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Bacteriocin Isolated from *Lactobacillus Rhamnosus* L34 Has Antibacterial Effects in a Rabbit Model of Infection After Mandible Fracture Fixation

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Rigid internal fixation (RIF) technology is a recently developed fracture fixation technique in which use of specific antibiotics before and after the operation and timely treatment of local infections is necessary.

Material/Methods: The bacteriocins were isolated from *Lactobacillus rhamnosus* L34. Twenty-four New Zealand White female rabbits were divided into 2 groups: bacteriocins and control group. After mandible fracture fixation, the rabbits were infected with *S. aureus* and subsequently injected with either bacteriocins or saline. The biofilm samples were harvested from rabbits euthanized on the 1st, 3rd, and 5th days and observed using a fluorescence microscope. Blood samples were collected at 1 h, 12 h, 1 day, 3 days, and 5 days after the injection of either bacteriocin or saline to test the level of C-reactive protein and TNF-α.

Results: Significant differences in the biofilm formation were evident between the bacteriocins and saline treatment group on days 1, 3, and 5. Moreover, the serum levels of TNF-α and CRP after treatment with bacteriocins were significantly lower than in controls.

Conclusions: Use of bacteriocins isolated from *Lactobacillus rhamnosus* L34 may be a promising way to control infections of mandible fracture after internal fixation *in vivo*.

MeSH Keywords: **Bacteriocins • Biofilms • Mandible • *Staphylococcus Aureus***

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Background

With the increasing incidence of open fractures due to high-energy injury, the number of resulting infections in hospitalized patients is also increasing [1]. Infection of fold open bone is difficult to prevent and to treat, so there is a critical need to address this problem in the clinical setting [2]. The incidences of mandible fracture are the first to be accounted in the accidents [3] and it is reported that mandible fractures account for 44.2% of all facial fractures [4]. *S. aureus* is one of the main pathogenic bacteria involved in bone fracture infections, and with the increasing trend of drug resistance in *S. aureus*, finding an effective antibiotic against *S. aureus* is urgent in treating the infection after fracture.

Rigid internal fixation (RIF) technology, a recently developed fracture fixation technique, has many advantages, such as less influence on the patient's diet and nutrition intake, and employs the use of titanium, which has good biological compatibility, shows no obvious adverse reactions and, can be implanted in the body for a long time [5]. Although RIF is a successful treatment strategy, application of specific antibiotics before and after the operation and timely local treatment are necessary. The sustained release of antibiotics to the local site of infection is considered as an effective method for the prevention and control of infection of open fractures [6].

Lactic acid bacteria are probiotics which are widely used in the food fermentation industry [7]. They can inhibit or kill some pathogenic bacteria and food spoilage organisms, and are conducive to health [8]. In recent years, studies have shown that some lactic acid bacteria can produce an antimicrobial substances called bacteriocin, which has bacteriostatic activity of precursor peptides or polypeptides, and because of it has high efficacy, is non-toxic, leaves no residue, and has no resistance characteristics, is seen as an effective alternative of antibiotics [9]. A variety of studies have reported that bacteriocin isolated from lactic acid bacteria have strong antibacterial effects on common clinical pathogens. Our previous study showed that bacteriocin isolated from *Lactobacillus plantarum* can control infection after mandible fracture [10].

In the present study, we isolated a broad range of bacteriocins from *Lactobacillus rhamnosus* L34. We analyzed its antibacterial effect on infection after mandible fracture fixation and measured the serum concentrations of C-reactive protein and TNF-α in rabbits with bacterial infections. The aim of the present study was to verify the effects of bacteriocins in treatment of infection after mandible fracture fixation *in vivo*.

Material and Methods

Strains and culture conditions

Lactobacillus rhamnosus L34 was obtained from the Department of Microbiology, Zhejiang University. It was maintained on MRS agar plates (MRSA, Difco, Detroit, MI, USA) and grown overnight in Rogossa broth (MRS, Difco, USA) at 20°C with gentle agitation [11]. *S. aureus* ATCC 29213 was used to screen for antibacterial activity. Cultures of *S. aureus* were grown overnight in brain-heart infusion broth (BHI, Difco, USA) at 37°C, centrifuged at 2000 rpm for 5 min. The final concentrations of the cultures were adjusted to 0.5×10^5 CFU/ml using Densicheck (BioMérieux, Lyons, France).

Isolation of bacteriocins

L. rhamnosus L34 cultures were grown in the MRS broth at 37°C for 24 h. The cultures were then centrifuged at 7000 g for 10 min and cell-free supernatants were isolated. The pH of the supernatants was adjusted to 6.5 and treated with catalase (5 mg/ml) to eliminate the effect of hydrogen peroxide, then filtered through 0.22-μm pore size filters (Millipore, USA) to obtain a crude bacteriocin preparation [12].

Crude bacteriocin preparations were saturated with 70% ammonium sulfate and stored at 4°C to precipitate the protein. After 24 h, the precipitates were recovered by centrifugation (10 000 g at 4°C for 30 min), vacuum-dried, and dissolved in 1 ml PBS. Suspensions were then separated in Sephadex G-100 columns (1.6×36 cm) and the fractions (1 ml each) containing bacteriocins were collected and stored at –70°C [13].

Animals

All animals were handled in strict accordance with good animal practice and the animal work was approved by the College of Medicine, Zhejiang University Chancellor's Animal Research Committee. Twenty-four New Zealand White female rabbits weighing 2.5–3.0 kg and aged 70–100 days were used in the study. Animals were housed in individual cages in a temperature-controlled room (23°C) with a 12-h light/dark cycle. Twelve rabbits were used for validation of the infection model and another 12 were assessed as the control group.

Model validation

Rabbits were initially anesthetized via inhalation of isoflurane (2%). The surgical procedure was then carried out as described in detail elsewhere [14]. The operation was performed on animals under general anesthesia induced by intramuscular injection of ketamine (25 mg/kg of body weight) followed by continuous inhalation of isoflurane (1%). A longitudinal gum

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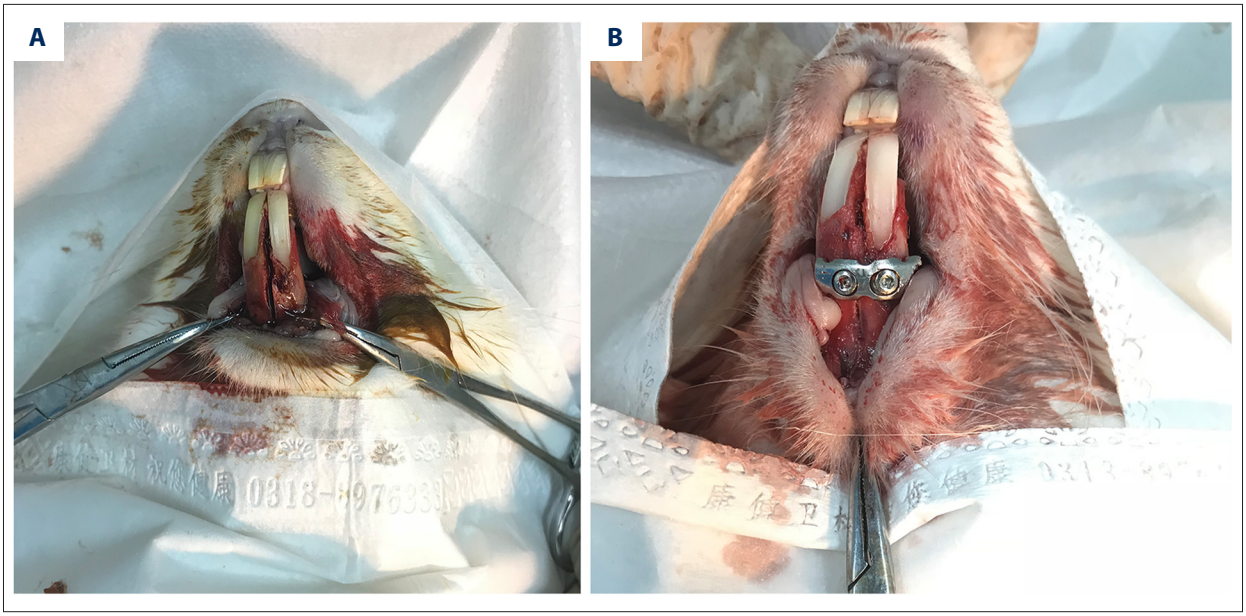

Figure 1. Mandible fracture fixation surgery in the rabbit model (**A**: exposure of the mandible and artificial mandible fractures; **B**: mandible fracture fixation).

incision was made and the periosteum was stripped to expose the mandible. The fracture of the mandible was made by means of a chisel or osteotome driven into the lines of the fracture. After fracture reduction, it was fixed with a mini-titanium board. The gums were finally closed to complete the procedure.

Immediately after the surgical wound was closed, rabbits were infected by injection of *S. aureus* (3×10^5 cfu/ml in a total of 0.5 mL inoculum) into the mandible fracture line. Subsequently, animals were divided into 2 groups: 12 rabbits were injected with 1 ml bacteriocins (0.5 mg/ml) and the other 12 rabbits were injected with 1 ml sterile saline solution as controls.

Detection of biofilm formation on the fixation

To evaluate the biofilm formation in our rabbit model, the biofilm samples were harvested from mini-titanium boards in rabbits euthanized on at days 1, 3, and 5 after infection. The samples were transferred to coverslips and fixed with 2.5% glutaraldehyde at 4°C. Tissues were then stained with 0.01% acridine orange solution (Sigma, USA) and observed at an excitation wavelength of 488 nm under a Nikon 80i microscope equipped with an argon laser [15]. All the images were analyzed and quantified using Image-Pro Plus 6.0 software (Media Cybernetics, USA). The fluorescence intensities of the biofilms are expressed as integrated optical density values (IOD) [16].

Determination of serum inflammatory cytokines

Blood samples from the 24 rabbits were collected at various time points (1 h, 12 h, 1 d, 3 d, and 5 d) after the injection of bacteriocins or saline. The blood samples were centrifuged at 3000 rpm for 5 min at 4°C and stored at –70°C. The protein levels of C-reactive protein (CRP) and TNF-α were measured using ELISA kits (Invitrogen, USA) [17]. All measurements were done in triplicate. The levels of CRP and TNF-α are expressed as mg/ml and pg/ml protein, respectively.

Statistical analysis

All tests were repeated 3 times, with consistent results. Data analysis was done using SPSS 14.0 software for Windows and the results were compared statistically using the *t* test. P values were considered statistically significant at $p<0.05$.

Results

The variation of biofilm formation on mini-titanium

The progress of the surgery of mandible fracture fixation is shown in Figure 1. *S. aureus* were imaged for biofilm formation using a fluorescence microscope. As can be seen in Figure 2, significant variability in biofilm formation appeared at day 1 after the injection of either bacteriocins or saline ($p<0.05$, Figure 2). Notably, significant differences were evident between the group of bacteriocins and saline groups at days 3 and 5 ($p<0.05$, Figure 2). The variation in the IOD is shown in Figure 2 and the fluorescence image of the biofilm is shown in Figure 3.

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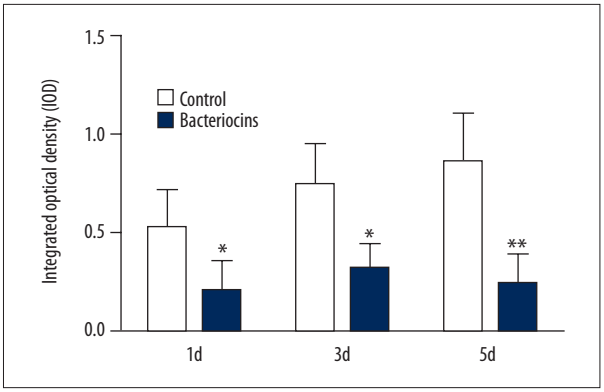

Figure 2. The integrated optical density (IOD) of biofilms in bacteriocins-injected rabbits and control group rabbits after 1 h, 12 h, 1 d, 3 d, and 5 d (mean ±SD, *, P<0.05)

Variations in the levels of serum inflammatory cytokines

After 1 h and 12 h of the bacteriocins or saline injections, the serum levels of TNF-α and CRP in the bacteriocins treatment group and control group all increased; however, the serum level of TNF-α and CRP did not show significant differences between the bacteriocins treatment group and the control group at 1 h and 12 h (Figure 4). However, at days 1 and 3 following bacteriocins treatment, the serum levels of TNF-α and CRP were significantly lower than in controls (p<0.05, Figure 4). Importantly, at day 5 after infection, the serum levels of TNF-α and CRP were reduced to normal level (as observed after 1 h) and were significantly lower than in controls (p<0.05, Figure 4).

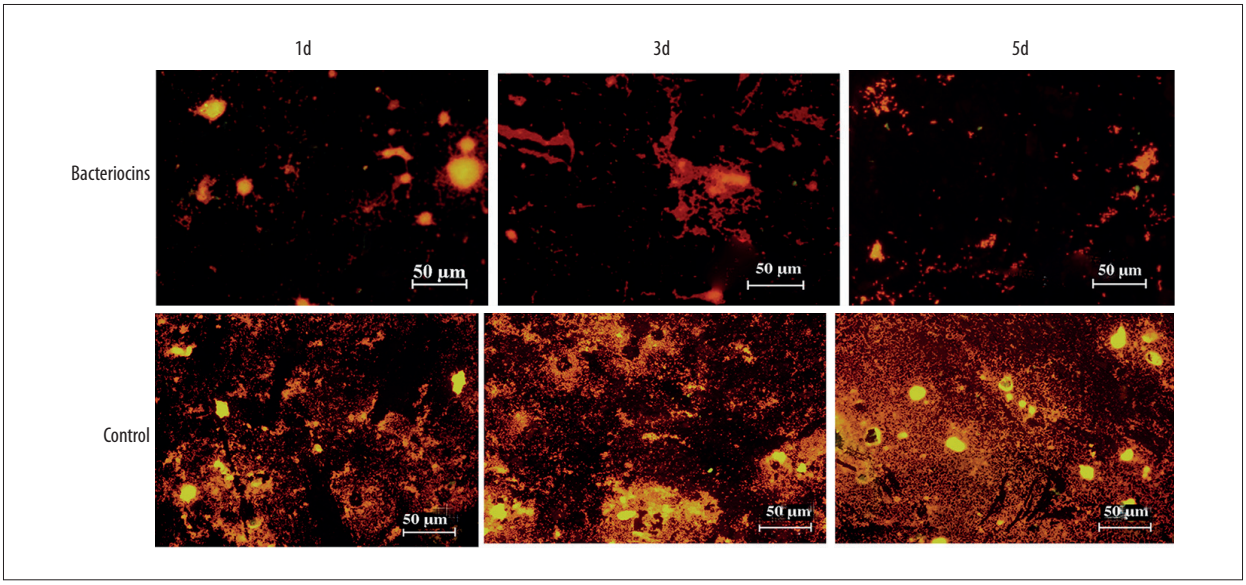

Figure 3. Fluorescent images of the biofilm of *S. aureus* in bacteriocins-injected rabbits and control group rabbits after 1 d, 3 d, and 5 d (stained with acridine orange).

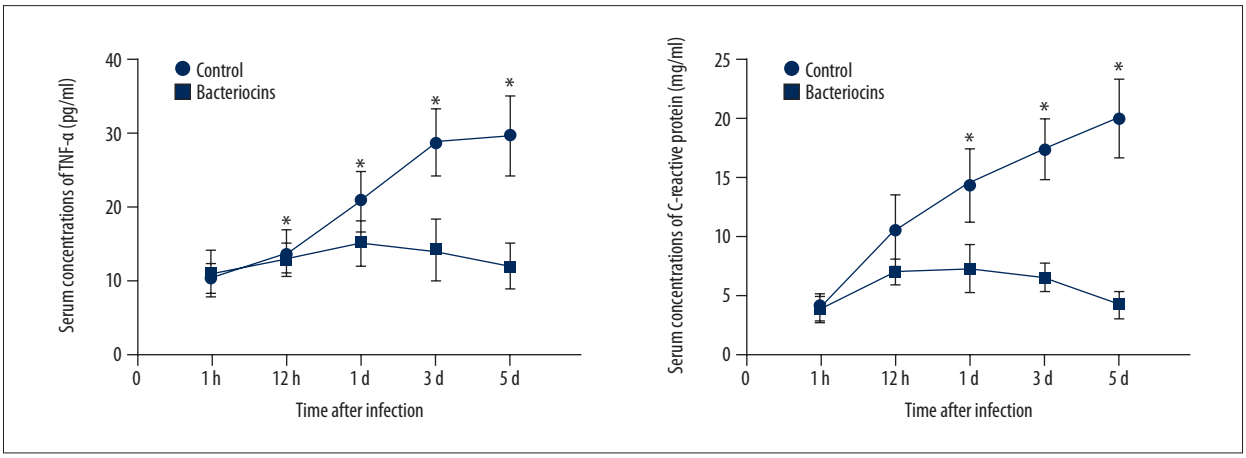

Figure 4. Serum concentrations of C-reactive protein (CRP) and TNF-α in bacteriocins-injected rabbits and control group at 1 h, 12 h, 1 d, 3 d, and 5 d following infection (mean ±SD, * P<0.05).

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Discussion

Clinically, the existence of *S. aureus* in infection is very common, and is the main pathogenic bacteria in nosocomial infections [18]. Owing to the extensive use of antibiotics, drug-resistant strains have developed [19]. Therefore, it becomes essential to identify safer and effective antibiotics or drug candidates.

Postoperative infection of internal fixation in orthopedic surgery is a catastrophic event, which not only increases the treatment cost, but also slows fracture healing, and in some cases can even led to deformity and dysfunction [20]. It is reported that the incidence of infection after orthopedics internal fixation implantation is 0.5–5% [21]; therefore, finding ways to prevent postoperative infection in orthopedics internal fixation implantation is a major problem. Lactic acid bacteria can produce a variety of antibacterial substances, such as organic acids, double b acyl, butanone, hydrogen peroxide, antifungal peptides, and bacteriocin [22]. Bacteriocins are bioactive proteins secreted by some bacteria; they can kill or inhibit other microbes in the same or similar living environment [23]. Compared to antibiotics, bacteriocins have added advantages as they can inhibit the closely related bacterial strains, and more importantly, they are non-toxic, with no adverse effect, no residue, and no drug resistance, and do not produce toxins when used for the treatment of infection [24]. In the present study, we found that bacteriocins exert a strong antibacterial effect on *S. aureus*.

The mandible is part of the lower region of the face, which is easily injured. There are several anatomical weak areas, which, when exposed to direct or indirect violence, become susceptible to breaks and fractures [25]. Mandible fracture fixation surgery is very common in China. Despite the routine use of antibiotics, postoperative infection occasionally occurs [26]. TNF-α and CRP are common inflammatory markers that can be used for monitoring infection [27]. We found that the levels of TNF-α and CRP were significantly lower after the injection of bacteriocins, which is in agreement with previous experimental results [28,29].

Although *S. aureus* is a standard strain used in testing, the bacterial species involved in mandible fracture infections after internal fixation are complicated and the pathogenic bacteria may be different from the standard strains. Therefore, our results may not reflect the true situation of infection in humans. In future, we will consider all aspects to conduct experiments *in vivo*.

Conclusions

In conclusion, bacteriocins isolated from *Lactobacillus rhamnosus* L34 significantly reduced the formation of biofilms and inflammation of mandible fractures after internal fixation. Thus, bacteriocins isolated from *L. rhamnosus* L34 may be promising in control of mandible fracture infection after internal fixation and could have potential clinical application in infection control in the future.

Conflicts of interest

None.

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