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MITIGATION OF THE TOXIC EFFECTS OF COMMON PATHOGENS BY PROBIOTICS

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***Abstract*:** In order to avoid the chronic forms of diseases development and to successfully treat patients with acute manifestations of infections it is necessary not only to fight pathogenic microorganisms with antibiotics, but also to learn how to influence the pathogens biofilm construction. *Pseudomonas aeruginosa* (*P. aeruginosa*) causes severe and persistent infections. The problem is further compounded by the ability of the pathogen to form a biofilm matrix. In addition it secretes pyocyanin, a potent virulence factor causing cell death and tissue damage. Pathologically, Pyocyanin and extracellular DNA (eDNA) are the key players dictating the development of biofilm formation and infection, which could be a promising target for developing new strategies against *P. aeruginosa* infection. *Staphylococcus aureus* (*S. aureus*) is amongst the most common causes of hospital-acquired infections having several virulence factors including coagulase production, ά-haemolysin production and biofilm formation. In this work the study of the antibiofilm activity of pathogens, *P. aeruginosa* pyocyanin production inhibition and reduction of *S. aureus* enzymes production by the original metabolites and living cell suspensions of *Lactobacillus reuteri* (*L. r.*) DSM17938, *Lactobacillus acidophilus* (*L. a.*), and *Saccharomyces boulardii* (*S. b.*) was carried out. All probiotics inhibited the growth of the test pathogens when tested using zone of inhibition assay. The cell adhesion and biofilm formation ability of both tested pathogens were studied and best methods for fast biofilm formation were chosen for the rest of the study. Statistically significant reduction ($p<0.05$) of the biofilms formation by pathogens' microbial cells occurred with the use of cell suspensions and metabolites of bacteria and of fungi. Cell free suspension (CFS) of all probiotics affects biofilm formation more than cell suspension (CS) of all probiotics on both pathogens tested. For *P. aeruginosa*, biofilm formation decreased 78% by *L. r.*, 76% by *L. a.*, and 70% by *S. b.* Additionally, *S. aureus* biofilm formation less affected than *P. aeruginosa* were it was decreased by 52% by *L. r.*, 56.5% by *L. a.*, and 43% by *S. b.* Moreover, *P. aeruginosa* pyocyanin production decreased significantly ($p<0.01$) when co-cultured with all probiotics for 72h where *L. r.* had the strongest effect. Conversely, there was no statistically significant difference in the activity of coagulase production of *S. aureus* cultivated with different probiotics CS and CFS when compared with the control group. Furthermore, haemolysin activity increased when *S. aureus* co cultured with CS and CFS of tested probiotics. In conclusion, all tested probiotics have a strong negative effect on very important virulence factors of *P. aeruginosa* and *S. aureus*, i.e. the ability to form biofilms on plastic surfaces and pyocyanin production of *P. aeruginosa*. Our findings provide new insights into the possible mechanisms involved in the protective effect of probiotics in the context of *P. aeruginosa* and *S. aureus* infections.

Key words: *Pseudomonas aeruginosa, Staphylococcus aureus, Probiotics, Biofilm.*

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INTRODUCTION

Bacteria are ubiquitous. Playing an important role in maintaining the surroundings in which we live. Only a small percentage of the world's bacteria cause infection and disease, these bacteria are known as pathogenic bacteria. Bacterial growth is characterized by two phenotypes, planktonic or sessile aggregates (biofilm). It has become apparent that bacteria exist predominately within biofilms in clinical settings. Biofilms are currently estimated to be responsible for over 65% of nosocomial infections and 80% of all microbial Infection (1, 2). The research on biofilms has increased dramatically, and there are also a lot of publications. Therefore, the research on this subject has become increasingly acceptable. The most important characteristic of bacterial biofilms is a decreased susceptibility to antimicrobial agents. This decreased susceptibility has two aspects, tolerance and resistance (3- 5). Tolerance means that bacteria are not killed, although they are unable to grow in the presence of the drug, whereas resistance allows bacteria to grow in the presence of antibiotics. Bacteria can become resistant, irrespective of their growth pattern (i.e. planktonic or biofilm), whereas only bacterial aggregates in biofilm exhibiting tolerance. Both phenomena are equally important and they may occur simultaneously. Tolerance may arise once a threshold number or density of bacteria has aggregated, whereas resistance will develop over time due to intrinsic and external factors such as mutations. The antimicrobial resistance of biofilm could be up to 1000-fold more than planktonic cells due to several mechanisms (5 & 6). Therefore, biofilm formation is a principal virulence factor for varieties of microorganisms that cause chronic infections. The nature of biofilm development along with resistance to antimicrobial agents develops at high speed. Therefore, great challenges and needs for

multi-targeted or combinatorial therapies. The biofilm mode of bacterial survival and growth is now being seen as a serious threat to public health and awareness about such cases draws importance among the scientific as well as social communities. The bacteria residing in biofilms have enhanced the extent of virulence and pathogenicity. For example, *Pseudomonas aeruginosa* (*P. aeruginosa*) can form biofilms in a wide variety of environmental conditions which can lead to chronic persisting infections (7). The control of the formation and development of biofilm becomes obligatory. But the increase in virulence and resistance against known antibiotics also poses a problem in the therapeutic designing of drugs. New strategies for the prevention of biofilm formation are necessary. Few strategies are being adopted for removal of biofilm-associated bacteria either by avoidance of attachment of the bacteria to the surface, use of compounds that can disrupt the biofilm formation, induction of dispersion, or degradation of the biofilm structure if they formed. Many evidence-based investigates suggest that the chronicity of persistent bacterial infections is due to bacterial biofilm formation, which contrasts with the planktonic bacteria found in acute infections (8-11). Most chronic infections involve numerous bacterial divisions so the accumulation of resistance and tolerance is a serious problem that further complicates underlying infection (7). The scope of this work is related to microorganisms isolated from diabetic foot ulcers chronic wounds. Chronic wounds are an ideal environment for biofilm formation. The necrotic tissue and debris allow bacterial attachment, and wounds are susceptible to infection due to impaired host immune response (12). Most studies demonstrate that the deep dermal tissues of all chronic wounds harbour multiple bacterial species (13-15). The common bacterial colonizers found is *Staphylococcus aureus* (*S. aureus*), while *P. aeruginosa* was observed in more than half of the chronic wounds investigated. Furthermore, *P. aeruginosa*-infected wounds were significantly larger in area than wounds without *P. aeruginosa*, and presence of *P. aeruginosa* also seemed to delay or even prevent the healing process (16 & 17). However, many chronic wounds will not heal, despite aggressive treatment; this is probably attributed to the presence of bacteria with biofilm growth. *P. aeruginosa* is an opportunistic bacterial pathogen of considerable medical importance that causes life-threatening infections in immunocompromised individuals such as patients suffering from burns, wounds, lungs infection, and urinary tract infections, those infections lead to high mortality rates as these organisms have several virulence factors which contribute to the seriousness of infection. (18). *P. aeruginosa* is a major wound colonizer known to manifest in chronic wounds (13 & 14), and their Infections are associated with significant rates of morbidity (18). These infections are difficult to treat due to biofilms limiting the diffusion of antibiotics (19). This is an alarming health concern as nosocomial acquired infections by multidrug-resistant *P. aeruginosa* continue to increase whilst treatment options become increasingly limited (9 & 19). In the case of burn injury infections, *P. aeruginosa* along with *S. aureus* is known as the principal pathogens responsible for serious complications (20). Regarding *P. aeruginosa* biofilm, exopolysaccharides, extracellular DNA (eDNA), Pyocyanin, rhamnolipids, and functional proteins are all factors that contribute to the formation of their biofilms (18). Pyocyanin is a redox-active, cytotoxic blue-green pigment that has bactericidal effects on other organisms. This Substance also can intercalate with eDNA thereby increasing the viscosity of DNA (21). Moreover, Pyocyanin can lead to the production of reactive oxygen species (ROS) and impaired wound healing. Pyocyanin accepts electrons from NADH and donates these electrons to molecular oxygen to form ROS such as hydrogen peroxide (H_2O_2). H_2O_2 is a strong inhibitor of many bacterial and fungal species, as well as altering the functions of the host cell. Besides the cell lysis by ROS production, pyocyanin inhibits bacterial growth by interacting with the cell membrane respiratory chain (21 & 22). Subsequent pyocyanin-eDNA interactions promote the protection of the bacterial biofilm from antibiotics and host cells (22). *P. aeruginosa is* most commonly implicated in wound infection, with the ability to form a biofilm, making them highly resistant to antibiotics (23). However, the development of a new strategy based on the control of the microbial growth in their biofilm and targeting pyocyanin may be foreseen as a breakthrough in medicine and will have a strong impact on the clinical practice and preventive medicine. Pathologically, *S. aureus* is currently the most common cause of infections in hospitalized patients. The bacterium infects diverse tissues and causes a wide spectrum of diseases. It is frequently the most common bacteria isolated from all types of wounds regardless of acute or chronic infection and is responsible for many other infections, such as scalded skin syndrome, toxic shock syndrome, cellulitis, septic arthritis, and osteomyelitis. It has many virulence factors which include adhesive surface proteins, immune modulators, toxins, and biofilm formation (24 & 25). The seriousness of the infections almost exclusively results from toxin activity, enzymes, and biofilm formation.

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit to the host (26). These tiny microorganisms belong to the natural human flora, which is vital to the development and maturation of human anatomy and physiology (27). However, commensal microorganisms cannot be called probiotics unless isolated, characterized, and proven for their health effects. They have been documented as "generally recognized organisms (GRAS)" by the Food and Drug Administration of the United States. They can tolerate gastric acidity, attach to the inner surface of the gastrointestinal tract (GIT), and modify the mucosal and systemic immune responses of hosts (28). The use of probiotics has become increasingly popular worldwide. Various mechanisms have been associated with their beneficial effects, some mechanisms are widespread in most studied probiotics such as interference with pathogen attachment and invasion to host cells (termed “colonization resistance” or the barrier effect), normalization of perturbed microbiota, the destruction of pathogenic toxins, reinforcement of the integrity of host cells (such as intestinal enterocytes), restoration of dysbiosis of the normal microbiota, gut barrier reinforcement, regulation of intestinal transit and production of useful metabolites and enzymes (29). Others such as modulation of the host immune system, production of specific bioactive compounds such mechanisms have been demonstrated for specific strains and species (30-32). There have been many studies of the negative impacts of probiotics against pathogens. Probiotic yeast *Saccharomyces boulardii* has been shown to be effective in inhibition of pathogen adhesion, degradation of bacterial toxins, and their receptors on the host mucosa (33) and consequently, provide a protective effect *against C. difficile*-induced inflammatory diarrhoea. Probiotic bacteria, *Lactobacilli,* are well known to have a positive effect on the maintenance of human health. Extensive researches have been done on *Lactobacilli species,* A study examined the effect of *Lactobacillus rhamnosus GR-1* and *L. reuteri RC-14* on growth and virulence factor expression in Uropathogenic *Escherichia coli* (UPEC) isolate found that compounds secreted by *Lactobacilli* likely protect the urogenital tract from UPEC colonization and infection by inhibiting growth, inducing stress and down-regulating proteins critical for host attachment (34). Another study shows that the biological activity of culture supernatant of toxigenic strains of *C. difficile* has been significantly reduced as a result of Co-culture with *Lactobacilli* strains which demonstrate the positive effect of such probiotics (35). Moreover, the use of probiotic *Lactobacilli* to combat microbial infections and biofilms is surpassing excellence in many studies (36-39). Therefore, continual researches are desired as the success of their uses is heavily dependent on scientific research substantiation. However, this study was focused on study attenuation of the virulent effects of

common pathogens, namely, *P. aeruginosa* and *S. aureus* by probiotics. Therefore, two species belong to the genus of *Lactobacillus* (*L. reuteri* and *L. acidophilus*), and probiotic yeast *S. boulardii* were selected for the study.

METHODS

STUDY DESIGN

In view of the fact that the virulence factors of pathogens are strongly associated with their pathogenicity. Thus, the knowledge about the relationship between probiotics and pathogenic microbes can contribute to the elaboration of strategies for the prevention or treatment of associated microbial diseases. This study was designed to study the effects of probiotic microorganisms related to inhibition and microbial biofilm formation and disruption of clinically isolated pathogens to assess and compare the effects of different probiotics namely, *Lactobacillus reuteri* DSM17938, *Lactobacillus acidophilus*, and *Saccharomyces boulardii* on growth inhibition and biofilm formation of two common bacterial pathogens namely, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, to assess the disruption potential of probiotics on *Pseudomonas aeruginosa* biofilm and to evaluate the effect of these probiotics on some virulence factors associated with these pathogens.

MICROBIOLOGIC METHODS

MICROORGANISMS USED IN THE STUDY

Probiotics used in this study were obtained from commercial products, including the following probiotic microorganisms: *Lactobacillus acidophilus* (ACIDO®), *Lactobacillus reuteri* (Biogia®), and yeast cell *Saccharomyces boulardii* (Bioflor®) were isolated from the following probiotic products: Acido®, Biogia®, and Bioflor® respectively. The product was firstly suspended in sterile distilled water, pure colonies isolated by streaking of *Lactobacillus* strains, and Saccharomyces boulardii on Rogosa Agar and Sabouraud Dextrose Agar (SDA) respectively. The pathogenic microorganisms' *P. aeruginosa*, and *S. aureus* were collected from patients with diabetic foot ulcer (DFU) in Diabetic and Endocrine diseases Hospital in Tripoli city / Libya. All isolates were identified by phenotypic characters, maintained in 50% glycerol, and stored at -80°C. Fresh cultures are prepared from frozen stocks before every experimental procedure. This was done by inoculating stocks over the surface of freshly prepared nutrient agar plates which were then incubated at 37°C for 18 hours, from which pure colonies were used to run every experiment.

IDENTIFICATION OF PATHOGENS

P. aeruginosa and *S. aureus* were previously collected from a diabetic foot infection. In the laboratory within aseptic techniques, isolates were streaked directly over the surface of freshly prepared Cetrimide agar and Mannitol salt agar respectively (HiMedia) which was then incubated at 37ºC for 24 hours. Further identification tests include gram stain, morphological characteristics, biochemical tests, and culture characteristics were done. Secondly, the isolates were quantitatively determined for their ability to produce biofilm on polystyrene plates (40). Briefly, isolated colonies of overnight grown bacteria were inoculated in brain heart infusion and incubated for 24hrs. Then, the grown bacteria were diluted and adjusted to 0.5 McFarland turbidity standards to reach 10^6CFU/ml. An aliquot of 10 ml of each diluted strain was added to polystyrene- made tissue culture flask and incubated for 48 hours at 37°C. After incubation, media with suspended bacteria were then removed; the plates washed carefully three times with distilled water, air-dried. After that, the formation of biofilm was inspected and the highest-producer isolate was selected to run the next experiments**.**

PREPARATION OF CELL-FREE SUPERNATANT (CFS)

Cell-free supernatant was prepared as described by Chew *et al.* 2015 (41). Broth cultures of *L. acidophilus* and *L. reuteri* in Rogosa, or Brain heart infusion media were adjusted to McFarland standard 0.5 (9.95ml of 1% sulphuric acid+ 0.05ml 1% barium chloride). Subsequently, 1 ml of the culture was added to 10 ml of fresh media and incubated under an anaerobic condition. The anaerobic jars were incubated for 48 hours at 37°C. Likewise, *S. boulardii* were adjusted to McFarland standard 0.5. Then, 1ml of the culture was added to 10ml of Brain heart infusion and incubated under the appropriate condition for 48 hours, after incubation, the cell supernatant was collected following centrifugation at 5000 rpm for 15 min, and the obtained CFS was stored at -20°C.

ZONE OF INHIBITION ASSAY

The Agar diffusion method was used to study antimicrobial activity (42). Pathogenic microorganisms were tested for inhibition by the probiotic supernatants. The studied pathogens were adjusted to McFarland standard 0.5, subsequently; adjusted suspension was swabbed on Muller Hinton agars, after which, wells of 6-mm-diameter were formed in each plate. To test the inhibition of probiotic by-product, the wells in each plate were filled with 100µl of the probiotic supernatant, fresh media was used as a negative control. The plates were incubated at 37°C for 48 hrs. Each probiotic strain was tested in triplicate to ensure accuracy and reproducibility and controls were treated the same way as to test samples. Following 48 hours, the diameters of the inhibition zones were measured for each well. Clearance zones were measured in millimetres from the edge of the wells to the edge of the clearance zones.

BIOFILM INHIBITION ASSAY

The inhibitory activity of probiotic supernatant and probiotic cell suspension against microbial biofilm was performed by using a modified microtiter plate method (MMTP) as described previously Stepanovic *et al.* 2007 and Sancineto *et al.* 2016 (40 & 43). This assay was performed by inoculating 200 µl of CFS or probiotic suspension into flat-bottom polystyrene wells contains 0.5 McFarland of the in 200 µl of BHI, and subsequently incubated for 24 hrs at 37°C. Negative control samples contained 200 µl of fresh media. After the incubation period, the contents of each well were aspirated, and wells were gently washed three times with

sterile distilled water. The plate was shaken well, so that, non-adherent bacteria were removed. The bacteria attached to the wells were then fixed with 70% ethanol, washed with distilled water, and dried for 20min. In each well, 200 µl of 1% crystal violet were added for 30 min. After this procedure, the wells were washed three times with distilled water. The attached stain was re-solubilized in 300 µl of 33% glacial acetic acid. The absorbance of the crystal violet solution was measured at 600nm by a UV-Vis spectrophotometer. The amount of formed biofilm was quantitatively assessed and comparing the absorbance values of CFS treated wells versus untreated control wells.

The biofilm formation rate (%) was calculated according to the following formula:

$$\text{Biofilm formation rate } (\%) = \left(\frac{OD_{Sample}}{OD_{Control}}\right) \times 100$$

BIOFILM DISRUPTION ASSAY

Biofilm disruption assays were performed only on *P. aeruginosa* biofilm using a modified protocol originating from Stepanovic *et al.* 2007 (40). The biofilms were formed by inoculating 400 µl of the *Pseudomonas* culture (0.5 McFarland) into flat-bottom polystyrene wells and incubating the plates for 48 hrs at 37°C. After the incubation period, the contents of each well were aspirated, and wells were washed with sterile distilled water, and subsequently replaced with 400 µl CFS or probiotic suspension, fresh media were used as a negative control. After that, the plates were re-incubated at 37°C for 24hrs. The reduction in the size of Biofilm adhered to the plates was quantified as described previously

EFFECT OF PROBIOTIC CULTURE ON PYOCYANIN PRODUCTION OF *P. aeruginosa*

The ability of probiotic cells to inhibit the production of pyocyanin was assessed. Briefly, 5 ml of Nutrient broth was inoculated with 50 µl of 24 hours old *P. aeruginosa* culture (10^8 CFU ml^{-1}) and 50 µl of 24-hour old probiotic culture (10^8 CFU ml^{-1}). Co-cultures were incubated for 72 h at 37°C in 5% CO_2 conditions (35). The assay of pyocyanin is based on the absorbance of pyocyanin at 520 nm in an acidic solution. 5ml of the co-culture was taken and put into a centrifuge tube and centrifuged for 10 min at 10000 rpm using a centrifuge. The spent culture supernatant was then mixed with 3ml of chloroform to precipitate organic matter and the mixture was vortexed 5 times. After that, the mixture was centrifuged for 5 minutes at 3000 rpm. The upper green layer was removed using a pipette. Then the bottom layer was mixed with 1.5ml of 0.2N HCL, and the mixture was vortexed as described previously. This gives us a pink layer that was measured at 520nm using a spectrophotometer. The concentration of pyocyanin is expressed as micrograms of pyocyanin per 5 ml of supernatant, this was determined by multiplying the absorbance at 520nm by 17.072 (Essar *et al*. 1990) (44). Pyocyanin production of co-cultures was compared with pure *P. aeruginosa* culture.

EFFECT OF PROBIOTIC CULTURE ON VIRULENT ENZYMES OF *S. aureus*

Besides the biofilm formation, the presence of membrane proteins, enzyme production such as coagulase and haemolysin are an important virulence factors produced by *S. aureus*. Five ml of Brain heart infusion broth was inoculated with 50 µl of 24 h *S. aureus* culture (10^8 CFU ml^{-1}) and 50 µl of 20 h old probiotic culture (10^8 CFU ml^{-1}). Co-cultures were incubated for 20 h at 37°C in 5% CO_2 conditions. After then, analysis of coagulase production was done by transferring 0.5ml of co-culture to sterile tubes containing 0.5 ml of human plasma. After incubation at 36±°C for 6h, the formation of clots was verified, considering the following criteria: no clot formation (negative reaction); small and disorganized clot (+); small and organized clot (++); big and organized clot (+++); coagulation of the entire contents of the tube (++++). For analysis of haemolysin activity, Blood agar was used. Aliquots of 5ul of co-culture were spotted on these media, after incubation for 48h at 37°C, the occurrence of translucent halo around the colonies was checked, indicating positive haemolytic activity. Enzyme activity (Pz) was evaluated according to previously described procedures (45) by measuring the ratio of the diameter of the colony and the diameter of the colony plus the zone of haemolysis. The smaller the value of Pz, is, the greater the enzyme activity. The enzyme activity was classified as: negative (Pz=1), positive (≥0.64 Pz< 1), and strongly positive (Pz<0.64).

STATISTICAL ANALYSIS

All experiments were carried out in triplicate (3 biological replicas and 3 technical replicas). The results are presented as means ±SD. Statistical analysis was conducted using the SPSS version20. After assumptions of normality, the variances of homogeneity were checked by one-way analysis of variance (ANOVA). The significance level was set at $p<0.05$.

RESULTS

ZONE OF INHIBITION ASSAY

Clearance zones were measured in millimetre (mm) for all samples, and results presented in Table 1. The negative controls of wells with fresh broth showed no zones of inhibition, demonstrating probiotic-specific inhibition.

Table (1) Inhibitory effects of cell-free supernatant (CFS) of *L. reuteri*, *L. acidophilus*, and *S. boulardii* on the growth of tested bacteria. Data represent mean values of triplicate experiments (n=9).

	S. aureus	***P. aeruginosa***
PROBIOTIC	Inhibition zone(mm)	Inhibition zone (mm)
L. reuteri	**7**	**5**
L. acidophilus	**5.8**	**6.33**
S. boulardii	**9**	**8**

BIOFILM ASSAY

The isolates were assayed for the formation of biofilm on polystyrene –made tissue culture flask. After periods of 24 hours, the formation of biofilm was observed. All isolates have the ability to form biofilm on polystyrene surface with a varying density. Highest- producer isolates were selected to run the next experiments.

BIOFILM FORMATION ASSAY

The results of biofilm inhibition are presented in (Figures 2 and 3), the supernatants collected from probiotic cultures (CFS) and cell suspensions (CS) of all included probiotics reduced the ability of the tested pathogen to adhere to polystyrene plates when incubated for 24h. The probiotic CS were less effective than CFS, which indicates that the effect on inhibition is more likely due to secreted metabolite during growth.

BIOFILM DISRUPTION ASSAY

Besides preventing the initial formation of biofilms, the ability of probiotic CFS and CS to disrupt and detach preformed biofilms on polystyrene surfaces *In-vitro* was investigated. As shown in (Figure 4), there is a considerable drop in the mass of preformed biofilm of *P. aeruginosa* on the polystyrene plate. Where all CFS of tested probiotics showed significant decrease in the size of the biofilms formed. Conversely, probiotic cells CS do not showed a significant reduction in the size of biofilm formed.

EFFECT OF PROBIOTIC CULTURE ON PYOCYANIN PRODUCTION

The effect of probiotic cells on the amount of pyocyanin production was determined, and used as 'proof-of-concept' experiments, before utilizing the more applicable PCR technique. The probiotics were incubated with P. aeruginosa to evaluate their ability to inhibit the production of pyocyanin. The pyocyanin test is done in nutrient broth media where pure P. aeruginosa culture and their co-culture with probiotics were tested for the amount of pyocyanin produced. As visually seen in (Figure 5), the co-culture with probiotics affect significantly $p<0.01$ the production of pyocyanin after 72 hours of co-incubation compared to pure Pseudomonas culture. Quantitatively, the concentration of pyocyanin is determined as micrograms of pyocyanin per 5 ml of supernatant, this was determined by multiplying the absorbance at 520nm by 17.072. As shown in (figure 6). The percentage of reduction of the pyocyanin production was as follows: L. reuteri (86.3%); L. acidophilus (79.5%) and S. boulardii (69.5%).

EFFECT OF PROBIOTIC CULTURE ON VIRULENT ENZYMES OF *S. aureus*

The effect of probiotic co-culture on the activity of coagulase and haemolysin production was assessed. After 6 hours of incubation, there was consistency in the production of coagulase in co-cultures, when compared to the control group (Results not shown) indicating that none of the studied probiotics can affect the ability of Staphylococcus aureus to produce free coagulase. Similarly, there was no statistically significant difference in the activity of the haemolysin of S. aureus cultivated with probiotic culture when compared to the control group.

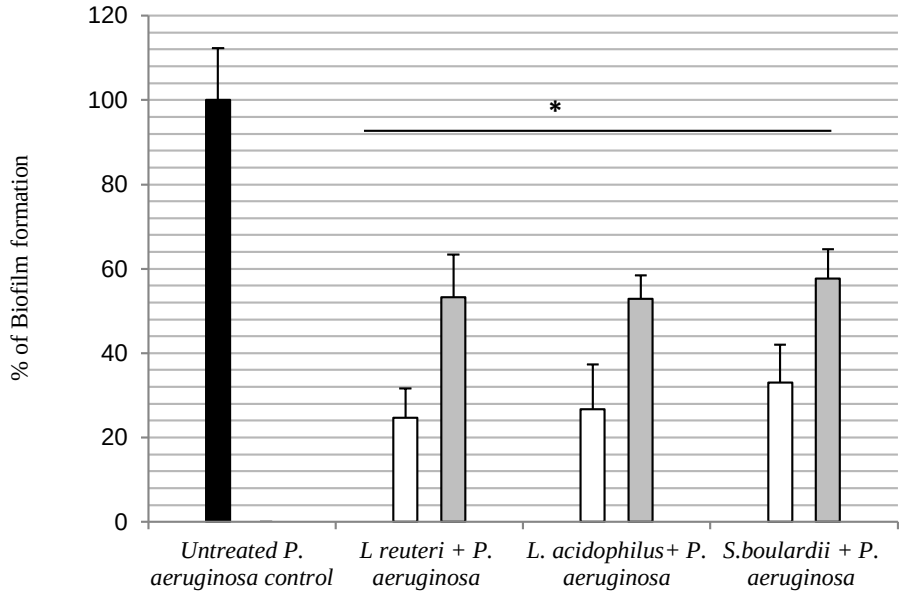

Figure (1) Inhibitory effects of different probiotics on the formation of biofilm by *Pseudomonas aeruginosa* determined by measuring the absorbance of crystal violet attached to biofilm formed on 48-well polystyrene plate. black bar, control (untreated biofilm); white bars, treated biofilms using cell-free supernatant (CFS) of probiotic culture. Grey bars, treated biofilms using probiotic cell suspension (PCS). Results are expressed as mean ±SD, n=9,***** p<0.0001.

DISCUSSION

P. aeruginosa and *S. aureus* are the most common pathogens isolated from acute and chronic wounds and are resistant to multiple antibiotics (23). The resistance to antibiotics imposes a rising risk to human health by increasing the disease burden and spread. Therefore, there is an urgent need to find a novel alternative to fight these pathogens. Probiotics have received a growing interest in the prevention and treatment of infectious and other human diseases such as gastrointestinal, urogenital, respiratory, and even periodontal diseases (46). This study demonstrates that cell-free preparations of *L. reuteri*, *L. acidophilus*, and *S. boulardii* supernatants after 48 hrs incubation were able to reduce the growth of *P. aeruginosa* and *S. aureus* isolated from diabetic foot ulcer with varying effect for each supernatant. The inhibitory activity of probiotics against bacteria was well-documented (47). Moreover, probiotics isolated from yogurts exhibited antibacterial effects against some common pathogens including *p. aeruginosa* and *S. aureus* (48) and antibacterial properties against gram-negative and gram-positive bacteria (49). In the same study, the evidence of antibacterial properties of cell-free supernatants was attributed to the production of organic acids that lowering the *p*H. Moreover, bioactive compounds released by probiotics such as bacteriocins and hydrogen peroxide were responsible for their antimicrobial properties (50 & 51).

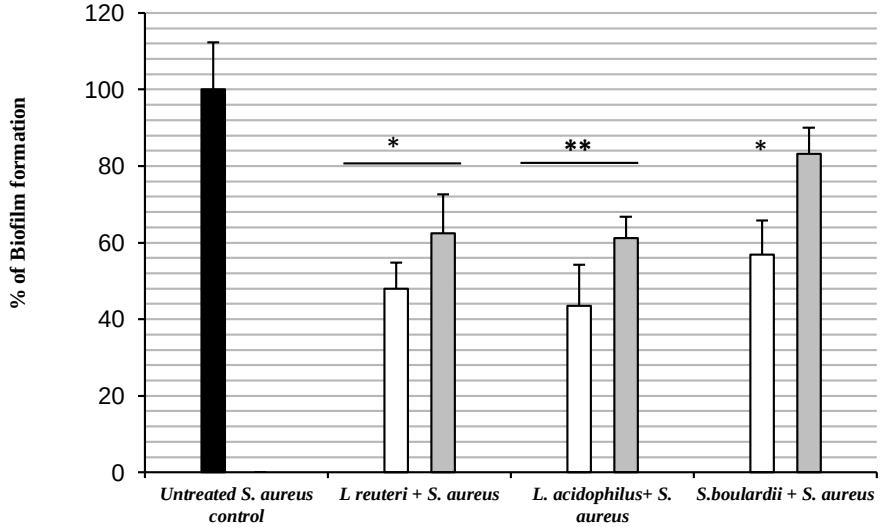

Figure (2) Inhibitory effects of different probiotics on the formation of biofilm by *Staphylococcus aureus* determined by measuring the absorbance of crystal violet attached to biofilm formed on 48-well polystyrene plate. Black bar; control (untreated biofilm); white bars, treated biofilms using cell-free supernatant (CFS) of probiotic culture. pattern bars, treated biofilms using probiotic cell suspension (PCS). Results are expressed as mean ±SD, n=9,***** $p<0.01$**, ****$p<0.001$.

Table (2) Antibiofilm potential of different probiotics against *P. aeruginosa.*

PROBIOTIC	**Mean OD_{600nm}** (%inhibition) **CFS***	**Mean OD_{600nm}** (% inhibition) **CS***
L. reuteri	0.212±0.066 (75.32%)	0.457±0.079 (46.79%)
L. acidophilus	0.229±0.010 (73.34%)	0.454±0.044 (47.14%)
S. boulardii	0.284±0.086 (66.93%)	0.496±0.053 (42.25%)
Control	0.859±0.084	

***CFS**= cell free suspension**, *CS=** cell suspension

Table (3) Antibiofilm potential of Probiotic against *S. aureus*

PROBIOTIC	**Mean OD_{600nm}** (%inhibition) **CFS***	**Mean OD_{600nm}** (% inhibition) **CS***
L. reuteri	0.406±0.047 (52.1%)	0.529±0.044 (37.55%)
L. acidophilus	0.369±0.025 (56.44%)	0.518±0.056 (38.85%)
S. boulardii	0.482±0.043 (43.1%)	0.708±0.069 (16.42%)
Control	0.847±0.067	

***CFS**= cell free suspension**, *CS=** cell suspension

Table (4) Disruption potential of Probiotic against *P. aeruginosa biofilm*

PROBIOTIC	Mean OD_{600nm} (% reduction) **CFS***	Mean OD_{600nm} (% reduction) **CS***
L. reuteri	0.435±0.081 (59.68%)	0.899±0.059 (16.68%)
L. acidophilus	0.369±0.062 (65.8%)	0.887±0.060 (17.79%)
S. boulardii	0.456±0.092 (57.73%)	0.967±0.060 (10.37%)
Control	1.079±0.20	

***CFS**= cell free suspension**, *CS=** cell suspension

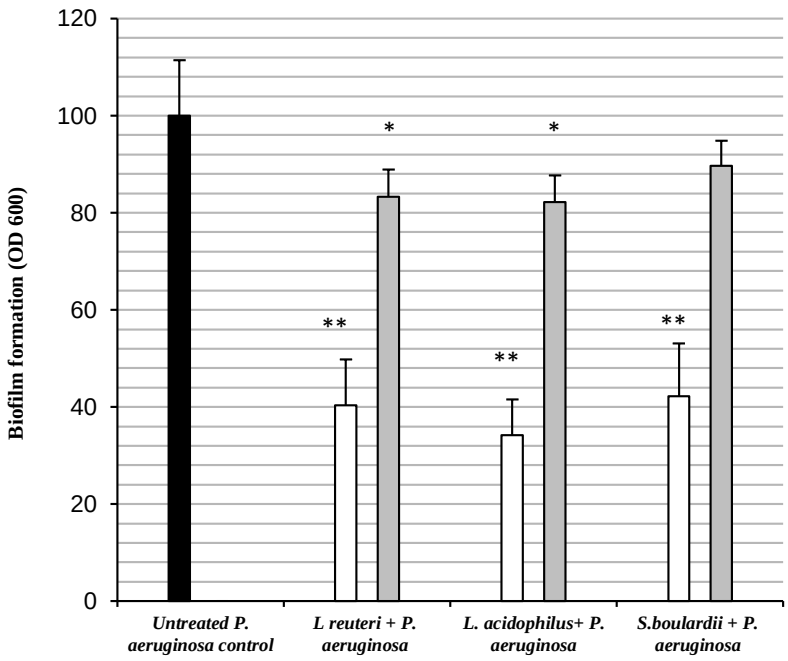

Figure (3) Effects of different probiotics on the disruption of 48-hour preformed biofilm of *Pseudomonas aeruginosa* determined by measuring the absorbance of crystal violet attached to biofilm formed on 48-well polystyrene plate. Black bar, control (untreated biofilm); white bars, treated biofilms using cell-free supernatant of probiotic culture. Pattern bars, treated biofilms using probiotic cell suspension. Results are expressed as mean ±SD, n=3,***** $p<0.05$**, **** $p<0.001$.

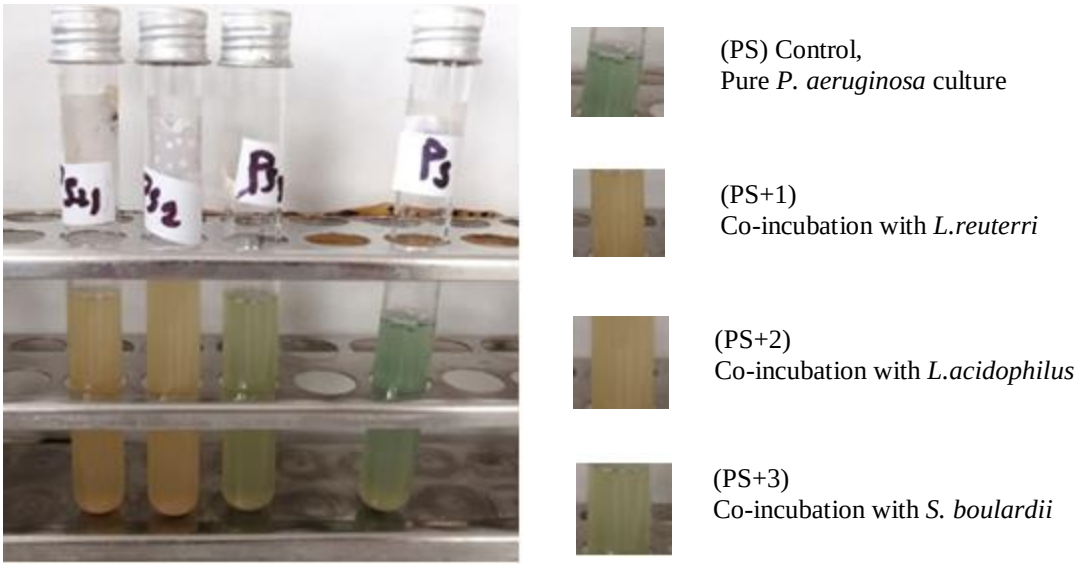

Figure (4) The effect of co-incubation of different probiotics with *P. aeruginosa* on the production of pyocyanin (Green pigment)

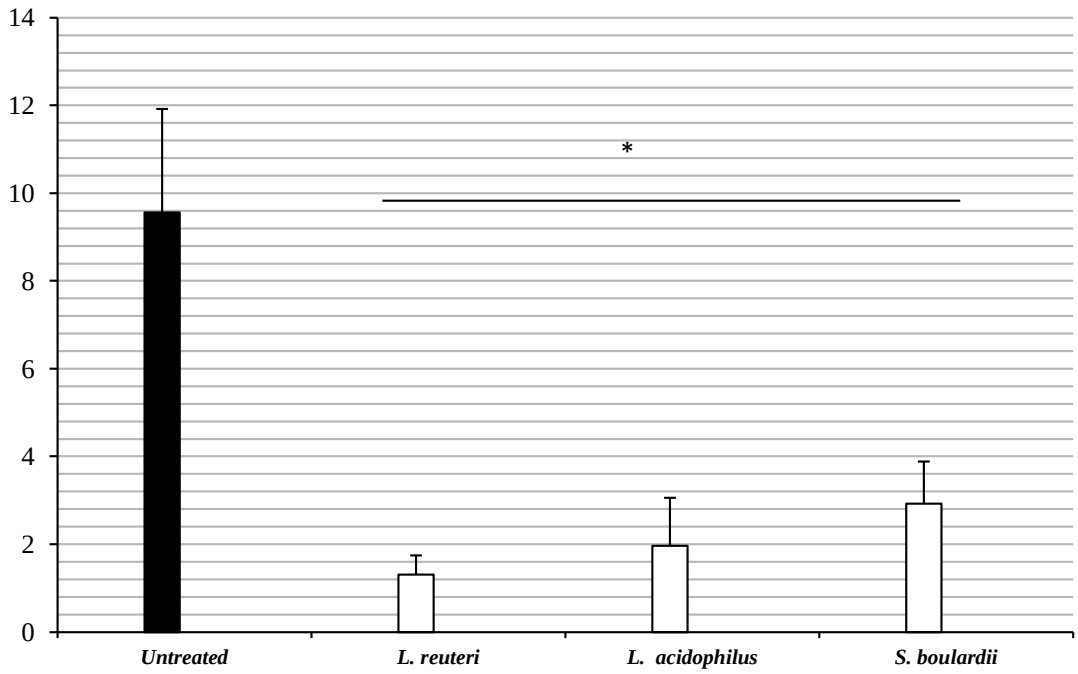

Figure (5) Amount of Pyocyanin in (µg/5ml) in pure *P. aeruginosa* culture and co-culture with different probiotics.
The percentage of reduction of the pigment production were *L. reuteri* (86.3%); *L. acidophilus* (79.5%) and *S. boulardii* (69.5%). n=9, * $p<0.05$

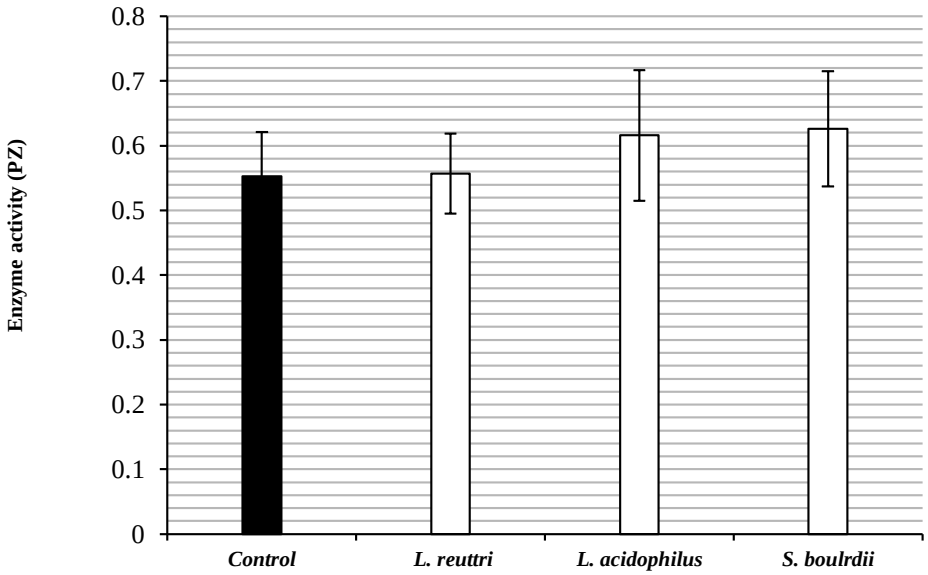

Figure (6) An average of enzyme activity (Pz) of haemolysin produced by *Staphylococcus aureus* in the presence or absence (control) of probiotics.

Additionally, a similar study showed that probiotic strains belonging to many genera had results support the potential use of probiotics/their bioactive compounds as antimicrobials. Biofilm formation is a phenomenon of microorganisms that results in a persistent microbial mass resistant to antimicrobial agents and related to about 80% of bacterial infections to humans. Infections due to biofilms formed by bacterial pathogens are difficult to diagnose and can contribute to high healthcare costs and morbidity rates (52). The antibiofilm properties of probiotics have been investigated, in this study; CFS of probiotics grown in Rogosa broth or brain heart infusion not only reduce the biofilm formation but have a potential to disrupt pre-formed biofilm of *P. aeruginosa*. In previous studies many probiotics successfully prevented biofilm formation, suggesting that they may be successfully used in the future as a complement to conventional therapies in the treatment of a range of skin disorders (53). Another study performed by sikorska and smoragiewicz (2013) demonstrated that many probiotics strains including *L. reuteri* inhibited methicillin-resistant *S. aureus* (MRSA) biofilm formation possibly by competition and production of acid and/ or bacteriocins (54). The *L. reuteri* and *L. acidophilus* have been also reported to affect both *Candida albicans* hyphae formation and mature biofilm development (55). Furthermore, In vitro investigations focusing on bacteriocins production, adhesion, growth inhibition, and co-aggregation of probiotics support their potential role in modulating microbial biofilms (46). In addition to biofilm, the virulence factors of bacteria play vital roles in pathogenicity, as observed in *P. aeruginosa*, with various virulence factors produced during biofilm formation which responsible for the seriousness of infection (56). With all of these observations, it has been suggested that *P. aeruginosa* metabolite pyocyanin plays an important role in bacterial resistance to antimicrobials through interactions with DNA. Pyocyanin binds directly to DNA and enhances DNA solution viscosity (57). This suggests that the DNA-pyocyanin interaction is one of the important factors contribute to resistant biofilms. In our study the effect of probiotics co-culture on the production of pyocyanin by *P. aeruginosa* was assessed and compared to the pure culture of *P. aeruginosa*. Interestingly, the production of pyocyanin was inhibited in probiotic co-culture compared to control, suggests that the tested probiotics contain some secreted factors during growth that affect pyocyanin production. In a previous study, Lee *et al*. (2020) observed decreased motility of *P. aeruginosa* as well as decreased production of pyocyanin and proteases as a result of probiotic *Pediococcus acidilactici* (58). In conclusion both pathogenic bacterial test cultures to various extents are sensitive to samples of *L. reuteri*, *L. acidophilus* and *S. boulardii*. Furthermore comparative characteristics of the *L. reuteri*, *L. acidophilus* and *S. boulardii* filtrates impact on the biofilm formation by the presented gram negative and gram positive pathogenic strains showed that, under the same conditions of the experiment performed, the statistically significant reduction in the biofilms formation by microbial pathogens cells occurred with the use of probiotics metabolites possesses high antibiofilm properties, which is established due to a significant reduction of the biofilms formation by selected test strains. Prospects for further research lie in the comprehensive study of disintegrates and original metabolic products tested probiotics in order to apply them in development of new generation antimicrobials against pathogenic and poly resistant to antibacterial drugs opportunistic pathogens. The antibacterial and antibiofilm effects of probiotic obtained from this study widen their applications in medicine and the possibility to eradicate microbial biofilms.

AUTHOR CONTRIBUTIONS STATEMENT

RM designed the study, supervised the project, co-wrote and contributed to data analysis. **AT** performed the laboratory-based analyses and co-wrote the manuscript and contributed to data analysis and **NE** incorporated in the laboratory work (Media and reagents preparation and other duties).

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFRENCES

1. Soto, Sara M. 2014. "Importance of Biofilms in Urinary Tract Infections : New Therapeutic Approaches." 2014.
2. Hurlow, J., Couch, K., Laforet, K., Bolton, L., Metcalf, D. et al. (2015). Clinical Biofilms: A Challenging Frontier in Wound Care. Advances in Wound Care, 4(5), 295-301.
3. Bjarnsholt, Thomas et al. 2005. "Pseudomonas Aeruginosa Tolerance to Tobramycin, Hydrogen Peroxide and Polymorphonuclear Leukocytes Is Quorum-Sensing Dependent." Microbiology (Reading, England) 151(Pt 2): 373–83.
4. Donlan, Rodney M, and J William Costerton. 2002. "Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms." Clinical microbiology reviews 15(2): 167–93.
5. Mah, T F, and G A O'Toole. 2001. "Mechanisms of Biofilm Resistance to Antimicrobial Agents." Trends in microbiology 9(1): 34–39.
6. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet. 2001 Jul 14;358(9276):135-8.
7. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents. 2010 Apr;35(4):322-32.
8. Costerton, J W et al. 1987. "Bacterial Biofilms in Nature and Disease." Annual review of microbiology 41: 435–64.
9. Costerton, J W, P S Stewart, and E P Greenberg. 1999. "Bacterial Biofilms: A Common Cause of Persistent Infections." Science (New York, N.Y.) 284(5418): 1318–22.
10. Burmølle, Mette et al. 2010. "Biofilms in Chronic Infections - a Matter of Opportunity - Monospecies Biofilms in Multispecies Infections." FEMS immunology and medical microbiology 59(3): 324–36.
11. Wolcott, R D et al. 2010. "Chronic Wounds and the Medical Biofilm Paradigm." Journal of wound care 19(2): 45-46,48-50,52-53.
12. Zhao, Ge et al. 2013. "Biofilms and Inflammation in Chronic Wounds." Advances in Wound Care 2(7): 389–99.
13. Gjødsbøl, Kristine et al. 2006. "Multiple Bacterial Species Reside in Chronic Wounds: A Longitudinal Study." International wound journal 3(3): 225–31.
14. Dowd, Scot E. et al. 2008. "Survey of Bacterial Diversity in Chronic Wounds Using Pyrosequencing, DGGE, and Full Ribosome Shotgun Sequencing." BMC Microbiology 8: 1–15.
15. Thomsen, Trine R et al. 2010. "The Bacteriology of Chronic Venous Leg Ulcer Examined by Culture-Independent Molecular Methods." Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 18(1): 38–49.
16. Madsen, S M, H Westh, L Danielsen, and V T Rosdahl. 1996. "Bacterial Colonization and Healing of Venous Leg Ulcers." APMIS : acta pathologica, microbiologica, et immunologica Scandinavica 104(12): 895–99.
17. Halbert, A R, M C Stacey, J B Rohr, and A Jopp-McKay. 1992. "The Effect of Bacterial Colonization on Venous Ulcer Healing." The Australasian journal of dermatology 33(2): 75–80.
18. Newman, John W, Rachel V Floyd, and Joanne L Fothergill. 2017. "The Contribution of *Pseudomonas aeruginosa* Virulence Factors and Host Factors in the Establishment of Urinary Tract Infections." (June): 1–11.
19. Drenkard, Eliana, and Frederick M Ausubel. 2002. "Pseudomonas Biofilm Formation and Antibiotic Resistance Are Linked to Phenotypic Variation." Nature 416(6882): 740–43.
20. Høiby, Niels et al. 2010b. "International Journal of Antimicrobial Agents Antibiotic Resistance of Bacterial Biofilms." 35: 322–32.
21. Parsons, James F et al. 2007. "Structural and Functional Analysis of the Pyocyanin Biosynthetic Protein PhzM from *Pseudomonas aeruginosa*." : 1821–28.
22. Das, Theerthankar, and Mike Manefield. 2013. "Phenazine Production Enhances Extracellular DNA Release via Hydrogen Peroxide Generation in Pseudomonas Aeruginosa." (June): 13–16.

23. Serra, Raffaele et al. 2015. "Chronic Wound Infections : The Role of *Pseudomonas aeruginosa* and *Staphylococcus aureus*." : 605–13.

24. Vergnano, Stefania et al. 2011. "Characteristics of Invasive *Staphylococcus aureus* in United Kingdom Neonatal Units." The Pediatric infectious disease journal 30(10): 850–54.

25. Percival, Steven L et al. 2016. "Antiseptics for Treating Infected Wounds: Efficacy on Biofilms and Effect of PH." Critical reviews in microbiology 42(2): 293–309.

26. FAO / WHO. 2001. "Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria." Expert consultation report, Córdoba, Argentina (October): 1–34.

27. McFarland, Lynne V. 2015a. "From Yaks to Yogurt: The History, Development, and Current Use of Probiotics." Clinical Infectious Diseases 60(May): S85–90.

28. Iannitti, T., and B. Palmieri. 2010. "Therapeutical Use of Probiotic Formulations in Clinical Practice." Clinical Nutrition 29(6): 701–25.

29. Kumar, Manoj et al. 2013. "Probiotic Metabolites as Epigenetic Targets in the Prevention of Colon Cancer." Nutrition Reviews 71(1): 23–34.

30. Heuvelin, Elise et al. 2009. "Mechanisms Involved in Alleviation of Intestinal Inflammation by Bifidobacterium Breve Soluble Factors." PLoS ONE 4(4).

31. Fernandez, Elise Macho et al. 2011. "Anti-Inflammatory Capacity of Selected Lactobacilli in Experimental Colitis Is Driven by NOD2-Mediated Recognition of a Specific Peptidoglycan-Derived Muropeptide." Gut 60(8): 1050–59.

32. McFarland, Lynne V., Charlesnika T. Evans, and Ellie J. C. Goldstein. 2018. "Strain-Specificity and Disease-Specificity of Probiotic Efficacy: A Systematic Review and Meta-Analysis." Frontiers in Medicine 5(May).

33. Castagliuolo, I et al. 2006. "Saccharomyces Boulardii Protease Inhibits Clostridium Difficile Toxin a and B-Induced Effects in Human Colonic Mucosa." Gastroenterology 114(1): A948–49.

34. Cadieux, P. A., J. P. Burton, E. Devillard, and Gregor Reid. 2009. "Lactobacillus By-Products Inhibit the Growth and Virulence of Uropathogenic Escherichia Coli." Journal of Physiology and Pharmacology 60(SUPPL.6): 13–18.

35. Antoni, Graciela L De. 2010. "Co-Culture with Potentially Probiotic Microorganisms Antagonises Virulence Factors of Clostridium Difficile in Vitro." : 19–29.

36. Ramos, Alberto N et al. 2012. "Antipathogenic Properties of Lactobacillus Plantarum on Pseudomonas Aeruginosa: The Potential Use of Its Supernatants in the Treatment of Infected Chronic Wounds." Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 20(4): 552–62.

37. Knysh, O V et al. 2019. "Influence of Cell-Free Extracts of Bifidobacterium Bifidum and Lactobacillus Reuteri on Proliferation and Biofilm Formation by Escherichia Coli and Pseudomonas Aeruginosa ." Regulatory Mechanisms in Biosystems 10(2 SE-).

38. Widyarman, Armelia Sari. 2019. "Persistent Endodontics Pathogens Biofilm Inhibited by Lactobacillus Reuteri Indonesian Strain Persistent Endodontics Pathogens Biofilm Inhibited by Lactobacillus Reuteri Indonesian Strain." 26(3): 160–64.

39. Jørgensen, Mette Rose et al. 2017. "Probiotic Lactobacillus Reuteri Has Antifungal Effects on Oral Candida Species in Vitro." Journal of Oral Microbiology 9(1): 1–8.

40. Stepanovic, Srdjan et al. 2007. "Quantification of Biofilm in Microtiter Plates: Overview of Testing Conditions and Practical Recommendations for Assessment of Biofilm Production by Staphylococci." APMIS : acta pathologica, microbiologica, et immunologica Scandinavica 115(8): 891–99.

41. Chew, S Y et al. 2015. "Probiotic Lactobacillus Rhamnosus GR-1 and Lactobacillus Reuteri RC-14 Exhibit Strong Antifungal Effects against Vulvovaginal Candidiasis-Causing Candida Glabrata Isolates." Journal of applied microbiology 118(5): 1180–90.

42. Fijan, Sabina. 2016. "Antimicrobial Effect of Probiotics against Common Pathogens." Probiotics and Prebiotics in Human Nutrition and Health.

43. Sancineto, Luca et al. 2016. "Diphenyl Diselenide Derivatives Inhibit Microbial Biofilm Formation Involved in Wound Infection." BMC Microbiology 16(1): 1–10.

44. Essar, David W, L E E Eberly, Ayele Hadero, and Irving P Crawford. 1990. "Identification and Characterization of Genes for

a Second Anthranilate Synthase in *Pseudomonas aeruginosa* : Interchangeability of the Two Anthranilate Synthases and Evolutionary Implications." : 884–900.

45. Alp, Fornitano et al. 2019. "Lactobacillus Rhamnosus versus Staphylococcus Aureus : Influence on Growth and Expression of Virulence Factors." 2: 29–33.

46. Vuotto, Claudia, Francesca Longo, and Gianfranco Donelli. 2014. "Probiotics to Counteract Biofilm-Associated Infections: Promising and Conflicting Data." International journal of oral science 6(4): 189–94.

47. Ramos, Alberto N et al. 2012. "Antipathogenic Properties of Lactobacillus Plantarum on Pseudomonas Aeruginosa: The Potential Use of Its Supernatants in the Treatment of Infected Chronic Wounds." Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 20(4): 552–62.

48. Hami Kaboosi. 2011. "Antibacterial Effects of Probiotics Isolated from Yoghurts against Some Common Bacterial Pathogens." African Journal of Microbiology Research 5(25): 4363–67.

49. Tejero-Sariñena, Sandra et al. 2012. "In Vitro Evaluation of the Antimicrobial Activity of a Range of Probiotics against Pathogens: Evidence for the Effects of Organic Acids." Anaerobe 18(5): 530–38.

50. Drider, Djamel, Farida Bendali, Karim Naghmouchi, and Michael L Chikindas. 2016. "Bacteriocins: Not Only Antibacterial Agents." Probiotics and antimicrobial proteins 8(4): 177–82.

51. Yang, Shih Chun, Chih Hung Lin, Calvin T. Sung, and Jia You Fang. 2014. "Antibacterial Activities of Bacteriocins: Application in Foods and Pharmaceuticals." Frontiers in Microbiology 5(MAY): 1–10.

52. Römling, U, and C Balsalobre. 2012. "Biofilm Infections, Their Resilience to Therapy and Innovative Treatment Strategies." Journal of internal medicine 272(6): 541–61.

53. Lopes, E. G. et al. 2017a. "Topical Application of Probiotics in Skin: Adhesion, Antimicrobial and Antibiofilm in Vitro Assays." Journal of Applied Microbiology 122(2): 450–61.

54. Sikorska, Hanna, and Wanda Smoragiewicz. 2013. "Role of Probiotics in the Prevention and Treatment of Meticillin-Resistant Staphylococcus Aureus Infections." International Journal of Antimicrobial Agents 42(6): 475–81.

55. Orsi, C F et al. 2014. "Inhibitory Effects of Different Lactobacilli on *Candida albicans* Hyphal Formation and Biofilm Development." Journal of biological regulators and homeostatic agents 28(4): 743–52.

56. Wagner, Victoria E, Richard J Gillis, and Barbara H Iglewski. 2004. "Transcriptome Analysis of Quorum-Sensing Regulation and Virulence Factor Expression in *Pseudomonas aeruginosa*." Vaccine 22 Suppl 1: S15-20.

57. Das, Theerthankar, and Mike Manefield. 2013. "Phenazine Production Enhances Extracellular DNA Release via Hydrogen Peroxide Generation in *Pseudomonas aeruginosa*." (June): 13–16.

58. Lee, Da-Hye, Bong Sun Kim, and Seok-Seong Kang. 2020. "Bacteriocin of Pediococcus Acidilactici HW01 Inhibits Biofilm Formation and Virulence Factor Production by *Pseudomonas aeruginosa*." Probiotics and antimicrobial proteins 12(1): 73–81.