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Research Article

Effect of Lactoferrin Loaded on Chitosan Against *Staphylococcus Aureus* and *Pseudomonas Aeruginosa*

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Abstract: The increasing resistance of bacterial strains to antimicrobial compounds presents a significant global health challenge. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two such bacteria that pose substantial risks, particularly in causing infections of the respiratory tract and bacteremia. Chitosan, a non-toxic and biocompatible polymer, has demonstrated inhibitory effects on bacterial growth. Lactoferrin, an iron-binding protein found in milk, exhibits antimicrobial properties. In this study, lactoferrin was purified from camel milk using CM Sephadex C-50 chromatography after removing casein through ion exchange chromatography. The purification process was confirmed using SDS-PAGE and the absence of color with tetramethylbenzidine. Subsequently, purified lactoferrin was loaded onto 1% chitosan using thiamine pyrophosphate (TPP). The successful loading of lactoferrin onto chitosan was verified using Scanning Electron Microscopy (SEM), Zeta Potential, Particle Size Determination, and Fourier Transform Infrared Spectroscopy (FTIR). After evaluating the cytotoxicity of various lactoferrin concentrations loaded on chitosan, the antimicrobial effects of lactoferrin (at concentrations of 300 and 350 µg/mL) loaded on chitosan were assessed using a microassay method. The results demonstrated a significant decrease (P-value < 0.01) in the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria compared to the control group when treated with 300 µg/mL of lactoferrin-loaded chitosan. Furthermore, a substantial decrease (P-value < 0.001) was observed with a concentration of 350 µg/mL.

Keywords: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, antimicrobial properties, chitosan, lactoferrin

1. Introduction

The growing repertoire of bioactive peptides derived from milk proteins has highlighted their potential applications [1]. Antimicrobial peptides (AMPs), produced by living organisms, play a crucial role in natural defense against infections [2]. Camel milk contains antibacterial compounds like lysozyme and lactoferrin, alongside beta-2 lactoglobulin and lower alpha casein levels, making it less allergenic for children [3]. Lactoferrin, a prominent glycoprotein found in milk and various bodily secretions, exhibits antimicrobial properties [4]. Its protein structure comprises two distinct lobes, each capable of binding to free iron. Lactoferrin's multifunctional properties make

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it a promising candidate for clinical and commercial use. Recent studies have demonstrated its beneficial effects, including immune system modulation, antimicrobial activity, and antioxidant properties, for both infants and adults [4]. Lactoferrins exert their antimicrobial effects through both bacteriostatic and bactericidal mechanisms against a wide range of Gram-positive and Gram-negative bacteria [5]. Beyond their direct antibacterial activity, lactoferrin and its derived peptides can potentially serve as natural antibiotics in human and veterinary medicine [6]. Moreover, lactoferrin's antimicrobial activity is mediated through both iron-dependent and iron-independent pathways [7]. Lactoferrin is a vital component of the immune system, acting as a primary defense barrier against microbial attacks [8, 9]. Gram-negative bacteria possess an asymmetric outer membrane composed of a phospholipid-rich inner leaflet and an outer leaflet primarily consisting of lipopolysaccharide (LPS). LPS structures in bacteria are modified in response to the requirement of different environment [10]. Lactoferrin has been shown to bind to the lipid A component of LPS. In bovine lactoferrin, the loop region corresponding to amino acids 19 to 36 is crucial for this interaction and is also present in the lactoferricin sequence [5]. Chitosan, a biocompatible, bioactive, and non-toxic polymer, has diverse applications [11]. Its primary amine groups contribute to its antibacterial and antitumor properties. Additionally, chitosan's ability to spontaneously form nanoparticles enhances its antimicrobial activity compared to its original form [12]. Derived from crustacean exoskeletons, chitosan is a versatile sugar with various applications [13]. *Staphylococcus aureus*, a bacterium commonly found on the skin and nasopharynx, poses a significant health threat by causing infections in multiple body regions and contaminating food. Despite advancements in healthcare, *Staphylococcus aureus* remains a formidable pathogen, leading to considerable morbidity and mortality in both community and hospital settings [14]. *Pseudomonas aeruginosa*, an opportunistic pathogen, primarily affects immunocompromised patients and is a major contributor to morbidity and mortality in individuals with cystic fibrosis and hospital-acquired infections. Its diverse mechanisms for adaptation, survival, and resistance to multiple antibiotics make *Pseudomonas aeruginosa* infections life-threatening and a substantial public health concern globally, particularly in specific food groups like milk and dairy products [15]. Antimicrobial peptides (AMPs) represent a growing area of research, alongside traditional antimicrobial agents [16]. This study aims to contribute to our understanding of the antimicrobial properties of lactoferrin (Lf) loaded on chitosan, particularly against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The research seeks to explore the potential of developing alternative treatments to address the escalating issue of bacterial resistance. This involves generating novel structures that can be effectively utilized in the development of therapies against these specific bacteria.

2. Methods

2.1 *Purification of lactoferrin*

Camel milk was sourced from a farm near Mashhad city, Iran. Lactoferrin (cLf) purification followed the protocols established by Raei et al. (2015) and the modified method of Yoshida (1991) [17, 18]. The process involved defatting through centrifugation, adjusting milk casein pH to 4.6, and subsequent centrifugation. Ammonium sulfate precipitation and dialysis were conducted to remove salts. Defatting and casein separation were followed by ion exchange chromatography using CM Sephadex C-50 resin. Fractions were collected based on salt concentration and analyzed for protein content using spectrophotometry at 260 nm. SDS-PAGE confirmed the presence of a single band with a molecular weight of around 80 kilodaltons. The Bradford test determined protein concentrations, and the presence of lactoferrin or lactoperoxidase in collected fractions was verified using the tetramethylbenzidine (TMB) color production test. Fractions containing lactoperoxidase were identified by the blue color produced in the presence of hydrogen peroxide and tetramethylbenzidine. The Bradford test determined protein concentrations. These fractions were then lyophilized to concentrate cLf.

2.2 *Bacterial strain preparation*

Pseudomonas aeruginosa (ATCC27853) and *Staphylococcus aureus* (ATCC25923) strains were obtained from the Razi Institute of Vaccine Research and Serum Manufacturing, Northeast Branch. Bacterial counts were determined, and dilutions of 10^6, 10^7, and 10^8 were prepared for subsequent experiments [19].

2.3 *Loading lactoferrin on chitosan*

Lactoferrin (cLf) was loaded onto chitosan (medium molecular weight, powder, Sigma-Aldrich) by adding thiamine pyrophosphate (TPP) to deionized water, adjusting the pH, and then adding 120 microliters of the nanobubble sample. Subsequently, 10 mL of medium molecular weight chitosan at pH 5 was introduced onto a shaker and incubated at 57 degrees Celsius for 10 minutes, following the methodologies outlined by Ren et al. (2018) and Yang et al. (2017) [20, 21].

2.4 *Confirmation of lactoferrin loading on chitosan*

To assess the loading of cLf on chitosan, various techniques were employed:

2.4.1 *Scanning electron microscope*

cLf samples were prepared for scanning electron microscopy by placing 20 microliters of the sample on an aluminium sheet and allowing it to dry at room temperature. A scanning electron microscope was utilized to analyze the size of cLf loaded on chitosan and generate magnified images of the sample.

2.4.2 *Zeta potential*

The measurement of cLf loading on chitosan was conducted using a Zeta model device, specifically the HORIBA NANO PARTICLE ANALYZER MODEL SZ-100.

2.4.3 *Particle size determination*

The investigation of the size distribution of cLf loaded on chitosan was carried out with a particle-size device, namely the NANO PARTICLE ANALYZER MODEL SZ-100 HORBIA.

2.4.4 *Dynamic light scattering*

Dynamic light scattering was employed to evaluate the dispersion and changes in light intensity resulting from the Brownian motion of particles. The size of cLf particles loaded on chitosan was determined under specific conditions using the NANO PARTICLE ANALYZER MODEL SZ-100 HORBIA.

2.4.5 *Fourier transform infrared spectrometer test:*

The quality of the sample was assessed using an infrared Fourier transform spectrometer test, recording the functional groups of the samples. Thermo Nicolet Avatar 360 FTIR spectrometer was utilized for this analysis. These techniques collectively confirm the successful loading of cLf onto chitosan and provide comprehensive insights into the physical characteristics of the loaded particles.

2.5 *Investigating the cytotoxicity of lactoferrin-loaded chitosan*

To assess the effects of lactoferrin-loaded chitosan on cell growth and proliferation, the MTT colorimetric assay was employed using the HeLa cell line [16]. The experiment was conducted in triplicate. The results obtained included the amount of cell debris and the concentration inhibiting cell growth by 50% (IC50). SPSS software and ANOVA statistical tests were utilized for data analysis [22].

2.6 *Investigating the effect of lactoferrin on bacteria*

To evaluate the impact of various lactoferrin (cLf) concentrations on the growth and proliferation of *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria, concentrations of 300 and 350 micrograms/mL were administered every two hours for 24 hours. The initial bacterial counts for *Pseudomonas aeruginosa* were 15.5×10^4, 15×10^5,

and 15×10^6, while for *S. aureus*, counts were 33×10^5, 33×10^6, and 33×10^7 milliliters. BHI medium (Brain Heart Infusion) was used for bacterial growth, with 100 microliters applied to each well. Growth controls included 10^6, 10^7, and 10^8 dilutions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria, along with phosphate-buffered saline (PBS) and BHI [1, 16].

2.6.1 *Statistical analysis*

The normality of the data was checked using Minitab 19 software. A split-plot design was employed for time-series analysis. The comparison of the average main and reciprocal effects was performed using the Least Significant Difference method at the $P < 0.01$ level.

3. Findings

3.1 *Lactoferrin purification results*

Following the defatting process, camel milk underwent purification using ion exchange chromatography. SDS-PAGE analysis revealed protein bands of approximately 80 kilodaltons (Figure 1), with a protein concentration of 433 micrograms per millilitre. The experimental results pinpointed fractions 0.6, 0.7, and 0.8 as being rich in cLf.

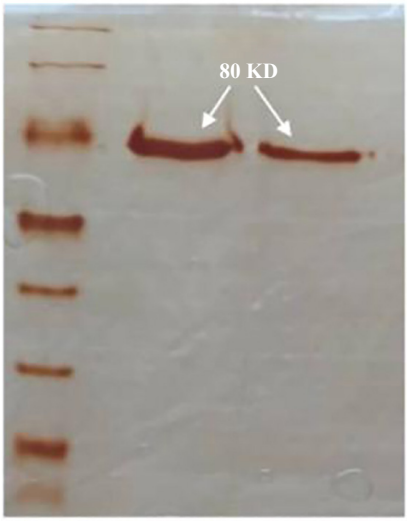


Figure 1. SDS-PAGE Electrophoresis Results

In Figure 1, the electrophoresis results of SDS-PAGE demonstrate the protein bands for specific fractions. Notably, bands for fraction 1, fraction 0.8, molar number 2, fraction 0.6, and molar number 5 are visible. The molecular weight marker (kilo Dalton) serves as a reference for estimating the sizes of the observed protein bands. The distinct bands at approximately 80 kilodaltons indicate the presence of lactoferrin in the analyzed fractions. This confirmation supports the successful purification of lactoferrin from camel milk during the experimental process.

3.2 *Confirmation results of lactoferrin loading on chitosan*

3.2.1 *Scanning electron microscopy results*

After the successful loading of cLf on chitosan, scanning electron microscopy (SEM) was employed to visualize the resulting structures. The SEM images revealed shapes with larger dimensions, providing insights into the morphological characteristics of the loaded entities. Below each image, specific details regarding dimensions and

surface type are presented, enhancing the understanding of the structural changes induced by the loading process.

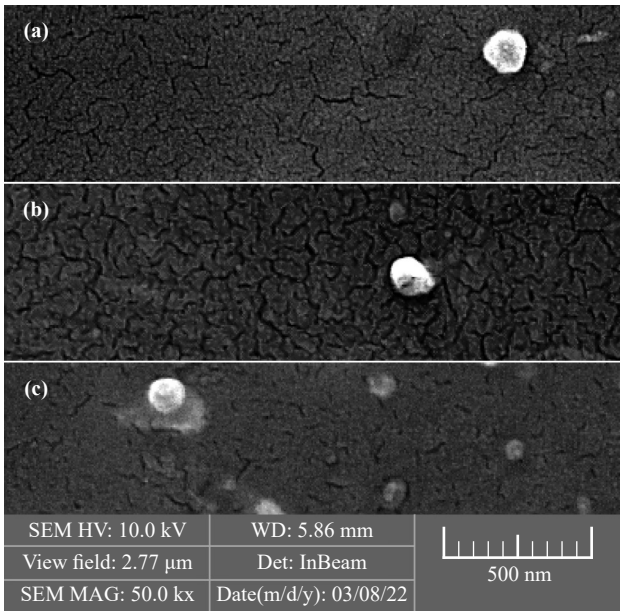


Figure 2. Scanning electron microscope image (a, Chitosan, approx70 nm)-(b, Lactoferrin 300 micrograms/mL, approx. 200)-(c, Lactoferrin 350 micrograms/mL, approx. 200) Loaded on chitosan Spherical shapes Surfaces was observed

3.2.2 *Load measurement results on the particle surface*

The surface charge of particles in the samples was quantified through zeta potential measurement. The results of measuring the surface charge are presented in Table 1, showcasing the zeta potential values obtained using a zeta potential measuring device.

Table 1. The results of the potential

Surface charge (mV)	Point A
-8.26	Chitosan nanoparticles
-4.4	Chitosan loaded with a concentration of 300 µg/mL cLf
-9.4	Chitosan loaded with a concentration of 350 µg/mL cLf

3.2.3 *Particle size measurement results*

The particle size in the mentioned samples was determined and the results are provided in Table 2. The measurements were conducted using a particle meter to quantify the number of particles at different sizes.

Table 2. Particle Size Measurement Results by Particle Meter

Average particle size (nm)	samples
965	chitosan
677	Chitosan loaded with a concentration of 300 µg/mL cLf
630	Chitosan loaded with a concentration of 350 µg/mL cLf

3.2.4 *Dynamic light scattering results*

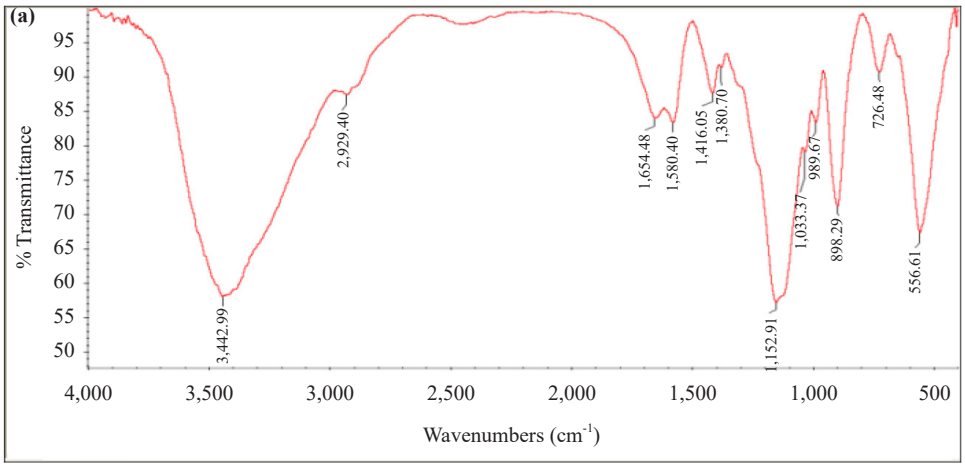
The analysis of results obtained through dynamic light scattering, utilizing cumulative operations to determine the polydispersity index and mean hydrodynamic diameters, is presented in Table 3. The average cLF loaded onto chitosan exhibited a trend of decreasing and then increasing with increasing cLf concentration. Notably, for all samples, the polydispersity index was higher than 0.451, indicating that the samples are polydisperse, containing particles of varying sizes.

Table 3. Dynamic Light Scattering Results

P.I	Z-averages	samples
0.451	0.1885	chitosan
0.359	9.489	Chitosan loaded with a concentration of 300 µg/mL cLf
0538	7.6267	Chitosan loaded with a concentration of 350 µg/mL cLf

3.2.5 *Fourier transform infrared spectrometer (FTIR) results*

The functional groups of cLf were examined through Fourier transform infrared spectroscopy (FTIR) and recorded using the Thermo Nicolet Avatar 360 FTIR spectrometer. The FTIR spectra are presented in the wavelength range of 1,400-4,000 cm.



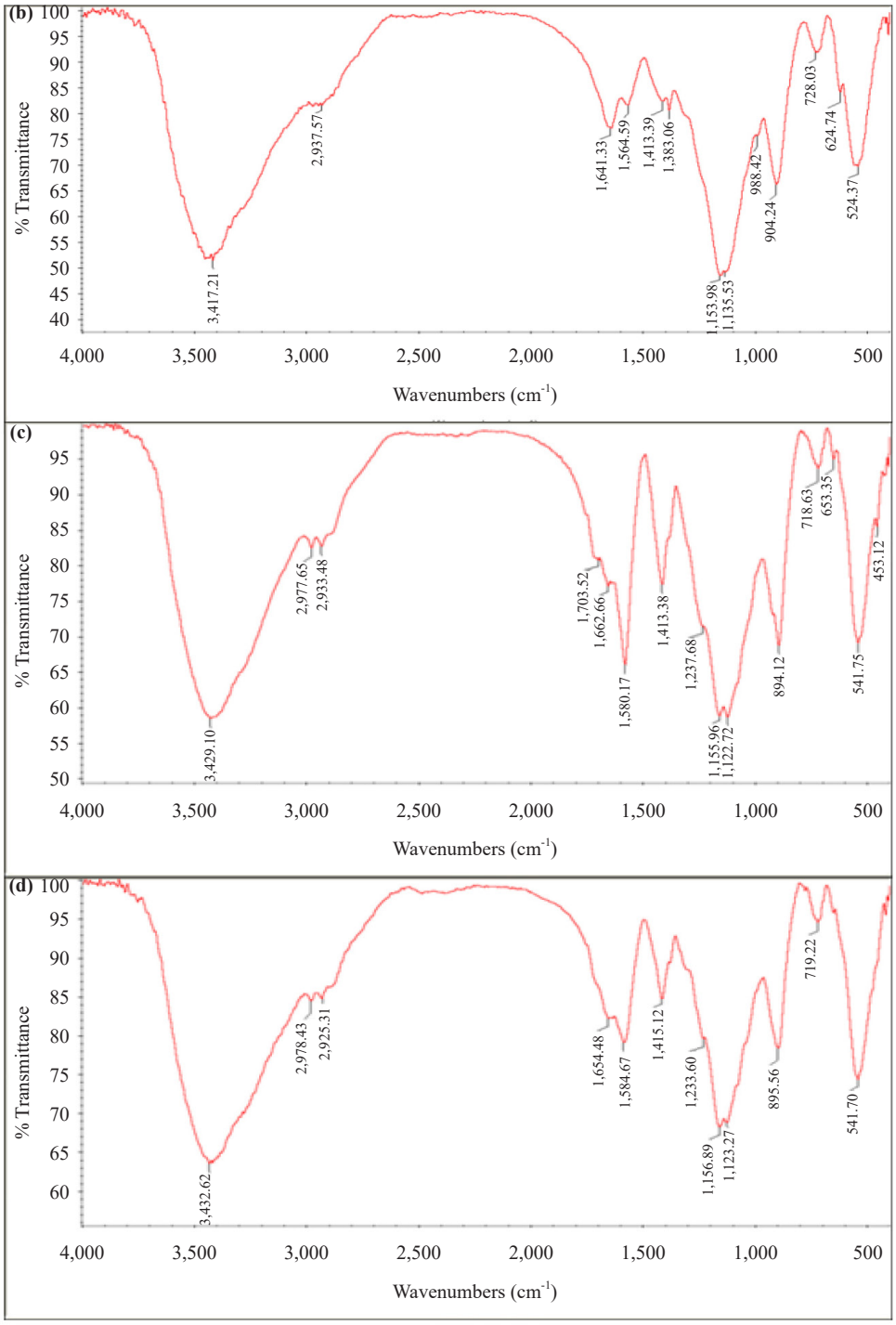


Figure 3. Results Fourier transform infrared spectrometer test; (a) Result of Lactoferrin; (b) Result of chitosan; (c) Result concentration of 300 µg/mLl cLf; (d) Result concentration of 350 µg/mL cLf

The existence of an OH peak with a broad peak with hydrogen bonding can be seen in the region of 3,300-3,400 cm^{-1}. The stretching N-H bond of amide type 2 is a peak around 3,300 which seems to be behind the broad peak and covered. Alkane C-H bond can be seen in the region of 2,850-3,000 cm^{-1}. Aromatic C-H bond is seen in the region above 3,000 cm^{-1}.

3.3 *MTT assay*

Statistical analysis revealed a significance level of less than 0.05 ($P < 0.05$), indicating that lactoferrin loaded on chitosan did not have a lethal effect on cells.

3.4 *Bacterial enumeration and cLf impact results*

Quantification of *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria yielded counts of 31×10^{10} and 33×10^{9} CFU (Colony Forming Units) per millilitre, respectively. The analysis demonstrated that cLf loaded on chitosan significantly reduced the growth rate of both *S. aureus* and *P. aeruginosa* compared to the control group. The reduction in the growth of *S. aureus* and *P. aeruginosa* after treatment with a concentration of 300 µg/mL of chitosan-loaded cLf was statistically significant (p-value < 0.01). Moreover, the reduction in the growth of *S. aureus* and *P. aeruginosa* after treatment with a concentration of 350 µg/mL of chitosan-loaded cLf was highly significant (p-value < 0.001). These results underscore the inhibitory effect of cLf loaded on chitosan, demonstrating its potential as an antimicrobial agent against both *S. aureus* and *P. aeruginosa*.

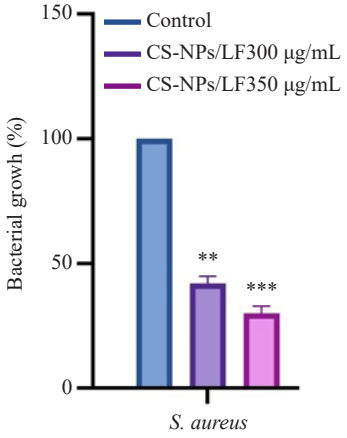


Figure 4. Comparison Diagram of the Effect on the Growth of Different Concentrations of cLf Loaded on Chitosan on *S. aureus* Bacteria Compared to the Control; ** P-value < 0.01 *** p-value < 0.001 LF = Lactoferrin Cs-NPs = Chitosan Nanoparticles

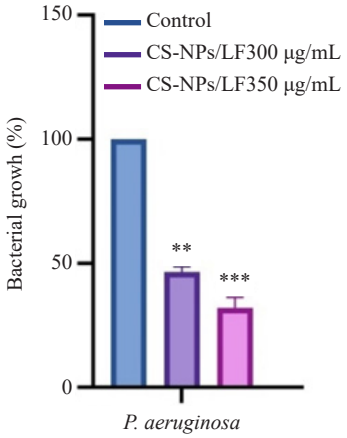


Figure 5. Comparison diagram of the effect on the growth of different concentrations of cLf loaded on chitosan on *P. aeruginosa* bacteria relative to control; ** P-value < 0.01 *** p-value < 0.001 LF = Lactoferrin Cs-NPs = Chitosan Nanoparticles

4. Discussion

The emergence of antibiotic resistance in various bacteria poses a significant global health threat. This necessitates the development of alternative antimicrobial agents. Over the past two decades, there has been growing interest in natural antimicrobial compounds, including antimicrobial peptides (AMPs) [23-25]. AMPs are a diverse group of molecules produced by living organisms that act as natural defences against infections. Lactoferrin (Lf), a key component of the innate immune system, plays a crucial role in host defence. Notably, peptides derived from Lf, such as lactoferricin, often exhibit more potent antimicrobial effects than the complete protein [16, 26]. In this study, lactoferrin was purified using CM Sephadex C-50 exchange chromatography and SDS-PAGE was used to determine protein purity. Lactoferrin with a molecular weight of 80 kDa was obtained (Figure 1). Other researchers have also used this method to purify lactoferrin [17]. Lf's antimicrobial properties are attributed to its cationic nature, with a positive isoelectric pH of around 9. This positive charge allows the separation of Lf from milk whey through cation exchange chromatography at neutral pH, a common method for its purification [15, 27]. In this study, Lf was loaded onto chitosan using TPP, similar to other research investigating the antibacterial effect of this combination [28]. Our findings demonstrate a significant reduction in the growth rate of both *P. aeruginosa* and *S. aureus* treated with cLf loaded on chitosan compared to the control group. The reduction in growth was statistically significant at a concentration of 300 µg/mL (p-value < 0.01) and even more pronounced at 350 µg/mL (p-value < 0.001) (Figure 4 and 5). These results are consistent with previous observations that Lf exhibits inhibitory effects on a broad spectrum of gram-negative and gram-positive bacteria [24]. In this research, the loading of lactoferrin on chitosan was confirmed by scanning electron microscopy (Figure 2). and zeta potential (Table 1), particle size (Table 2), dynamic light scattering (Table 3) methods. Also, the Fourier transform infrared spectroscopy test (Figure 3) was investigated. Particle size, zeta potential, scanning electron microscopy and Fourier transform infrared spectroscopy were used in Kintura's research. In the aforementioned research, loading was confirmed with these methods [28]. Lf antibacterial mechanisms include iron sequestration, membrane destabilization, targeting bacterial virulence mechanisms, and host cell invasion strategies. By blocking bacterial adhesion and invasion, Lf helps prevent infections [28]. The antibacterial properties of AMPs often involve direct interaction with the negatively charged bacterial membrane, leading to membrane depolarization or lysis. While earlier studies attributed the inhibition of bacterial growth by Lf to iron sequestration, recent evidence suggests that depolarization and subsequent loss of membrane integrity are the primary causes of bacterial death [29, 30]. The mechanism against gram-positive bacteria involves interaction with lipoteichoic acid, reducing the membrane's negative charge and allowing lysozyme access to peptidoglycan, ultimately creating a breach in the bacterial structure [29, 30]. The positive charge of many AMPs enables them to electrostatically interact with the negatively charged bacterial cell membrane. The net charge of known natural AMPs varies within the range of +16 to -6 [31]. Further supporting the potential of this approach, research by Varela et al. (2021) suggests promise for bovine lactoferrin loaded on chitosan as an alternative treatment for keratoconus [32]. Several studies have investigated the antibacterial effects of Lf against various bacterial strains. Kutila et al. (2003) found the highest inhibitory activity against Escherichia coli and *P. aeruginosa* [33]. AMP contains can make them more specific towards different classes of bacteria and are characterised by several advantages such as an unlimited reservoir of organisms that represent a good starting point for developing new antimicrobial agents [34]. Zibaee et al. observed that low concentrations (100 and 200 µg/mL) of Lf did not decrease *S. aureus* growth but even promoted it compared to the control. However, at higher concentrations, Lf inhibited *S. aureus* growth [35]. Alkhulaifi et al. (2024) reported that Lf at 4 mg/mL inhibited *S. aureus* and 27 mg/mL inhibited P. aeruginosa, which aligns with our findings of a greater effect against *S. aureus* [36]. Zibaee et al. also observed that 300 µL of Lf inhibited *S. aureus* growth by 37% [35]. The present study demonstrates that 300 µL of cLf loaded on chitosan *inhibited S. aureus* growth by 60%, suggesting a significantly enhanced effect compared to Lf alone on P. aeruginosa. Lipopolysaccharide (LPS), a crucial component of the outer membrane in gram-negative bacteria, plays a vital role in host-pathogen interactions and chronic infection establishment. Lf binds to LPS's hydrophobic anchor, Lipid A, forming a complex that neutralizes LPS's toxic effects [37]. This interaction with the lipid part of the LPS in gram-negative bacteria is highlighted as a mechanism leading to membrane damage and changes in outer membrane permeability, ultimately resulting in LPS release. These insights further emphasize the multifaceted antimicrobial actions of Lf against different bacterial strains [5]. The Lf-LPS complex also communicates with the bacterial cell, dampening the host's inflammatory response triggered by LPS [37]. Studies by researchers like Duarte et al., Hedyeloo et al., and Niu et al. have explored the antibacterial properties of Lf loaded on chitosan. The consensus from these studies suggests that loading Lf onto chitosan enhances its antibacterial

effectiveness. The interplay between chitosan and Lf is complex, but when properly harnessed, it can boost lactoferrin's antibacterial prowess [38-40]. Ostan et al. (2017) point out that lactoferrin binding protein B (LbpB) is a bi-lobed outer membrane-bound lipoprotein that comprises part of the lactoferrin (Lf) receptor complex in Gram-negative pathogens. Recent studies have shown that LbpB protects bacteria against cationic antimicrobial peptides due to its large regions [41]. Metal chelators like EDTA bind membrane-stabilizing cations such as Ca^{2+} and Mg^{2+}, releasing LPS and disrupting the outer bacterial membrane. High concentrations of Ca^{2+} and Mg^{2+} block this release of LPS by EDTA [42]. The binding of Ca^{2+} by bovine lactoferrin has been reported (a similar mechanism) [43]. Divalent ions (Ca^{2+} and Mg^{2+}) can block the release of LPS from the outer membrane of Gram-negative bacteria by lactoferrins [5]. However, our research suggests that lactoferrin loaded on chitosan is unlikely to bind to protein B. Alkhulaifi et al. (2024) found that lactoferrin at a concentration of 4 mg/mL had an effect on *Staphylococcus aureus* and 27 mg/mL on P*seudomonas* [36]. This result aligns with the present study's findings regarding the greater effect of lactoferrin loaded on chitosan against *Staphylococcus aureus* compared to *Pseudomonas aeruginosa*. Zibaee et al. have studied that 300 microliters of lactoferrin on *Staphylococcus aureus* have an inhibitory effect of 37% growth [35]. Our research demonstrates that 300 microliters of lactoferrin loaded on chitosan has an inhibitory effect of 60% growth on *S. aureus*, highlighting a significant increase in the effect of lactoferrin loaded on chitosan compared to lactoferrin alone on P. aeruginosa.

5. Conclusion

This study investigated the efficacy of camel milk lactoferrin loaded on chitosan nanoparticles against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The findings demonstrated a significant reduction in the growth rate of these bacteria at concentrations of 300 and 350 µg/mL compared to the control group. These results suggest that chitosan-loaded camel milk lactoferrin holds promise as a potential therapeutic strategy for *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections. This study highlights the potential of chitosan-loaded camel milk lactoferrin nanoparticles as a promising alternative for treating diseases caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Further research is warranted to translate these findings into clinical applications optimize the loading process and determine the most effective concentration of chitosan-loaded lactoferrin treatment. Investigating the mechanisms by which chitosan-loaded lactoferrin exerts its antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* would provide valuable insights for future development. We believe that our research involves generating new structures that can be successfully used in the development of therapies against these infections.

Conflict of interest

The authors declare no competing financial interest.

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