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Original Article

The effect of bacteriocins derived from lactic acid bacteria on growth and biofilm formation of clinical pathogenic strains

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Abstracts: Objective: The purpose of this study was to evaluate the effects of bacteriocins derived from lactic acid bacteria on the growth and biofilm formation of clinical pathogenic strains of bacteria. Methods: Ten patients with culture-proven staphylococcal and *Streptococcus pneumoniae*re spiratory tract infections were selected and *S. aureus*or *S. pneumoniae* were isolated fromtheir sputum. The antibacterial effect of the bacteriocins on the growth and biofilm formation of these strains was evaluated by colony counting and scanning electron microscopy. Results The bacteriocins showed significant antibacterial effects on 9 strains of *S. aureus* and *S. pneumoniae*. For the biofilm experiments, counts of the *S. aureus* and *S. pneumoniae* strains in biofilms significantly decreased after 24 h ($P<0.05$). Conclusions: In conclusion, the bacteriocins derived from lactic acid bacteria strongly reduced the growth and biofilm formation of *S. aureus* and *S. pneumoniae in vitro*, and the use of this class of antimicrobial agents may be an important new approach in controlling respiratory tract infections.

Keywords: Bacteriocin, biofilm, antibacterial effects, respiratory tract infection

Introduction

For the last 30 years chronic staphylococcal and *Streptococcus pneumoniae* lung infection in the respiratory tract have been regarded as biofilm-based infections [1, 2]. *Streptococcus pneumoniae* is widely distributed in the environment and is an important nosocomial pathogen. In recent years, *Streptococcus pneumoniae* has become one of the most important nosocomial pathogens of the lower respiratory tract [3].

Lung infection caused by *S. pneumoniae*can often be a recurrent, protracted illness that is refractory to treatment [4, 5]. Even with the repeated application of antibioticsit is still difficult to eliminate *S. pneumoniae* [6]. Most scholars believe that *Streptococcus pneumoniae* in lung tissue survives in a biofilm and is one of the key factors in recurrent infections [7, 8]. Hospital acquired pneumonia tops the list of hospital acquired infections. The death rate of hospital infection could reach as high as 40%, the main pathogenic bacteria of whichare drug resistance of *S. aureus*. Therefore, it is vitally important to find a drug to which resistance is not easily developed [9].

The development and application of antibiotics is essential to treat bacteria-associated infectious diseases, however allergic reactions, secondary infections, acquired drug resistance and other side effects can limit their application [10]. Bacteriocins are a type of polypeptide or precursor peptides with antibacterial activity produced by *Lactobacillus* [11]. As bacteriocins are generally non-toxic to animals, have no antigenicity, are heat stable, easily damaged by some of the proteases in the human gut, do not lend themselves to the rapid development of resistance and do not accumulate in the body, in recent years, there has been increasing interest in the study of bacteriocins [12, 13]. In this paper we have evaluated the effects of a bacteriocin derived from lactic acid bacteria on the growth and biofilm formation of clinical pathogenic strains, and to explore the practical value

Table 1. Inhibitory effects of bacteriocin on clinical isolated *S. aureus* after 24 h ($\times 10^6$ CFU/ml, means ± SD)

Strains	CFU (control)	CFU
1	12.76±3.21	3.41±0.91*
2	11.25±2.82	2.38±0.79*
3	8.91±2.19	2.44±0.97**
4	9.53±1.09	1.75±0.76**
5	11.83±1.64	2.38±0.21*

*P<0.05, **P<0.01 compared with PBS controls (paired-*t* test).

Table 2. Inhibitory effects of bacteriocin on clinical isolated *S. pneumoniae* after 24 h ($\times 10^6$ CFU/mL, means ± SD)

Strains	CFU (control)	CFU
1	14.32±3.81	4.81±1.21*
2	14.25±2.98	3.38±1.69*
3	16.91±4.21	3.49±1.87**
4	14.59±3.58	12.45±4.98
5	12.89±1.84	3.38±1.21*

*P<0.05, **P<0.01 compared with PBS controls (paired-t test).

of bacteriocins in the treatment of respiratory tract infections.

Materials and methods

Bacterial isolation and culture

Sputum samples were collected from 10 patients with culture-proven staphylococcal and *Streptococcus pneumoniae* respiratory tract infections who had experienced a short duration of symptoms (exclusion limit <1 year; actual experience 0-21 days) and the strains were isolated. Isolates were incubated on Columbia sheep blood agar (BioMérieux, France) at 37°C under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) for 24 h. *S. aureus*and *S. pneumoniae* were verified by Gram-staining and to the species level using a Vitek (BioMérieux, France). After identification, the *S. aureus*and *S. pneumoniae* strains were purified once again on Columbia sheep blood agar and stored at -80°C.

Preparation of culture supernatants and bacteriocins

Production of culture supernatants: Lactobacillus plantarum ST71KS was first inoculated into MRS broth at 37°C for 24 h under anaerobic conditions. Cells were removed by centrifugation and the supernatant were used for further processing. To eliminate interference from acid and hydrogen peroxide, supernatants were adjusted to pH 6.5, and treated with catalase (5 mg/ml). Finally the supernatants were filtered through a 0.22 μm pore size filter (Millipore, USA).

Isolation for bacteriocins

Bacteriocins from *Lactobacillusplantarum* ST-71KS were isolated according to the methods by Martinez *et al* [14]. In brief, a 100 ml culture of *L. plantarum* was precipitated using 60 g ammonium sulfate. The crude precipitate was centrifuged for 20 min at 10,000 ×g at 4°C. The resulting pellet was resuspended in 2 ml of 10 mM Tris-HCl pH 7.4 and the resuspended pellet was concentrated using an Amicon Ultra-4 Centrifugal Filter device (Millipore, USA) with a molecular weight (MW) cut-off of 140 kDa to a final volume of 0.5 ml at 4°C. The final suspension was concentrated by freeze-drying and stored at 4°C.

Antibiotic sensitivity testing

Isolated *S. aureus* and *S. pneumoniae* strains were cultured in brain heart infusion (BHI) medium (Oxoid, UK). After incubation, the concentration of the *S. aureus* and *S. pneumoniae* cultures was adjusted to 0.5 McF (1.5×10^8 CFU/mL) using a Densicheck (BioMérieux, France). Finally, 50 μl of the *S. aureus* and *S. pneumoniae* cultures and 0.1 mg of the bacteriocin preparation were combined with 2 mL BHI broth and incubated under microaerophilic conditions at 37°C for 24 h. PBS (100 μL) was used as a control.

After incubation, the suspensions were vortexed for 1 min and diluted serially to 10^{-3}. Then, 100 μl of the suspension was incubated under microaerophilic conditions on brain-heart blood agar at 37°C for 24 h and the number of colonies was counted.

Inhibitory effects on biofilm formation of the two test strains

For this assay 10 *S. aureus* and *S. pneumoniae* clinical isolates were used. An overnight culture was diluted in PBS to obtain 0.5 MCF (1.5×10^8 CFU/mL) and 0.1 mL of the culture were trans-

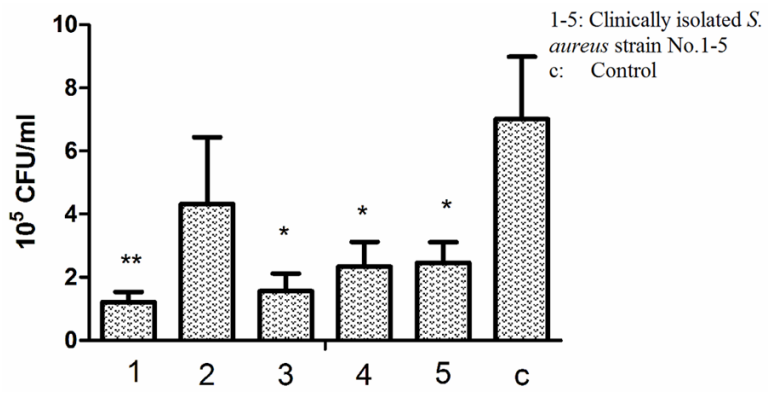

Figure 1. Viable *S. aureus* cells in the biofilms after 24 h in the presence of bacteriocin or PBS. *P<0.05, **P<0.01 compared with PBS controls (paired-*t* test).

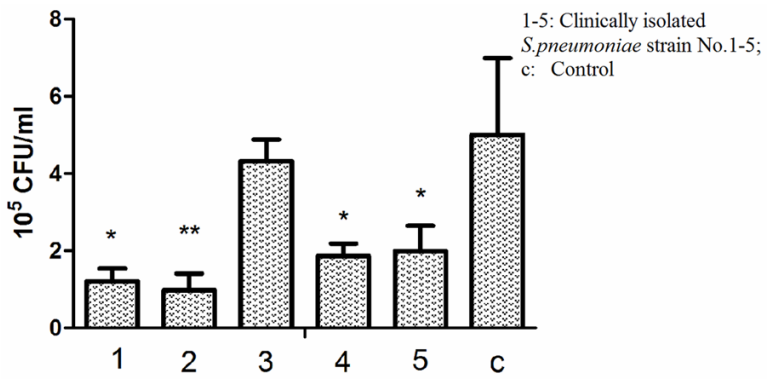

Figure 2. Viable *S. pneumoniae* cells in the biofilms after 24 h in the presence of bacteriocin or PBS. *P<0.05, **P<0.01 compared with PBS controls (paired-t test).

ferred to a sterile 15 ml petri dish with cover glass to which 0.1 mg bacteriocin was added. As a control 100 mL of PBS was used instead. The petri dishes were mounted in an overhead shaker and incubated at 37°C for 24 h. After incubation the cover glass was washed for 5 s in washing buffer and the cells were carefully removed using sonication and suspended in 1 mL PBS. The suspensions were vortexed for 1 min and serially diluted to 10-3. Subsequently, 100 µL of the suspension was incubated under microaerophilic conditions on MSB at 37°C for 24 h and the number of colonies was counted.

For scanning electron microscopy, after incubation, each cover glass was gently washed three times with PBS. The cover glass were first fixed with 2.5% (wt/vol) glutaraldehyde in filter-sterilized 0.1 M phosphate buffer (pH 7.4) at room temperature for 2 hours and then rinsed 3 times for 15 minutes in a 0.1 M sodium cacodylate buffer. Next, a postfixation step was performed for 1 hour with 1% (wt/vol) osmium tetroxide in a 0.1 M sodium cacodylate buffer. The dried samples were fixed onto metal holders with double-sided adhesive tape and finally coated with platinum and palladium in an evaporator. The samples were observed at 3 kV with a scanning electron microscope (SU-70; Hitachi, Tokyo, Japan). Five fields of view were randomly chosen from the surface of each sample.

Statistical analysis

All experiments were performed at least three times and expressed as means ± SD. SPSS 14.0 software for Windows was used for data analysis. Data were analyzed for statistical significance using Student's test. A *p* value of <0.05 was considered as significant by using Student's test.

Results

Interference test

Among the 5 clinically isolated strains of *S. aureus*, bacteriocins showed significant antibacterial effects on 4 strains (P<0.05, **Table 1**), while the bacteriocins had inhibitory effects on 5 clinically isolated strains of *S. pneumoniae* (P<0.05, **Table 2**).

Inhibition of biofilm formation

After 24 h of incubation, the bacterial counts in the *S. aureus* biofilmswere significantly different from the counts in the control group (P<0.05; **Figure 1**). Furthermore, the number of *S. pneumoniae* in the biofilms decreased significantly (P<0.05, **Figure 2**), while there was no obvious difference with the bacterial count in the strain 4 biofilm (P>0.05). The results of scanning electron microscopy are shown in the **Figures 3** and **4**.

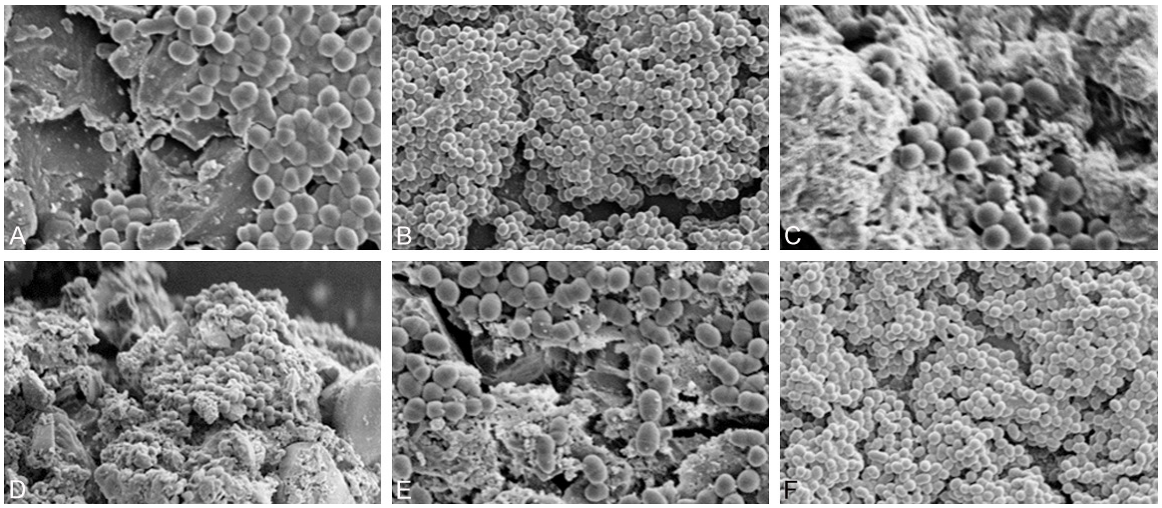

Figure 3. Scanning Electron Microscope (SEM) images of the biofilm of *S. aureus* in the presence of bacteriocin (A-E: Clinically isolated *S. aureus* strain No.1-5; F: Control).

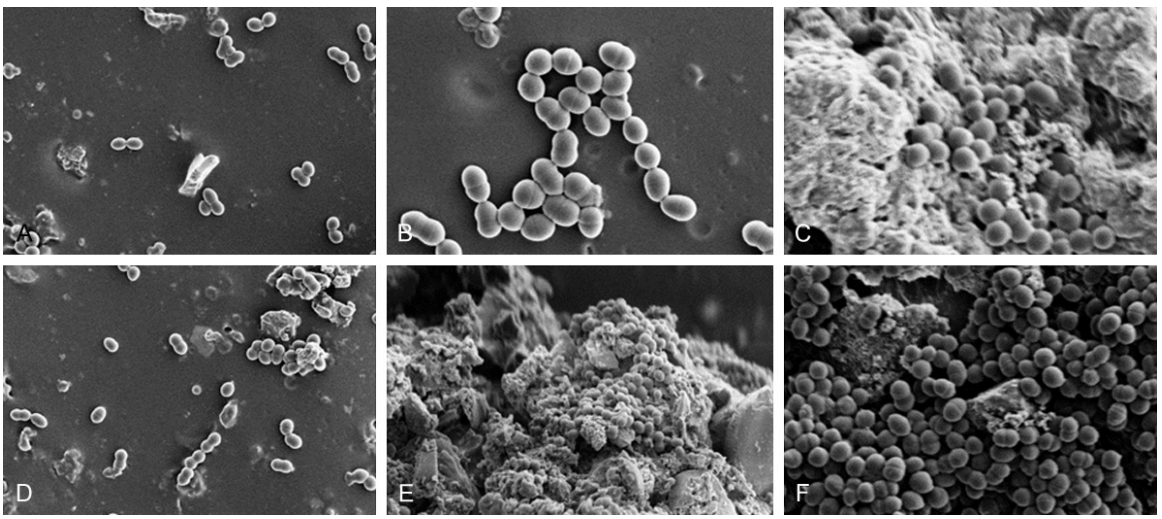

Figure 4. Scanning Electron Microscope (SEM) images of the biofilm of *S. pneumoniae* in the presence of bacteriocin (A-E: Clinically isolated *S. pneumoniae* strain No.1-5; F: Control).

Discussion

S. aureus and *S. pneumoniae* are the predominant pathogens of the human respiratory tract [15, 16]. Many microorganisms exist in biofilms, including a number of pathogens and pathogenic bacteria can cause chronic infections and relative infection with respiratory tract infections [17].

Many studies have provided strong evidence in favor of biofilm-based infections in the respiratory tracts of patients [18-20]. In our study, bacteriocins derived from lactic acid bacteria showed strong inhibitory effects on clinically isolated pathogenic strains, which was in accordance with the results of the study conducted by Lewus et al [21] and Arqués, et al [22].

Bacteriocins are antibacterial compounds that have strong antibacterial activity against Gram-positive and Gram-negative bacteria. Bacteriocins have been shown to have good antibacterial activity against a variety of pathogenic bacteria [23, 24]. Biofilms are complex structures formed by adhered bacteria on various surfaces, including the respiratory tract. Increased tolerance to antimicrobial agents is

thought to be largely a consequence of biofilm formation, and as such, finding a simple and effective way to control biofilms is important. Studies have shown that bacteriocins can inhibit biofilm formation by pathogens [25] and reduce the occurrence of drug resistance [26]. In our study, the bacteriocins had a strong inhibitory effect on clinically-isolated pathogenic bacteria, indicating that the bacteriocins have potential value in treating respiratory tract infections.

It is interesting to observe the antibacterial effect of bacteriocins on the growth of *S. aureus* and *S. pneumoniae* isolated from respiratory tract infection patients. However, the respiratory tract is inhabited by highly diverse and abundant bacteria that form a complex ecosystem, which is difficult to simulate *in vitro*. Therefore, to study the effect of these bacteriocins on respiratory pathogens *in vivo*, it is still necessary to understand the role of bacteriocins in the treatment of respiratory tract infections. In addition, any additional mechanisms of action of bacteriocins have not yet been thoroughly investigated. In future experiments, we will conduct the animal experiments to verify the antibacterial effect of these bacteriocins.

In conclusion, the bacteriocin extract derived from lactic acid bacteria strongly reduced the growth and biofilm formation of *S. aureus*and *S. pneumoniae in vitro*, and the use of this class of antimicrobial agents may be an important new approach in controlling respiratory tract infections.

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Disclosure of conflict of interest

None.

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