Antibacterial activity of probiotic lactobacilli enhanced by adding olive oil to crude *Lactobacillus acidophilus*

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Abstract: In the present study, antibacterial activity of crude and modified *L. acidophilus* supernatant against 28 clinical isolates of gram negative and gram positive pathogens have been evaluated using the cup-cut plate and time-killing curve methods. The isolates were isolated from different patients and selected as the most resistant strains when challenged with antibiotics already in clinical practice. The crude cell-free *L. acidophilus* supernatants demonstrated wide antibacterial spectrum against the vast majority of the pathogenic strains where the diameter of the zone of inhibition around the cups containing the supernatants ranged between 10 and 15.5 mm. Modifying the MRS media by adding 1%v/v olive oil to *L. acidophilus* culture during the incubation period resulted in a supernatant with significantly weak antibacterial activity as compared to crude supernatant. In that almost most of the isolates were completely resistant to the antibacterial activity of the modified *L. acidophilus* supernatant. In contrast, the antibacterial activity of the crude supernatant was significantly enhanced by adding 1%v/v olive oil to the cell-free *L. acidophilus* supernatants collected 48 hours post-incubation as compared to the crude 48hrs supernatant without olive oil. This is evident by the remarkable differences of the diameter of the zone of inhibition among the conditions. In a kinetic study the lactobacillus sensitive MRSA and *P. aeruginosa* showed comparable and significant susceptibility to the antibacterial activity of crude and modified *L. acidophilus*, although they were isolated from different patients. In conclusion, the mixing of cell-free *L. acidophilus* supernatant with olive oil (1%v/v) produced a new compound with very good antibacterial activity as compared to controls.

Keywords: *L. acidophilus* supernatant, Anti-bacterial, MRSA, Olive oil

Introduction

Lactobacillus acidophilus (L. acidophilus) is one of the predominant probiotic bacteria found in the normal intestinal microflora of animals and humans. Several studies have indicated that *L. acidophilus* confers a wide range of health benefits to hosts (1, Fuller 1989). In particular, the capability of such bacteria to inhibit the growth of various Gram-positive or Gram-negative bacteria is well known. For instance, *L. acidophilus* is used to treat infection with *Helicobacter pylori* (2) and to prevent respiratory infections in children attending daycare centers (3). A study published in 2010 suggests that probiotics may lower the risk of common childhood illnesses such as ear infections, strep throat and colds. It is also being tested to prevent serious infections in people on ventilators (4). Women sometimes use lactobacillus suppositories to treat vaginal infections and urinary tract infections (UTIs) (5). This inhibitory effect is related to the ability of *L. acidophilus* to release several antimi-crobial substances (6), reduce gut pH by secreting lactic acid, improve immune activities by enhancing cytokine produ-ction (7), produce hydrogen peroxide which is active against wide range of pathogens (8) and maintain the healthy intestinal microbiota through competitive exclusion and antagonistic action

against pathogenic bacteria in the animal intestine (9). In addition, oral administration of *L. acidophilus* enhanced mitogen-induced murine lymphocyte proliferation and serum levels of IgG and IgM (10) and gut mucosal IgA secreting cells (11). Therefore, treatment with *L. acidophilus* can modify the concentrations of gut microbial populations and control gut bacterial overgrowth.

Previous published data have shown that *Lactobacillus* derived products including culture supernatants have been used for their wound healing and antiviral properties and to reduce cholesterol levels and the risk of colon cancer (2-4). A recent result by *Fahed* and *Radeef* has revealed that cell free supernatant of *L. acidophilus* was found to be very effective in inhibiting the production of lipase from biofilm and planktonic cells of MRSA isolates (12)**.** This supernatant was also proved to be effective against *Helicobacter pylori in vitro* and *in vivo* in people and was shown to have antimicrobial activities against *Enterobacter aerogenes, Salmonella enteric Bacillus anthracis* and *E. coli (*2, 13, 14).

On the other hand, olive oils has been proven to exert significant bactericidal activity *in vitro* against Gram-positive and Gram-negative bacteria (15). This activity is not only against the enteric microorganisms of the intestine *Helicobacter pylori*, *Escherichia coli* and *Clostridium perfringens*, but also against the beneficial bacteria *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (16-18). The bioactivities of olive oil have been related to the phenolic compounds, oleuropein and hydroxytyrosol (19). We therefore hypothesized that the combination of olive oil plus *L. acidophilus* supernatant may provide rapid bactericidal activity against pathogenic bacteria. Accordingly, the objectives of the study were to evaluate the bactericidal activity of *L. acidophilus* supernatant, and to address the influence of olive oil on the antimicrobial activity of *L. acidophilus* supernatant.

Materials and methods

Materials: All the chemicals were purchased from Sigma Chemical Co. (Tripoli, Libya), unless otherwise indicated.

Bacterial strains: The organisms were obtained from different patients. 10 *MRSA* and 7 *pseudomonas aeruginosa* isolates were selected from the stock of isolates obtained from renal transplant recipients. 6 different strains were isolated from biopsies obtained from patients diagnosed with GIT tumors. 5 different strains were isolated from patients with diabetic foot lesions. All isolation and identification procedures were done at department of microbiology and immunology – faculty of pharmacy according to NCCL protocols.

Preparation of supernatants from Lactobacillus cultures: The procedure for the preparation of LS has been previously reported (20). Briefly, *L. acidophilus* (NCAIM B 01075) were grown in MRS broth (pH 6.2; Liofilchem Laboratories, Tripoli, Libya) at 37°C in different condition as indicated below under microaerophilic conditions. This medium contains a rich nutrient base as well as polysorbate, acetate, magnesium, and manganese, which are known to promote the growth and proliferation of lactobacilli. Overnight bacterial cultures contained 2.5 x 10^8 colony-forming units, and these cultures were centrifuged at 10,000 g for 15 mins at 4°C to obtain cell-free supernatant. Supernatants were filter-sterilized by passing through a sterile 0.2 µl pore size filter. The pH of the supernatants was adjusted to 6.5 with

NaOH. The resulting supernatants were stored at - 20°C. At the time of the experiments, the *L. acidophilus* supernatant was thawed and used in agar cut-cup technique. Four different supernatant conditions were prepared; MRS-24 hrs, MRS-48 hrs, MRS-olive oil 24 hrs, and MRS-48 hrs + olive oil. MRS + olive oil without bacteria and phenol were served as negative and positive controls, respectively.

Screening for antibacterial activity: Agar diffusion cup-plate method described by (21) was followed to detect *L. acidophilus* supernatant inhibition activity. Muller Hinton agar was used to study the antibacterial activity of the *L. acidophilus* supernatants. 25 ml of freshly prepared Muller Hinton agar media (Oxoid, Tripoli, Libya) was poured in each Petri dish of 9 cm diameter to obtain 3-4 mm thickness layer of media. After solidification, using the swab, the Mueller-Hinton agar plate was streak to form a bacterial lawn. The plate was allowed to dry for approximately 5 minutes. After that, a sterile cork borer was used to prepare six cups of 4 mm diameter in the medium of each Petri-dish. An accurately measured 50 µl of the tested conditions and phenol 5% as positive control, were added to the cups with the help of micropipette on Mueller- Hinton-agar plates previously seeded with the respective bacteria. The study was performed in triplicate. All the plates were kept at room temperature for effective diffusion of *L. acidophilus* supernatants and then they were incubated at 37 ± 1° C for 24 hrs. The diameter of the zone of inhibition around the cup containing the tested conditions was measured.

Time-kill curve method: Cell free supernatant of LAB was obtained as described above. Time-kill curve method and the criteria for classification as bacteriostatic or bactericidal effect, synergism or antagonism were carried out according to previously reported modification (22). MHB was prepared and added to tubes containing cell-free supernatant of LAB incubated for 24 hrs, 48 hrs, 24 hrs in presence of olive oil (1%v/v) and 24 hrs supernatant mixed with 1% olive oil v/v. MHB and cell-free supernatants were mixed in a ratio 1:1 (5 ml each). Tubes containing MHB, MRS alone and in presence of 1% v/v olive oil were used as negative controls, while tube containing phenol 5% was used as positive control. All tubes were inoculated with the target bacteria and adjusted at 10^{8} CFU/mL(CLSI 2009). Surviving bacteria were counted after 0, 6, 12, 18, 24, 30, and 36 hrs of incubation at 37 °C by sub-culturing 50 µL serial dilutions (10-1, 10-2 and 10-4 in order to eliminate potential carryover effect) of samples (in 0.9% sodium chloride) on MH agar plates. The analysis was carried out in triplicates and the mean was taken. Bacteriostatic and bactericidal effects were defined as a decrease of < 3 log CFU/mL and ≥ 3 log CFU/mL after 24 hr of incubation, respectively, compared to the size of the initial inoculums (24).

Statistical analysis

The results were interpreted with the standard deviation. Student *t*-test was applied to know significant differences between the antimicrobial effectiveness of each condition. A *p*-value less than 0.05 were taken as the critical criterion for statistical significance.

Results

Antibacterial activity of L. acidophilus supernatants against pathogenic bacteria

The results of the antibacterial activities *L. acidophilus* supernatants on multi-drug

resistant hospital strains are presented in Tables below. The cup-cut agar technique results of present study, suggest that the cell-free-supernatants exerted varying inhibitory effect on the selected pathogenic strains. Table 1 shows that cell-free supernatants collected from the culture after 24 and 48 hours have almost similar antibacterial activity against hospital MRSA strains isolated from renal transplant recipients. The diameters of zone of inhibition around the cups were comparable to the positive control (phenol). However, a lower or almost no antibacterial activity was observed with cell-free supernatant collected from *L. acidophilus* incubated with (1% v/v) olive oil over 48 hours. Interestingly, adding the olive oil (1%v/v) to 48 hours cell-free supernatant did not reduce the antibacterial activity as compared to 24 and 48 hours supernatant without oil. Collectively, cell-free supernatants collected after 24 hours demonstrated antibacterial activity against 80% of the strains and this activity dropped down to 20% with cell-free supernatant collected from *L. acidophilus* bacteria incubated 48 hrs in presence of (1%v/v) olive oil.

Table 1: Inhibitory effect of *Lactobacillus acidophilus* supernatants on methicillin resistant *Staphylococcus aureus* (MRSA)

Strains	Diameter of inhibition zone (mm) Conditions of *L. acidophilus* supernatant					
	A	**B**	**C**	**D**	**E**	**F**
MRSA-1	1.4±0.57	3.7±1.5	8.7±0.58	1.4±0.6	11.4±1.1	10.6±0.5
MRSA-2	2.3±0.6	11.3±0.6	11.3±0.55	2±0.8	12.5±0.9	11.2±0.9
MRSA-3	1.3±0.55	10±0.9	10.5±0.6	5.4±0.57	12.7±0.57	12.2±0.6
MRSA-4	3±0.9	10.7±1.5	12.4±0.78	1.7±1.1	11.4±1.1	10.2±0.89
MRSA-5	1.4±0.57	10±0.9	11.7±0.6	10.6±0.57	10.5±1.1	11.4±1.1
MRSA-6	3.4±1.5	4.4±1.5	10.4±0.57	1.7±0.54	9.7±0.57	11.7±0.57
MRSA-7	2.3±0.6	10.3±1.5	14.3±0.64	2.5±0.87	11.7±0.57	12.4±1.2
MRSA-8	2.4±1.2	13.7±0.58	15.5±0.54	1.7±1.1	17.6±	11.3±1.1
MRSA-9	1.7±0.6	14.3±1.5	15±1.1	2.5±0.56	11.2±1.2	14.7±0.57
MRSA-10	2±0.89	11±0.8	11.7±1.15	1.7±0.55	13.5±1.1	13.1±0.6

***A**= negative control (MRS broth + olive oil), **B**= supernatant collected 24 hours post-incubation, **C**= supernatant collected 48 hours post-incubation, **D**= supernatant collected 48hours post-incubation with 1% olive oil, **E**= supernatant collected 48hours post-incubation + 1% olive oil, **F**= positive control (phenol 5%)*

Results in Table 2 demonstrate the antimicrobial activity of *L. acidophilus* supernatants against *P. aeruginosa*. All of the strains were highly susceptible to 24
and 48 hours supernatants as compared to the positive control (phenol 5%). Adding olive oil to *L. acidophilus* during incubation (48 hours) resulted in a supernatant with reduced antibacterial activity against some but not all *P. aeruginosa* strains, where 3 of 7 strains were highly resistant. Interestingly, adding the olive oil (1%v/v) to 48 hours cell-free supernatant enhanced the antibacterial activity against some strains as compared to 48 hours supernatant without oil.

Table 2: Inhibitory effect of *Lactobacillus acidophilus* supernatants on *Pseudomonas aeruginosa*

Strains	Diameter of inhibition zone (mm) Conditions of L. acidophilus supernatant					
	A	**B**	**C**	**D**	**E**	**F**
P. a-1	1.1±0.9	7.3±1.5	14.7±0.58	1.1±0.7	16.4±0.57	14.3±0.5
P. a-2	1.3±1.2	14.3±0.6	14.3±0.95	11.3±1.2	12.5±0.6	15.7±0.6
P. a-3	1.1±0.95	12±0.9	14±0.9	8.3±0.57	12.7±0.57	18.2±0.57
P. a-4	2.7±0.57	11±1.1	12.4±0.58	1.3±1.1	15.7±0.5	11.2±0.89
P. a-5	2.2±0.9	12.4±0.57	12.3±0.9	8.6±1.2	10.5±1.1	11.4±1.1
P. a-6	2.4±0.6	13.2±1.2	12.7±0.57	1.3±1.1	14.7±0.57	12.7±0.57
P. a-7	1.7±0.6	11.3±1.1	11.3±0.64	9.3±0.57	12.7±0.9	15.4±1.5

***A**= negative control (MRS broth + olive oil), **B**= supernatant collected 24 hours post-incubation, **C**= supernatant collected 48 hours post-incubation, **D**= supernatant collected 48hours post-incubation with 1% olive oil, **E**= supernatant collected 48hours post-incubation + 1% olive oil, **F**= positive control (phenol 5%)*

Table 3 shows the antibacterial activity of *L. acidophilus* supernatants against hospital strains collected from patients diagnosed with GIT tumors. As shown in the above, 24 and 48 hours supernatants have shown remarkable antibacterial activity against all strains as compared to the positive control (phenol 5%). Adding olive oil to *L. acidophilus* during incubation (48 hours) reduced the antibacterial activity of the cell-free supernatants against the tested strains. Interestingly, adding the olive oil (1%v/v) to 48 hours cell-free supernatant enhanced the antibacterial activity against most of the strains as compared to 48 hours supernatant without oil.

Table 3: Inhibitory effect of *Lactobacillus acidophilus* supernatants on bacterial strains isolated from patients with GIT tumors

Strains	Diameter of inhibition zone (mm) Conditions of L. acidophilus supernatant					
	A	**B**	**C**	**D**	**E**	**F**
proteus spp	1.1±0.9	11.3±0.57	14.7±0.58	8±0.7	21.1±1.1	11.7±1.5
E. sakazaki	0.6±1.2	13.3±0.6	13.7±1.2	9±1.2	13.7±0.9	13.1±0.9
E. sakazaki	0.6±0.57	12±0.9	13.6±0.9	8.3±0.57	12.7±0.57	13.2±0.57
P. areugi.	1.3±0.57	10.7±1.1	12.4±0.58	1.3±1.1	15.7±0.5	16.2±0.89
E. sakazaki	1.3±0.9	12.4±0.57	12.3±0.9	1.5±0.6	13.7±1.1	20.7±1.1
P. areugi.	1±0.6	11.7±0.6	11.7±0.57	1.3±1.1	16.7±0.57	17.7±0.57

***A**= negative control (MRS broth + olive oil), **B**= supernatant collected 24 hours post-incubation, **C**= supernatant collected 48 hours post-incubation, **D**= supernatant collected 48hours post-incubation with 1% olive oil, **E**= supernatant collected 48hours post-incubation + 1% olive oil, **F**= positive control (phenol 5%)*

Table 4 shows the antibacterial activity of *L. acidophilus* supernatants against strains collected from patients with diabetic foot lesion. All of the strains have similar susceptibility to 24 and 48 hours supernatants as compared to the positive control (phenol 5%). Interestingly, adding olive oil to *L. acidophilus* during incubation (48 hours) reduced but did not inhibit the antibacterial activity of the supernatants. Remarkable decrease in diameter of zone of inhibition around the cup containing the supernatant was observed for all the strains which in contrast to the other strains involved in this study. Adding the olive oil (1%v/v) to 48 hours cell-free supernatant enhanced the antibacterial activity against most of the strains as compared to 48 hours supernatant without oil.

Table 4: Inhibitory effect of *Lactobacillus acidophilus* supernatants on bacterial strains isolated from patients with diabetic foot lesions

Strains	Diameter of inhibition zone (mm) Conditions of L. acidophilus supernatant					
	A	**B**	**C**	**D**	**E**	**F**
Proteus spp.	1.1±0.9	13.3±0.57	12.7±0.58	8.3±0.7	14.7±1.1	13.3±0.57
S. aureus	0.4±1.2	11.3±1.6	10.7±1.2	9±1.2	13.7±0.9	15.1±0.9
S. aureus	0.3±0.57	12.3±0.9	11.6±0.9	8.7±1.17	13.7±0.57	11.2±0.57
MRSA	1.3±0.57	11.7±1.1	11.7±0.58	9.3±1.1	14.7±0.5	12.7±0.89
P. areugi	0.7±0.9	11.4±0.97	10.7±0.9	8.7±0.6	14.7±1.2	12.7±1.1

***A**= negative control (MRS broth + olive oil), **B**= supernatant collected 24 hours post-incubation, **C**= supernatant collected 48 hours post-incubation, **D**= supernatant collected 48hours post-incubation with 1% olive oil, **E**= supernatant collected 48hours post-incubation + 1% olive oil, **F**= positive control (phenol 5%)*

It was interesting to compare the sensitivity of MRSA and *P. aeruginosa* to *L. acidophilus* supernatant, since they represented the majority of the tested strains in the study (figure 1). The results in figure 1 are expressed as diameter of zone of inhibition around cups containing supernatants. In the case of MRSA, adding 1%v/v olive oil to 48 hrs cell-free supernatant resulted in slight insignificant increase in diameter of zone of inhibition as compared to 48hrs cell-free supernatant without oil (*p=0.1362; 95% CI=-2.482-0.359*). On the other hand, *P. Aeruginosa* strains showed remarkable susceptibility to 48 hrs cell-free supernatant plus 1%v/v olive oil. In this condition, there was significant increase in diameter of zone of inhibition as compared to 48 hrs cell-free supernatant without oil (*p=0.002; 95% CI=3.452-0.9084*). As shown previously, significant reduction in antimicrobial activity of to *L. acidophilus* supernatant was observed against both strains when 1% v/v olive oil added to *L. acidophilus* culture. Generally, both strains showed comparable results to the other conditions as shown in figure 1.

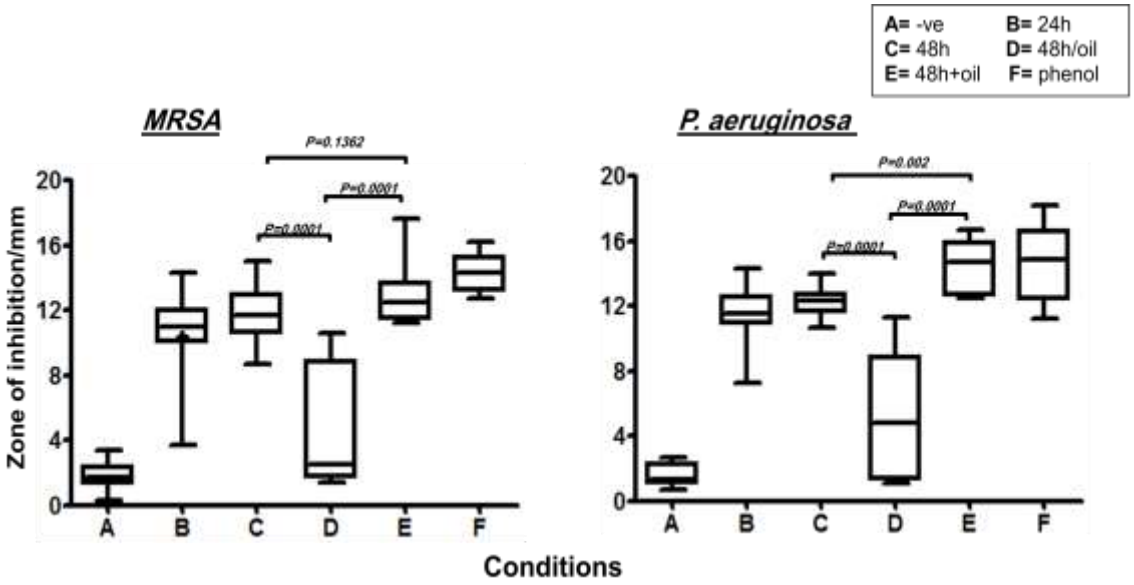

Figure 1: Antibacterial activity of to *L. acidophilus* supernatants against 13 hospital isolates of MRSA and 10 of *P. aeruginosa*. Sensitivity was deduced by comparing the inhibition zone diameter produced by different conditions using cup-cut plate method. Each experiment was conducted in triplicate. The data are expressed as mean values ± standard deviation of three experiments. Statistical analysis was performed with a Student *t* test.

Kinetic of antimicrobial activity using time-kill curve method

In the present study, the antimicrobial effectiveness of *L. acidophilus* supernatant was also confirmed by time-kill curve method. The strains *MRSA-1* and *P. areuginosa -4* were selected for the time-kill curve study because they had the highest antimicrobial resistant profile among the strains involved in this study. Bacterial growth was assessed by counting colonies to determine the number of c.f.u. Figures 2a and b show growth curves for MRSA-*1* and *P. areuginosa -3* strains with and without the cell-free supernatant of LAB. In general, addition of LAB supernatants to target bacteria resulted in reduced growth of both strains compared to control (figures 1 and 2). 24 and 48-hrs LAB supernatants appeared to have bacteriostatic activity within 24 hrs of incubation and reduced growth of *MRSA-1* and *P. areuginosa -3* (< 3 log CFU/mL decrease) and reached the bacteriocidal activity after 36 hrs of incubation (≥ 3 log CFU/mL decrease). Remarkably, addition of olive oil 1% v/v to 24 hrs cell-free supernatant improved the bacteriocidal activity against both strains. This combination was the most effective condition where it showed a clear bacteriocidal activity within 18 hrs of incubation (≥ 3 log CFU/mL) and completely inhibited growth of both strains after 24 hrs of incubation. In contrast, co-incubation of *L. acidophilus* with 1% v/v olive oil reduced the bacteriocidal activity, and showed only bacteriostatic activity of LAB supernatant against both strains (< 3 log CFU/mL decrease).

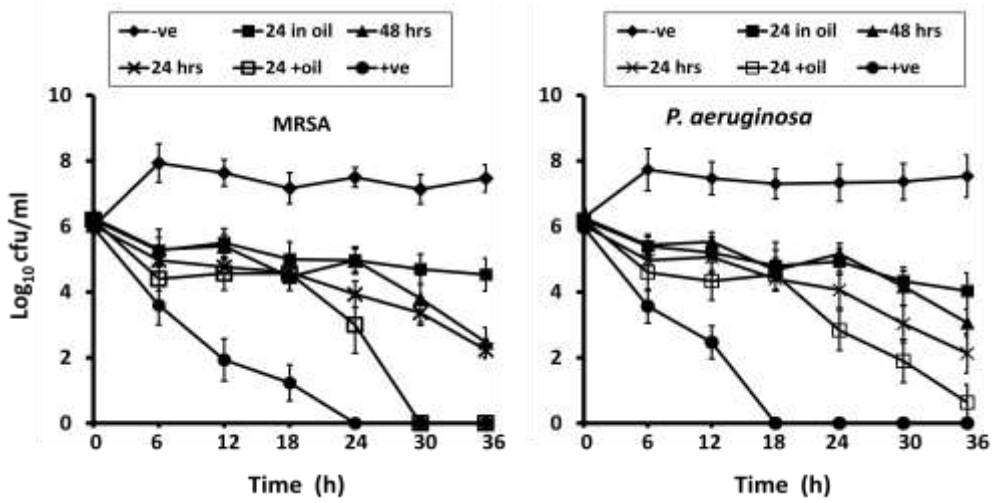

Figure 2: the killing curve **activity of *L acidophilus* against *P. aeruginos and MRSA.*** The numbers of viable bacteria were determined (log CFU/ml) after different time points. Each experiment was conducted in triplicate. The data are expressed as mean values ± standard deviation of three experiments. Statistical analysis was performed with a Student *t* test

Discussion

As previously reported, *L. acidophilus* possess and release several bioactive substances such as organic acids hydrogen peroxide, carbon dioxide and bacteriocins which have high antimicrobial activity and can act non-specifically against broad ranges of pathogens (9, 20, 24, 25). It is well known that antimicrobial activity is one of the most important selection criteria for probiotics. Therefore, the current study was designed to; 1-) address the antimicrobial activity of *L. Acidophilus* supernatant against several clinical bacterial pathogens isolated from patients with different infectious diseases, and 2-) address the antimicrobial activities of the novel combination of olive oil plus *L. acidophilus* supernatant against highly antibiotic resistant clinical strains *in vitro*.

The effectiveness of each condition was determined by agar disc diffusion method and inhibition zone diameters were measured against tested strains. Based on the observations, 80% and 100% of MRSA strains isolated from renal transplant recipients were susceptible to the antibacterial activity of 24 and 48 hrs LAB supernatants, respectively. This insignificant difference might be due to the variation in the concentration of the antimicrobial products released during the period of *L. Acidophilus* incubation. In agreement with our results, cell free supernatant of LAB was found to be very effective in inhibiting the production of lipase from biofilm and planktonic cells of MRSA isolates (12). Furthermore, cell-free supernatant of *L acidophilus* succeeded in eliminating wound infection caused by *S. aureus* (26). Additionally, cell-free supernatants of *L. acidophilus* showed very good antimicrobial activity against the target *P. aeruginosa* strains isolated from the same patients with inhibitory zone greater between 7 ± 3.53 to 14 ± 0.60 mm diameter. Therefore, Gram negative and Gram-positive pathogens involved in the present investigation were remarkably inhibited by cell-free supernatants

of *L. Acidophilus.* In supporting to our observations, earlier reports showed that *L. acidophilus* isolated from human intestine have antimicrobial activity against a wide range of Gram negative and Gram-positive pathogens *in vitro* and in vivo (27-29). We extended the investigation to involve 6 bacterial strains isolated from patients with GIT tumors and 5 strains from patients with diabetic foot lesion. Although, the strains utilized in this study produced different results, likely due to their unique clinical backgrounds, they were susceptible to the antimicrobial activity of cell-free supernatant of *L acidophilus*. Similar results were reported in that *L acidophilus* have been shown to inhibit the *in vitro* growth of many enteric pathogens and have been used in both humans and animals to treat a broad range of gastrointestinal disorders (9). Besides this, cell-free supernatant of *L acidophilus* was highly efficient in controlling wound infection caused by *S. Aureus*. In consistency, elsewhere study has concluded that the highest inhibitory activity of *L acidophilus* supernatant observed was against *Bacillus subtilis*, *P. aerogenosa*, *S. pyogenes, P. vulgaris, S. aure* (30). Noteworthy, all tested strains were highly pathogenic with highly antibiotic resistance profiles and classified based on their sites of collection. The results of present study, suggest that the cell-free-supernatants of *L acidophilus* exerted reliable inhibitory effect on these pathogenic strains. Collectively, in this study, we have shown that cell-free supernatant of *L. acidophilus* have significant antibacterial effect and this effect was independent of bacterial strains and sites of samples collection.

The main intention of our study is designed to address the effect of olive oil on the antibacterial activity of *L. acidophilus.* In this contest, olive oil at the concentration of 1% v/v was added to; 1-) the MRS media containing *L. acidophilus* and incubated for 24 hrs followed by supernatant collection, and 2-) the cell-free supernatant of the *L. acidophilus* after 24 hrs of incubation. Modification of media as carried out in this study allows us to test and compare the influence of olive oil during and after incubation. The incubation of *L. acidophilus* in presence of 1% v/v olive oil overnight produced supernatant with significantly low, if any, antibacterial activity against the target bacteria compared to the same supernatant in absence of olive oil. This is evident by absence of the zone of inhibition around the cups containing the supernatant. Very few strains were still susceptible with reduced diameter of zone of inhibition, which may be because the strains are heterovariant to the combination. This observation can be, to some extent, explained by susceptibility of *L. acidophilus* to the inhibitory effect of olive oil. As a result of losing its viability, *L. acidophilus* will also lose its ability to release bioactive and antibacterial compounds in the media, therefore its supernatant will lose the antibacterial property. The presence of antimicrobial compounds in olive fruits and olive oils has been proven in many literatures (19, 31, 32) . In particular, Oleuropein and its derivatives such as the aglycon of oleuropein and the elenolic acid showed strong bactericidal activity against LAB (33-35). Therefore, our observations were in consistent with others in this context. Interestingly, adding 1% v/v olive oil to 24-hrs cell-free supernatant resulted in producing a product with significantly higher antibacterial activity compared to 24-hrs cell-free supernatant without olive oil. Importantly, no change regarding the pH was recorded in the mixture; therefore, the effect of pH on the growth of the tested bacteria was excluded. As mentioned

earlier, cell-free supernatant of *L. acidophilus* has been proven to have antimicrobial activity (21, 23, 24). Besides this, several studies revealed that olive oil and fruits exerted a strong bactericidal action against a broad spectrum of microorganisms (33-35). Therefore, the presence of these two products together will be additive and/or synergistic effect, which is a clear explanation of our observation. These findings opened up the possibility of using olive oil and LAB supernatant as an effective antibacterial product to prevent and/ or delay the onset of growth of several pathogens.

Further effectiveness of combination of olive oil with *L. acidophilus* supernatant was also confirmed by time-kill curve against MRSA-1 and *P. areuginosa -3* strains. The strains in this study, however, were specifically chosen because they were highly resistant to wide range of clinically used antibiotics. To our knowledge, the present study is the first to study and compare the efficacies of this combination against highly pathogenic bacteria. Time-kill curve technique, which measures bactericidal activity, appears to be more relevant and provide a dynamic picture of antimicrobial action and interaction over time, as opposed to the agar diffusion technique, which is usually applied only once (after 24 h of incubation) (36, 37). A starting inoculum of 1×10^6–1×10^7 cfu/mL was chosen as representative of those observed in severe clinical infections. However, the combination of olive oil plus *L. acidophilus* supernatant demonstrated both synergistic and enhanced bactericidal activity against two clinical MRSA -1 and *P. areuginosa -3* strains (≥ 3 $\log_{10}$ CFU/ml decrease). With this regard, the time to bactericidal activity was generally lesser than 24 h, compared to 24-hrs cell-free supernatant alone. In contrast, supernatant collected after 24 hrs incubation in presence of olive oil did not display enhanced antimicrobial activity (< 2 $\log_{10}$ CFU/ml decrease) where the regrowth of both strains continued to occur after 36 hrs. This may be because the olive oil had suppressed L. *acidophilus* and reduced the antimicrobial compounds in the surroundings, as cited earlier. To our knowledge, the rapid and sustained bactericidal activity of olive oil plus *L. acidophilus* supernatant obtained in this study is a novel finding. The time-kill curve results was in consistent with the results obtained by agar diffusion plate technique. Collectively, the results obtained support the hypothesis that the combination of olive oil plus *L. acidophilus* supernatant is a likely mechanism for the reduction of several pathogenic bacteria in vivo. However, the clinical significance of these observations remains to be established. These data support the need for *in vivo* investigations to validate the interaction observed *in vitro* between olive oil and *L. acidophilus* supernatant.

In conclusion, the novel combination of olive oil plus *L. acidophilus* supernatant provided rapid bactericidal activity and provides a therapeutic option for treating highly pathogenic bacteria, especially when bactericidal activity is desired. Last, further deep investigations with these combinations are warranted.

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