Short Communication

Antibacterial and Antibiofilm Activity of Some *Lactobacillus* spp. Isolated from Natural and Commercial Sources Against XDR *Pseudomonas aeruginosa* Clinical Isolates

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Abstract

Background: The emergence of biofilm-forming- and antibiotic-resistant *Pseudomonas aeruginosa* has renewed efforts to identify safe and natural alternative agents such as probiotics. **Objectives:** This study aimed to assess both the antibacterial and antibiofilm efficacy of some lactobacilli probiotics isolated from natural and commercial sources against pathogenic *P. aeruginosa*. **Materials and Methods:** Clinical *P. aeruginosa* isolates were isolated and identified from Baghdad hospitals, which were later tested for their ability to resist antibiotics and produce biofilms. Lactobacilli species (*Lactobacillus acidophilus* and *Lactobacillus plantarum*) were also isolated and identified from natural and commercial sources, and their effect on antibacterial and antibiofilm production were studied. **Results:** The results showed the apparent efficacy of lactobacilli against bacteria and biofilms, and the possibility of using *L. acidophilus* and *L. plantarum* as effective probiotics to deal with multidrug-resistant *P. aeruginosa*, in addition to their role as antibiofilm. The results showed that naturally isolated lactobacilli have more excellent probiotic properties than commercially isolated ones. Finally, the results supported the idea of using probiotics as an alternative to antibiotics to treat antibiotic-resistant pathogenic bacteria, and hence treat some diseases associated with this pathogenic bacterium. **Conclusion:** The results showed the possibility of using *L. acidophilus* and *L. plantarum* as effective probiotics to deal with multidrug-resistant *P. aeruginosa*, and hence treat some diseases associated with this pathogenic bacterium such as nosocomial infections. *Lactobacillus* spp. can be good anti-virulence agents (antibiofilm).

Keywords: Bioactivity, *Lactobacillus* spp., MDR, *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa is an opportunistic bacterial pathogen in invertebrates, plants, and human as well as within the immunocompromised patient is poisonous and easily developed antibiotic resistances,[1] whereas these pathogenic bacteria have various virulence factors, including hemolysin, pyocyanin, and biofilm formation.[2,3]

The World Health Organization (WHO) warns that antibiotic resistance poses a major threat to global public health. Humanity has been doomed to greater morbidity and mortality from microbial diseases, and the emergence of multidrug-resistant bacteria has only made the situation worse.[4,5] The growth of multidrug resistance is related to the lack of new and potent antimicrobials. Also, there were international attempts to develop new and more potent antimicrobial medications as well as novel and potent medication delivery and targeting methods.[6] Therefore, there has been growing interest in finding antimicrobial compounds from natural sources such as probiotics and medicinal plant extracts as an alternative approach to discover new antimicrobial compounds.[7]

Probiotics are described as "live bacteria which when provided in suitable proportions confer a health benefit for the host" by the Food and Agriculture Organization (FAO) and the WHO. The human digestive system reaps

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many benefits from probiotic bacteria. One category of organisms thought to have probiotic effects is lactobacilli.[8]

Probiotics can compete with other microorganisms using different mechanisms such as inhibiting pathogens by competing with them on the limited substrates necessary for their metabolites. Some probiotics prevent the adherence of pathogens to the host cell. Other probiotics can secrete metabolites with antimicrobial activity such as bacteriocins, H_2O_2, and organic acids.[9] The aforementioned advantages provide great potential in the utilization of probiotics as an antibiofilm agent.

The current study aimed to assess both the antibacterial and antibiofilm activity of some lactobacilli probiotics isolated from natural and commercial sources against pathogenic *P. aeruginosa* clinical isolates in Baghdad, Iraq.

Materials and Methods

Bacterial isolates

In the present study, out of a total of 130 clinical samples (obtained from various clinical sources including urine, sputum, ear, wound, and burn), 30 *P. aeruginosa* isolates were found to be resistant to extensively-drug resistant (XDR). The clinical samples were collected from patients with ages ranging between 18 and 75 years, at Al Kadimyia Teaching Hospital, Al-Karkh Hospital, and Medical City Hospitals (including Burns Specialized Hospital, Baghdad Teaching Hospital, Al-Shahid Ghazi Al-Hariri Hospital For Surgical Specialties). The study period was from October 2022 to December 2022.

Two XDR and biofilm-producing *P. aeruginosa* isolates (P53 and P80) were selected for this study [Figures 1 and 2]. In addition, two *Lactobacilli* spp. (*Lactobacillus acidophilus* and *Lactobacillus plantarum*) were selected that were isolated from 31 samples of dairy products, including Arabic cheese, sweet cheese, and dried yogurt, during the period from January 2023 to April 2023. Different commercial probiotics obtained from some pharmacies, Baghdad, were also included in this study. All bacterial isolates were subjected to preliminary diagnostic laboratory tests using Gram stain, biochemical tests, and the VITEK2 system for the identification of bacterial isolates.

Antibiotics susceptibility test (AST)

Antibiotics susceptibility test was done via disk diffusion method (Kirby–Bauer method) according to Clinical

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Patient Name: 53 R, . Patient ID: RTUTRU
Location: Physician:
Lab ID: 143 Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: **Collected:**

Comments:	

Susceptibility Information	**Analysis Time:** 13.83 hours	**Status:** Final

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
+Amoxicillin		R	+Cefepime		R
+Ampicillin		R	Aztreonam		
+Amoxicillin/Clavulanic Acid		R	Doripenem	>= 8	R
Ampicillin/Sulbactam			+Imipenem		R
+Carbenicillin		R	Meropenem/Vaborbactam		
+Piperacillin		R	+Amikacin		
Piperacillin/Sulbactam	>= 64	R	+Gentamicin		
+Piperacillin/Tazobactam		R	Tobramycin	>= 16	R
+Cefsulodin		R	+Cinoxacin		R
+Cefadroxil		R	+Ciprofloxacin		R
+Cefazolin		R	+Enoxacin		R
+Cefradine		R	Levofloxacin	>= 8	R
+Cefotetan		R	+Marbofloxacin		
Cefoxitin			Doxycycline		
+Cefditoren		R	Minocycline		
+Cefixime		R	Tetracycline		
Cefotaxime			Tigecycline		
Ceftazidime/Avibactam	>= 16	R	Chloramphenicol		
Ceftolozane/Tazobactam	>= 32	R	Colistin	4	I

AES Findings	
Confidence:	Consistent

Figure 1: The antibiogram of XDR *P. aeruginosa* isolates P53 selected in this study

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Patient Name: 80 R, . Patient ID: WETGD
Location: Physician:
Lab ID: 144 Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: **Collected:**

Comments:	

Susceptibility Information	**Analysis Time:** 9.92 hours	**Status:** Final

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
+Amoxicillin		R	+Cefepime		R
+Ampicillin		R	Aztreonam		
+Amoxicillin/Clavulanic Acid		R	Doripenem	>= 8	R
Ampicillin/Sulbactam			+Imipenem		R
+Carbenicillin		R	Meropenem/Vaborbactam		
+Piperacillin		R	+Amikacin		
Piperacillin/Sulbactam	>= 64	R	+Gentamicin		
+Piperacillin/Tazobactam		R	Tobramycin	>= 16	R
+Cefsulodin		R	+Cinoxacin		R
+Cefadroxil		R	+Ciprofloxacin		R
+Cefazolin		R	+Enoxacin		R
+Cefradine		R	Levofloxacin	>= 8	R
+Cefotetan		R	+Marbofloxacin		
Cefoxitin			Doxycycline		
+Cefditoren		R	Minocycline		
+Cefixime		R	Tetracycline		
Cefotaxime			Tigecycline		
Ceftazidime/Avibactam	>= 16	R	Chloramphenicol		
Ceftolozane/Tazobactam	>= 32	R	Colistin	<= 0.5	S

AES Findings	
Confidence:	Unknown

Figure 2: The antibiogram of XDR *P. aeruginosa* isolates P80 selected in this study

Laboratory Standard Institute recommendations[10] and the results were confirmed by Vitek 2 System.

Detection of biofilm formation

MDR bacterial isolates (*P. aeruginosa*) biofilm forming potential was evaluated using a crystal violet staining-based 96-well microtiter plate assay.[11]

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

In accordance with the study,[12] the cell-free supernatants were prepared as follows: Bacteria were cultivated to the mid-exponential phase in MRS broth for 24 h at 37°C in anaerobic conditions after being extracted off an agar plate. McFarland standard no. 0.5 turbidity was used to modify the optical density (OD) 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 h at 37°C. Centrifugation (10,000 × *g* for 15 min at 4°C), filtration through a sterile 0.22 m hole-size membrane, and subsequent plating on MRS agar revealed no lactobacilli growth. The inhibitory activity of this recently made cell-free supernatant (stock solution) was tested.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MICs) are the lowest antimicrobial agent concentrations that would prevent the observable growth of a microbe following overnight incubation. The experiment was done according to the study of Elshikh *et al.*[13] as follows: Column 1 contained 100 µL of the cultured broth from cultivating each *Lactobacillus* isolate in Muller Hinton broth (MHB) with *P. aeruginosa* at a pH of 7, whereas Columns 2–10 contained 50 µL of MHB broth alone. As can be seen on the processed plate, column 11 contained 100 µL of the medium broth (as a control to check sterility) and column 12 included 100 µL of diluted standardized inoculum. After transferring and mixing surfactants from columns 1 to 10 using a multichannel pipette, we got 50 µL per well. The suspension of standard microorganisms was then diluted 100-fold in MHB broth. All wells with surfactant and the control wells received an additional 50 L of the bacterial solution with the modified OD600. 5×10^5 CFU

mL^{-1}. The whole time spent on making and dispensing the OD-adjusted bacteria was less than 15 min. After 24 h at 37°C, resazurin (0.015%) was added to all wells (30 µL per well), and the plates were incubated again for 2 to 4 h so that the color change could be seen. At the end of the incubation period, columns in which the blue resazurin color had not altered were considered to be at or above the MIC value. Sub-MIC wells are those that come after MIC.

Evaluation of *Lactobacillus* spp. potential as antibiofilm

This experiment was done according to the study of Blando *et al.*,[14] with some modification: Briefly, 100 µL bacterial suspension (*P. aeruginosa*) was inoculated and cultured with or without 100 µL of *Lactobacillus* spp. (at MIC concentrations), without shaking at 37°C. After incubation for 24 h, samples were dipped three times in 200 µL of sterile PBS to remove nonadherent cells. Crystal violet in water at a concentration of 0.1% was used to stain the biofilms. After adding 150 µL of crystal violet (0.1%) to each well and letting it sit for 15 min at room temperature, the nonadherent, free-floating bacteria were eliminated. Five times of washing with distilled water to eliminate any trace of the removed color. Destaining was accomplished by soaking the preparations in 200 µL of 95% ethanol for 3 min. Finally, each sample was transferred in a volume of 150 µL to a new microtiter plate. At 600 nm, an ELISA reader was used to measure the OD of the ethanol dye solution. The formula was used to calculate the percentage of biofilm inhibition, as follows:

$$\text{Bioflim reduction}\,(\%)=\left(\frac{OD_{control}}{OD_{sample}}\right)\times 100\%$$

Statistical analysis

The R statistical programming was utilized for statistical analysis of data. The analysis of variation (ANOVA) was utilized to determine the statistical variation for the studied isolates and their resistance to antibiotics. Also, Hierarchical Clustering analysis was utilized to limit isolates similar in their resistance to antibiotics and similar types of antibiotics working on the types of bacteria under study. The value of probability (*P* value) was also calculated to determine the level of statistical significance.[15]

Ethical approval

This study was approved by a local committee of publication ethics at University of Baghdad, Iraq, under reference number 0327/016 on June 11, 2022.

Results

Antimicrobial susceptibility test and biofilm formation

Two XDR of *P. aeruginosa* isolates were selected, namely *P. aeruginosa* P53 and *P. aeruginosa* P80. The results showed that both of these isolates were resistant to all the antimicrobial agents tested. Regarding the biofilm formation, both XDR isolates of *P. aeruginosa* (P53 and P80) were strong biofilm producers.

Determination of MIC of cell-free culture supernatants of *Lactobacillus* isolates

This experiment is conducted to determine the lowest dilution of CFCS of the examined lactobacilli isolates to suppress the growth of *P. aeruginosa* isolates. The results revealed that the strongest inhibitory effects for the *P. aeruginosa* P78 growth were MIC and sub-MIC (25 and 12.5 µg/mL) of both natural *L. acidophilus* and natural *L. plantarum*, respectively. On the other hand, all examined isolates of lactobacilli showed the same MIC and sub-MIC (50 and 25 µg/mL), respectively, for *P. aeruginosa* P50 [Table 1 and Figure 3].

Evaluation of *Lactobacillus* spp. potential as anti-biofilm activity

The antibiofilm potential of the examined *Lactobacillus* isolates was evaluated. The results in Figures 4 and 5, the direct effect of all isolates of lactobacilli showed a strong antibiofilm potential against isolates of *P. aeruginosa* P53 and P80, while their MIC and sub-MIC show moderate effect, except commercial *L. acidophilus*, which show weak effect. In addition, both natural isolates (*L. acidophilus* and *L. plantarum*) showed a stronger effect than commercial

Table 1: The results of MIC of *Lactobacillus* Spp. against XDR *P. aeruginosa* isolates

No. of *P. aeruginosa*	100	50	25	12.5	*Lactobacillus* spp.
P53	–	MIC	Sub-MIC	–	Natural *L. acidophilus*
	–	MIC	Sub-MIC	–	Commercial *L. acidophilus*
	–	MIC	Sub-MIC	–	Natural *L. plantarum*
	–	MIC	Sub-MIC	–	Commercial *L. plantarum*
P80	–		MIC	Sub-MIC	Natural *L. acidophilus*
	–	MIC	Sub-MIC	–	Commercial *L. acidophilus*
	–		MIC	Sub-MIC	Natural *L. plantarum*
	–	MIC	Sub-MIC	–	Commercial *L. plantarum*

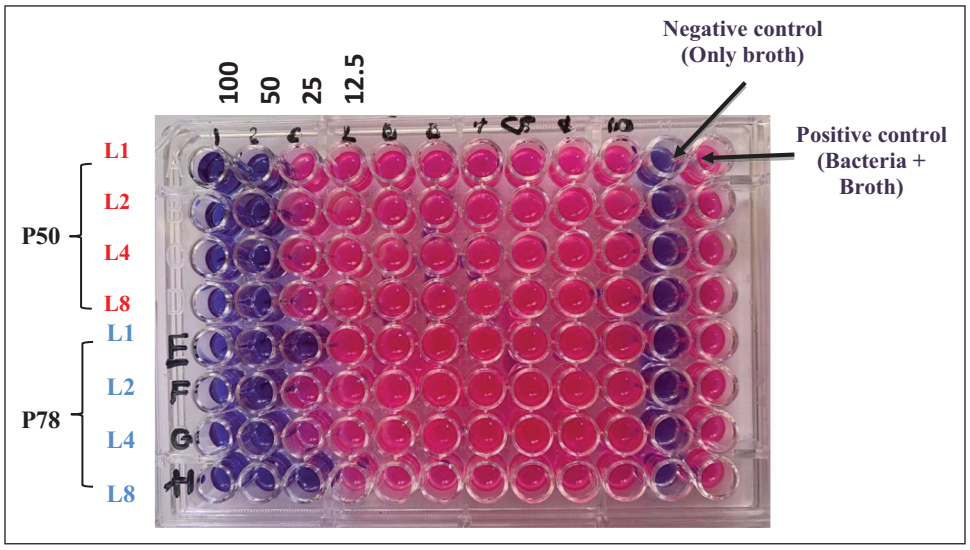

Figure 3: Determination of MIC and sub-MIC for CFCS of Lactobacilli against MDR *P. aeruginosa* isolates (P53 and P80). L1: Natural *L. acidophilus*, L2: Commercial *L. plantarum*, L4: Commercial *L. acidophilus*, L8: Natural *L. plantarum*

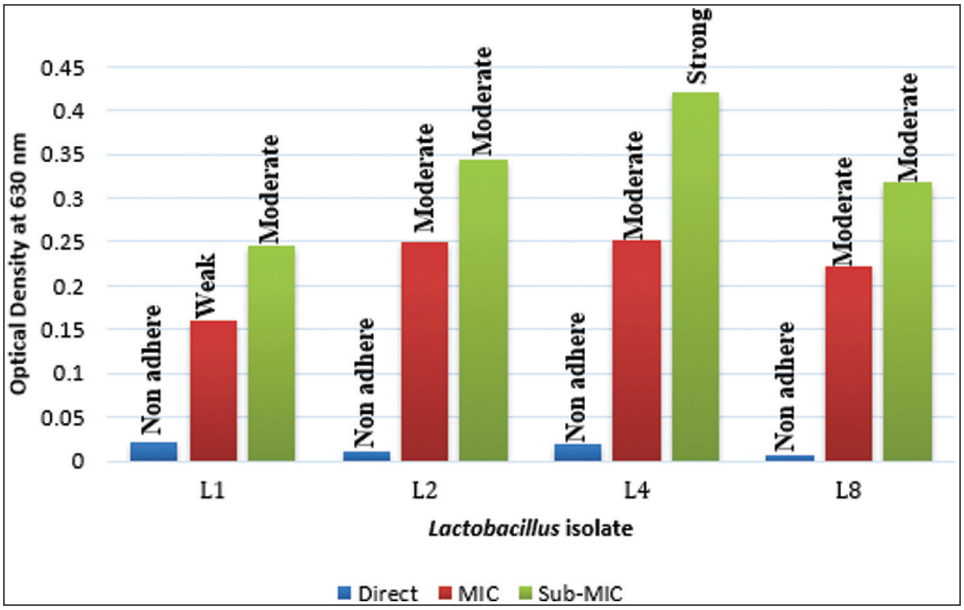

Figure 4: The antibiofilm potential of lactobacilli isolates against tested *P. aeruginosa* P53. L1: Natural *L. acidophilus*, L2: Commercial *L. plantarum*, L4: Commercial *L. acidophilus*, L8: Natural *L. plantarum*

stains. Indeed, *L. acidophilus* isolate showed a stronger antibiofilm effect than *L. plantarum* in both types (natural and commercial) [Figures 4 and 5].

Discussion

In this study, two isolates of *P. aeruginosa* were considered as XDR which were resistant to three classes of antimicrobials making it difficult to choose appropriate suitable antimicrobial therapy, and the overuse of antibiotics has resulted in a rise in *P. aeruginosa*'s resistance to several antibiotics, which in turn has led to an increase in the prevalence of MDR strains of the bacteria.[16]

As a result of this study, all isolates of lactobacilli show a strong antibiofilm potential against isolates of *P. aeruginosa* P50 and P78. In a study reported by Haghighatafshar *et al.*,[17] The bacteriocin isolated from *L. rhamnosus* was effective against *P. aeruginosa*. The minimum bactericidal concentration (MBC) was 62.5 µg/mL, whereas the minimal inhibitory concentration (MIC) was 31.25 µg/mL. Also, it has been reported by Gaspar *et al.*[18] that *L. acidophilus* inhibited *Streptococcus agalactiae* and *P. aeruginosa*, but it had no impact on the *Escherichia coli*, *S. aureus*, or *Candida albicans* strains that were tested.

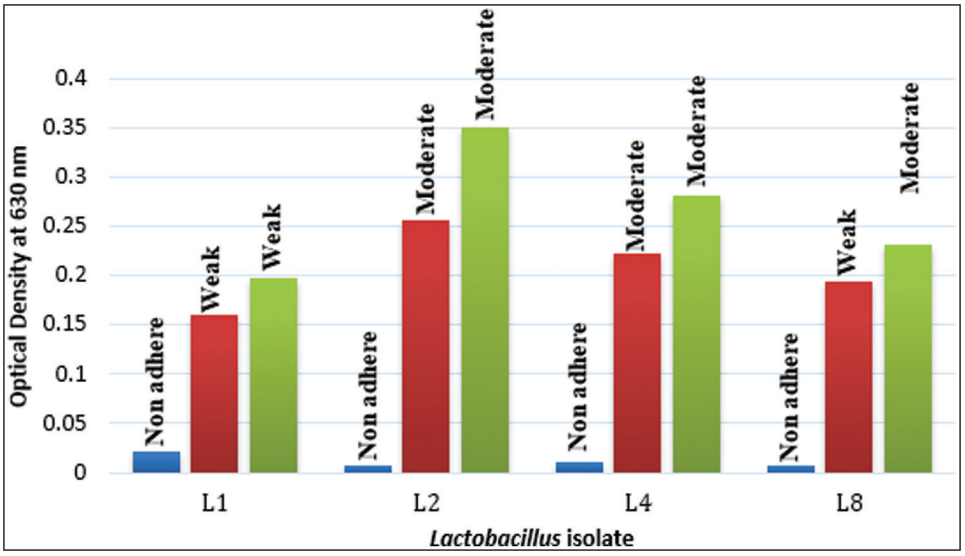

Figure 5: The antibiofilm potential of lactobacilli isolates against tested *P. aeruginosa* P80. L1: Natural *L. acidophilus*, L2: Commercial *L. plantarum*, L4: Commercial *L. acidophilus*, L8: Natural *L. plantarum*

The mechanisms of antimicrobial activity of CSF *lactobacillus* strains include (i) competitive exclusion of bacteria to adhere and compete for nutrients and adhesion receptors, (ii) the process through which microbial communities assemble into separate, interconnected structures known as co-aggregation, and (iii) powerful antibacterial chemical synthesis, including lactic acid (which reduces the reaction environment's pH), hydrogen peroxide (H_2O_2), biosurfactants, and bacteriocin-like substances.[19] Bacteriocins are small antimicrobial peptides that have lethal or inhibitory effects against other types of bacteria. Their adsorption to specialized receptors on the surface of bacteria, causes vital and phenotypic metabolic changes, killing those bacteria.[20-23]

Pseudomonas aeruginosa has been shown to be capable of biofilm formation. An infection's persistence and resistance are largely attributable to biofilm, a virulence component.[24] Many studies have focused on the use of *Lactobacillus* spp. as an alternative agent in the treatment of biofilm-associated illnesses because of their anti-biofilm activities against antibiotic-resistant strains of *S. aureus* and *P. aeruginosa*.[25]

Because it may prevent *P. aeruginosa* biofilm development, *L. acidophilus* may be a useful tool in the fight against this infection.[26] When comparing CFS to exclusively pathogenic strains (*P. aeruginosa*), biofilm development was considerably (P = 0.05) inhibited. Increasing the concentration of SLp759 in the wells increases the bacteria's resistance to adhesion inhibition.[27]

The study conducted by Shokri *et al*.[28] showed that the biofilm production of *P. aeruginosa* was inhibited or eliminated by two *Lactobacillus fermentum* strains. *Bacillus cereus* and *P. aeruginosa* biofilms were shown to be inhibited by metabolites from *Lactobacillus pentosus* and *L. plantarum* isolated from fermented dairy products.[29] Melo *et al*.[30] revealed that Cocoa fermentation produced compounds with antibiofilm activity, including those from *L. fermentum* TCUESC01 and *L. plantarum* TCUESCO2.

Conclusion

The results showed the possibility of using *L. acidophilus* and *L. plantarum* as effective probiotics to deal with multidrug-resistant *P. aeruginosa*, and hence treat some diseases associated with this pathogenic bacterium such as nosocomial infections. Also, *Lactobacillus* spp. can be a good anti-virulence agent (antibiofilm). In addition, the results showed that naturally isolated lactobacilli have more excellent probiotic properties than commercially isolated ones.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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