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Brief Report

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Antagonistic Effect of Probiotics on Drug Resistant *Pseudomonas aeruginosa* Isolated From Burn Wound Infection

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Abstract

Background and Objectives: *Pseudomonas aeruginosa* is an opportunistic bacterium and is one of the main causes of infection in burn patients. As this bacterium gets resistant to various types of antibiotics through different acquiring mechanisms, it can easily induce skin deformities and even patients' death.

Methods: This descriptive study was carried out on 200 patients with burn injuries hospitalized in specialized burn hospitals. Culture methods and diagnosis tests were used to separate and diagnose *P. aeruginosa*, and disk diffusion Agar method with the Kirby-Bauer standard was applied to determine the pattern of drug-resistance. The antagonist effect of lactic bacteria isolated from 14 samples of milk and yogurt on the growth of *P. aeruginosa* was also assessed using Agar well diffusion method.

Findings: Out of 30 strains diagnosed and isolated as *P. aeruginosa* 45.2% were resistant to gentamicin, 51.6% to cefotaxime, 48.8 to imipenem, and 45.2 to ciprofloxacin. Study of probiotics revealed that *Lactobacillus casei*, with the average diameter of 20.3 mm, has the highest inhibitory effect against *P. aeruginosa*.

Conclusions: The results of this study showed that the spread of *P. aeruginosa* resistant to medicine is very high in the surveyed hospitals, and that the isolated lactobacilli have a significant inhibitory effect on drug resistant *P. aeruginosa* strains. Our results hence suggest that, using probiotic products would help better control of *P. aeruginosa* infection in burn patients.

Keywords: *Pseudomonas aeruginosa*, Drug resistance, Probiotic, Burn, Antagonistic effect

Background and Objectives

Burn is one of the most critical conditions in medicine that could hurt patients in all physical and mental aspects and involve them at any age. Wounds caused by burn are proper environments for growth of various bacteria species such as *Pseudomonas aeruginosa*.[1]

Pseudomonas aeruginosa is an opportunistic, gram-negative, aerobic bacterium which owns polar flagella and exotoxin, and is able to grow in all environments.[2,3] This bacterium has been diagnosed as the third cause for hospital infections and the second reason for infections of burn injuries.[4,5] Because patients become susceptible to infection due to injury of the skin, which is the first defense barrier of the body.[6] Unfortunately, due to excessive consumption of antibiotics, the spread of infection to hospital *P. aeruginosa* resistant to numerous antibiotics has been widely increased globally, causing various problems in treating infections resulting from these bacteria, especially in important hospital wards such as burn and intensive care units (ICUs).[7,8]

The acquiring resistance in this bacterium takes place through various mechanisms, including changes in cell membrane, production of beta-lactamases and efflux pumps.[9,10] Due to its special internal systems such as resistance transfer system, the bacteria have got resistant to various antibiotics, causing the spread of infection and septicemia in patients' bodies.[11] Moreover, treating the infections and selection of proper antibiotics for this bacterium is very difficult as it is resistant to many antimicrobial combinations and antiseptics such as ammonia, hexachlorophene, soaps and iodized solutions.[12] Making use of various antimicrobial combinations such as probiotics as a replacement for commonly-used antibiotics seems reasonable to help the treatment process of patients infected by *P. aeruginosa.*

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The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have defined probiotics as live micro-organisms whose proper consumption leaves positive effects on the host's body. These bacteria are able to produce antimicrobial combinations called bacteriocin, which have a wide range of effect against pathogens. With due regards to the proper antimicrobial effects of bacteriocins, it is likely that they will be used as supplements with new replacements for current antibiotics in the future. In recent years, antagonism of the bacteria which produce bacteriocin or the like, and their usage in controlling the growth of pathogen microbes have been considered so that in future they could replace chemical preservatives such as sulfur dioxide, benzoic acid, sorbic-acid, nitrate and nitrite.[13,14]

The word "bacteriocin" includes a wide range of proteins or antimicrobial extracellular ribosomal peptides known as bactericidal materials produced by bacteria which have inhibitory or cytotoxic effect on similar strains or dependent strains. In general, they affect cytoplasmic membrane and make holes in 2 phospholipids layers of membrane by creating proton-motive force. Their range of activity and their protein characteristics distinguish them from antibiotics.[15]

Most recent reports on bacteria generating these bacteriocins are related to the bacteria belonging to lactic acid group, especially *Lactobacillus, Lactococcus*, *Pediococcus*, *Leuconostoc*, and *Enterococcus*.[16-18] This study has been carried out with the aim to review the antibacterial effects of probiotic combinations over the growth of *P. aeruginosa* isolated from burn wounds in laboratory conditions.

Methods

Collecting *Pseudomonas aeruginosa* Samples

In this cross-sectional study, samples were collected from 200 patients hospitalized in burn Mazandaran province hospitals in January-May 2016. Specifications of the patients, including their age, gender and percentage of burn were recorded. The data were used anonymously, and they were not charged for participating in this study. The isolated samples were identified using gram-stain method and biochemical tests such as oxidase test, catalase test, glucose- and lactose-fermentation pattern in TSI medium, consumption of glucose through oxidation in OF medium, growth in 42ºC, pigment production and growth on Cetrimide agar.

Determining Sensitivity to Antibiotics

The sensitivity of *P. aeruginosa* isolates to imipenem (10 μg), ciprofloxacin (50 μg), gentamicin (10 μg) and cefotaxime (30 μg) produced by Padtan-Teb were determined using disk diffusion agar method (Kirby-Bauer). To this end, after 24-hour cultivating of the bacteria, a suspension with turbidity of 0.5 McFarland was produced in the physiologic serum. Resistant to antibiotics being studied and were cultured in the agar Muller-Hinton medium (Merck, Germany) in the form of spread sheet using sterile swap and then disk diffusion was carried out. After 16-18 hours of incubation in 37ºC, the inhibition diameter was measured using standard tables defined by the Clinical and Laboratory Standards Institute (CLSI) and the results were categorized as resistant, semi-sensitive and sensitive.[19] In this study, the *P. aeruginosa* ATCC27853 was used as the standard strain for evaluation.

Isolating Probiotic Strains

To isolate probiotic bacteria, 12 samples of milk and yogurt were collected from local yogurt-producing workshops and 2 samples of pasteurized yogurt produced by Pegah Company and Amol's Kalleh Company in sterile screw-cap containers. The yogurt and milk samples were transferred to laboratory in maximum 6 hours in cold conditions, and isolating conditions were implemented on them; so that 10 mL of each sample were mixed with 90 mL of physiologic serum completely, and a dilution was prepared.

Dilutions of yogurt and milk in MRS broth culture medium (Sigma-Aldrich, Germany) were inoculated for lactobacilli and in broth M17 (Sigma-Aldrich, Germany) for streptococci, and were incubated in anaerobic conditions in 37ºC for 48 hours. Solutions enriched in the MRS agar and M17 agar special medium were then cultured and the isolates were studied in terms of microscopic and macroscopic specifications. Species were identified based on the pattern of carbohydrates fermentation. Finally, the colony of isolated lactic bacteria was inoculated to the special broth medium and were incubated for 4 days (the required time for production of antibacterial material) in 37ºC after adding paraffin to media. After 4 days, the paraffin was extracted and the contents of the tubes were transferred to sterile glass tubes after being combined, and were centrifuged per 2800 round for 10 minutes. Sediments were removed in sterile conditions and the upper solution was preserved for further studies.

Investigating Antimicrobial Effect of Probiotics

To investigate the antimicrobial effects of probiotics, agar well diffusion method was applied. To this end, a suspension equal to 0.5 McFarland was prepared from *P. aeruginosa* resistant to all antibiotics being studied and were cultured in the agar Muller-Hinton medium in the form of spreadsheet. Then, wells in 6 mm diameter were drilled in the medium using sterile pipette Pasteur, and 100 λ of the upper solution of the bacteria isolated from

yogurt and milk were poured in these wells. The plates were then incubated for 24 hours in 37ºC. After 24 hours, the inhibition diameter was measured and recorded in millimeters. Each step of the test was repeated 3 times. To study any significant difference in the results, the chi-square test was carried out. $P<0.001$ was regarded as the significant level. In this study, the standard strain of *Leuconostoc mesenteroides* PTCC1663 was considered for comparison.

Results

In this study, of the total 200 samples, 30 strains of *P. aeruginosa* were isolated which were mainly collected from male patients (71%) at the age range of 21-40 and with the burn percentage of 16%-30% (Figures 1 and 2). Of the isolated strains, 45.2% showed resistance to gentamicin, 51.6% to cefotaxime, 48.4% to imipenem and 45.2% to ciprofloxacin. The most sensitivity cases were seen to ciprofloxacin antibiotic (Table 1). The chi-square test showed that the frequency and sensitivity of *P. aeruginosa* to different antibiotics.

Results of Isolating Probiotic Bacteria

In this study, of the total 40 lactic bacteria isolates most strains contained lactobacilli (85%) and least of them contained *Lactococcus* (15%). Among the *Lactobacillus* strains, most of the isolated strains were of *L. casei* (45%), and other strains included *Lactobacillus plantarum, Lactobacillus bulgaricus* and *Lactobacillus fermentum* (Table 2).

Results of Well Diffusion Method

Upon cultivating *Pseudomonas aeruginosa* on Muller-Hinton agar medium, drilling wells and adding bacteriocin after incubation, plates were investigated in terms of the diameter of their inhibition zone.

After injecting *L. casei*, *L. plantarum* and *L. bulgaricus* to the growing *P. aeruginosa* resistant to all four antibiotics, inhibition zone was created (Figure 3). The average diameter of the inhibition zones were 20.3, 18.2, 17.1 mm respectively which shows the inhibitory effect of these probiotics on the resistant strains. By injecting *L. fermentum* and lactococci to the *P. aeruginosa* resistant to all 4 antibiotics, no inhibition zone was created which means that these probiotics have no inhibitory effect on these strains (Table 3).

Moreover, the isolated *Lactococcus* strains only prevented from production of *P. aeruginosa* pigments, having no inhibitory effect on the growth of this pathogen. The standard strain of *L. mesenteroides* also had the inhibitory effect on *P. aeruginosa*, as no bacterial growth was observed around the wells in the strain resistant to all 4 antibiotics upon injection of *Leuconostoc* probiotic.

Discussion

Pseudomonas aeruginosa is a gram-negative bacillus, and is an opportunistic pathogenic bacterium which is one of the main causes for hospital infections. Although the infections caused by this bacterium mainly occurs in the hosts whose immune systems have been weakened, it causes a major threatening disease for the patient upon being deployed.[12] The pathogens of infections resulting from *P. aeruginosa* is very complicated due to the clinical

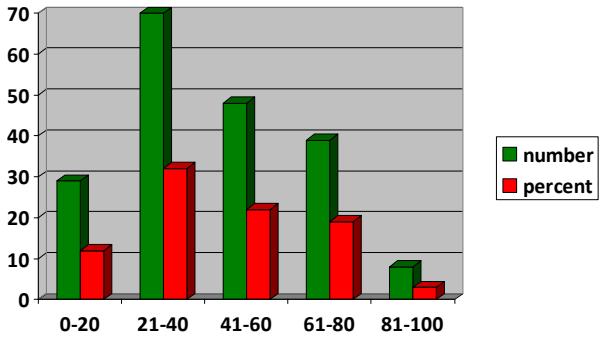

Figure 1. The Frequency and Percentage of Distribution of Patients' Age.

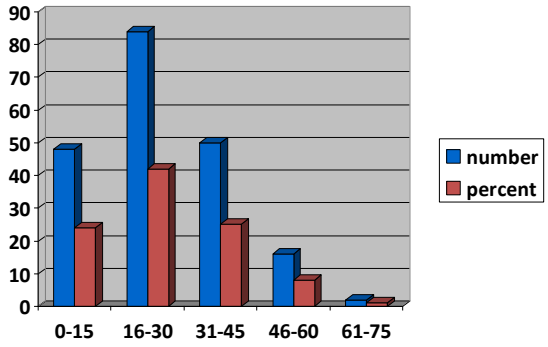

Figure 2. The Frequency and Percentage of Burn in Patients.

Table 1. Frequency distribution of antibiotic resistance among *Pseudomonas aeruginosa* isolates

Antibiotics	Susceptible		Semi-susceptible		Resistant	
	No.	%	No.	%	No.	%
Cefotaxime	8	25.8	7	22.6	16	51.6
Ciprofloxacin	16	51.6	1	3.2	14	45.2
Gentamicin	15	48.4	2	6.5	14	45.2
Imipenem	16	51.6	0	0	15	48.4

Table 2. Relative and Absoluate Frequency of Lactic Bacteria Isolated From 12 Sampels of Milk and Yogurt

Lactic Bacteria	No. of Samples	Absolute Abandonce	Relative Abandonce
L.casei	4	18	45%
L. plantarum	2	8	20%
L.bulgaricus	2	5	12.5%
L. fermentum	1	3	7.5%
Lactococcus	3	6	15%
Total number	12	40	100%

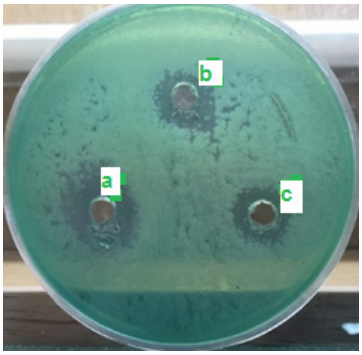

Figure 3. The Antimicrobial Effect of the Combination Produced by (a*) Lactobacillus casei* (b) *Lactobacillus plantarum* and (c) *Lactobacillus bulgaricus* on Growth of *Pseudomonas aeruginosa.*

changes and multiplication of the pathogen. This organism rarely causes disease in healthy people, though it might cause malignant transformations due to the changes in skin and mucous membrane as the natural defensive barriers and ultimately their permeability.[20]

This bacterium shows resistance to common antibiotics, but some species respond to gentamicin, tobramycin, colistin, norfloxacin, ciprofloxacin and amikacin. Gentamicin and carbenicillin are mainly used to treat severe infections. According to bacteriologic studies, the best antibiotic which could be used for pseudomonastic infections, especially in critical circumstances, is imipenem. However, it is usually difficult to control the spread of *P. aeruginosa* as it has intrinsic resistance to various antimicrobial treatments. In this study, of the 30 isolated *P. aeruginosa* strains, 48.4% showed resistance to imipenem.

In a study conducted by Shahcheraghi et al in Shahid Motahhari burn hospital, the percentage of resistance to ceftazidime, gentamicin, amikacin, tetracycline and ciprofloxacin were 96%, 93.7%, 93.4%, 91% and 86.7% respectively.[12] These results showed a considerable difference with our results, as the resistance to ciprofloxacin and gentamicin were lower in our study. Studies conducted in other countries show high resistance of *P. aeruginosa* isolates in the burn ward.[21,22] The important point about the high amount of resistance in this isolates collected from burn ward is that researchers are on the belief that these infections are mainly due to environmental causes, and infection occurs following hospitalization of burn patients and the increase of susceptibility to bacterial contaminations. In other words, this bacterium can easily replace in hospitals as it is among the bacteria which is resistant to unfavorable environmental conditions.

In the study carried out by Karimi Estahbanati over the 4 months from 22 December 1998 until 20 April 1999, patients hospitalized in Shahid Motahhari hospital who were suspected to wound infection were studied. Of the 205 various samples of bacteria found in their study, *P. aeruginosa* was found to be the most frequent with 117 cases. The frequency for resistance of these *Pseudomonas* bacteria to the antibiotics of gentamicin, ceftizoxime, carbenicillin, cephalothin and ceftazidime was very high. The most sensitive and effective antibiotics were amikacin and tetracycline. This shows that *Pseudomonas* is the most common bacteria in creating infection in burn wound.[23]

Furthermore, the high resistance of this bacteria to the above-mentioned antibiotics has made it very difficult to treat these infections with the present antibiotics. So prevention from these infections is the best way to defeat them. Vaccination of the susceptible patients with anti-*Pseudomonas* polyvalent antiserum could create immunity in almost half of these patients or delay infection. In the present study, most cases of resistance were related to cefotaxime (51.6%) and most sensitivity was towards ciprofloxacin.

In the present study, the number of patients who showed sensitivity to ciprofloxacin, gentamicin and imipenem was more than the ones who were resistant to the same antibiotics or have average sensitivity. The number of patients who showed resistance to cefotaxime was also more than the ones who were sensitive or semi-sensitive

Table 3. Comparison of Growth and Inhibition of *Pseudomonas aeruginosa* Strains When Encountering to Different Probiotics

Lactobacilli	Strain 1.5×10^8 CFU/mL	Growth		No Growth		Comparison
		No.	%	No.	%	
L. casei	*P. aeruginosa*	4	22.2	14	77.8	$\chi^2=0$, NS
L. plantarum	*P. aeruginosa*	3	37.5	5	62.5	$\chi^2=0$, NS
L. bulgaricus	*P. aeruginosa*	2	40	3	60	$\chi^2=0$, NS
L. fermantum	*P. aeruginosa*	3	100	0	0	$\chi^2=0$, NS

to the same antibiotic.

In light of above, experts are looking for more effective medicine which lack side-effects for treating infections caused by *Pseudomonas*. As today many cases of drug resistance have been spotted and confirmed in various infections, it is recommended to use combinations or antimicrobial material to stop or control such resistance. Probiotics are part of these combinations which are not only economically justifiable, but they are also easier to access and their application in medicine and pharmacy is confirmed. In addition, they lack the side effects resulting from consumption of antibiotics.[18]

In another study, some metabolites are reported to be created through the activities of a bacteriocidal called bacteriocin in some homofermentative and heterofermentative species such as *Streptococcus lactis*. Bacteriocin is produced faster within the first 24-48 hours as its ability to deactivate bacteriophages will be reduced after this time.[24] Researches have shown that *Lactobacillus* and their metabolic products are effective in preventing infections or delaying colonization of gastric mucosa with *Helicobacter pylori*, and that they are related to production of organic acids, free fatty acids, ammonia, ethanol, hydrogen peroxide and bacteriocins.[25]

Heidari and Ghaemi studied the activity of bacteriocin produced by lactic acid bacteria separated from diaries. The inhibition zone around 10 pathogen bacteria next to the bacteriocin of *Lactobacillus* isolated through well diffusion assay were studied and their antimicrobial effect were confirmed.[26] In the present study, the lactic bacteria isolated from diaries were able to produce bacteriocin and their antimicrobial effect was also confirmed.

Khonafari and Esmaeilzadeh studied production of lactocins by probiotic strains in local yogurt samples. The results of this study showed isolation of 21 strains of lactic acid bacteria respectively. Production of antimicrobial combinations by strains in the logarithmic phase of their growth and their antimicrobial effects were also observed.[27]

In a study conducted using well diffusion method, it was found out that a mixture of fermented milk which contained L. *casei* halts the growth of intestinal pathogens such as *Shigella dysenteriae* (8 mm), *Salmonella typhimurium* (9 mm) and *Escherichia coli* (2.8 mm). pH reduces in the foods containing these microorganisms and this way prevents diarrhea in the mice.[28] In another study comparing Kefir extract and gentamicin antibiotic, it was found out that the diameter of the inhibition zone for Kefir in 37ºC and with fermentation time of 48 hours (with density of 300 mg/mL) was 14 mm on *P. aeruginosa*; while the amount was equal to almost 9 µg/mL of gentamycin sulfate.[29]

Mojgani and Esmail Khanian identified and studied bacteriocin produced by *Lactobacillus acidophilus* isolated from local cheese in Karaj. In their study, lactocin which was produced by *L. acidophilus* was studied and its inhibitory effect against gram-positive and gram-negative bacteria was investigated, and the inhibitory range of this bacteriocin against pathogen bacteria was also observed and confirmed.[30]

Taj-Abadi and Hejazi et al realized that the variety of microbes is very high in dairies, therefore the microflora study of these products is very difficult. The direct screening method could be a suitable method for isolating strains containing probiotic potential by preventing the growth of sensitive bacteria, and will make it easier to study probiotic features of dairies.[31] In the present study, lactobacilli were isolated after being cultivated in the specific medium and gram staining method.

Kazemi Darsanaki and Ghaemi studied the antimicrobial activity of lactic acid bacteria separated from probiotic products (*Lactobacillus* and *Bifidobacterium*). They isolated lactic acid bacteria from yogurt samples and probiotic pills and identified them using biochemical methods. In this study, metabolites produced by lactic acid bacteria were successful in preventing the growth of pathogen bacteria, which means these bacteria have a positive role for the health of human beings.[32] In the present study the antagonistic effect of lactobacilli, especially *L. casei*, showed very good antimicrobial effects on the growth of *P. aeruginosa* resistant to all four medicines. In another study it was reported that the solution creates bactericide effect against a broad spectrum of gram-positive and gram-negative bacteria of the pathogen when it is consumed over culture of *L. fermentum*, *L. casei, L. acidophilus* and *L. lactis*.[27]

In the present study, *P. aeruginosa* isolated from burn wound which were resistant to gentamicin, ciprofloxacin, cefotaxime and imipenem was easily stopped by *Lactobacillus*. Therefore, according to results, the metabolites isolated from probiotic bacteria are able to prevent pathogens from growing.

Conclusions

The results of this study showed that the spread of *P. aeruginosa* resistant to medicine is very high in the surveyed hospitals, and that the isolated lactobacilli have a significant inhibitory effect on drug resistant *P. aeruginosa* strains. Our results hence suggest that, using probiotic products would help better control of *P. aeruginosa* infection in burn patients.

Author's Contributions

LF contributed to study concept, and designed, supervised, and edited the final manuscript. MA performed sample collection and laboratory examinations and interpreted the

data. All authors discussed the results and implications and provided their comments during all the stage.

Competing interests

Authors have no conflict of interests.

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