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Research Article

Antimicrobial Activity of Chitosan Scaffold Loaded with Soluble Factors of Different Probiotic Strains Against Multidrug Resistant *Pseudomonas aeruginosa*

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Background: Bacterial infection remains the most frequent complication of burn injury, which can lead to sepsis, even if antibiotics are used topically and systemically. *Pseudomonas aeruginosa* (*P. aeruginosa*) is the main causative agent in many cases. The emergence of antibiotic-resistant strains in recent years has increased the need to find novel alternative therapies, such as probiotics. Therefore, this study aimed to examine the antimicrobial properties of probiotic cell-free supernatant (CFS), along with the potential use of a chitosan scaffold both as an antimicrobial agent and as a carrier for the delivery of these complexes.

Objective: Evaluation of the antimicrobial properties of cell-free soluble factors of probiotic bacteria both alone and in combination with chitosan scaffolds

Materials and Methods: Nine isolates of *P. aeruginosa* previously identified by standard diagnostic tests were investigated. The antimicrobial effects of probiotics in the form of Pedilact® oral drop which contained three probiotic strains, Kidilact® sachet, which contained seven probiotic strains, and strains of *Lactobacillus casei* (*L. casei*) and *Lactobacillus acidophilus* (*L. acidophilus*) isolated from yogurt were studied by an agar well diffusion assay and by using CFS harvested at various growth stages, without pH neutralization. Chitosan with different concentrations of glutaraldehyde (GA) as a crosslinking agent was fabricated to produce a suitable scaffold for loading cell-free supernatants of probiotic strains. The scaffolds were then characterized using scanning electron microscopy. The antimicrobial properties of the CFS, chitosan, and chitosan scaffolds loaded with CFS were analyzed against MDR *P. aeruginosa.*

Results: In the agar well diffusion assay, CFS obtained from probiotic strains effectively inhibited the growth of a clinical strain of *P. aeruginosa*. This effect was observed when CFS was assessed without pH neutralization. Kidilact® was the most promising synbiotic formulation based on its inhibitory activity. The chitosan scaffold was successfully fabricated, as shown by SEM, and its structure was not affected by acidic CFS. The fabricated scaffolds were able to deliver CFS and, interestingly, antibacterial activity against *P. aeruginosa* when CFS was loaded on the chitosan scaffold was enhanced significantly.

Conclusion: The results of this study showed chitosan scaffold loaded with cell-free probiotics metabolites can be considered to be a promising antimicrobial dressing in wound healing applications

Keywords: Antibiotic resistance, Antimi crobial effect, Chitosan, Probiotics, *Pseudomonas aeruginosa*

1. Background

Burn wound infections are, by far, the most frequent complications encountered by patients in the acute phase of thermal injury. Most hospital infections in burn wards are caused by opportunistic bacteria and extensive wounds after burns, leaving a susceptible route for bacterial invasion. *Pseudomonas aeruginosa* is the most common burn wound pathogen, and also the most likely one to be responsible for sepsis leading to burn-linked death (1, 2).

The emergence of antibiotic-resistant bacterial strains is one of the greatest threats to public health. The mechanisms of antimicrobial resistance are as varied as the bacteria themselves and could be intrinsic or acquired from other microorganisms (3).

P. aeruginosa has intrinsic resistance to a wide range of antibiotics and because of this, infections caused by this bacterium are becoming increasingly challenging. In addition, the widespread use of antibiotics in recent years has caused this bacterium to become resistant to broad-spectrum antibiotics of different classes. Therefore, antibiotic options for infection control have become increasingly limited and expensive (4, 5). Since the world is heading toward a post-antibiotic era, many strategies are now focused on finding effective natural agents (6).

Probiotics are living microbial species and, when administered rationally, confer health benefits to the host. Important mechanisms by which probiotics affect the health of the host include regulating the immune system of the intestine, improving the function of physical and immunological barriers, and competitive elimination of pathogenic microorganisms by producing antimicrobial peptides (7-9). Moreover, previous studies have reported the effects of synergistic administration of probiotics and prebiotics, known as synbiotics, on general infectious complications in various patients (10).

Numerous studies have shown that probiotics have beneficial effects in the treatment of gastrointestinal diseases. Probiotic treatment in periodontal medicine and for respiratory and urogenital infections has been successful. Probiotics are, therefore, widely used for the prevention and treatment of skin diseases; specifically, wound infection is one of the most promising areas (11). Bacteria belonging to the Bifidobacterium and Lactobacillus genera are the most widely used probiotic microorganisms. Lactic acid bacteria especially have attracted particular attention because they can outcompete pathogens while also regulating the immune response by inhibiting neutrophil and macrophage apoptosis and enhancing phagocytosis (12). It seems that probiotic therapy against Pseudomonas is particularly promising, with evidence that *L. plantarum* can interfere with quorum sensing, inhibit biofilm formation, and reduce pseudomonal bioburden in burn wounds (13, 14). Since burn wound infection is a localized disease, local treatments that can achieve proper inhibitory concentrations in infected areas are preferred over systemic treatment (15). However, despite the clear advantages of local antimicrobial treatment, there are still several challenges posed by the wound environment that impede the efficient delivery of therapeutic agents (16). In this context, topical delivery of bioactive functional probiotics can treat or prevent infection; however, improved delivery systems are required for an optimal release profile and efficacy.

The development of delivery carriers based on natural or synthetic polymers is gaining importance for enhancing therapeutic efficacy. However, natural products are more biocompatible than synthetic products for use in biomedical research.

The natural biopolymer, chitosan, has been used in many forms, including films, coatings, beads, and hydrogels, to deliver antibiotics and antimicrobial agents. Chitosan scaffolds are among the most important drug carriers for the delivery of therapeutic agents, especially in infected wounds. It is one of the most important biopolymers involved in producing wound dressings, which not only accelerates wound healing but also prevents infection, an important aspect of wound healing (17).

Although numerous studies have demonstrated similar results, the mechanism by which chitosan inhibits microbial growth is not fully understood. Scientists have therefore proposed several hypotheses, including cell wall disruption and intracellular component leakage as electrostatic interactions, affecting protein synthesis processes, and interfering with nutrient chelation. Moreover, as a wound dressing, chitosan stimulates the natural healing process and is non-toxic to mammalian cells (18).

This study was therefore performed to evaluate the antimicrobial properties of cell-free soluble factors of different probiotic strains both alone and in combination with chitosan scaffolds.

2. Objective

In this study, we evaluated the antimicrobial properties of cell-free soluble factors of different probiotic strains,

both alone and in combination with fabricated chitosan scaffolds (both as delivery means and antibacterial treatment), against MDR *P. aeruginosa* isolated from clinical samples as a potential candidate to address infection in skin wounds.

3. Material and Methods

3.1. Bacterial Isolation, Identification

Nine *P. aeruginosa* isolates from burn wounds were previously identified in a study by Rudy et al. (19). All isolates were identified by biochemical analysis and confirmed by the detection of the mexB gene using PCR gene amplification technique. Each specimen was named S1–S9 for further processing.

3.2. Antibacterial Susceptibility Testing

Antibacterial susceptibility testing of these isolates was performed using the Kirby–Bauer disc diffusion method, and the results were interpreted according to the 2020 Clinical and Laboratory Standards Institute (CLSI) guidelines. The diameters of complete inhibition zones were measured (judging by unaided eyesight). The diameters of the inhibition zones were compared with the critical values of each antimicrobial disc to qualify the target bacteria as sensitive, resistant, or intermediate.

3.3. Synbiotic and Probiotic Strains and Preparation of Cell-Free Supernatant from Pre-Cultures

Synbiotics in the form of Pedilact® oral drop (Zist-takhmir Company, IR), Kidilact® sachet (Zist-takhmir Company, Iran), and *L. acidophilus* (purified from yogurt), and *L. casei* ATCC 393 were used. Each sachet and 15mL oral drop contained 109 colony-forming units (CFU) of probiotic strains. The oral drop contained high levels of three bacterial strains of *Lactobacillus rhamnosus*, *Lactobacillus reuteri, Bifidobacterium infantis*, and Kidilact® sachet, which contained high levels of seven bacterial strains of *Lactobacillus acidophilus (L. acidophilus), Lactobacillus casei (L. casei), Lactobacillus rhamnosus, Bifidobacterium infantis, Bifidobacterium breve, Lactobacillus bulgaricus,* and *Streptococcus thermophiles.*

Bacterial pre-culture preparation to provide optimal conditions for bacteria to present their potential antibacterial activity is a fundamental step. Strain suspensions in phosphate-buffered saline (PBS) were adjusted to achieve an optimal density of 0.22 at 600 nm. One mL of bacterial suspension was sub-cultured on 9 mL Man-Rogosa-Sharpe (MRS) broth and then incubated for 12, 24, 36, 48, 72 h, and 6 days at 37 °C in shaking condition. Upon incubation, the bacteria were harvested by centrifugation (10 min, 4000 rpm), and the supernatant was separated and filtered through a 0.22 µm filter. To prepare a cell-free supernatant (CFS) of *L. acidophilus*, an isolated colony was obtained on blood agar, and the remaining process was similar to that of the other bacteria.

3.4. Antimicrobial Assay (Well diffusion method)

The microbial suspensions prepared from *P. aeruginosa* equal to 0.5 McFarland (corresponding to 1.5 × 108 $CFU.mL^{-1}$) standard were cultured on MHA. Then, wells with a diameter of 5.5 mm were created, and 90 µL of CFS of probiotic bacteria was added to the wells. The plates were then incubated at 37 °C for 24 h. A control experiment was performed by replacing CFS with MRS broth.

3.5. Preparation of Chitosan Scaffold

Chitosan powder (Sigma-Aldrich, St Louis, MO, USA) with medium molecular weight was used to prepare the scaffold. Chitosan solution (2%, w/v) was dissolved in 1% acetic acid and the resulting solution was mixed by mechanical stirring at 250rpm for 2 h. After complete dissolution, the chitosan solution was divided into two aliquots.

F1: The solution was filtered through a 0.45 µm filter. First, 500 µL of the chitosan blend was aliquoted into 48-well plates and placed at -20 °C for 24 h and then at -70 °C for another 24 h. Finally, the solution was freeze-dried at -70 °C. The scaffolds were then treated with glutaraldehyde (GA) (2.5%, w/v) for 4 h at room temperature (RT), and after proper washing, they were placed in a freeze dryer at -50 °C for 24 h.

F2: The chitosan blend was placed on a magnetic stirrer and GA solution (0.25 %, w/v) was added drop-wise until the final volume of GA in the chitosan blend was 8%. Subsequently, it was aliquoted to 500 µL in 48-well plates and incubated for 24 at -20 °C and 24 h at -70°C respectively, followed by another 24 h incubation at -70 °C freeze dryer. The formed scaffolds were treated with GA solution (0.25%, w/v) at RT for 4 h, and after proper washing, they were placed again at -50 °C freeze dryer for 24 h.

Table 1. Inhibition zones of *P. aeruginosa* isolate by chitosan scaffold coated with probiotics supernatant using well diffusion method†

	Mean zone of inhibition (mm)		P-value
	F1	F2	
Pedilact®	15.8 ± 0.6	19.6 ± 1.5	0.002
Kidilact®	18 ± 0.41	24 ± 2	0.004
Lactobacillus acidophilus	17.2 ± 0.9	22 ± 2.1	0.004
Lactobacillus casei	17.5 ± 1.11	21.3 ± 0.9	0.004

†Data are expressed as mean± standard deviation of results from three replicate experiments

All Scaffolds were treated according to the following steps: 1. Three times wash for 15 minutes in sterile water. 2. Leave the scaffold in glycine solution (0.5 M) for 15 minutes and 3. Three times wash for 15 minutes in sterile water.

3.6. Preparation of Chitosan Scaffolds Loaded with Probiotic Cell-Free Factors

The S4 isolate was cultured on MHA. Two groups of scaffolds, F1 and F2, were impregnated with CFS of strains obtained from 48-hour pre-culture of Pedilact®, Kidilact®, *L. acidophilus,* and *L. casei,* placed on the surface of plates and then incubated for 24 h.

The antimicrobial potency of the probiotic cell-free factors was determined by measuring the inhibition zone, as described previously. Control samples were scaffolds impregnated with sterile water.

3.7. Morphology of Scaffolds

Scanning electron microscopy (MIRA3, TESCAN CO., Czech Republic) was used to evaluate the morphological characteristics of the chitosan scaffolds. Prior to imaging, two dry cylindrical samples from each scaffold group were sputter-coated with gold-palladium (Q150RES; Quorum Technology, UK) for 1 min.

3.8. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc). Continuous variables are expressed as mean ± standard deviation and between-group comparisons were based on the Mann–Whitney U test. The level of statistical significance was set at $P < 0.05$.

4. Result

4.1. Characterization of P. Aeruginosa

In the present study, nine isolates that were confirmed to be *P. aeruginosa* according to their biochemical profile and molecular technique (mex B gene PCR test) were used (19).

4.2. Determination of Antibiotic Susceptibility of Clinically Isolated P. Aeruginosa

The MHA-based resistance profiles of nine *P. aeruginosa* isolates from patients with burn wounds are shown in **Table 1**. Antimicrobial resistance was most common against piperacillin and meropenem (100%). Levofloxacin was the most active drug against the isolated *P. aeruginosa* with 67% of the isolates being sensitive to it. According to the results, S4 isolate exhibited higher antibiotic resistance and was selected as the main strain for further analysis (**Fig. 1**).

4.3. Determination of the Antibacterial Effect of Soluble Factors of Probiotics Strains

To determine the antibacterial effects of the soluble factors of different probiotic strains against *P. aeruginosa*, a well diffusion method was used. The growth inhibition zone values for different strains of *P. aeruginosa* supernatants were measured using CFSs obtained from strain pre-cultures at different time frames (i.e., 12 h, 24 h, 36 h, 48 h, 72 h, and 6 days of incubation).

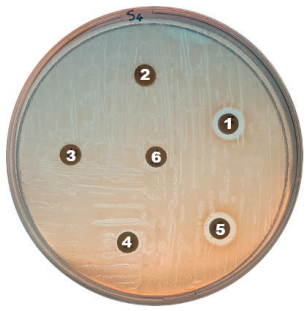

Figure 1. ***Pseudomonas aeruginosa*** **isolate 4 (S4) antibiotic susceptibility pattern. 1**) levofloxacin, **2**) ceftazidime, **3**) meropenem, **4**) aztreonam, **5**) gentamicin, **6**) piperacillin.

The results showed that the highest inhibition zone, which indicates the most robust anti-pseudomonal activity, was produced by 48 h pre-culture CFSs. Therefore, these CSFs were selected for further analysis. CFS had different pH values at different time points. We used 48h culture and the pH at this point was 5 for (Pedilact®, Kidilact®) and 4.5 for *L. acidophilus*, and *L. casei.* Comparing the antibacterial activity of Kidilact® and Pedilact®, Kidilact® showed significantly greater activity ($P = 0.02$), while no significant differences were found between the other strains' supernatants (**Fig. 2**).

4.4. Evaluation of the Antibacterial Effect of Chitosan Scaffold Loaded with Symbiotic Supplements and Probiotics Strains

Based on previous studies by Chhabra *et al*. (18) and Yang *et al*. (20), two approaches were used to prepare chitosan scaffolds. However, the prepared scaffolds dissolved and lost shape upon loading the CFSs, probably due to the acidic pH of the secretions. To stabilize and optimize scaffold fabrication, different concentrations of GA were used.

The effectiveness of the chitosan scaffold as a carrier for different supernatant strains was examined using a diffusion method (such as disk diffusion).

The results of the diffusion method are shown as growth inhibition zones of *P. aeruginosa* using chitosan scaffolds loaded with the probiotic supernatant (**Table 1 and Fig. 3**).

The well-diffusion method confirmed the incorporation and delivery of different supernatants from the chitosan scaffold (**Fig. 3**). CFSs loaded scaffolds provided significantly greater bacterial inhibition than the supernatants alone. Moreover, the F2 group showed better antibacterial activity than the F1 group regardless of the loaded complex ($P < 0.05$).

4.5. Morphology of Scaffolds

The surface morphology and microstructure of the

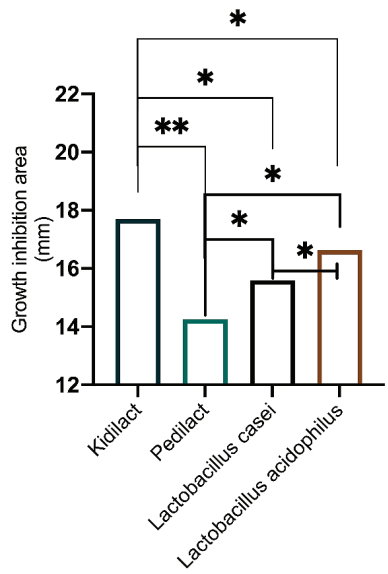

Figure 2. Comparative bar graph of antibacterial activity of CFSs provided from 48 h pre-cultures. Kidilact® presents significantly greater activity ($P = 0.02$), no significant differences were found between the other strains CFS. (*$P < 0.05$, ** $P < 0.05$).

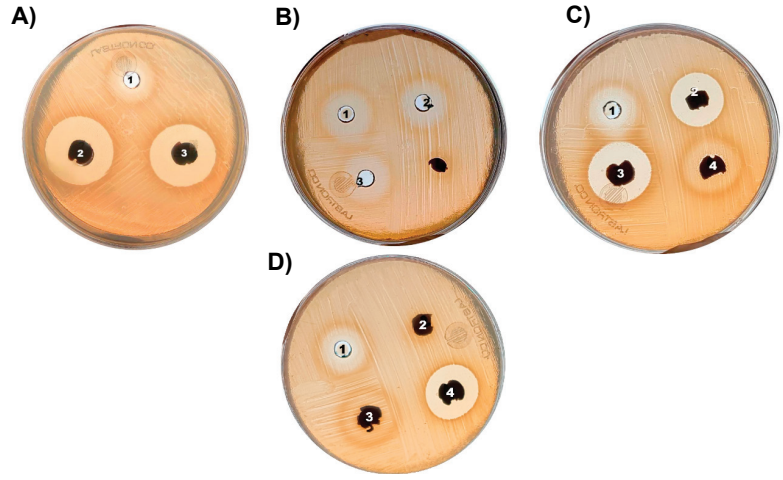

Figure 3. I**nhibition zones of *P. aeruginosa* isolate by chitosan scaffolds loaded with synbiotics and probiotic supernatant (48 h). A1**) *Lactobacillus acidophilus* (*L. acidophilus*) supernatant. **A2**) F2 chitosan scaffold loaded with Kidilact® supernatant. **A3**) F2 chitosan scaffold loaded with *L. acidophilus* supernatant. **B1**) Kidilact® supernatant. **B2**) *L. acidophilus* supernatant. **B3**) *Lactobacillus casei* (*L. casei*) supernatant. **B4**) F1 chitosan scaffold loaded with water (control). **C1**) Kidilact® supernatant. **C2**) F1 chitosan scaffold loaded with Kidilact® supernatant. **C3**) F2 chitosan scaffold loaded with *L. casei* supernatant. **C4**) F2 chitosan scaffold loaded with water (control). **D1**) Pedilact® supernatant. **D2**) F1 chitosan scaffold loaded with water (control). **D3**) F2 chitosan scaffold loaded with water (control). **D4**) F2 chitosan scaffold loaded with Pedilact® supernatant.

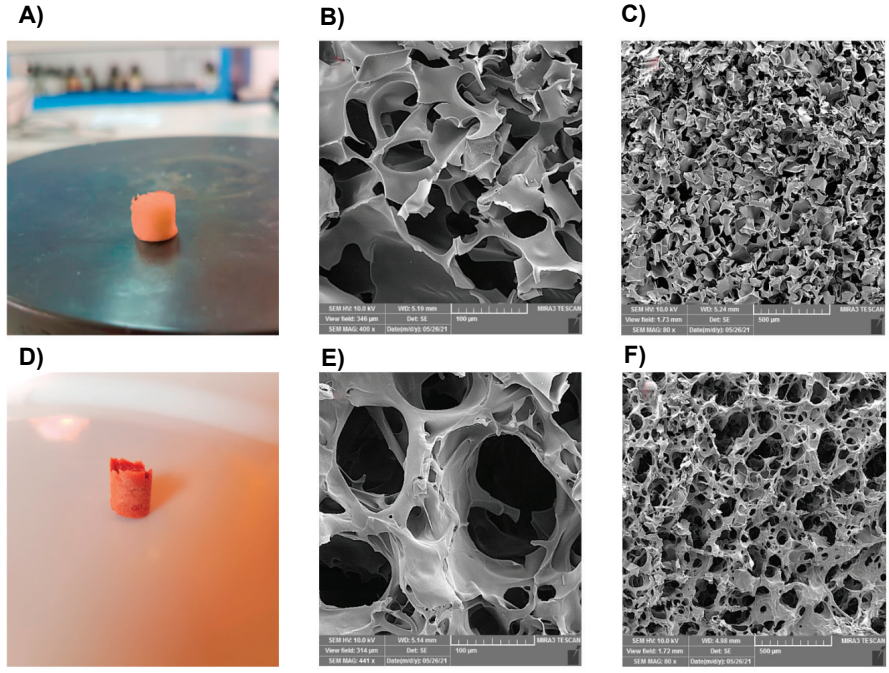

Figure 4. Freeze-dried chitosan scaffolds produced with crosslinking and the corresponding Scanning Electron Microscopy images of unloaded scaffolds. F1 scaffold group (**A, B, C**) treated with GA (2.5%, w/v) at the final step of fabrication, and F2 (**D, E, F**) treated with GA (0.25%, w/v) before freeze-drying.

prepared chitosan scaffolds were analyzed using SEM. The fabricated scaffolds showed a porous structure. The pores exhibited irregular and segregated structures (**Fig. 4**). It seems that adding glutaraldehyde, as a cross-linking and stabilizer agent, does not affect the structure of chitosan scaffolds.

5. Discussion

The continual emergence of antibiotic resistance following chronic wound infection poses a serious threat to public health (1), and novel therapeutic approaches to address this challenge are essential and need to be continuously developed. Recently, the use of novel agents, such as natural products with antibacterial characteristics, bacteriophages, nanoparticles, nanofibers, and probiotics, has received considerable interest. Probiotics and prebiotic antimicrobial metabolites possess strong antimicrobial activity with a minimum risk of developing bacterial resistance. However, the successful local application of these agents owing to their vulnerability to degradation is a significant challenge (11). In the current study, we designed an *in vitro* study to examine the effect of antimicrobial metabolites of two commercial synbiotics and two probiotic strains, both alone and in combination with a chitosan scaffold as a carrier to deliver and possibly enhance antibacterial activity.

The inhibitory effect of probiotic strains against some resistant clinical isolates of pathogenic skin bacteria, including *Staphylococcus aureus* (*S. aureus*) and *P. aeruginosa*, has been extensively reported (21). However, studies on the antimicrobial capacity of synbiotics and their metabolites are limited. The results of the present study revealed that synbiotic supplements and commercial probiotic strain supernatants showed strong inhibitory activity against the resistant clinical isolates of *P. aeruginosa*. Using the well diffusion method, the highest inhibitory zone was recorded by the supernatant of Kidilact®, followed by *L. acidophilus, L. casei*, and Pedilact® which exhibited the lowest inhibitory activity but did not reach a statistical significance level. Other methods such as the disk diffusion assay and agar dilution method were also used to confirm the antimicrobial activity of CFS, but the best results and more reproducibility were achieved by the agar diffusion method, which is most likely due to better solubility and hence the availability of CFS in well diffusion. These findings are consistent with the study by Diaz *et al*. that showed the metabolites (extracts from culture supernatants) of *L. casei* and *L. acidophilus* effectively disrupt the production of virulence factors and interfere with quorum sensing of *P. aeruginosa* strains (22). Similarly, the results of a study by Valdez *et al*. indicated that *L. plantarum* and/or its by-products inhibited *P. aeruginosa* pathogenic activity and colonization both in vitro and in infected burned mouse models (14). Another detailed study by Ramos *et al*. further indicated that *L. plantarum* mediates this anti-pathogenic activity through a quorum-quenching mechanism (13). The other inhibitory mechanism proposed involves altering pH through the production of acetic acid and lactic acid by probiotic strains that inhibit some adhesive molecules of pathogens (23).

The antimicrobial activity of Kidilact® was significantly greater than the other synbiotic, Pedilact®. This could be due to the greater number of probiotic and prebiotic strains that are used within this compound. Abbasi *et al*. showed the antimicrobial activity of CFS of three commercial synbiotics, Kidilact®, Vitalact®, and Protexin®, against methicillin-resistant *S. aureus* (24). They also observed different antimicrobial activities among the synbiotics used, probably due to the diversity and particular combination of probiotic strains in each commercial product.

Several studies have confirmed that probiotics secrete and release antimicrobial compounds such as organic acids, diacetyl, hydrogen peroxide, and anti-microbial peptides. These molecules are selectively active against several pathogenic bacteria. Numerous reports have demonstrated that the CFS of probiotics contains volatile organic compounds that can excrete antibacterial compounds. Bacterial AMPs (Bacteriocins) are a heterogeneous group of ribosomally synthetized peptides that show bactericidal and bacteriostatic effects against pathogens. Therefore, CFS of probiotics could be considered a compound with a combination of different antimicrobial molecules with different mechanisms of action that potentially reduce the chance of resistance induction (25, 26).

Another aspect of this study was the assembly and use of chitosan scaffolds to load and deliver cell-free probiotic metabolites. Chitosan and its derivatives are among the most investigated biomaterials for biomedical applications owing to their biodegradable, biocompatible, non-toxic, and non-allergenic nature. Meanwhile, drug-loaded chitosan scaffolds, owing to their easy processability, have emerged as viable

antimicrobial dressing to control and reduce infection (27, 28).

Chitosan scaffolds loaded with water were not able to inhibit bacterial growth, suggesting a lack of antimicrobial characteristics against resistant *P. aeruginosa*. Sandri *et al*. made a similar observation and reported that unloaded chitosan scaffolds failed to show antibacterial properties. Whereas silver nanoparticle-loaded scaffolds possess antibacterial activity against *E. coli and S. aureus* (29).

Different formulations were used to produce stable chitosan scaffolds. It seems that adding glutaraldehyde provides sufficient mechanical stability to the scaffold to bear the acidic pH of the probiotic metabolites. In addition, the F2 group scaffolds loaded with synbiotics or probiotic supernatants presented higher antibacterial activity than their F1 counterparts. This could be due to the different GA treatment methods used in the F1 and F2 groups. GA concentration possibly alters the cross-linking degree of the prepared scaffold and, as a result, affects the release profile of the loaded agents. Interestingly, despite the lack of antimicrobial activity of unloaded chitosan, CFS-loaded chitosan showed enhanced activity in comparison to CFS alone. We know from other studies that chitosan shows some limited antimicrobial activity, which in combination with CFS, the antibacterial effect is boosted most likely due to the different mechanisms of action of the two compounds. To evaluate the different ingredients, present in CFS released from chitosan, analysis like GC-MS (for volatile compounds) and LC-MS (for AMPs) should be done.

The present study showed that commercial synbiotics in the form of oral drops or tablets and isolated probiotic strains display relatively robust inhibitory activity against resistant clinical isolates of *P. aeruginosa*. Moreover, we developed a fabrication method for a chitosan scaffold that could be used for the effective incorporation of these antibacterial agents and to enhance their profiling release.

6. Conclusion

In conclusion, a chitosan scaffold loaded with cell-free probiotic metabolites can be considered a promising antimicrobial dressing for wound-healing applications. The chitosan scaffold was developed successfully to retain, deliver, and enhance antibacterial activity. Further analysis, such as gene and protein expression studies, would be beneficial to evaluate the impact of synbiotic and probiotic supernatants on the molecular mechanisms of antibiotic resistance. It is also recommended to evaluate the effectiveness and dressing activity of probiotic-loaded chitosan scaffold in animal models *in vivo*. Scaffolds with antimicrobial activity, especially against MDR *P. aeruginosa* (which is a major cause of wound infection)) could potentially be used for dressing wounds in wound care clinics or burn wards to improve the wound healing process. Indeed, limitations such as safety and mechanical stability of the scaffold need to be addressed in further studies.

Declarations

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Conflict of Interest

None.

Ethics approval

The study protocol was reviewed and approved by the ethics committee of the Mashhad University of Medical Sciences (ID: IR.MUMS.MED.REC.1398.188) on 2019/04/30.

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