

An Experimental Study on the Potential of Honey Lactobacilli to Combat Multidrug-Resistant *Acinetobacter baumannii* (MDRAB) in Northern Iran

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ABSTRACT

Backgrounds: Infections caused by *Acinetobacter baumannii* represent a global health threat in medical settings. This research aimed to investigate the phenotypic characteristics of antimicrobial resistance in *A. baumannii* strains isolated from post-burn infections as well as the antagonistic effects of honey probiotics on multidrug-resistant isolates.

Materials & Methods: A total of 220 post-burn wound samples were analyzed to identify *A. baumannii* strains using microbiological and molecular tests. The Kirby-Bauer test was conducted according to CLSI (Clinical and Laboratory Standards Institute)-2021 guidelines to determine resistance phenotypes. Additionally, probiotics were isolated from 11 honey types through phenotypic and molecular methods, and their antagonistic properties were evaluated using the disk diffusion method.

Findings: Out of the 63 *A. baumannii* isolates identified, 76.19% exhibited multidrug-resistance. Most of the isolates were obtained from patients with second-degree burns, accounting for 23.81% of the cases. The highest antibiotic sensitivity was against amikacin, with a sensitivity rate of 58.73%. In the disk diffusion tests, the average growth inhibition zone diameter for all multidrug-resistant *A. baumannii* isolates was 29 mm when treated with *Lactobacillus plantarum* derived from Camelthorn honey. This probiotic effect was significantly 1.7 times greater than that of *L. delbrueckii* and 1.9 times greater than that of *L. acidophilus*.

Conclusion: Although this *in vitro* study did not investigate the biochemical properties of honey, certain native Iranian honeys were shown to possess desirable anti-*Acinetobacter* properties due to the presence of lactobacilli.

Keywords: *Acinetobacter baumannii*, Nosocomial infection, Drug resistance, Honey, probiotic

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Introduction

Acinetobacter is an aerobic, Gram-negative bacterium commonly found in various environments, such as soil, water, and air. *A. baumannii* is an opportunistic pathogen that could cause both community-acquired and hospital-acquired (nosocomial) infections, including central nervous system infections, wound infections, post-burn infections, and bacteremia in immunocompromised patients^[1]. It could also colonize the skin, oral cavity, gastrointestinal tract, conjunctiva, respiratory tract, and genitourinary tract of healthy individuals. Nosocomial infections are mostly transmitted through direct contact with hospital staff and equipment, highlighting the role of factors such as proper disinfection of medical equipment, personnel awareness of infection risks, and the type of disinfectant used. Numerous studies have indicated that critically ill patients in different hospital wards, such as intensive care units, burn wards, and neonatal units, are more susceptible to infections caused by *A. baumannii*. These environments provide optimal conditions for rapid transmission of *A. baumannii* among patients^[2, 3]. According to CHINET's bacterial drug-resistance surveillance data, *A. baumannii* ranks among the top five surveilled strains of concern and is the second most prevalent non-fermenting, Gram-negative bacterium. The indiscriminate and overuse of antibiotics has led to a global epidemic of multidrug-resistant *A. baumannii* (MDRAB)^[4]. MDR *A. baumannii* strains exhibit resistance to at least three antibiotic classes, including cephalosporins, fluoroquinolones, and aminoglycosides. Extensively drug-resistant (XDR) *A. baumannii* strains are resistant to the above antimicrobials and carbapenems^[5]. Due to its ability to evade the effects of antimicrobials, *A. baumannii* has been classified by the World Health Organization (WHO) as one of the most

dangerous ESKAPE pathogens. The rise of antibiotic resistance in *A. baumannii* has created significant challenges for clinical treatment. Additionally, the global increase in the prevalence of multidrug-resistant bacteria poses a significant challenge to patient care, particularly in countries with limited access to antimicrobial resistance data^[6, 7]. Unlike traditional antibiotics, alternative antimicrobial agents, such as probiotics, have fewer side effects and lower toxicity. These agents may help control the spread of drug-resistant bacteria. Probiotics prevent pathogens from reaching and binding to target host cells by aggregating and forming complexes with pathogens. As a result, pathogens could not attach to the cells. Various studies have demonstrated that probiotics could inhibit the growth of pathogenic microorganisms through multiple mechanisms, including stimulation of the host immune system, competitive inhibition of pathogen adhesion, and production of compounds inhibiting pathogens, such as organic acids, hydrogen peroxide, and bacteriocins^[8, 9]. Honey, as an acidic substance, has bacteriostatic and bactericidal effects due to its osmolality, hydrogen peroxide content, low pH, phenolic acid level, and flavonoids. Some probiotics may be present in honey, potentially transmitted through the honey production process from the gut of honeybees. These probiotics may remain viable for a certain period of time. Therefore, both honeybees and honey could provide probiotics that may be beneficial for future use. Probiotics found in honey have several health advantages, including revitalizing, enhancing, and strengthening the host's immune system against pathogens and harmful environmental factors, aiding in digestion, providing vital nutrients, and detoxifying and removing harmful substances^[10, 11].

Objectives: Given the warnings regarding

drug resistance and the importance of *A. baumannii* transmission from the environment to clinical settings, especially in burn and trauma wards, this study aimed to assess the inhibitory effects of lactobacilli isolated from various honey types on multidrug-resistant *A. baumannii* isolates derived from different burn phenotypes.

Materials and Methods

Research population and microbiological isolation procedure: In this experimental laboratory study, a total of 220 post-burn wound samples were collected from patients (age range: 9 to 78 years, mean $\pm$ standard deviation: 43 ± 7.1) hospitalized in the burn wards of six hospitals in the northern provinces of Iran (Mazandaran and Golestan) by experienced nurses during 2022-2023. In compliance with ethical standards and after obtaining permission from the hospital ethics committee, a questionnaire was prepared to collect information about the patients' gender, age, burn degree, and length of hospitalization. Patients with autoimmune diseases and those under 7 years of age (both male and female) were excluded from this study.

Burn wound samples were cultured on blood agar to identify bacteria and then on MacConkey agar to examine bacterial growth. Gram-negative and lactose-negative bacteria were selected for further investigation. *Acinetobacter* strains were identified based on microbiological procedures, including Gram-staining, indole, methyl red, Voges-Proskauer, motility, triple sugar iron agar, oxidative-fermentative with 10% glucose, and DNase (for differentiation from *Stenotrophomonas*) tests. *A. baumannii* ATCC 19606 was used as a positive control in all experiments. *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were used as positive controls in oxidase and

DNase tests, respectively. For molecular identification and final confirmation of *A. baumannii* isolates, genomic DNA was extracted by boiling method, and then PCR (polymerase chain reaction) was performed based on the identification of the *blaOXA-51* gene using forward and reverse primers (F: 5'-TAATGCTTTGATCGGCCTTG-3' and R: 5'-TGGATTGCACTTCATCTTGG-3')^[12, 13]. The PCR reaction was carried out in a final volume of 25 μ L, containing 1 μ L of DNA sample, 1 μ L of each primer, 12 μ L of 2X Master Mix (containing 20 μ M dNTP and 1.5 μ M $MgCl_2$), and 11 μ L of distilled water. The reaction was performed in a thermocycler (Eppendorf, Germany) with the following cycling conditions: an initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. The resulting PCR products were then electrophoresed on 1.5% agarose gel. Detection of 353 bp fragments confirmed the presence of *A. baumannii* isolates (Figure 1). The PCR product supernatant was measured at 260-280 nm in the range of 2-1.8% by a Nanodrop device, and then the resulting bands were visualized using gel electrophoresis. *P. aeruginosa* ATCC 27853 and *A. baumannii* ATCC 19606 were considered as negative and positive controls, respectively.

Antimicrobial susceptibility testing: Antimicrobial susceptibility was assessed using the disk diffusion (Kirby-Bauer) method. First, a bacterial suspension with a turbidity of 0.5 McFarland was prepared from fresh cultures of *A. baumannii* and then cultured on Mueller Hinton agar (MHA: Merck, Germany). Next, the following antibiotic disks (purchased from Padtan Teb, Iran) were placed on MHA medium: levofloxacin (LVX5), cefepime (CP30), amikacin (AN30), ticarcillin-clavulanic acid (TCC 75+10), imipenem (IMP10),

meropenem (MEM10), Ceftazidim (CTZ30), aztreonam (ATM30), gentamicin (GM10), fosfomycin (FOS200), ciprofloxacin (CIP5), and colistin (CS10). After 18-24 hours of bacterial culture at 37 °C, the growth inhibition zone diameter was measured, and the results were interpreted as susceptible, intermediate, and resistant according to the Clinical and Laboratory Standards Institute (CLSI) document M100- 2021^[14].

Identification of lactobacilli in honey: To isolate probiotic bacteria, 45 g of each of 11 different honey samples collected from various regions of Iran (including: Citrus honey, Faruj mountain honey, natural Comb honey, Konarak honey, Thyme honey, Ardabil honey, Camelthorn honey, Eucalyptus honey, Yazd Astragalus honey, Sunflower honey, and Coriander honey) were separately combined with 180 mL of 0.1% (w/v) peptone water in a flask. The mixtures were centrifuged at 1800 rpm for 10 min. The resulting pellets were resuspended in 10 mL of MRS broth (Merck, Germany) and incubated at 37 °C for 48 hours.

After incubation, 1 mL of each culture was transferred onto MRS agar plates and incubated anaerobically at 37 °C for another 48 hours. Colonies suspected of being *Lactobacillus* underwent phenotypic examinations, including carbohydrate fermentation tests (arabinose, cellobiose, galactose, gluconate, lactose, maltose, mannitol, D-mannose, melibiose, raffinose, salicin, sorbitol, sucrose, trehalose, xylose, melezitose, ribose, and esculin) as well as ammonia (NH₃) production test. The results were obtained after 24 hours of incubation at 37 °C. To identify the dominant *Lactobacillus* strains, genomic DNA was extracted using the boiling method, followed by PCR using the forward and reverse primers listed in Table 1. The PCR assay was performed in a 25 µL volume, consisting of 1 µL of genomic DNA, 1 µL of each primer,

12 µL of 2× Master Mix (20 µM dNTPs and 1.5 µM MgCl₂; Amplicon, Denmark), and 11 µL of distilled water. The PCR procedure was conducted in a thermal cycler (Eppendorf, Germany) with the following conditions: an initial denaturation at 95 °C for 10 min, followed by 40 amplification cycles consisting of denaturation at 95 °C for 25 s, annealing at 59-63 °C for 30 s, and extension at 72 °C for 30 s. The PCR products were subsequently separated by 1.5% agarose gel electrophoresis.

Evaluation of acid resistance: To determine the acid resistance of the probiotic antimicrobial compound, 100 µL of the 0.5 McFarland-adjusted *Lactobacillus* suspension was introduced into 10 mL of MRS broth with pH values of 4, 3, and 2, adjusted with 1 N hydrochloric acid. After 24 hours of incubation at 37 °C on MRS agar, strains producing colony counts exceeding 10⁶ CFU/mL were identified as acid-resistant.

Determining the anti-Acinetobacter potential of lactobacilli: The disk diffusion method was used to assess the inhibitory effect of *Lactobacillus* strains as probiotics against MDRAB isolated from post-burn infections. Initially, *Lactobacillus* isolates were inoculated into MRS broth, and paraffin was added to maintain anaerobic conditions and enhance the production of antimicrobial compounds. The cultures were then incubated at 37 °C for four days in anaerobic jars. After incubation, the paraffin was removed, and the samples were mixed thoroughly. They were transferred into sterile glass tubes and centrifuged at 2800 rpm for 10 min. The supernatant was collected aseptically for further experiments. Sterile 6 mm blank paper disks were soaked in the probiotic supernatant for five min and then dried at 37 °C for 15 min. Standardized suspensions of *A. baumannii* (10⁸ CFU/mL) were spread onto Mueller-Hinton agar plates (Merck, Germany), and then probiotic-

impregnated disks were placed on the agar surface. The plates were incubated at 37 °C for 24 hours, and the growth inhibition zone diameters were recorded. Growth inhibition zones less than 7 mm were considered as resistant, between 8-9 mm as moderately sensitive, and over 10 mm as sensitive to probiotics (18). Blank paper disks were used as negative controls, while ceftaroline disks (Mast, UK) served as positive controls.

Data analysis: All statistical analyses were conducted using IBM SPSS Statistics Version 23.0. The Chi-square distribution was utilized to test for independence with a null hypothesis at a significance level of 0.05.

Findings

Demographic details of bacterial isolates and determination of antibiotic susceptibility: A total of 63 isolates (28.64%) were identified as *A. baumannii*, with a higher prevalence among female patients (58.75%)

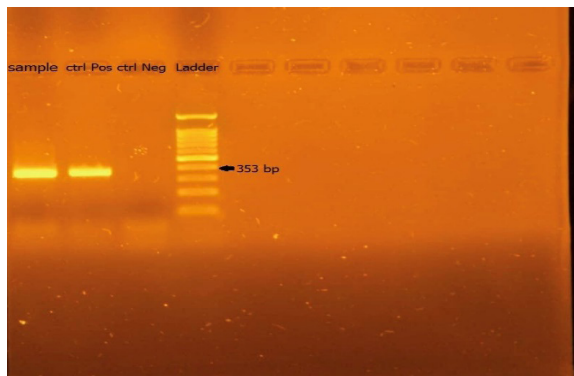


Figure 1) PCR amplification of the *blaOXA-51* gene

and those aged 50-78 years (25.40%). The highest frequency of *A. baumannii* isolates was among patients over 50 years of age (49.21%), particularly among those with second-degree burns (23.81%) and a hospital stay of three weeks (27%) (Table 2). Statistical analysis showed no significant differences in prevalence between different age groups and genders ($p = .06$). In the Kerby-Bauer test, the lowest resistance rates were observed against cefepime and levofloxacin (both at 14.29%), while the highest resistance was noted against aztreonam (42.86%) (Figure 2). The prevalence rate of MDRAB isolates was 76.19%, and the frequency of extensively drug-resistant (XDR) *A. baumannii* isolates was 25.39%.

Probiotic properties of lactobacilli and their potential against MDRAB: Among the 11 honey types studied, Faruj and Comb honeys contained the lowest probiotic levels. *L. acidophilus* and *L. plantarum* were the most prevalent probiotic isolates (81.81% each), followed by *L. delbrueckii* with a frequency of 63.63% (Table 3). In terms of acid resistance, 88.89% of *L. acidophilus* strains, 55.56% of *L. plantarum* strains, and 86.42% of *L. delbrueckii* strains demonstrated resistance at pH 4. The results indicated that the growth rate was higher at pH 4 compared to the other pH levels ($p < .05$), although no significant differences were observed between the other pH levels. The results of the disk diffusion method indicated that *L. plantarum* isolates

Table 1) General characteristics of study primers ^[15-17]

Primer Name	Sequence (5'>3')	Length (bp)
Lplan-F	AAAATCATGCGTGCGGGTAC	210
Lplan-R	ATGTTGCGTTGGCTTCGTCT	
Lbrevis-F	GCAGTTGCCGAGGTCCAA	64
Lbrevis-R	CCAACGCATTTTCAGCATCA	
Lacido-F	GTAATCGTGTTCTACATATACATAG	152
Lacido-R	GGTTATAAAGTTAACAGCATTGTTC	

Table 2) Characteristics of the study population and frequency of *A. baumannii* isolates

Variables	Age Groups	9-25 Years	25-50 Years	50-78 Years	X ²	P-Value
Gender	Woman	9 (14.28 %)	12 (19.04 %)	16 (25.40 %)	0.17	.061
	Man	3 (4.76 %)	8 (12.70 %)	15 (23.81 %)	0.20	.063
Burn degree	Second degree	7 (11.11 %)	7 (11.11 %)	15 (23.81 %)	0.19	.040*
	Third degree	2 (3.17 %)	9 (14.28 %)	11 (17.46 %)	0.15	.043*
	Fourth degree	0	0	12 (19.04 %)	0.11	.058
Duration of hospitalization	One week	1 (1.58 %)	5 (7.94 %)	7 (11.11 %)	0.13	.060
	Two weeks	2 (3.17 %)	4 (6.35 %)	7 (11.11 %)	0.18	.030*
	Three weeks	9 (14.28 %)	11 (17.46 %)	17 (27 %)	0.21	.020*

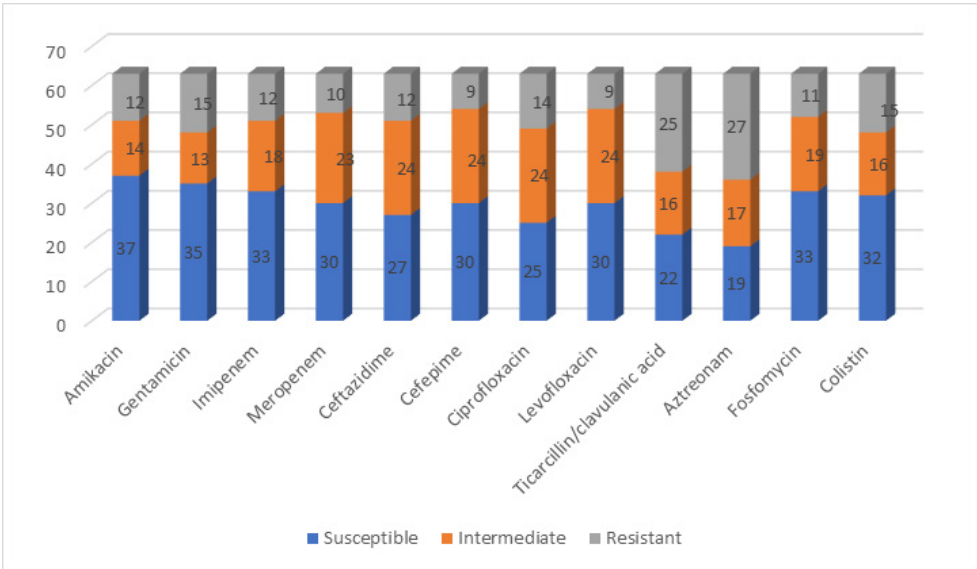


Figure 2) Absolute abundance of resistance to 12 different antibiotics among clinical isolates of *A. baumannii*

from Camelthorn honey exhibited the best anti-bacterial properties against all multidrug-resistant *A. baumannii* isolates, creating an average inhibition zone of 29 mm. However, they were only effective against five of the 16 extensively drug-resistant isolates (31.25%). *L. delbrueckii* had an average inhibition zone of 17 mm, and *L. acidophilus* had an average inhibition zone of 15 mm. Neither of these two *Lactobacillus* species was able to inhibit any of the

extensively drug-resistant strains. As shown in Table 4, the average growth inhibition zone diameter produced by *L. plantarum* when tested against multidrug-resistant *A. baumannii* isolates was significantly 1.7 -fold greater than that of *L. delbrueckii* and 1.9-fold greater than that of *L. acidophilus*. Notably, 36 to 75% of multidrug-resistant isolates displayed sensitivity to *L. plantarum* supernatant ($p= .03$).

Table3) Distribution and abundance of *Lactobacillus* species in 11 honey types

Probiotic	Honey										
	Citrus	Faruj	Sunflower	Comb	Konarak	Thyme	Ardabil	Camelthorn	Eucalyptus	Astragalus	Coriander
<i>L. acidophilus</i>	+	-	+	+	+	+	+	+	+	+	+
<i>L. reuteri</i>	-	-	+	-	-	-	-	+	-	-	-
<i>L. brevis</i>	+	-	+	-	+	+	+	+	-	+	-
<i>L. gallinarum</i>	-	-	-	-	-	-	-	-	-	-	-
<i>L. plantarum</i>	+	+	+	-	+	+	+	+	+	+	+
<i>L. delbrueckii</i>	+	-	+	+	-	+	+	+	+	+	+
<i>L. fermentum</i>	-	-	+	-	+	-	-	+	-	-	-

Table 4) Inhibitory effects of three probiotics on the growth of MDRAB

Probiotic	Standard Deviation + Average Inhibition Zone Diameter(mm)	P-Value
<i>L. plantarum</i>	21.5 ± 4.07	
<i>L. delbrueckii</i>	11.5 ± 3.60	.03
<i>L. acidophilus</i>	11.8 ± 0.00	

Comparison of groups expressed as mean ± standard deviation using one-way analysis of variance

Discussion

Wound healing could be delayed or stopped by numerous factors, including diabetes, skin infections, trauma, and burns. The number of patients with burn wounds is increasing rapidly due to lifestyle changes and aging problems. Burns are one of the most devastating medical conditions, with long-term physical and psychological effects on patients. Colonization of the wound site by pathogens and opportunistic bacteria significantly contributes to the chronicity of wounds. Burn injuries are very common in our country, and previous studies have identified *A. baumannii*, *P. aeruginosa*, and *S.*

aureus as the most common bacteria found in burn wounds [18- 20]. In this study, the frequency of *A. baumannii* isolates from burn wounds was reported to be approximately 28.64%. This figure is slightly lower than the frequency (29%) reported in the same region in 2025 [19] and significantly lower than that (55%) reported in 2019 [7]. The prevalence of bacterial agents responsible for post-burn infections could vary depending on the infection site. Given that antimicrobial resistance patterns may differ between regions and even between different hospitals within the same community, this study aimed to determine the frequency of multidrug-resistant phenotypes of *A. baumannii* isolates from burn wounds in the northern provinces. In this study, *A. baumannii* isolates exhibited resistance to all tested antibiotics. The highest resistance rates were observed against aztreonam (42.86%) and ticarcillin-clavulanic acid (39.68%). Additionally, 76% of the isolates were classified as multidrug-resistant, which is higher than that reported in a study in Iran in 2013 [21].

In two other studies in Iran, the frequency of MDRAB was reported to be 60 and 45%,

respectively [22, 23], which are lower compared to studies conducted in Spain and Italy [24, 25]. According to the latest report from the WHO and the European Centre for Disease Prevention and Control (ECDC), there was significant variation in the prevalence rates of carbapenem-resistant *Acinetobacter* spp. across Europe in 2020. Among the 38 countries and areas that provided data, three countries reported incidence rates of less than 1%. In contrast, 35 countries had rates of 50% or more. Ireland, the Netherlands, and Norway had the lowest rates, while 21 countries, particularly in southern and eastern Europe, reported carbapenem resistance rates of 50% or more [26]. In this study, resistance of MDRAB isolates to carbapenems was lower than 20%. Given the widespread nature of this problem, it is reasonable to establish guidelines for the sensible use of systemic antimicrobials and alternative treatments in the management of burn and wound infections. These guidelines should be applied not only across different countries but also within various regions of each country.

Due to its bactericidal and bacteriostatic power, honey affects a wide range of bacteria, including aerobic, anaerobic, Gram-negative, and Gram-positive bacteria. Compounds in honey, such as flavonoids and aromatic acids, attack the bacterial cytoplasm, leading to the loss of potassium ions and damage by stimulating cell autolysis and increasing bacterial membrane permeability, leading to the loss of bacterial capacity to synthesize ATP, which in turn inhibits bacterial growth [27]. In this study, *L. plantarum* isolated from Camelthorn honey showed the best antibacterial properties against MDRAB. A study by Li et al. (2023) showed that honey probiotics not only controlled bacterial infections but also promoted wound healing [28]. In a 2016 study, the antimicrobial and anti-adhesion effects of *Lactobacillus* on

uropathogenic *Escherichia coli* bacteria were confirmed in all microbial tests used [29]. In 2021, the same researchers showed that honey compounds were effective in controlling infections caused by surgery, wounds, burns, eye diseases, skin diseases, oral mucosa problems, and necrotic areas [30]. In 2017, lactobacilli isolated from honey were shown to inhibit the growth of *S. aureus* isolates [31]. Studies have also examined the antibacterial effects of honey and confirmed a direct relationship between honey concentration and storage time with its antimicrobial effects (zone of inhibition) [30, 32]. Although this property could vary depending on the type of honey, in the present study, probiotics isolated from Camelthorn honey showed the greatest anti-*Acinetobacter* effect. Research has indicated that honey-containing foods significantly enhance probiotics. Both monofloral honey (chestnut, acacia, lime) and polyfloral honey (eucalyptus, greenbrier) could serve as effective prebiotics that support the growth of bifidobacteria strains in yogurt [29, 31]. A 2018 study in Iran cited the presence of peroxide (hydrogen peroxide) and non-peroxide (antioxidants) components in honey as the reason for inhibiting the growth of *Shigella sonnei*, *Listeria monocytogenes*, *S. aureus*, and *Bacillus cereus* [32]. Matzen et al. (2018) showed that honey derived from Danish rose had a greater antibacterial effect, inhibiting the growth of *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa* in all cases. [33] In a study in Finland, commercially available organic honeys had significant antimicrobial activity against several bacterial pathogens, including *P. aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, and *Bacillus* [34]. The diverse antibacterial effects of honey probiotics on different bacteria could be attributed not only to the acidity and diverse antimicrobial properties of various honey types but also to the diversity

and resistance capabilities of the pathogens involved.

The present study had some limitations. The limitations of this study were the small number of isolates by type of infection (it should be noted that it was part of the sampling process during the COVID-19 pandemic), potential bias, and lack of generalizability to other populations. However, the strength of the present study is the monitoring and measurement of drug resistance of *A. baumannii* isolates from the burn wards of several hospitals in the north of the country in recent years and the confirmation of the anti-*Acinetobacter* potential of Camelthorn honey *in vitro*.

Conclusion

Given the high prevalence of drug-resistant *Acinetobacter* in various hospital wards worldwide [35] over the past decade and considering the current study findings, it is essential to continuously detect and monitor *A. baumannii* strains exhibiting high levels of drug resistance. This approach is crucial for reducing the threat of antimicrobial resistance in Iran. Although this *in vitro* study did not explore the biochemical properties of honey, certain native Iranian honeys were shown to have desirable anti-*Acinetobacter* properties due to the presence of lactobacilli. Additionally, since honey is a natural product with minimal toxicity and side effects, it could be effective in controlling post-burn infections if appropriate local drug protocols are established. Therefore, it seems promising to investigate the physicochemical properties and potential effects of honeys with probiotic characteristics.

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Ethical approval : This study was approved by the Ethics Committee of Islamic Azad University, CHALUS Branch, Mazandaran, Iran (code No. 1401023).

Authors' Contribution: L. Fozouni contributed to the conceptualization, investigation, data curation, formal analysis, visualization, and writing (original draft preparation). Both L. Fozouni and H. Barzegar worked on the methodology, investigation, data curation, and writing (original draft preparation). All authors contributed to conceptualization, methodology, funding acquisition, project administration, resources, supervision, validation, and writing (review and editing).

Conflict of interest: The authors declare that there is no conflict of interest.

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Data Availability: The data that support the findings of this study are available upon request from the corresponding author.

Consent to participate: Patients were satisfied to participate in the study.

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