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Effect of *Lactobacillus plantarum* on virulence factors of *Pseudomonas aeruginosa* isolated from wound infection

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ABSTRACT

Background: *Pseudomonas aeruginosa* represents a major concern in nosocomial infections. It possesses many virulence factors that aid its pathogenicity and antimicrobial resistance. Non-antibiotic therapy against this pathogen includes probiotics, phages, phytomedicine and other new modalities. These agents alone or in combination with antibiotics can be highly effective against these virulent strains. **Objectives:** To investigate the in-vitro effect exerted by the probiotic "*Lactobacillus plantarum*" on two virulence factors of *Pseudomonas aeruginosa* (pyocyanin pigment production and elastase enzyme activity). **Methods:** The study was carried out on 40 *Pseudomonas aeruginosa* strains isolated from pus collected from wound infection using sterile swabs. The effect of *Lactobacillus plantarum* on pyocyanin production and the elastolytic activity of *Pseudomonas aeruginosa* were detected using pyocyanin extraction method and Human Elastase ELISA Kit respectively before and after the addition of *Lactobacillus plantarum*. **Results:** Among the forty strains investigated, the pyocyanin production and elastolytic activity of *Pseudomonas aeruginosa* isolates were significantly decreased after addition of *Lactobacillus plantarum*. **Conclusions:** *Lactobacillus plantarum* has an important invitro anti-pathogenic effect against *Pseudomonas aeruginosa* as it significantly interferes with two of its main virulence factors (pyocyanin & elastase).

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an ubiquitous pathogen that represents a significant problem in all health-care systems especially those requiring prolonged hospitalization [1]. The presence of such pathogen in medical micro-environments e.g. wounds exhibits major concerns in patients' post-operative care [2].

There are several virulence contributing factors of *P. aeruginosa* including cell-related factors e.g (endotoxin, flagella, pili) and extra-cellular ones e.g (alginate, exotoxin A, exoenzyme S, pyocyanin, elastase, etc.) [3].

Pyocyanin is a bluish cytotoxic pigmentation that acts by blocking the wound

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healing process through promoting the oxidative-stress process and activating the pathway of P38 mitogen-activated-protein-kinase (MAPK) in the infected tissues [4].

Pseudomonas is resistant to many antimicrobial agents either naturally or through acquired pathways and it becomes increasingly resistant to many discovered anti-*Pseudomonas* agents. Therefore, treatment of wounds or burn patients infected with *P. aeruginosa* became very problematic due to limited therapeutic alternatives [5] making it necessary to advance the search for other new non-antibiotic alternatives that are economically available to the developing countries [6].

Non-antibiotic therapeutic modalities against this multi-drug resistant (MDR) pathogen include probiotics, phages and phytomedicines. These agents by themselves or combined with currently used antimicrobials can be highly efficient against MDR *P. aeruginosa* strains [7].

Probiotics are viable non-pathogenic organisms capable of altering the flora of the medium where they exist e.g. gastrointestinal tract (GIT). Generally, they obtain a positive impact on the host health and physiology of the body [8]. They have also shown antiviral, anti-mycobacterial and anti-cancer effects [9].

Lactic acid bacteria (LAB) are a famous example of probiotics widely distributed in nature. Many studies designated their several health promoting and immunity function influences like regulation of gut microbiota disorders, stimulation of IgA production, enhancing T-cell production and providing a shield against other enteric organisms through inhibiting their growth, secreting acidic metabolites, bacteriocins and other products with antimicrobial effects [10-13].

Lactobacillus plantarum (*L. plantarum*) possesses anti-pathogenic ability against various pathogens e.g. *Escherichia coli*, *Shigella* and *Salmonella* [14]. It also shows immune-modulatory action, tissue repair, and angio-genic properties; suggesting that its use in the treatment of infected wounds is reasonable [15]. In this study we aimed to investigate the invitro effect of *L. plantarum* on two of the main extracellular virulence determinants of *P. aeruginosa* (pyocyanin pigment production and elastase enzyme activity).

Methods

Study population, setting, and data collection

The present study was carried out on forty pathogenic *P. aeruginosa* strains isolated from wound pus collected from patients admitted to Surgical and Burn Units of Tanta University Hospitals using sterile cotton swabs. The laboratory identification and procedures were conducted in Medical Microbiology and Immunology Department, Faculty of Medicine, Tanta University. Ethical approval for this study (no. 31186/11/2016) was provided by the Ethics and Research Committee, Tanta Faculty of Medicine. Written informed consents were obtained from all participants in this research. A code number was put to each sample for adequate provision to maintain privacy of participants and confidentiality of data.

Subjects

The study included clinically suspected patients who showed evidence of local signs for wound infection (Redness, hotness, swelling, purulent discharge or delayed healing of wound) and/or systemic manifestations (fever, chills, or hypotension) with no other apparent source of infection except the wound. Patients showing any other apparent or suspected source of infection were excluded.

Specimen collection

All samples were collected with sterile swabs in sterile containers under complete aseptic conditions. Swabs were introduced into the depth of lesion and rolled to aspirate pus or exudation from the wound. Samples were transported as soon as possible to Medical Microbiology and Immunology Department, Faculty of Medicine, Tanta University.

Specimen processing

Swabs were cultured on nutrient agar and MacConkey agar (Oxoid, UK) for evaluation of the colony size, shape, edge, color, opacity, elevation and surface. The organisms showing characteristic colony morphology of *P. aeruginosa* were obtained followed by microscopic identification by Gram stained films to clarify the morphology of the bacterial cells (size, shape and arrangement). *P. aeruginosa* was identified as Gram-negative bacilli of variable size, non-sporing and non-capsulated. Biochemical identification was performed with a simplified scheme of biochemical tests such as sugar fermentation test, triple sugar iron test (to confirm its non-fermentation activity) and oxidase test [16].

Preparation of *Lactobacillus plantarum* strain

A control strain of *L. plantarum* was kindly supplemented by Microbiology Department, Faculty of Pharmacy, Tanta University. It was

grown on blood agar at 37°C in a candle jar overnight. The *L. plantarum* colonies were identified as very minute alpha hemolytic colonies, Gram positive bacilli and catalase test negative [16].

- **Pyocyanin extraction:** The compound was isolated from cellular debris in an aqueous suspension formed by the addition of chloroform. Because of the unique pigmentation this compound was quantified with a spectrophotometer before and after addition of *L. plantarum*. All isolated *P. aeruginosa* strains which had the ability of pyocyanin pigment production were grown in presence and absence of *L. plantarum* for detection of ability of *L. plantarum* to interfere

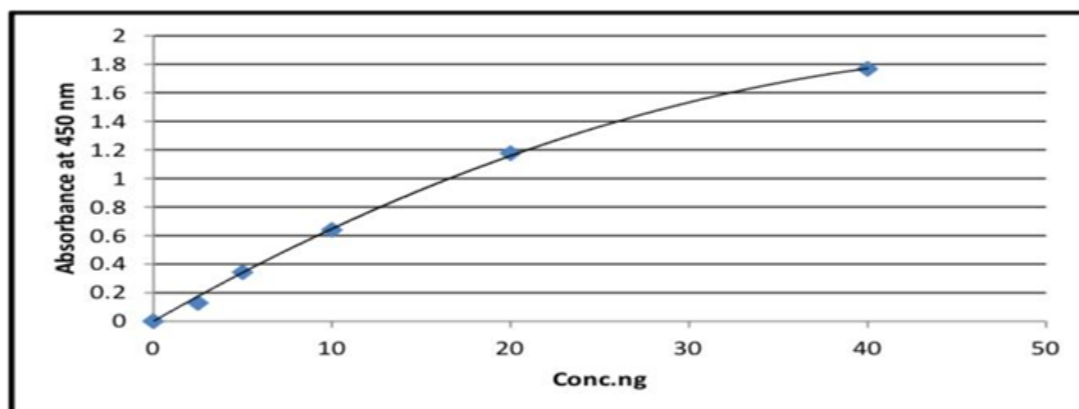
with pyocyanin pigment production before and after its addition [17].

- **Elastase assay:** The elastolytic activity of the isolated strains were investigated by measuring elastase levels in all samples, before and after addition of *L. plantarum* using Elastase ELISA kit (Assay Kit Co., LTD) which is based on a standard sandwich enzyme-linked immunosorbent assay technology [18]. Steps were followed according to manufacturers' instructions and O.D. absorbance was measured at 450 nm by an ELISA reader as shown in **table (1)** and **figure (1)**. A standard curve was drawn and elastase concentration in the samples was determined before and after addition of *L. plantarum*.

Table 1. Typical standards of the Elastase ELISA Kit.

Conc. ng/ml	2.5	5	10	20	40
Absorbance 450 nm	0.128	0.343	0.638	1.176	1.767

Figure 1. Standard curve Elastase ELISA Kit.



Statistical analysis

The raw data were coded, entered and analyzed using SPSS system files (SPSS package version 18).

Results

Pyocyanin production of *P. aeruginosa* isolates before and after addition of *L. plantarum*

out of the 40 *P. aeruginosa* isolates only 20 isolates showed pyocyanin production). The pyocyanin production in *P. aeruginosa* isolates was significantly decreased after addition of *L.*

plantarum compared to pyocyanin production before addition of *L. plantarum* (p value < 0.01*). This is shown in **table (2)** and **figure (2)**.

Elastolytic activity of *P. aeruginosa* isolates before and after addition of *L. plantarum*

The elastolytic activity of *P. aeruginosa* isolates significantly decreased after addition of *L. plantarum* compared to elastase production before addition of *L. plantarum* (p value < 0.01*). This is shown in **table (3)** and **figure (3)**.

Table 2. Comparison between pyocyanin production of *P. aeruginosa* isolates before and after addition of *L. plantarum*.

Effect of pyocyanin production	Before	After	<i>p</i> value
Mean \pm SD	31.9 \pm 13.19	23.11 \pm 8.07	<0.01*

Figure 2. Comparison between pyocyanin production of *P. aeruginosa* isolates before and after addition of *L. plantarum*.

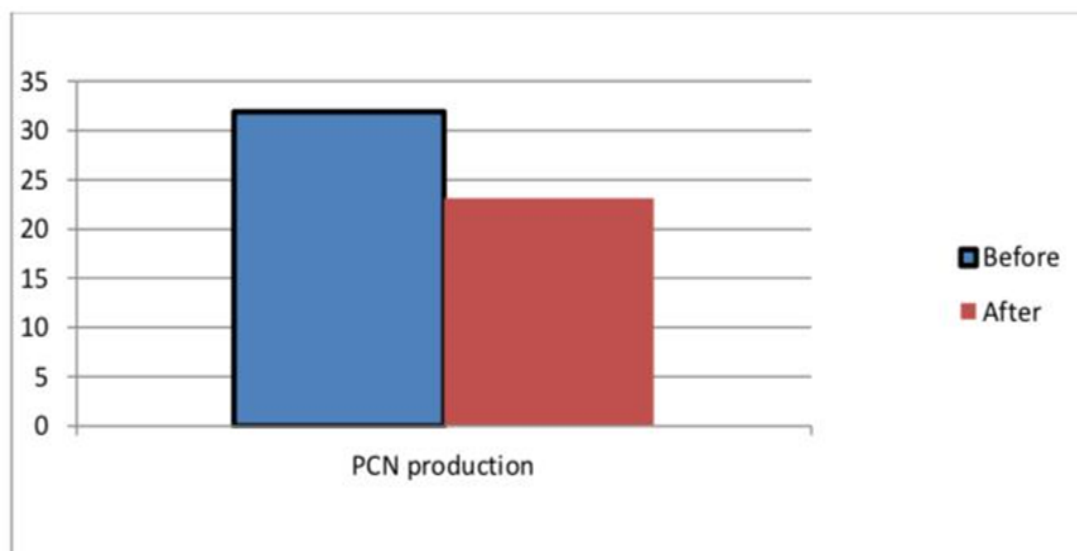
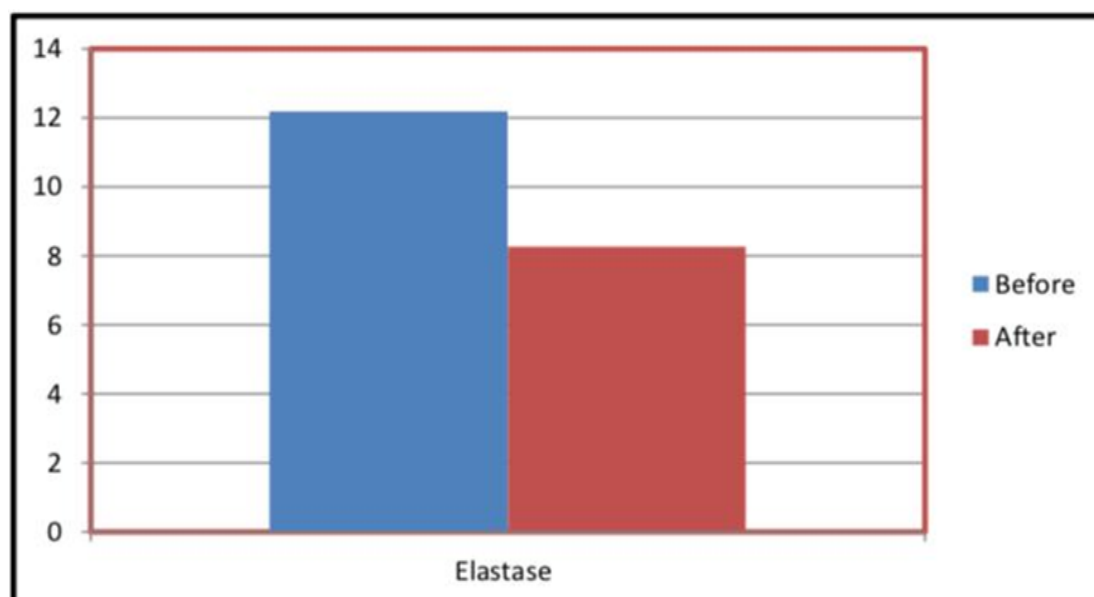


Table 3. Comparison between elastolytic activity of *P. aeruginosa* isolates before and after addition of *L. plantarum*.

Effect on elastase activity	Before	After	<i>p</i> value
Mean \pm SD	12.18 \pm 9.14	8.27 \pm 6.43	0.007 (<0.01*)

Figure 3. Comparison between elastolytic activities of *P. aeruginosa* isolates before and after addition of *L. plantarum*.



Discussion

Multidrug resistant infections caused by virulent *P. aeruginosa* strains are increasing worldwide and the urgent need for novel yet nontoxic available antimicrobial agent is clear and mandatory.

This study investigated the invitro effect of the probiotic, *L. plantarum*, on two important *P. aeruginosa* virulence factors; we revealed that both pyocyanin production and the elastolytic activity in the examined *P. aeruginosa* isolates significantly decreased after addition of *L. plantarum*. Our results are concurrent with results of an Argentinean study [15] which also reported that both pyocyanin production and elastase enzyme concentration was significantly inhibited by *L. plantarum* suggesting that this significant inhibition of these two vital virulence factors was attributed to interference with the quorum-sensing (QS) mechanism which regulates the production of both pyocyanin and elastase enzyme as well as having a significant role in bacterial growth. Many recent studies have also confirmed *L. plantarum* role in controlling QS process exerting an inhibitory function against virulent strains of *P. Aeruginosa* and other related species e.g. *Aeromonas* [19-21].

For further clarification, another Egyptian study [22] reported that *L. plantarum* showed the capacity not only to inhibit the bacterial growth *P. aeruginosa* but also to interfere with the biological activity of acyl-homoserine lactone hormone responsible of the QS process [23] making it logical to observe an inhibition in the production of pyocyanin and elastase enzyme.

Moreover, our study is in partial agreement with another study [24] which reported significant inhibition of the elastolytic activity of *P. aeruginosa* after addition of *L. plantarum*. The inhibitory effect shown was associated with the inhibition of QS molecules, but an inhibitory action of *L. plantarum* secondary metabolites on elastase couldn't be ruled out.

On the other side, a French study [13] reported that when more than eighty *Lactobacilli* isolates were tested for their capacity to inhibit the elastolytic activity of *P. aeruginosa* control strain (PAO1), only five *Lactobacilli* strains could significantly reduce the activity of elastase. The discrepancy in results may be explained by the different *Lactobacilli* strains used and also the authors of the previous study explained that PAO1

is sensitive to pH and acid in a dose-dependent manner, growth inhibition increase in parallel with any increase in acid concentration and pH decrease. For that reason, the inhibitory activities of *Lactobacilli* towards *P. aeruginosa* elastolytic activity were noticed in those five strains in particular as they could induce a much higher pH decrease while other strains were poorly acidifying.

Conclusion

This study investigated the effect of *L. plantarum*, one of the probiotics, on pathogenic *P. aeruginosa* which became a major cause of nosocomial infections facing healthcare systems. Probiotics are thought to be a safe and economic alternative to antimicrobial agents that became highly resistant and ineffective against many pathogens during the last decades. Based on our results we can conclude that the in-vitro activity of two of the main virulence factors *P. aeruginosa*'s was significantly diminished after addition of *L. plantarum*. It is possible that in near future it can be used as an in-vivo alternative medicine for the treatment of *P. aeruginosa* infections. However, there were some limitations of this study. Firstly, the study could have been generalized, if further samples were collected from other departments beside the surgical department as further sources of *P. aeruginosa* infection. Moreover, it would be more conclusive, if we investigated larger number of patient samples for better statistical analysis.

Conflict of interest

The authors report no conflicts of interest.

Authors' Contribution

All authors contributed equally to this work.

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