane-hydrochloride (Oxoid) buffer (pH 7.2), 60% sorbitol (wt/wt), 1.0 µM $ZnCl_2$, and 10 mM NPPC (Sigma Aldrich) plus 10 µL of the tested CFS [24]. Plates were then incubated at 37 ◦C for 17 h before the absorbance of the solution contained in wells at 405 nm was measured using a microtiter plate reader. Control wells, each containing 10 µL fresh culture medium and 90 µL NPPC reagent were treated similarly. Phospholipase C (PLC) activity was determined using a calibration curve constructed by Elleboudy *et al.* [24] and expressed in units/µL.

2.3.5. Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to examine the changes in biofilm structures caused by interactions between LAB and test pathogens. For this assay, biofilms were allowed to form in 6-well polystyrene microtiter plates. After 48 h of incubation, the medium was aspirated and the non-adherent cells were removed by washing twice with PBS. The biofilms were then fixed according to Fischer *et al.* [25] by adding a solution of glutaraldehyde (El-Nasr Chemicals Co., Qalyubiah, Egypt) in 0.1 M phosphate buffer at a final concentration of 2.5% to the wells and storing the plate overnight at 4° C. The films were then dehydrated using an ethanol series (25, 50, 75 then 100% each for 15 min) and air dried for 20 min. The bottoms of the wells were then cut and kept in a desiccator before analysis. For examination, the discs were mounted onto aluminum stubs, sputter-coated with gold and imaged using a Scanning Electron Microscope (JEOL®, Tokyo, Japan).

2.4. Testing the Effect of LAB as an Immunomodulatory Agent

Production of gamma interferon (IFN-γ) from peripheral blood mononuclear cells (PMBC) was used as a marker for testing the immunomodulatory effect of LAB and was measured using ELISA kit (Biolegend®, San Diego, USA). This was carried out by monitoring IFN-γ level expressed by PBMC in response to exposure to test pathogens in presence and absence of LAB. The experiment was done according to Panagiota Xaplanteri *et al.* [26]. PBMC were isolated from healthy donors on Ficoll Histopaque® (Sigma Aldrich Co., Taufkirchen, Germany) by density gradient centrifugation. The cells were then washed and resuspended in RPMI-1640 medium (Lonza group Ltd., Basil, Switzerland) supplemented with 10% fetal bovine serum (Lonza) and antibiotics (Lonza). PMBC were then seeded in 96-well microtiter plates at a density of 10^5 cells/ well and incubated overnight at 37°C in a 5% CO_2 atmosphere. PBMC were either left untreated (addition of fresh RPMI-1640) or treated with LAB or NCFS for 1 h. This was conducted as follows:

(i) Treatment with viable LAB cells at a multiplicity of infection (MOI) 10:1

(ii) Treatment with killed LAB cells at a density equivalent to MOI of 10:1

(iii) Treatment with NCFS of LAB

After the treatment period, the test pathogens were added at a MOI 10:1 and incubation was continued under the same conditions for 6 h. Control wells were exposed to the same conditions except that they were incubated only with fresh RPMI-1640. Supernatants were assayed for the produced IFN-γ.

2.5. Statistical Analysis

The results of all biofilm experiments were statistically analyzed using two-way ANOVA followed by the Bonferroni post-hoc test at a 95% confidence level.

ELISA results were analyzed using one-way ANOVA followed by the Tukey post-hoc test at a 95% confidence level.

All tests were performed using *GraphPad Prism* Version 5.0.

3. RESULTS

3.1. Antimicrobial Activity Against the Target Pathogenic Strains

The cell free culture supernatants (CFS) of 24 h and 48 h cultures of *Lactobacillus* strains were examined for their antimicrobial activity against *P. aeruginosa, E. coli* and *S. aureus,* by radial agar diffusion method; the antimicrobial activity was recorded as the diameter (measured in mm) of the inhibition zone (I.Z) showing no growth around the wells. The results are shown in Table **1**.

Although the CFS of the tested LAB showed antimicrobial activity, these preparations were devoid of antimicrobial activity when neutralized with sodium hydroxide indicating that this activity was due to acid production only.

Table 1: Antimicrobial Activity of 24 h and 48 h Cell Free Culture Supernatants of Tested Lactobacilli Against some Bacterial Pathogens

LAB strain (24 h CFS)	Antimicrobial activity (I.Z. in mm) against			LAB strain (48 h CFS)	Antimicrobial activity (I.Z. in mm) against		
	P. aeruginosa	*E. coli*	*S. aureus*		*P. aeruginosa*	*E. coli*	*S. aureus*
L. gasseri	13	15	13	*L. gasseri*	22	18	16
L. rhamnosus	16	15	16	*L. rhamnosus*	22	17	16

3.2. Testing the Effect of LAB as Antibiofilm Agents

3.2.1. Quantification of Total Biofilm Biomass as Determined by CV Staining

The results in Figure **1** show that the values of crystal violet (CV) absorbance for 24 h single-species biofilms of test pathogens showed nearly similar values to their corresponding dual-species biofilms with LAB except for *E. coli* where the CV absorbance value of its dual-species biofilm with *L. rhamnosus* was significantly higher than that of its corresponding single-species biofilm ($p < 0.05$).

Also depending on CV absorbance, the total biomasses of 48 h dual-species biofilms of all test pathogens with the tested LAB strains highly increased to an extremely significant level ($p < 0.001$) compared to the corresponding single-species biofilms of the test pathogens.

Overall changing the growth condition from single-species biofilms to dual-species biofilms and the time of incubation had an extremely significant effect on the total biofilm biomass ($p \leq 0.0001$) except for *E. coli* where the time effect had a relatively lower significance ($p = 0.0097$). Also the interaction between the growth condition and time affected the total biomass significantly ($p = 0.0001$-0.0003).

3.2.2. Quantification of Biofilm Cells as Determined by Viable Count Technique

The tested pathogens were allowed to form biofilms either single-species biofilms or dual-species biofilms with the test LAB strains for 24 h or 48 h. As shown in Figure **2**, growing the pathogens in dual-species biofilms with either *L. gasseri* or *L. rhamnosus* caused an extremely significant reduction on the log CFU mL^{-1} values of all the tested pathogens compared to the values obtained with their single-species biofilms at both time intervals ($p < 0.001$ each). According to the statistical analysis, both variables (addition of LAB and increasing contact time) affect the biofilm formation of the tested pathogens to an extremely significant level ($p < 0.0001$).

3.2.3. Effect of LAB on Extracellular Enzymes Production

Both protease and phospholipase C (PLC) of the test pathogens were measured in CFS collected after biofilm formation experiments.

3.2.3.1. Protease Assay

The proteolytic activities of *S. aureus* showed a very significant reduction upon co-culturing with LAB ($p < 0.01$) with post-hoc tests showing an extremely significant reduction of *S. aureus* protease activity with

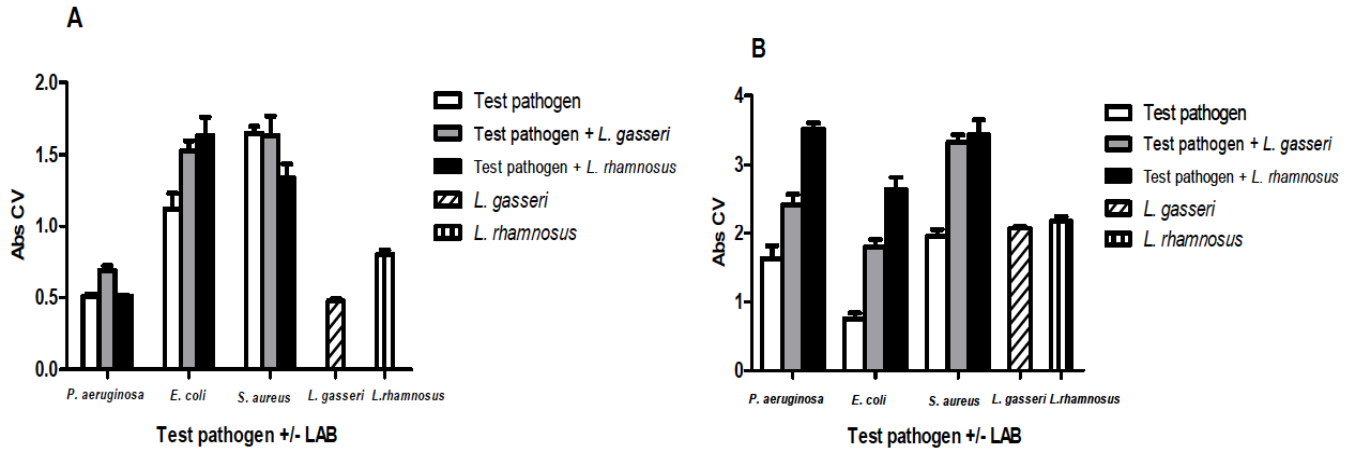

Figure 1: Antibiofilm activities of *L. gasseri* and *L. rhamnosus* against *P. aeruginosa*, *E. coli* and *S. aureus* after 24 h (**A**) and 48 h (**B**) culture periods as determined by crystal violet staining.

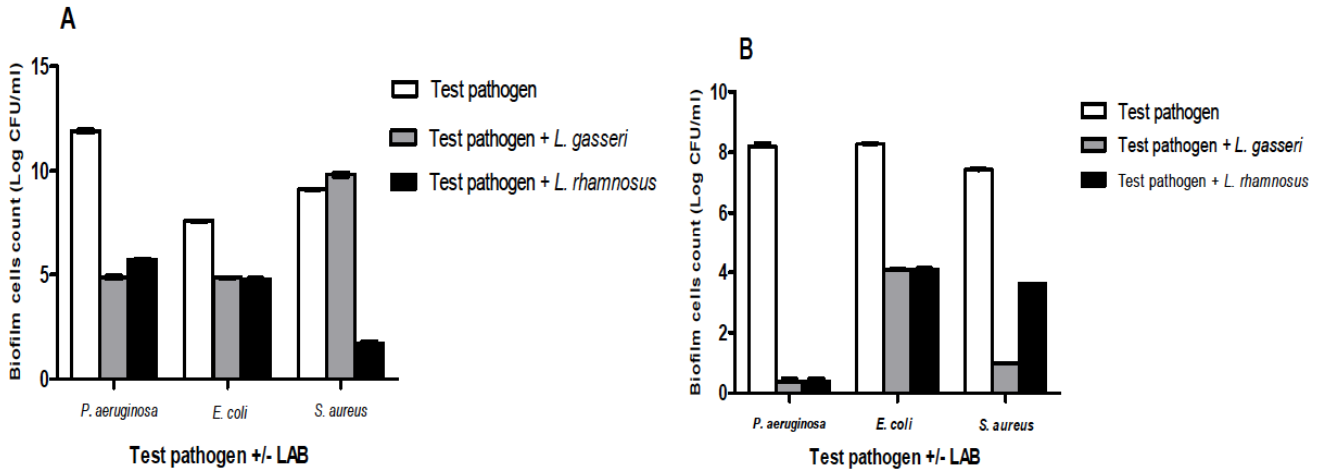

Figure 2: Antibiofilm activities of *L. gasseri* and *L. rhamnosus* against *P. aeruginosa*, *E. coli* and *S. aureus* after 24 h (**A**) and 48 h (**B**) culture periods as determined by viable count technique.

both LAB strains at both points of time ($p < 0.001$). *P. aeruginosa* also showed a significant reduction of its proteolytic activity ($p < 0.05$) however post-hoc tests revealed a significant effect only with *L. gasseri* 24 h dual-species biofilm ($p < 0.001$). Although the interaction p-value was not significant during assessment of LAB and time effects on *E. coli* protease activity, the proteolytic activities of the CFS obtained from 24 h *E. coli- L. rhamnosus* dual-species biofilm and that from 48 h *E. coli- L. gasseri* dual-species biofilm were significantly lower than that of *E. coli* single-species biofilm at the same points of time ($p < 0.05$). The results of protease assay are shown in Figure **3**.

3.2.3.2. Phospholipase C Assay

The PLC activities of the supernatants of both single- and dual-species biofilms were nearly the same and did not change with time ($p > 0.05$).

The PLC activities of CFS prepared from 24 h and 48 h biofilms of either single-species biofilms of test pathogens or their corresponding dual-species biofilms with the tested LAB were nearly the same and did not change with time ($p > 0.05$) as shown in Figure **4**.

3.2.4. Scanning Electron Microscopy

The test pathogens in single-species biofilms and in the corresponding dual-species biofilms with LAB were examined using scanning electron microscopy (SEM) to detect the effect of LAB on the pathogen biofilm formation upon co-culturing. The SEM images showed that LAB could largely replace the pathogens in their biofilms upon co-culturing as shown in Figure **5**.

3.3. Immunomodulatory Effect of LAB when Added to Co-Cultures of Test Pathogens with PBMC

The expression of IFN-γ by PBMC *in vitro* in response to stimulation by pathogens in absence or

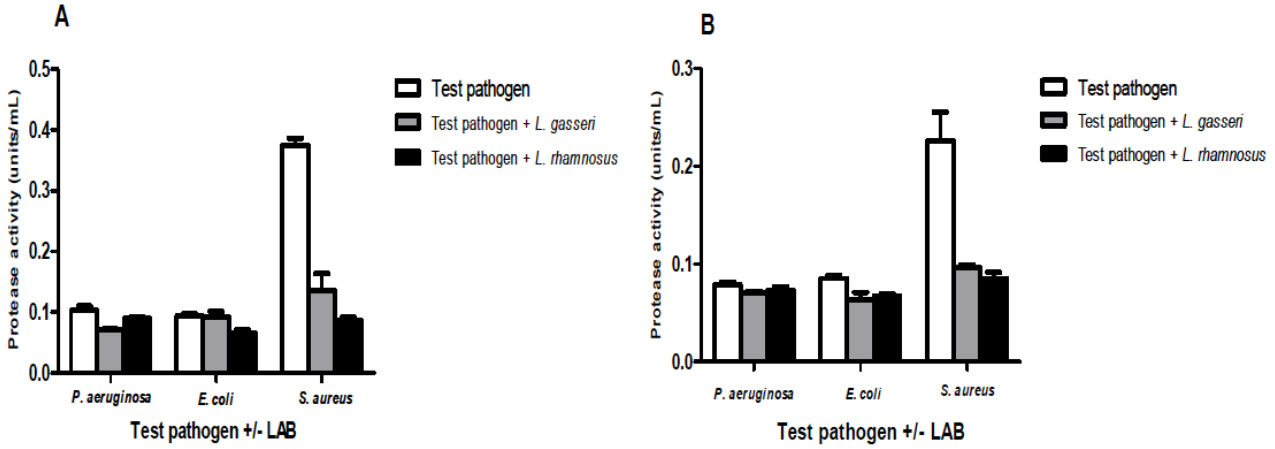

Figure 3: Protease activities of cell free culture supernatants obtained from 24 h (**A**) and 48 h (**B**) single-species biofilms of test pathogens and their corresponding dual-species biofilms with LAB.

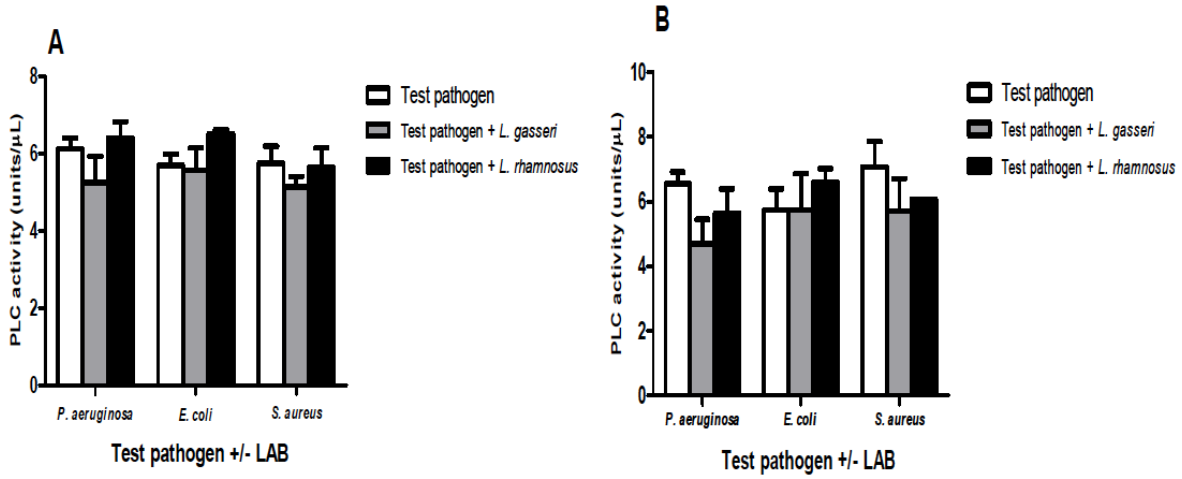

Figure 4: Phospholipase C (PLC) activities of cell free culture supernatants obtained from 24 h (**A**) and 48 h (**B**) single-species biofilms of test pathogens and their corresponding dual-species biofilms with LAB.

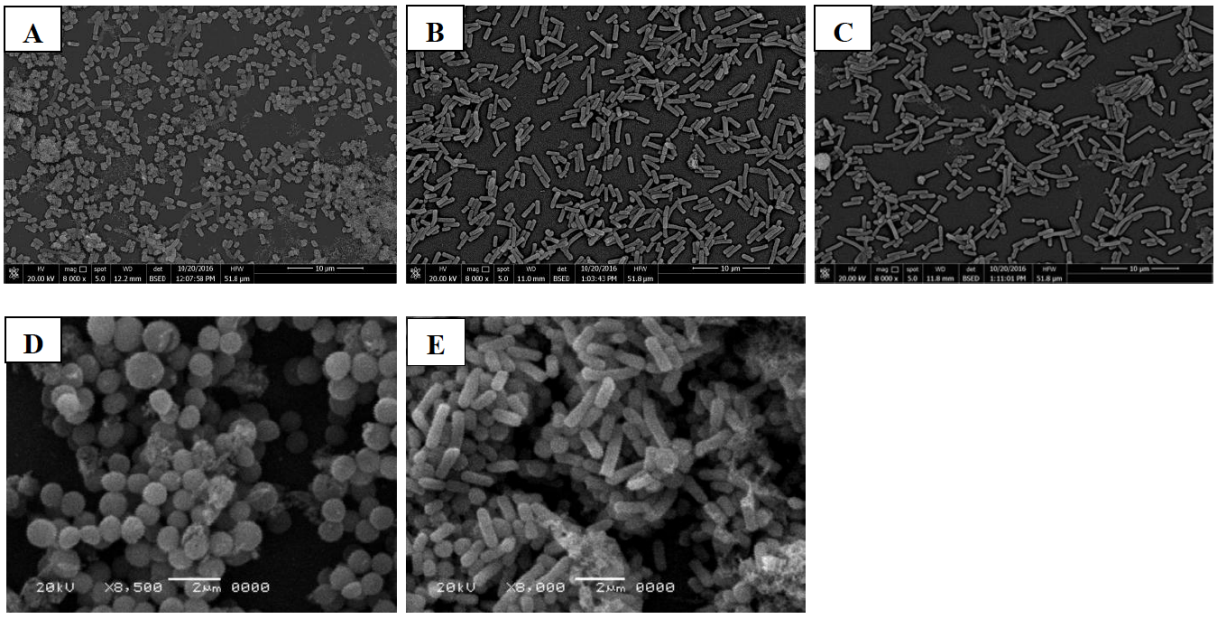

Figure 5: Scanning electron micrographs of 48 h single-species biofilms of test pathogens and their corresponding dual-species biofilms with LAB. (**A**) *E. coli alone,* (**B**) *E. coli* + *L. gasseri,* (**C**) *E. coli* + *L. rhamnosus,* (**D**) *S. aureus alone,* (**E**) *S. aureus* + *L. gasseri.*

presence of LAB was recorded. The effect of LAB on this response was recorded as percentage increase or decrease from the corresponding response to the tested pathogen in the absence of LAB. The results are shown in Table **2**.

Both *P. aeruginosa* and *S. aureus* increased the expression of gamma interferon (IFN-γ) by PBMC by 16.21% and 21.59%, respectively compared to the control experiment. While only 12.42% reduction of IFN-γ expression was observed with *E. coli.*

Although *L. gasseri* preparations increased IFN-γ production by ≥ 20% when added to a culture of PBMC stimulated with *P. aeruginosa* (Table **2**), these differences together with the differences obtained with other pathogens in presence of different LAB preparations were statistically non-significant ($p > 0.05$).

4. DISCUSSION

Bacterial biofilm infections are often very difficult to treat thanks to the biofilm mechanisms that act synergistically to protect the bacteria rendering them more resistant to the antimicrobial agents and to the host immune defense mechanisms compared to their planktonic counterparts [15,16]. Moreover, the

Table 2: Gamma Interferon (IFN-γ) Production in Peripheral Blood Mononuclear Cells (PBMC) Stimulated with Test Pathogens with or without Added Lactobacilli (LAB) Preparations

	% Increase or decrease of IFN-γ production by PBMC in response to test pathogens in presence of different LAB preparations					
	L. gasseri preparations			*L. rhamnosus* preparations		
	Viable cells (MOI 10:1)*	Killed cells**	48 h NCFS***	Viable cells (MOI 10:1)*	Killed cells**	48 h NCFS***
P. aeruginosa	+24.93%	+22.61%	+20.10%	+12.84%	+13.62%	-0.097%
E. coli	-3.49%	-2.807%	+3.57%	-5.047%	-6.143%	-15.06%
S. aureus	+5.86%	+2.93%	-0.46%	+8.07%	-2.7%	+6.267%

*MOI: multiplicity of infection.
**Used at a count corresponding to MOI 10:1.
***NCFS: neutralized cell free culture supernatant. Well diameter=8 mm.

prevalence of antibiotic resistance is rapidly rising accompanied with seriously declining rates of new antibiotics discovery [27]. This strongly boosts the need for finding alternative agents with anti-infective properties.

The current trend of promoting health by natural means led to a special interest in natural anti-biofilm agents including bacterial derived anti-biofilm agents. Recently, probiotics defined as 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' [28] have gained much attention due to their promising antagonistic activities against a wide range of microbial pathogens [17]. They, particularly lactobacilli are also being investigated for their potential to be used as a tool to prevent biofilm formation or even to disperse preformed ones and have shown promising effects in management of oral, wound and vaginal infections *in vitro* and in clinical trials [14]. LAB may exert their antibiofilm activity through a variety of mechanisms. For example, they may inhibit the bacterial growth via secretion of antimicrobial peptides or lactic acid [29]. Some LAB produce biosurfactants which can act effectively to prevent the first step in biofilm formation by modifying the cell surface properties or by being adsorbed to solid surfaces thus impairing the bacterial adherence [30]. They may also alter the biofilm integrity via interference with cell-to-cell aggregation and surface attachment processes. This effect may be mediated by the exopolysaccharides release by some LAB or by the physicochemical properties of their cells surface [31,32]. However, these effects seem to be highly strain specific [14].

The present study investigated some of the anti-infective properties of two *Lactobacillus* (LAB) strains, *Lactobacillus rhamnosus* EMCC 1105 and *Lactobacillus gasseri* EMCC 1930 against *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* (*S. aureus*) which are known to be the bacterial pathogens most commonly implicated in biofilm infections [13] .

The antimicrobial activity of the 24 h- and 48 h- cell free culture supernatants (CFS) of both LAB strains was measured using radial diffusion assay and could inhibit the growth of the tested pathogens forming a clear inhibition zone (IZ). However, the shown activity is most probably due to the produced acids only as no IZ were observed upon testing neutralized cell free culture supernatant (NCFS) instead of the acidic CFS.

The total biomass of dual-species biofilms of test pathogens with LAB as determined by crystal violet (CV) staining was significantly higher than that of their corresponding single-species biofilms after 48 h ($p < 0.001$) while no significant change was observed after 24 h ($p > 0.05$) except for the dual-species biofilm of *E. coli* with *L. rhamnosus* whose biomass significantly increased ($p < 0.05$) compared to its corresponding single-species biofilm. Since single-species biofilms of LAB showed a high absorbance indicating their ability to form biofilms successfully, it was assumed that the increase in the total biomass of dual-species biofilms was probably due to the contribution of LAB rather than the test pathogens.

In order to elucidate the effect of LAB on the pathogens in biofilms, the count of the test pathogens in single-species biofilms and in their corresponding dual-species biofilms with LAB was quantified using viable count technique and calculated as log $CFU.mL^{-1}$. The biofilms were also examined using scanning electron microscopy (SEM).

After 24 h, *L. gasseri* and *L. rhamnosus* were able to reduce the counts of both *P. aeruginosa* and *E. coli*

($p < 0.001$). *L. rhamnosus* could also cause a decrease in the viable count of *S. aureus* ($p < 0.001$), however the count slightly increased with *L. gasseri*. After 48 h, the three test pathogens showed an extremely significant reduction of count with both *L. gasseri* and *L. rhamnosus* ($p < 0.001$). This data confirmed the potential of LAB to compete successfully with the test pathogens in biofilms. This was further substantiated by SEM images which showed that LAB were able to replace the test pathogens in their biofilms.

Since bacterial extracellular hydrolytic enzymes, protease and phospholipase C (PLC) were shown to play a pivotal role in infection establishment and spreading in addition to their direct damaging effects on host cells, the effect of LAB on production of these enzymes by test pathogens in biofilms was evaluated [33,34]. Both strains of LAB could significantly reduce the proteolytic activity of *S. aureus* at both points of time ($p < 0.01$). Also, *L. gasseri* caused an extremely significant reduction in *P. aeruginosa* proteolytic activity in 24 h biofilms ($p < 0.001$). The proteolytic activity of *E. coli* showed a slight reduction in its 24 h dual-species biofilm with *L. rhamnosus* and its 48 h dual-species biofilm with *L. gasseri* ($p < 0.05$).

However, growing the test pathogens in dual-species biofilms with *L. gasseri* or *L. rhamnosus* did not affect the PLC activity of any of the three test pathogens at both points of time. This may be due to low basal level of PLC activity of the test pathogens which may be strain related or may be a consequence of the growth conditions used in the experiment. Changing the growth conditions in further experiments may reveal a more pronounced effect of LAB on the pathogens PLC activity.

The immunomodulatory effect of the two test LAB strains in this study was investigated. Gamma interferon (IFN-γ) was chosen as a marker due to its important role in developing an effective innate host response against microbial infections [35]. The pretreatment of peripheral blood mononuclear cells (PBMC) with different LAB preparations before stimulation with the test pathogens did not cause a significant change in the amounts of gamma interferon expressed by the PBMC ($p > 0.05$). However, this cannot be used as a conclusive evidence for the inefficiency of these LAB probiotics as immunomodulatory agents as only one marker was used to assess the immunomodulatory effect. Additionally, the *in vivo* studies may show a different result. Thus monitoring the expressed levels of other cytokines and assessing these effects *in vivo* can reveal the whole picture of the immune responses affected by these two LAB strains.

In conclusion, the highly significant antibiofilm activity observed for the LAB strains used in this study augmented with their inhibitory effect on proteolytic activities of the tested pathogens in biofilm in addition to the antimicrobial effect of their produced acids reflect their potential antagonistic activities against pathogens. They may be used effectively as antibiofilm and anti-infective agents for controlling *P. aeruginosa*, *E. coli* and *S. aureus* infections.

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