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ANTI- PYOCYANIN AND ANTI- HEMOLYSIN IMPACT OF SOME NATURAL AND COMMERCIAL PROBIOTICS AGAINST MULTIDRUG- RESISTANT *Bseudomonas aeruginosa*

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ABSTRAC

The aim of this study is to evaluate the anti-pyocyanin and anti-hemolytic efficacies of lactobacilli probiotics isolated from natural and commercial sources against pathogenic *Pseudomonas aeruginosa (P. aeruginosa)*.

Two isolates of *P. aeruginosa* (P50 and P78), which were determined as multidrug-resistant bacterial strains, were obtained from one hundred-thirty samples, including burn, wound, ear, sputum, and urine, collected from patients attending a number of hospitals in Baghdad, including Al-Karkh Hospital, Al Kadimyia Teaching Hospital, and Medical City Hospitals (Al-shahid Ghazi Al-Hariri Hospital for Surgical Specialties, Baghdad Teaching Hospital and Burns Specialized hospital) during the period of October and December 2022. The direct antibacterial effect of undiluted CFCS (cell-free culture supernatants) of *Lactobacillus* isolates and their anti-pyocyanin and anti-hemolytic were assessed.

The antibacterial effect of a natural isolate of *Lactobacillus acidophilus (L. acidophilus)* was higher than that of other isolates. The findings revealed that spontaneously isolated lactobacilli outperform commercially isolated lactobacilli regarding probiotic characteristics. All Lactobacillus isolates showed a strong anti-hemolytic and anti-pyocyanin effects against *P. aeruginosa* at 100 µg/ml, but this effect is not confirmed due to their antimicrobial activity against both strains of *P. aeruginosa*. Also, both isolates of *Lactobacillus plantarum (L. plantarum)* (natural and commercial) did not show an anti-hemolysin effect against both isolates of *P. aeruginosa* P50 and P78. The findings supported using probiotics instead of antibiotics to treat antibiotic-resistant pathogenic bacteria and, consequently, various disorders linked with this pathogenic bacterium.

Key words: Pyocyanin, Hemolysin, Pseudomonas aeruginosa, Probiotics.

[*]**The article is taken from the master's thesis of the first researcher.**

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التأثير المضاد للبيوسيانين والهيموليسين لبعض المعززات الحيوية الطبيعية والتجارية ضد عزلات الزائفة الزنجارية المقاومة للمضادات الحيوية

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الخلاصة

هدف الدراسة هو تقييم الفعالية المضادة للبيوسيانين والمضادة لانحلال الدم للمعززات الحيوية من العصيات اللبنية المعزولة من المصادر الطبيعية والتجارية ضد الزائفة الزنجارية المسببة للأمراض.

تم الحصول على عزلتين من الزائفة الزنجارية (P50 و P78)، والتي تم تحديدها على أنها سلالات بكتيرية مقاومة للأدوية المتعددة، من مائة وثلاثين عينة، شملت الحروق والجروح والأذن والبلغم والادرار، تم جمعها من لمرضى المترددين على عدد من المستشفيات في بغداد، بما في ذلك مستشفى الكرخ ومستشفى الكاظمية التعليمي ومستشفيات المدينة الطبية (مستشفى الشهيد غازي الحريري للاختصاصات الجراحية، مستشفى بغداد التعليمي ومستشفى الحروق التخصصي) خلال فترة تشرين الأول وكانون الأول 2022. تم تقييم التأثير المضاد للبكتيريا المباشر لمعلق النمو الخالية من الخلايا لعزلات (*Lactobacillus*) وقابليتها كمضادات للبيوسيانين ومضادات الهيموليسين.

كان التأثير المضاد للبكتيريا لعزلة (*L. acidophilus*) الطبيعية أعلى من العزلات الأخرى. كشفت النتائج أن العصيات اللبنية المعزولة من مصادر طبيعية تتفوق على العصيات اللبنية المعزولة من مصادر تجارية فيما يتعلق بخصائص المعززات الحيوية. أظهرت جميع عزلات العصيات اللبنية تأثيرًا قويًا مضادًا للبيوسيانين والهيموليسين ضد بكتيريا الزائفة الزنجارية عند 100 ميكروغرام/مل، ولكن لم يتم تأكيد هذا التأثير بسبب نشاطها المضاد للميكروبات ضد كلتا السلالتين من الزائفة الزنجارية. أيضًا، لم تظهر عزلتان (*L. plantarum*) (الطبيعية والتجارية) تأثير مضاد للهيموليزين ضد كل من عزلات P50 و P78.

دعمت النتائج استخدام المعززات بدلًا من المضادات الحيوية لعلاج البكتيريا المسببة للأمراض المقاومة للمضادات الحيوية، وبالتالي الاضطرابات المختلفة المرتبطة بهذه البكتيريا المسببة للأمراض.

الكلمات المفتاحية: البيوسيانين، الهيموليسين ، الزائفة الزنجارية ، المعزز الحيوي.

INTRODUCTION

Pseudomonas aeruginosa is considered an opportunistic pathogenic bacterium in humans, animals, and plants as well and this bacterium can exist in patients with immunocompromised diseases and can develop their resistance to antimicrobial agents **(Jeong *et al.*, 2023)**. This bacterium invades and destroys host tissues by producing a wide variety of toxic chemicals, pigments, and enzymes, including pyocyanin and hemolysin **(Allam *et al.*, 2020)**. Pyocyanin may cause an increase in intracellular Reactive Oxygen Species (ROS) and H2O2, causing oxidative stress and disrupting the cell cycle, enzymes, and DNA, ultimately leading to cell lysis **(Hall *et al.*, 2016)**. Hemolysins are a class of proteolytic enzymes that are responsible for red blood cell lysis and the disruption of lecithin and fat (**MKK *et al.*, 2019**).

These virulence factors provided specific properties, which increase the pathogenicity of *P. aeruginosa*. Hence, making these traits a target of treatment may contribute to reducing the severity of diseases caused by these bacteria. The rise of multidrug resistance is closely tied to the absence of novel and potent antimicrobial agents. Globally, efforts have been underway to develop advanced antibacterial drugs and innovative delivery methods **(AL-jumaily & Turkie, 2018).** As a result, there's a growing interest in exploring natural sources, such as medicinal plant extracts, and probiotics to discover alternative antibacterial and anti-virulence compounds **(Mohammed *et al.*, 2017).**

There is a broad trend towards the use of natural compounds as anti-pyocyanin and anti-hemolytic agents. One of these notable agents is probiotics, which are defined as "live

bacteria which when provided in suitable proportions confer a health benefit for the host" according to the World Health Organization (WHO) and the Food and Agriculture Organization (FAO). These natural living organisms include bacteria, such as lactobacilli genus **(Rasheed *et al*., 2020)**. These probiotics are characterized by their ability to prevent the pathogens' attachment to the cells of the host as well as competing with other microorganisms on the nutrients, which are limited and important for their growth. Also, these probiotics can produce antimicrobial compounds, such as organic acids, H2O2, and bacteriocins (**Hussein & Luti, 2020)**.

When considering these properties, there is a possibility of using these living organisms as an anti-pyocyanin and anti-hemolytic agent. Thus, the present work aimed to estimate the anti-pyocyanin and anti-hemolytic efficacies of some natural and commercial lactobacilli isolates, that are considered effective probiotics, against pathogenic *Pseudomonas aeruginosa.*

MATERIALS AND METHODS

1. Bacterial isolates

Two isolates of *P. aeruginosa* (P50 and P78), which were determined as multidrug-resistant bacterial strains, were obtained from one hundred-thirty samples, including burn, wound, ear, sputum, and urine, collected from patients attending a number of hospitals in Baghdad, including Al-Karkh Hospital, Al Kadimyia Teaching Hospital, and Medical City Hospitals (Al-shahid Ghazi Al-Hariri Hospital for Surgical Specialties, Baghdad Teaching Hospital and Burns Specialized hospital) during the period of October and December 2022. In addition to these pathogenic bacterial isolates, two isolates of Lactobacillus spp., including *L. plantarum* and *L. acidophilus*, were obtained from thirty-one samples of dairy products, including dried yogurt, sweet cheese, and Arabic cheese, during the period of January and April 2023. Also, two isolates of Lactobacillus spp., including *L. plantarum* and *L. acidophilus*, were obtained from commercial probiotics, which were collected from some pharmacies. All bacterial isolates were identified using different microscopically, cultural, and biochemical tests and the VITEK2 system.

2. Antibiotics susceptibility test (AST):

Kirby-Bauer method (disk diffusion method) was performed to determine the antimicrobial susceptibility as described by **(Höring *et al*., 2019)** and the results were determined according to Clinical Laboratory Standard Institute recommendations **(CLSI, 2022)**, and confirmed by Vitek 2 System **(Yoo *et al*., 2020).**

3. Preparation of *Lactobacillus* isolates cell-free supernatants (CFCS)

In accordance with **(Sousa *et al*., 2008),** the cell-free supernatants were prepared as follows: Bacteria were cultivated to the mid-exponential phase in De Man, Rogosa and Sharpe (MRS)broth for 24 hours at 37 °C in anaerobic conditions after being extracted from an agar plate. McFarland standard no. 0.5 turbidity was used to modify the optical density of the standard cell solution. The supernatant was made by adding 0.1 ml of the standard cell suspension to a tube containing MRS broth and incubating the mixture for 24 hours at 37°C. Centrifugation (10,000xg for 15 minutes at 4 degrees Celsius), filtration through a sterile 0.22 m hole-size membrane, and subsequent plating on De Man- Rogosa – Sharpe Agar (MRS) agar revealed no lactobacilli growth. The inhibitory activity of this recently made cell-free supernatant (stock solution) was tested.

4. Antibacterial activity of *Lactobacillus* isolates

The screening of the antibacterial activity was performed by using the agar well diffusion method as described by **(Hindal & Ali, 2015)** as follows: In this method, culture was done on Mueller Hinton agar medium with a sterile swab from a suspension of *P.aeruginos* in a nutrient broth medium, and wells with 6 mm diameter that has been cut in Mueller Hinton agar plates. Different concentrations of cell-free culture supernatant (CFCS) (50µl) were placed into the wells of the Mueller Hinton agar and incubated at 37°C for 18 hours. Tthe sterile MRS broth was used as a negative control. The diameters of the clear zones resulting from growth inhibition were measured.

Determination of minimum **inhibitory concentration (MIC)**

Antimicrobial agents are measured by their minimum inhibitory concentrations (MICs), which are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The experiment was done according to **(Elshikh *et al*., 2016).**

5. Evaluation of Anti-pyocyanin potential

The plates with glycerol-supplemented nutrient agar (GSNA) medium was utilized. *P. aeruginosa* and the chosen Lactobacillus spp. were cultured and then put into the plates' medium after they had solidified. After incubation for 72 hours at 28 ^{o}C., plates were checked for the presence of pyocyanin-producing isolates. As the bacteria multiply on the substrate, a bluish-green halo forms surrounding each colony **(Jaleel & Al-Shaibani, 2017).** The results were indicated as the presence or absence of pyocyanin on an agar plate.

6. Evaluation of Anti-hemolysin potential

Both *P. aeruginosa* and *Lactobacillus* spp. bacterial isolates were cultivated on blood agar plates. The plates were then stored in an anaerobic jar in an incubator at 37 ^{o}C. for 48 hours. Hemolysis was seen on a blood agar plate after incubation, and the result was either positive (presence of hemolysis) or negative (absence of hemolysis) **(Pienz *et al*., 2014).**

STATISTICAL ANALYSIS

Data analysis was performed statistically using the R programming. Antibiotic resistance among the tested isolates was analyzed statistically using an analysis of variance (ANOVA). In addition, a Hierarchical Clustering analysis was used to restrict the sample size to isolates sharing a high degree of antibiotic resistance and/or being susceptible to the same classes of antibiotics. The degree of statistical significance was also determined by calculating the value of probability (p-value). Coefficient variation, mean and standard deviation (SD) was utilized to estimate the effect of direct CFCS on *P. aeruginosa* isolates **(Thomas *et al*., 2013)**.

RESULTS AND DISCUSSION

Based on the results of this test, both isolates of *P. aeruginosa* (P50 and P78) demonstrated resistance to all utilized antimicrobials. Both of these isolates were defined as MDR, which means that these bacterial isolates were resistant to three or more antimicrobial classes. This, in turn, complicates the choice of a suitable antimicrobial agent for treatment. The emergence of these MDR isolates is attributed to random overuse of antimicrobials **(Yayan *et al*., 2015)**. The results were illustrated in (Table 1); whereas these results *P. aeruginosa* exhibited diversity in resistance phenomena and indicated that isolates were resistant to Tobramycin, Levofloxacin, Lomefloxacin, Gentamycin, Norfloxacin and

Netilmicin with 52.33, 50, 50, 61.63, 48.84 and 53.49%. In addition, 52.33, 52.33, 50, 47.67, 52.33, 52.33, 51.16 and 50% of isolates were sensitive to Ciprofloxacin, Amikacin, Imipenem, Tobramycin, Levofloxacin, Doripenem, Meropenem, Azithromycin and Gemifloxacin. The half of isolates (50%) were resistant to Lomefloxacin and Ofloxacin and others were sensitive.

Table (1): Antibiotics susceptibility test for P. aeruginosa isolates by Kirby- Bauer Method.

Antibiotic	*P. aeruginosa*						*p*-value
	Resistance		Intermediate		Sensitive		
	No.	%	No.	%	No.	%	
Ciprofloxacin	39	45.35	2	2.33	45	52.33	0.081
Amikacin	41	47.67	0	0.00	45	52.33	0.086
Imipenem	40	46.51	3	3.49	43	50.00	0.078
Tobramycin	45	52.33	0	0.00	41	47.67	0.050 *
Levofloxacin	43	50.00	1	1.16	42	48.84	0.053
Lomefloxacin	43	50.00	0	0.00	43	50.00	0.053
Norfloxacin	42	48.84	2	2.33	42	48.84	0.065
Gentamicin	53	61.63	0	0.00	33	38.37	0.042 *
Doripenem	39	45.35	2	2.33	45	52.33	0.081
Ofloxacin	43	50.00	0	0.00	43	50.00	0.053
Netilmacin	46	53.49	0	0.00	40	46.51	0.047 *
Meropenem	40	46.51	1	1.16	45	52.33	0.092
Aztreonam	27	31.4	15	17.44	44	51.16	0.24
Gatifloxacin	42	48.84	1	1.16	43	50.00	0.065
*** (P≤0.05)**							

The direct antibacterial effect of undiluted CFCS (cell-free culture supernatants) of Lactobacillus isolates was assessed. The cells of Lactobacillus isolates were incubated with pathogenic *P. aeruginosa* for 24 hours at 37°C. The results indicated that the antibacterial effect of a natural isolate of *L. acidophilus* was higher than that of other isolates with mean ± SD (21.0 ± 1.0) mm, followed by commercial *L. acidophilus* with mean ± SD (17.0 ± 1.0) mm, natural *L. plantrum* with mean ± SD (14.3 ± 0.57) mm and commercial *L. plantarum* with mean ± SD (13.0 ± 0.00), as illustrated in (Figure 1 and Table 2).

Table (2): The antibacterial activity of Lactobacillus isolates against isolate of *P. aeruginosa.*

Isolate	No.	Mean of diameter (mm)	SD	SE	Coefficient of Variation
Commercial *L. acidophilus*	3	17.000	1.000	0.577	0.059
Commercial L. plantarum	3	13.000	0.000	0.000	0.000
Natural *L. acidophilus*	3	21.000	1.000	0.577	0.048
Natural *L. plantarum*	3	14.333	0.577	0.333	0.040

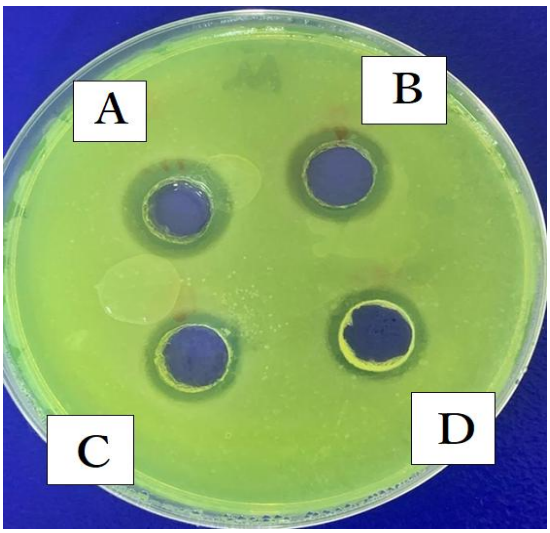

Figure (1): The antibacterial activity of *Lactobacillus* isolates against *Pseudomonas aeruginosa (*A: Natural *L. acidophilus,* B: Commercial *L.acidophilus,* C: Natural *L.platarum,* D: Commercial *L. platarum).*

The minimum dilution of CFCS required to inhibit the development of *P. aeruginosa* while using the evaluated *Lactobacillus* isolates was estimated. Table 3 demonstrates that the growth of *P. aeruginosa* P78 was most effectively restrained by the Minimum Inhibitory Concentration (MIC) and sub-MIC (12.5 µg/ml) of both natural *L. acidophilus* and natural *L. plantarum*. (Table 3); indicates that, for *P. aeruginosa* P50, the MIC and sub-MIC values for all tested Lactobacillus isolates were 50 and 25 µg/ml, respectively.

Table (3): The results of MIC of *Lactobacillus* spp. against *P. aeruginosa* isolates.

***P. aeruginosa* isolate**	**100**	**50**	**25**	**12.5**	***Lactobacillus* spp.**
P50	-	MIC	Sub MIC	-	Natural *L. acidophilus*
	-	MIC	Sub MIC	-	Commercial *L. acidophilus*
	-	MIC	Sub MIC	-	Natural *L. plantarum*
	-	MIC	Sub MIC	-	Commercial *L. plantarum*
P78	-		MIC	Sub MIC	Natural *L. acidophilus*
	-	MIC	Sub MIC	-	Commercial *L. acidophilus*
	-		MIC	Sub MIC	Natural *L. plantarum*
	-	MIC	Sub MIC	-	Commercial *L. plantarum*

The anti-hemolytic potential of the examined Lactobacillus isolates was evaluated. There was no clear anti-hemolytic activity of all lactobacilli strains, as shown in (Table 4). All Lactobacillus isolates showed a strong anti-hemolytic effect against *P. aeruginosa* at 100 µg/ml, but this effect is not confirmed due to their antimicrobial activity against both strains of P. aeruginosa. Also, both isolates of *L. plantarum* (natural and commercial) did not show an anti-hemolysin effect against both isolates of *P. aeruginosa* P50 and P78. This effect may be

attributed to the inhibition of bacterial growth of *P. aeruginosa* isolates at low concentrations of 50, 25, and 12.5 µg/ml (the last concentration only for natural L. acidophilus and L. plantarum against P78 isolate).

Table (4): The anti-hemolysin activity of lactobacillus isolates against *P. aeruginosa* isolates.

***P. aeruginosa* P50**							
lactobacilli isolates	**Bacterial growth**			**Hemolysin**			
	100	**50**	**25**	**100**	**50**	**25**	
Natural *L. acidophilus*	-	Low	+	-	-	-	
Commercial *L. acidophilus*	-	Low	+	-	-	-	
Natural *L. plantarum*	-	Low	+	-	+	+	
Commercial *L. plantarum*	-	Low	+	-	+	+	
***P. aeruginosa* P78**							
lactobacilli isolates	**Bacterial growth**				**Hemolysin**			
	100	**50**	**25**	**12.5***	**100**	**50**	**25**	**12.5**
Natural *L. acidophilus*	-	Low	+	+	-	-	-	-
Commercial *L. acidophilus*	-	Low	+		-	-	-	
Natural *L. plantarum*	-	Low	+	+	-	+	+	+
Commercial *L. plantarum*	-	Low	+		-	+	+	
Conc. 12.5 for natural *L. plantarum* and *L. acidophilus	**(+): Good growth (-): No growth**				**(+): Good hemolysis (-): No hemolysis**			

The anti-pyocyanin effect was investigated in the current study. The results were illustrated in (Table 5); and indicated that all Lactobacillus isolates exhibited a strong anti-pyocyanin effect against *P. aeruginosa* at 100 µg/ml. However, this effect is not confirmed due to their antimicrobial activity against both strains of *P. aeruginosa*. Also, both isolates of *L. plantarum* (natural and commercial) did not show an anti-pyocyanin effect against both isolates of *P. aeruginosa* (P50 and P78). This effect may be attributed to the inhibition of bacterial growth of *P. aeruginosa* strains to a low percentage at concentrations of 50, 25, and 12.5 µg/ml (the last concentration only for natural *L. acidophilus* and *L. plantarum* against P78 isolate).

Table (5): The anti-pyocyanin activity of lactobacilli isolates against tested *P. aeruginosa* P50, and P78.

***P. aeruginosa* P50**							
lactobacilli isolates	**Bacterial growth**			**Pyocyanin**			
	100	**50**	**25**	**100**	**50**	**25**	
Natural *L. acidophilus*	-	Low	+	-	-	-	
Commercial *L. acidophilus*	-	Low	+	-	-	-	
Natural *L. plantarum*	-	Low	+	-	+	+	
Commercial *L. plantarum*	-	Low	+	-	+	+	
***P. aeruginosa* P78**							
lactobacilli isolates	**Bacterial growth**				**Pyocyanin**			
	100	**50**	**25**	**12.5***	**100**	**50**	**25**	**12.5**
Natural *L. acidophilus*	-	Low	+	+	-	-	-	-
Commercial *L. acidophilus*	-	Low	+		-	-	-	

Natural *L. plantarum*	-	Low	+	+	-	+	+	+
Commercial *L. plantarum*	-	Low	+		-	+	+	
Conc. 12.5 for natural *L. plantarum* and *L. acidophilus	**(+): Good growth (-): No growth**				**(+): Good pyocyanin (-): No pyocyanin**			

In a study conducted by **(Khanmohammadi Otaghsara *et al*., 2020)**, *L. acidophilus* show antibacterial activity against *P. aeruginosa* isolate with a diameter (of 14 mm); this effect may attribute to that this bacterium can produce lactic acid, reduce pH, and produce antimicrobial compounds such as hydrogen peroxide, bacteriocins, ethanol, antibiotics, and other compounds **(Tilocca *et al*., 2020)**. It was demonstrated that both unmodified and heat-treated CFS produced by the commercial probiotic *L. acidophilus* were inhibitory towards *P. aeruginosa* **(Fredua-Agyeman & Gaisford, 2019)**. Moreover, the inhibition activity of bacteriocin obtained from *L. plantarum* against many pathogenic bacteria. In addition, bacteriocin inhibits closely related Lactobacillus strains and can affect yeast **(Lei *et al*., 2020).** The mechanism of the antibacterial activity of Lactobacilli may attribute to the production of biosurfactants and it may be associated with the producer's strain and biosurfactant type **(Ciandrini *et al*., 2016).**

It has been reported that the *L. plantarum* – containing crude extract exhibit anti-hemolytic activity **(Aguilar-Toalá *et al*., 2017)**. **(Soheili *et al*., 2019)** have determined the effect of natural antimicrobial compound 3-Phenyllactic acid (PLA) produced by *Lactobacillus* spp. on hemolysin. The results of the study showed that hemolysin was decreased in *P. aeruginosa* treated with PLA. In the study reported by **(Saroj *et al*., 2016)**, the viable cells of *Lactobacillus* spp. and their CFS inhibit hemolysin production of *Streptococcus pyogenes*. Also, this anti-hemolytic effect may attribute to some compounds that are produced by *Lactobacillus* spp. such as biosurfactants, which inhibited the hemolytic activity of *S. aureus* at 50 mg/ml, in the study conducted by **(Jiang *et al*., 2019)**. It is reasonable to believe that bioactive peptides from fermented milk extracts could be responsible for hemolysis prevention by building a surface coating on erythrocytes that would repair pores in the membrane caused by elevated temperature **(Aguilar-Toalá *et al*., 2017)**.

Probiotics have been demonstrated to suppress or kill pathogenic bacteria via a variety of mechanisms (such as toxin destruction). Some probiotic bacteria, however, are unable to use these pathways, which helps to explain why some probiotics are only effective against some pathogens and not others **(McFarland *et al*., 2018)**. According to **(Fuochi *et al*., 2019),** many of the postbiotics produced by Lactobacillus have either partial or complete characterization.

However, it has been speculated that each strain of Lactobacillus is capable of producing its own unique postbiotics with favourable effects on the host. The result showed that 3M004 cells/lysate inhibited biofilm and pyocyanin production of *P. aeruginosa* PA002 **(Liang *et al*., 2022)**. Different Lactobacillus spp. isolates have widely varying degrees of success in suppressing *P. aeruginosa*'s pyocyanin pigment synthesis. The clinical isolate of Lactobacillus spp., in comparison to the ambient strain, is distinguished by a greater capacity to create inhibitor compounds for the pigment. There is also no correlation between the amount of culture filtrate and the efficiency with which Lactobacillus isolates suppress pyocyanin formation **(Kasoob & Hummadi, 2022)**.

CONCLUSIONS

According to the results of this study, nosocomial infections and other disorders linked to multidrug-resistant *P. aeruginosa* may be treatable with the use of probiotics, including *L. acidophilus* and *L. plantarum*. Additionally, Lactobacillus spp. can act as effective anti-virulence agents (anti-pyocyanin and anti-hemolytic, respectively). Furthermore, the findings demonstrated that naturally isolated lactobacilli had better probiotic characteristics than those of commercially isolated lactobacilli.

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