SUSCEPTIBILITY PATTERN OF PSEUDOMONAS AERUGINOSA PRODUCING ENZYMES AGAINST ANTIMICROBIAL AGENT CELL FREE SUPERNATANT OF LACTOCOCCUS WITH THE FOCUS ON ITS DETERMINING QUANTITATIVELY BY OD (ENZYME LINKED IMMUNE SORBENT ASSAY)

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ABSTRACT
Objective: The present work was conducted to explore the prevalence of Pseudomonas aeruginosa that produce lipase and protease enzymes in 40 samples, as well as detection of quality and quantity production and determine its susceptibility to antimicrobial.

Methods: The antimicrobial activity of Lactococcus cell free supernatant (CFS) on P. aeruginosa growth and quantity production of enzymes by two methods (special media and OD) were also studied.

Results: A total of 8(20%) and 13(32%) isolates were found to be positive to P. aeruginosa producing lipase and protease, respectively. The in vitro antimicrobial activity results revealed that all isolates producing enzymes showed sensitive to CFS of Lactococcus, only one isolate exhibited low sensitivity to CFS 4 mm, however, these isolates varied in their sensitivity to CFS ranged 4-15 mm.

Conclusion: Results of the quantity production of P. aeruginosa enzymes with CFS of Lactococcus showed and exerts growth inhibitory activity and reduce the production of enzymes. We could be concluded that CFS of Lactococcus has great potential antimicrobial activity against P. aeruginosa growth and its ability on enzymes production.

Keywords: Pseudomonas aeruginosa, Lactococcus, Cell-free supernatant, Optical density, Lipase, Protease.

INTRODUCTION
Pseudomonas aeruginosa is an opportunistic pathogen as it initiates resistance to many antibiotics and disinfectant, in addition to its array of putative virulence factors plus plasmid acquired resistance [1,2]. Pseudomonas aeruginosa produced as the large array of colonized and virulence factors like lipase and protease enzymes production allowing it to make different types of infections [3].

It is well recognized that the intensive use of antibiotics contributes to the development of antibiotic resistance; moreover, antibiotics can be harmful to human and are associated with increased rates of mortality and morbidity [4]. In another side, the intensive use of probiotics lactic acid bacteria (LAB) has received increased during the last few decades [5]. Probiotics are defined as living microorganisms, which are safe for human, the major strain Lactococcus is the most dominant bacteria in the small and large intestine of humans [6]. LAB including Lactococcus spp. is gaining increasing interesting worldwide to be used in the prevention, control and treatment of disease, and health maintenance [7]. Considering the above facts in mind, this study was undertaken to determine the antimicrobial activity of Lactococcus casei against P. aeruginosa growth and its quality and quantity productivity to lipase and protease by two methods (using special media and optical density (OD) method).

METHODS
Collection of the tested bacterium
A total of 40 isolates from P. aeruginosa are used in this study which brought from Medical City Hospital from wounded patients. The isolated bacteria were identified according to Forbes et al. [8] and stored until used in the study.

Lipase enzyme productivity of P. aeruginosa test
The investigation of lipase production (LP) by P. aeruginosa isolates using tween 80 medium (Peptone 10 g + NaCl 5 g + CaCl₂ 0.1 g + agar 20 g and tween 80 ml with water 1000 ml) which inoculated with colonies that grow on brain heart infusion, these plates were incubated in 37°C for 24 hrs. The emergence of sediment around the colony indicating a positive result [9].

Protease enzyme productivity of P. aeruginosa test
Furthermore, to investigation of protease production (PP) by P. aeruginosa isolates were performed using skim milk Columbia medium (Peptone 2.3 g + starch 0.1 g + NaCl 0.5 g, agar-agar 4 g and 100 ml DW with Ph 7.5 + 100 ml skim milk and mixing for 5 minutes, and put in plates) which inoculated by streaking with colonies that grown on brain heart infusion, these plates were incubated in 37°C for 24 hrs. The emergence of transparent areas around the colony indicating a positive result [9].

Lactococcus isolate
From Al-Mustansiriyah University/Science Collage/high studies laboratories, this isolate was obtained and identified again according to Forbes et al. [8].

Preparation of cell-free supernatant from Lactococcus strain
Cell-free supernatant (CFS) was prepared according to Aminnezhad et al. [10]. Lactococcus was grown in MRS broth (pH 5.7) for 48 hrs at 37°C in anaerobic condition. CFS was obtained by centrifugation the culture at 15,000 rpm for 15 minutes at 4°C and then filtered through 0.45 µm filters (Millipore, Bedford, MA).

Agar well diffusion assay
P. aeruginosa isolates after testing were submitted to enzymes productivity antibacterial assay of Lactococcus CFS by agar well diffusion that done as follows:
A. Petri plates were prepared by pouring 20 ml of respective sterile Mueller-Hinton media for test *P. aeruginosa* isolates and allowed it to solidify.

B. Spreading of agar plates with 100 ml of each standardized tested *P. aeruginosa*.

C. Plates were allowed to dry and two wells (each 7 mm in diameter) made into agar plates with sterile borer.

D. Wells loaded with 100 ml of isolated bacterial culture filtrate supernatant and 100 ml sterile broth.

E. Plates were incubated at 37°C for 24 hrs for test bacterial.

F. Measurement of the diameter of the zone of inhibition with (mm) [11].

### Estimate the quantity production of *P. aeruginosa* enzymes

The quality of *P. aeruginosa* enzymes productivity was estimated using (mixing with culture media) method [12]. The test was done by adding CFS of *Lactococcus* (10%) enzyme to productivity special medium each of lipase and protease that put in small vials [after sterilized and cooled]. CFS was added to vials quietly and gently to prevent the formation of bubbles, then they were mixed well with vortex, pour the plate and left to cool, and harden, each plate was inoculated with *P. aeruginosa* isolate that grown activity and incubated in 37°C for 24 hrs. After that, it has to know whether the isolates retained had the productivity of enzymes through the appearance or absence of a special indicator of each enzyme around the colony like it was mentioned previously. Medium without CFS used as negative control and the medium with CFS without inoculum used as positive control.

### Estimate the quantity production of *P. aeruginosa* enzymes

The quantity of *P. aeruginosa* enzymes productivity was estimated using tissue culture plate method crystal violate staining (CV).

The experiment was done according to Hassan [13]. Briefly, the inoculum was prepared as described earlier, for each isolate, a loop full of activity growing cells was transferred to sterile Mueller-Hinton broth media. The OD of cells was determined for each suspension by enzyme-linked immune sorbent assay (ELISA) reader and adjusted to final OD 630 nm. These cell suspensions were then used to grow and enzymes (L.P.-PP) production. For each strain, 200 ml of the suspension was inoculated into individual wells of polystyrene of 96 well plates. MH broth not containing inoculum was used as a negative control. The plates were incubated at 37°C for 24 hrs under aerobic conditions. Supernatant including planktonic cells and liquid medium was then discarded, and wells were gently washed twice with phosphate-buffered saline to a get ride on any non-adherent cells.

Adherent cells were fixed in 95% ethanol for 5 minutes and then ethanol was removed by air drying and stained with 1% CV for 45 minutes. The OD values of stained adherent cells were determined by ELISA reader at 630 nm. *Lactococcus* CFS and broth only was used as positive control.

### Effect of *Lactococcus* CFS on *P. aeruginosa* enzymes productivity was quantified according to the above procedure. The extent of enzymes productivity degree was calculated according to the following equation:

Enzyme production (expressed as OD) = OD test- OD control.

The percentage of inhibition of productivity was calculated by the following formula:

\[
\text{Enzyme productivity} = \frac{\text{OD test}}{\text{OD control}} - \frac{\text{OD test} \times 100}{*100}
\]

Chursi et al. [14] in all experiment, three replicates per treatment were used.

### RESULTS AND DISCUSSION

In this study, it was obtained 8 (20%) isolates of *P. aeruginosa* producing of lipase and 13 (32.5%) isolates PP out of 40 isolates, while the same isolate did not repeat to possess both of enzymes in the same time (Table 1). This factor (enzymes production) is one of the virulence factors that added to other factors owned *Pseudomonas* especially *P. aeruginosa* [15,16].

The antimicrobial activity of *Lactococcus* CFS is studied by well diffusion agar against 8 isolates (producing lipase) that called L.P isolates and 13 isolates (P.P) also called PP isolates (Table 2).

A total of 21 tested isolates (L.P 8 + P.P. 13) were found to exhibit its effect. CFS, the isolate of probiotic (*Lactococcus*) showed high inhibitory activity against two isolates (L.P. - 4 and P.P- 1) with 15 minutes of both, whereas CFS gave lower inhibitory activity against just one isolate (L.P. 7) with 4 mm, the rest of the pathogenic isolate showed inhibition ranged 4-15 mm for L.P isolates and 7-15 mm for PP isolates (Fig 1). The antimicrobial activity of *Lactococcus* CFS was may be due to the production of organic acids, reuterin, hydrogen peroxide, proteinaceous compounds, hydroxyl fatty acids, and phenolic compound [17]. Whereas Pundir et al. [11] pointed out the antibacterial activity may be due to the production of acetic and lactic acids that lowered the pH of the medium or competition for nutrients, or due to the production of bacteria or antibacterial compounds.

After not the effect of *Lactococcus* CFS on growth of the isolates (21) of *P. aeruginosa*, the qualitative detection for isolates productivity to enzymes was measured by mixing with media method where results indicated that 6 L.P. isolates out of 8 became not L.P when exposed to CFS and just 2 stay produced, whereas 12 PP. isolates out of 13 convert to not P.P, 1 isolate still resist the CFS of *Lactococcus* (Table 3).

Many researches showed the high antibacterial activity of LAB and *Lactococcus* [18-20]. Some of the researchers refer to find many acids and H₂O₂ in the medium, the released hydrogen ions during the dissociation reduce the trans membrane gradient and neutralize the proton motive force, change the internal pH and cause denaturation of proteins and loss of viability, however these weak acids exhibit antimicrobial activity due to the combined effect of the un dissociated molecules and the dissociated ions. Un dissociated molecules and dissociated ions induced cell damage [21,22].

The possible effect of CFS on the quantitative productivity of *P. aeruginosa* enzymes was evaluated; the study of CFS in reducing the production of CFS against *P. aeruginosa* especially (L.P + P.P) isolates of *P. aeruginosa* was evaluated.

### Table 1: The total number of *P. aeruginosa* and percentage of producing L.P and P.P

| Total isolates number | L.P. isolates | P.P isolates |
|-----------------------|--------------|--------------|
| 40%                   | 8            | 13           |
| %                     | 20           | 32.5         |

L.P. isolates: Lipase-producing isolates; P.P isolates: Protease producing isolates

### Table 2: Antimicrobial activity of *Lactococcus* CFS against (L.P + P.P) isolates of *P. aeruginosa*

| Isolates number | L.Z. (mm) | Isolates number | L.Z. (mm) |
|-----------------|-----------|----------------|-----------|
| L.P 1           | 10        | P.P 1          | 15        |
| L.P 2           | 9         | P.P. 2         | 10        |
| L.P 3           | 12        | P.P 3          | 7         |
| L.P 4           | 15        | P.P. 4         | 12        |
| L.P 5           | 7         | P.P. 5         | 10        |
| L.P 6           | 11        | P.P 6          | 12        |
| L.P 7           | 4         | P.P 7          | 12        |
| L.P 8           | 10        | P.P 8          | 12        |
| P.P 9           | 11        | P.P 9          | 11        |
| P.P 10          | 7         | P.P 10         | 7         |
| P.P 11          | 9         | P.P 11         | 9         |
| P.P 12          | 14        | P.P 12         | 14        |
| P.P 13          | 8         |                |           |

L.Z (mm): Inhibition zone in micrometer; L.P. isolates: Lipase-producing isolates; P.P isolates: Protease producing isolates, *P. aeruginosa: Pseudomonas aeruginosa*
enzyme produced might prove the benefit of using a CFS to council the production of enzymes, so it can reduce the virulence of pathogen bacteria.

In Table 4 we notice that all L.P isolates were reduced after treatment with CFS, also all P.P isolates respond to inhibition by CFS (Table 4).

In this study, we put forward the hypothesis that whether treatment with CFS has higher antimicrobial activity against *P. aeruginosa* or not. The first criteria which *Lactococcus* isolates should need to fulfill is that should be resistant to that particular virulence factors to avoid the direct killing of the probiotic properties. The second criteria *Lactococcus* has special properties make it stronger than other bacteria.

### Table 3: Qualitative detection of effect the *Lactococcus* CFS on lipase and protease productivity of *P. aeruginosa*

| L.P. | L.P.+CFS | P.P. | P.P.+CFS |
|------|----------|------|----------|
| 1    | -        | 1    | -        |
| 2    | -        | 2    | -        |
| 3    | -        | 3    | -        |
| 4    | -        | 4    | -        |
| 5    | -        | 5    | +        |
| 6    | +        | 6    | -        |
| 7    | -        | 7    | -        |
| 8    | -        | 8    | -        |
| 9    | -        | 9    | -        |
| 10   | -        | 10   | -        |
| 11   | -        | 11   | -        |
| 12   | -        | 12   | -        |
| 13   | -        | 13   | -        |

L.P: Lipase production, P.P: Protease production, *P. aeruginosa*: *Pseudomonas aeruginosa*

### Table 4: Antimicrobial activity of *Lactococcus* CFS against L.P. and P.P. isolates of *P. aeruginosa* by (TCP-CV) method

| Isolate number | Without CFS | With CFS | Isolate number | Without CFS | With CFS |
|----------------|-------------|----------|----------------|-------------|----------|
|                | OD          |          |                | OD          |          |
| L.P. 1         | 0.207       | 0.09     | P.P. 1         | 0.24        | 0.015    |
| L.P. 2         | 0.1         | 0.049    | P.P. 2         | 0.126       | 0.005    |
| L.P. 3         | 0.122       | 0.054    | P.P. 3         | 0.166       | 0.067    |
| L.P. 4         | 0.164       | 0.078    | P.P. 4         | 0.175       | 0.031    |
| L.P. 5         | 0.153       | 0.086    | P.P. 5         | 0.226       | 0.087    |
| L.P. 6         | 0.126       | 0.005    | P.P. 6         | 0.218       | 0.001    |
| L.P. 7         | 0.115       | 0.019    | P.P. 7         | 0.13        | 0.013    |
| L.P. 8         | 0.151       | 0.06     | P.P. 8         | 0.1         | 0.081    |
| L.P. 9         | 0.251       | 0.06     | P.P. 9         | 0.251       | 0.06     |
| L.P. 10        | 0.261       | 0.016    | P.P. 10        | 0.261       | 0.016    |
| L.P. 11        | 0.175       | 0.001    | P.P. 11        | 0.175       | 0.001    |
| L.P. 12        | 0.1         | 0.081    | P.P. 12        | 0.1         | 0.081    |
| L.P. 13        | 0.142       | 0.077    | P.P. 13        | 0.142       | 0.077    |

OD: Optical density. These numbers is the final after calculated with equation, *P. aeruginosa*: *Pseudomonas aeruginosa*

In previous studies, an antimicrobial agent was conducted that the presence of organic acid acts by collapsing the electrochemical proton gradient, and H2O2 by peroxidation of membrane lipids thus altering the cell membrane permeability which results is disruption of substrate transport system [23-25].

The effect of *Lactococcus* CFS was a variety from isolate to another because it is obscure of the production levels and properties among its compounds depend on the biochemical properties of the strain used and physical and chemical conditions of growth [5,26].

### CONCLUSIONS

The antimicrobial compound produced by *Lactococcus* was effectiveness against the test pathogen *P. aeruginosa* that used in this study. CFS was able to prevent enzymes production so it can attenuate the virulence of *P. aeruginosa*.

### RECOMMENDATIONS

1. Since emerging reports showed increased prevalence of resistance against these drugs as observed, it seems necessary to use natural alternative (lactic acid bacteria), do as antimicrobial agents against *P. aeruginosa*.
2. These compounds could serve as an alternative to chemical preservatives additives used in food preservation, after checking safety of the LAB on the basis of criteria laid down by the WHO for potential probiotics, these organisms may be used as human or animal probiotics.

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**Fig. 1:** Antibacterial activity of cell free supernatant of *Lactococcus* against *Pseudomonas aeruginosa*. L.P: Lipase production, P.P: Protease production, C: Control
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