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Molecular Study of Antimicrobial Bacteriocins Isolated from *Lactobacillus* on *Pseudomonas aeruginosa*

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Abstract

Out of 50 samples of yogurt and vaginal swabs, 20 isolates of *Lactobacillus* (LAB) were isolated for the current investigation. Additionally, the bacteriocin produced by lactic acid bacteria that produced against *Pseudomonas aeruginosa* bacteria was screened. The results of morphological, microscopical, and biochemical tests used to identify these isolates showed that they are all of the *Lactobacillus* genus. Moreover, screening was performed for the best producer isolate and the results showed the yogurt-isolated *Lactobacillus* bacterium was the best bacteriocin activity according to screening results for the best producer isolate. To isolate the *P. aeruginosa*-tested bacterium about 100 urine samples from females patients at AlBatool teaching hospital (Diyala/Iraq) with ages ranging from 19 to 40 years were gathered. 34 investigated bacteria in all were isolated. When two antibiotics (Piperacillin 5 mg and Ciprofloxacin 10 mg) were tested for antibiotic susceptibility using the agar disk diffusion method, all isolates appeared to be resistant to Piperacillin and 16% to Ciprofloxacin. Using the micro-titer plate method on four different isolates of *P. aeruginosa*, both with and without bacteriocin treatments, the producing biofilm was observed. The results showed that bacteriocin treatment resulted in a decrease in biofilm thickness (i.e., biofilm absorbency). Additionally, the minimum inhibitory concentration (MIC) of bacteriocin, determined using the good diffusions method, was 12.5 mg/ml.

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The expression of genes *PA-ss* (species-specific gene) and *Ps1A* (polysaccharide synthesis locus) before and after bacteriocin treatment was compared using reverse transcription-quantitative quantitative polymerase chain reaction (RT-qPCR). The findings indicate that this gene's folding was reduced following bacteriocin treatment, indicating that bacteriocin inhibits the gene expression in the bacteria that form biofilms to isolate them.

Keywords: *P. aeruginosa*, Biofilm, *Lactobacillus*, bacteriocin, *PA-ss and Ps1A* gene, RT-qPCR.

دراسة جزيئية للتأثير الضد مايكروبي للبكتريوسين المعزول من العصيات اللبنية على الزائفة الزنجارية

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الخلاصة

تضمنت الدراسة الحالية عزل ٢٠ عزلة من بكتريا (LB *Lactobacillus*) من ٥٠ عينة من الزبادي والمسحات المهبلية لفحص النشاط المضاد للبكتيريا من العصيات اللبنية التي تنتج البكتيريوسين bacteriocin ضد بكتيريا الزائفة الزنجارية *Pseudomonas aeruginosa*. تم التعرف على هذه العزلات باستخدام الاختبارات المظهرية و المجهرية والكيميائية الحيوية ، وأظهرت النتائج أن جميع العزلات تنتمي إلى جنس *Lactobacillus*. علاوة على ذلك ، تم إجراء الفرز لأفضل عزلة منتجة للبكتريوسين وأظهرت النتائج أن بكتريا *Lactobacillus* المعزولة من اللبن كانت أفضل منتج للبكتريوسين واعلى نشاطا ضد بكتريا الزائفة الزنجارية *P. aeruginosa*. تم جمع حوالي ١٠٠ عينة بول من الإناث اللائي حضرن مستشفى البتول التعليمي بأعمار مختلفة من ١٩-٤٠ سنة لعزل بكتيريا الزائفة *P. aeruginosa* المختبرة. تم عزل ٣٤ عزلة من *P. aeruginosa* في هذه الدراسة و تم إجراء اختبار الحساسية للمضادات الحيوية باستخدام طريقة نشر قرص أجار ضد ٢ من المضادات الحيوية (بيبراسيلين ٥ ملغ وسيبروفلوكساسين ١٠ ملغ). اظهرت النتائج ان ١٠٠٪ من العزلات كانت مقاومة للبيبراسيلين و ١٦٪ اثبتت مقاومتها للسيبروفلوكساسين. تم فحص القدرة على إنتاج غشاء حيوي بطريقة microtiter plate method لأربعة من عزلات *P. aeruginosa* قبل وبعد المعالجة بالبكتريوسين, حيث أظهرت النتائج انخفاضًا في سمك الأغشية الحيوية (أي امتصاص الغشاء الحيوي) بعد العلاج بالبكتريوسين. أيضا ، تم إجراء التركيز المثبط الادنى MIC للبكتريوسين بطريقة الانتشار بالحفرة لتقييم أقل تركيز يثبط نمو البكتيريا والذي كان ١٢,٥ مجم / مل. تم استخدام النسخ العكسي لتفاعل البلمرة المتسلسل الكمي (RT-qPCR) لمقارنة التعبير الجيني لـ *PA-ss* (جين محدد للأنواع) و

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Ps1A (موضع تخليق عديد السكريد) قبل وبعد العلاج بالبكتيريوسين. أظهرت النتائج أن طي (folding) هذا الجين قد انخفض بعد العلاج بالبكتريوسين مما يعني أن البكتريوسين يثبط التعبير الجيني عن تكوين الغشاء الحيوي لبكتريا *Pseudomonas aeruginosa*

الكلمات المفتاحية: الزائفة الزنجارية, الغشاء الحيوي, بكتريا حمض اللاكتيك, البكتريوسين, تفاعل البلمرة المتسلسل الكمي

Introduction

The bacterium *Pseudomonas aeruginosa*, which is Gram-negative, is an opportunistic pathogenic cause of human illnesses, particularly urinary tract infections (UTI) [1]. In order for *P. aeruginosa* to live and develop antibacterial resistance, biofilm development is crucial [2]. Most bacterial issues are connected to biofilm [3]. According to Billings et al., [4] polysaccharide synthesis locus (*Psl*.) provides bacterial biofilm defenses against anti-microbial of many immature biofilm features, and the first gene *pslÁ* is the most important role in biofilm formation with regulation of *psl.* gene in cell[5].There are numerous recognized species of lactic acid bacteria (LAB) [6]. Following that, a species created bacteriocin, which has antifungal and antibacterial properties [7]. The current work used gene expression on *P. aeruginosa* biofilm genes (*PA-ss* and *Ps1A* gene) before and after bacteriocin treatments to assess the antibacterial effects of bacteriocin on *P. aeruginosa*

Materials and Methods

Sampling

Fifty samples were collected from Yogurt and vaginal swabs (25 from each) for isolation of *Lactobacillus* sp. One hundred samples were collected from urine for isolation of *P. aeruginosa* during the period from November 2021 to February 2022. All samples were collected from females of different ages who attended Al-Batool Teaching Hospital. The clinical diagnoses was done by a consultant obstetrician and gynecologist. The samples were cultured immediately after sampling for diagnostic purposes.

Lactobacillus isolation and identification

The vaginal swabs were streaked on Man Rogosa Sharp (MRS) agar whereas the yogurt samples was inoculated in MRS broth 1 ml of yogurt in 9 ml broth) then incubated at 37ºC for 48hrs using microaerophilic conditions. Then, pure isolates were subjected to identification as macroscopic and microscopic appearance and those isolates readily identified as Gram-positive rods, catalase negative and oxidase negative [8].

Isolation and identification of *P. aeruginosa*

The collected samples were cultured after collection on blood and MacConkey agar and incubated at 37°C for 24hrs, then the developing bacterial colonies were transported to Pseudomonas agar and incubated at 37°C for 24hrs. The isolates that carry *P. aeruginosa* attributes are then picked up for further macroscopically, microscopically, and biochemical tests**.**

Detection of Biofilm

P. aeruginosa isolates were tested for their capability to produce the biofilm by using microtiter plate method, according to [9].

Antibiotics susceptibility test

The disk diffusion susceptibility method for antibiogram testing (Kirby- Bauer method) was performed according to the Clinical and Laboratory Standards Institute (CLSI) [10]. Antibiotic discs (Bioanalyse – Turkey) used in this study includes Ciprofloxacin (CIP) and Piperacillin (PRL).

Lactobacillus screening for bacteriocin production:

Primary screening by agar-plug diffusion method

Primary screening was done by using agar-plug diffusion method according to Garcia, E.F.*et al*., [11].

Secondary screening by agar-wells diffusion method

Agar-wells diffusion method were done for secondary screening according to Biyari, S. and Fozouni, L. [12].

Bacteriocin activity assay

To quantify the bacteriocin activity, crude bacteriocin was serially diluted two-fold using saline solution. These dilutions were used to examine the antibacterial activity of bacteriocin against the examined bacteria by agar well diffusion assay (as previously described). Bacteriocin activity was expressed as AU/ml and defined as the reciprocal of the highest dilution showing a distinct inhibition zone of the yeast under study. AU was calculated as: (1000 / 100) × D, where 1000: constant, 100: volume of supernatant in a well (µl), and D : the dilution factor [13].

Partial purification of bacteriocin

MRS broth was inoculated with the bacterial isolate and incubated at 37°C for two days. Cells were harvested by centrifugation at 6000 rpm for fifteen minutes. Cell-free supernatant (CFS) was heated at 80°C for 10 minutes, then cooled and centrifuged at 6000 rpm for 15 minutes. The supernatant was mixed thoroughly with n-butanol at a ratio of 1:1.The mixture was centrifuged at 4000 rpm for 10 minutes to achieve phase separation. The organic phase was evaporated at 65°C by rotary evaporator, then the sediment was re-suspended in 1.0 mM sodium phosphate buffer (pH 6) and referred to as partially purified bacteriocin (PPB) [14]. The antimicrobial activity of bacteriocin was determined, as previously described, by the agar well diffusion method.

Extraction and purification of bacteriocin

The bacteriocin was extracted and purified using three steps: ammonium sulfate precipitations, ion exchange chromatography, and gel filtration.

Determination of (MIC) of bacteriocin

Serial dilutions were performed for bacteriocin and well diffusion method was used to determine the minimum concentration of bacteriocin that inhibits the *P. aeruginosa* growth. The results have been determined by the inhibition zone formation and measured the diameter of the zone.

Molecular study -Quantitative RT-PCR

Detection of *PA*-ss and *psl*A

A- Extraction of DNA and Polymerase Chain Reaction

Amplification DNA was extracted from 3 *P. aeruginosa* selected isolates according to the protocol of ABIOpure Extraction. The purity and concentration of the DNA were estimated by Nanodrop.

B- Primer Selection

Specific primers that were needed for amplifying a fragment of *PA*-ss and *psl*A are listed in Table- 1. Primers were provided in a lyophilized form and dissolved in sterile nuclease-free water to give a final concentration of 100 pmol /µl. Afterward, they were stored in a deep freezer until use.

Table 1: Primers used in *Pseudomonas* gene detection

Primer Name	Sequence 5`-3`	Annealing Temp. (°C)	Product size (bp)
pslA-F	CACTGGACGTCTACTCCGACGATAT	55	1119
pslA-R	GTTTCTTGATCTTGTGCAGGGTGTC		
PA-SS-F	GGGGGATCTTCGGACCTCA	58	956
PA-SS-R	TCCTTAGAGTGCCCACCCG		

Gene expression

A- RNA Extraction from *P. aeruginosa* isolates

RNA was isolated from *P. aeruginosa* planktonic cells using Trizol reagent (Promega, USA) according to the protocol described by the manufacturer.

B- Quantitative reverse transcription-PCR

The outcome was compared to 16S rRNA. Quantifying expression levels and comparing differences in ΔCt and fold changes between treated and untreated groups for specific genes [15].

Results and Discussions

Identification of *Lactobacillus*

About 15 (30%) isolates of *Lactobacillus* were obtained from vaginal swabs cultured on MRS agar after the incubation period and 5 (20%) isolates from yogurt samples as in table 2, these isolates were identified microscopically and biochemically. *Lactobacillus* isolates appeared as Gram-positive rods, non-spore-forming having white colored, soft, large or small, convex, creamy, smooth, and circular colonies on MRS agar, oxidase-positive and catalase-negative.

Identification of *P. aeruginosa*

A total of 34 isolates of *Pseudomonas aeruginosa* were isolated from urine samples. Morphologically all of these isolates were gram negative, non-sporing, capsulated, and motile bacilli, produced typical grapes like odor. They were also positive for oxidase, pyocyanin production as well as growth test at 4 and 42°C. To confirm the identification, the isolates were cultured on *Pseudomonas* base agar selective medium and the result was glamorous green colonies with distinctive odor proof that the identified isolates, table 2.

Table 2: The frequency of bacterial isolates under study.

Samples	No. of samples	No. of positive isolates	Frequency %
Vaginal swabs	30	15	30 %
Yogurt	20	5	10 %
Total	**50**	**20**	**40 %**
Urine	100	34	34 %

The results showed that all isolates were resistant to Piperacillin and only 4 (11.8%) isolates were resistant to Ciprofloxacin, this is due to the ability of *P. aeruginosa* to produce beta-lactamase such as the enzymes of the Extended-spectrum β-lactamase (ESβLs), which work on the analysis of penicillin's and cephalosporins in particular, whose genes are either on

chromosomes or on plasmids in many types of bacteria, which leads to multiple resistance to various antibiotics . Modifying the structure of the proteins binding to penicillin PBPs, which are the primary target of beta-lactam antibiotics of Abd and Luti [16].

After primary and secondary screening for bacteriocin production, the results showed that yogurt samples were the best and higher activity bacteriocin producer and one isolate was picked up for further purification and extraction. The purification steps of bacteriocin are depicted in Table 3.

Table 3: Purification and activity of bacteriocin produced by *Lactobacillus*

Steps of purification	Volume of bacteriocin (ml)	Activity (Au/ ml)	Protein Con. (mg/ml)
Crude extract	200	640	34.96
Ammonium sulfate precipitate 80%	50	1280	32.28
Ion exchange	15	2560	31.45
Sephadex -S-1000 for gel filtration	15	2560	22.82

AU: (Arbitrary unit) was expressed as the reciprocal highest dilution inhibiting the growth of the indicator strain.

The bacteriocin of *Lactobacillus* sp. was recovered with an increase in specific activity from 640 to 2560 AU/mg after precipitation by 80% saturation of the culture broth with ammonium sulphate. These results agreed with findings of Mojgani *et al* [17], and Ogunbanwo *et al* [18].reported that the increase in activity could be due to release of active monomers from bacteriocin complexes.

MIC is used to assess the antimicrobial capability of bacteriocins against four isolates from each tested isolate with a concentration of bacteriocin about 100mg/ml to 1.5 mg/ml for each using well diffusion method. It was found that at bacteriocin concentrations 12.5mg/ml is the lowest concentration that inhibits the growth of bacteria.

At the present study; all *P. aeruginosa* were biofilm producers with variable strength value depended on the optical density (OD) value with using crystal violate. Treating the biofilm

formed by *P. aeruginosa* with crude and purified bacteriocin caused a reduction in absorbency (i.e. biofilm thickness). The difference between the results of the current study and the previous studies may be due to the different components used in the medium, microbial concentration, or incubation period (24 hours), as the cell density increases in the biofilm as the incubation period increases, and the concentration of the dye affects the results as the 0.5% concentration gives better results when compared to 1% [19] .

Concentration and purity of DNA for bacteria:

The bacterial genomic DNA was extracted from over-night cultures of isolates. It was found that the purity ranged from 1.88 to 2.01 ng/µl.

The DNA of the three *P. aeruginosa* isolates (p14, p18 and p21) was extracted by using the (DNA extraction kit). Measurement of the concentration of DNA samples was found by Quantus Fluorometer. The concentration for all the twelve DNA samples was between (25, 28 and 24) ng/µl respectively. These concentrations were sufficient amounts for PCR amplification.

Three *P. aeruginosa* isolates from urine were subjected to PCR (Polymerase Chain Reaction) for the identification of two virulence genes (*PSIA* and *PA-SS*). Based on the annealing temperature and the molecular weight of the products (amplicon), the amplified virulence genes were identified.

The purpose of this step is to measure the expression of virulence genes (*PA-ss* and *PS1A*) for *P. aeruginosa* genes and compare the gene expression before and after bacteriocin treatment. In this study only three *P. aeruginosa* isolated from urine were choose contain the virulence genes relative quantification expression ratios of these genes were measured in comparison to the housekeeping gene (*fbp*) using RT- qPCR technique. The isolates were characterized by different sub-MIC levels of bacteriocin.

The folding value (gene expression) for each of the virulence genes was determined as dependent on the mathematical models is very widely applied (ΔΔCt model) which is known also as Livak equilibrium.

Table 4: *P. aeruginosa* biofilm gene expression values (*PA-ss*).

Sample	fbp	PA-ss	DCT	DDCT	Folding
P14	27.42	24.94	-2.48	0.00	1.00
P18	23.98	23.65	-0.32	0.00	1.00
P21	26.86	25.80	-1.06	0.00	1.00
PB14	30.20	29.84	-0.35	2.13	0.23
PB18	30.45	29.86	-0.58	-0.26	1.20
PB21	30.26	28.29	-1.98	-0.91	1.88

P= *Pseudomonas* isolates before bacteriocin treatment, PB= *Pseudomonas* isolates after bacteriocin treatment

Table 5: *P. aeruginosa* biofilm gene expression values (*PS1A*).

Sample	Fbp	PS1A	Dct	ddct	Folding
P14	27.42	32.526	5.105	0.000	1.000
P18	23.98	23.832	-0.146	0.000	1.000
P21	26.86	27.258	0.395	0.000	1.000
PB14	30.20	31.179	0.984	-4.121	17.406
PB18	30.45	30.114	-0.334	-0.188	1.139
PB21	30.26	31.168	0.904	0.508	0.703

P= *Pseudomonas* isolates before bacteriocin treatment, PB= *Pseudomonas* isolates after bacteriocin treatment

Although all biofilms were affected by bacteriocin at sub-MIC, the results of fold change presented a wide variation. For instance, the gene (*PS1A*) have suffered an increase in the isolate P14 and P18 after bacteriocin treatment. Nevertheless, the isolate P21 showed a decrease in fold change of *pslA* table 4 and 5. On the other hand, the gene (*PA-ss*) suffered a decrease in folding after bacteriocin treatment in P14 isolate and an increase in both P18 and P21 isolates.

The results of the current study were somewhat in agreement with the previous study of Maita and Boonbumrung [16], who revealed that, the biofilm formation is accompanied by drastic changes in gene regulation.

Conclusions

Lactobacillus sp. provided a good source of bacteriocin. Bacteriocin that was extracted from yogurt was more effective than vaginal isolates when it was isolated from yogurt and vaginal swab. Only 4 *P. aeruginosa* isolates were CIP-resistant, but all isolates were PRL-resistant. After being treated with bacteriocin, *P. aeruginosa* isolates showed decreased gene expression for the genes involved in biofilm formation.

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