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Antibacterial effects of bacteriocins isolated from *Lactobacillus rhamnosus* (ATCC 53103) in a rabbit model of knee implant infection

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Abstract. Infection following orthopedic surgery is a major complication that can have serious implications on patient health. The present study aimed to investigate the antibacterial effects of bacteriocins obtained from *Lactobacillus rhamnosus* on a rabbit model of *Staphylococcus aureus* infection following knee replacement surgery. Blood samples were collected 1, 2, 3, 4 and 5 days after bacteriocin injection, and C-reactive protein (CRP) and interleukin (IL)-6 levels were measured using commercial ELISA kits. In addition, biofilm formation was evaluated by fluorescence microscopy. Bacteriocins were identified to exhibit significant inhibitory effects on *Staphylococcus aureus* biofilm formation, and on CRP and IL-6 levels in the serum, following surgery and infection (all $P<0.05$ vs. the control group). The results of the present study indicate that bacteriocins are a potential agent for the prevention of orthopedic postoperative infections.

Introduction

Orthopedic infections, which are a major complication of medical implant surgery, can have serious implications on patient health. Orthopedic infections can cause delayed recovery, chronic osteomyelitis complications or even failure of surgery (1). Orthopedic infections are primarily caused by *Staphylococcus aureus* and *Streptococcus pyogenes* (2). The severity of *S. aureus* orthopedic infection is directly associated with the toxins that it generates. *S. aureus* bacteria can produce a variety of toxins, including bowel poison element, toxic shock toxin, coagulase proteases and haemolysin (3), and has toxin a specific pathogenicity. *S. aureus* can also express adhesive molecules, which allow bacterial adhesion to host cells or material surfaces. This facilitates the formation of biofilms that makes it increasingly difficult to treat infections in medical implants (4).

According to the World Health Organization, the prevalence of joint disease in people >55 years old is 80%, and joint disease, including arthritis and femoral head necrosis, are an important public health issue (5). Artificial joint replacement, which began in the 20th century, is one of the most successful orthopedic surgeries for the treatment of severe joint diseases. Joint replacement can help relieve joint pain and restore joint function. However, the most serious complication following artificial joint replacement is infection around the implant (6). As cases of artificial joint replacement increase each year, so does the incidence of infections around the implant. The use of antibiotics to control surgical infections has significant clinical efficacy. However, bacterial drug resistance is becoming increasingly common, making it more difficult to treat infections. Therefore, there is an urgent requirement to identify novel methods to treat such infections. Bacteriocins, which are secreted by lactic acid bacteria, are small molecule peptides that exhibit a unique antibacterial mechanism that bacteria rarely develop resistance to (7).

The present study aimed to isolate broad-range bacteriocins from *Lactobacillus rhamnosus* (ATCC 53103) and investigate their antibacterial effect on *S. aureus* in a rabbit model of knee implant infection. The serum concentrations of C-reactive protein (CRP) and interleukin (IL)-6 were measured in order to analyze the antibacterial effect of bacteriocins. The results of the present study highlight a potential novel method for the treatment of knee implant infections *in vivo*.

Materials and methods

Strains and culture conditions. L. rhamnosus (ATCC 53103) was purchased from the American Type Culture Collection (Manassas, VA, USA). The bacteria were maintained on de Man, Rogosa and Sharpe (MRS) agar plates (Difco; BD Biosciences, Franklin Lakes, NJ, USA) and cultured

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overnight in MRS broth (Difco; BD Biosciences) at 20˚C with gentle agitation. *S. aureus* (ATCC 29213; American Type Culture Collection) was used in the present study to screen for the antibacterial activity of bacteriocins from *L. rhamnosus*. Cultures of *S. aureus* were grown overnight in brain-heart infusion broth (Difco; BD Biosciences) at 37˚C, centrifuged (3,000 x g at 4˚C for 5 min), and the final concentrations of the cultures were adjusted to match the turbidity of a McFarland 0.5 standard ($0.5x10^5$ CFU/ml) using a spectrophotometer (Densimat; BioMérieux UK Ltd., Basingstoke, Hampshire, UK).

Production of crude bacteriocins. Crude bacteriocin preparations were prepared as previously described (8). Briefly, *L. rhamnosus* cultures were initially grown in MRS broth at 37˚C for 24 h. The cultures were then centrifuged (7,000 x g at 4˚C for 10 min) and cell-free supernatants were then aspirated. The pH of the supernatants was adjusted to 6.5, treated with catalase (5 mg/ml) and filtered through 0.22 μm pore size filters (EMD Millipore, Billerica, MA, USA).

Purification of bacteriocins. Crude bacteriocin preparations were saturated with 70% ammonium sulphate at 4˚C to precipitate the proteins. The pellets were recovered by centrifugation at 10,000 x g at 4˚C for 30 min, vacuum-dried and dissolved in 1 ml deionized water. The 10 ml suspensions were then applied to Sephadex G-100 columns (1.6x36 cm) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) that were pre-equilibrated with a phosphate buffer (pH 7.0), as previously described (9). The flow rate was adjusted to 24 ml/h, and the proteins were collected and adjusted to a total protein concentration of 2.5 mg/ml. The protein suspension was stored at -70˚C until required. A bacteriocins suspension (10 μl; 1 mg/ml) was loaded into a 12.5% Tricine-SDS-PAGE and the molecular weight of the bacteriocins was determined according to the methods of Biosa G (10). A total of 10 μl of the low molecular weight (10-120 kDa) BenchMark marker (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used as a standard.

Animals. A total of 12 New Zealand White female rabbits provided by the Zhejiang Province Academy of Medical Sciences (Hangzhou, China) weighing 2.5-3.0 kg and aged 70-100 days old were used in the present study. Animals were housed in individual cages in a temperature-controlled room (23˚C) with a 12 h light/dark cycle. A total of 6 rabbits were used for validation of the infection model, while the other 6 were assessed as part of the control group. All animals were handled in strict accordance with good animal practice as defined by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (11) and all animal work was approved by the College of Medicine of Ningbo University Chancellor's Animal Research Committee (Yuyao, China).

Model validation. The right knee of each rabbit was replaced with a tibial component using a Silastic implant (Dow Corning, Midland, MI, USA). The surgery was performed as described previously (12). Briefly, the rabbits were anesthetized via inhalation of 2% isoflurane, which was maintained by the intramuscular injection of ketamine (25 mg/kg) followed by continuous inhalation of 1% isoflurane. The right leg of each rabbit was shaved 24 h prior to the surgery and the skin was cleaned with an iodine solution just before surgery. A longitudinal skin incision was made to expose the knee. The epiphyseal plates were removed following dislocation of the tibia. The metaphysis was exposed and the cancellous bone of the medullary cavity of the proximal metaphysis was reamed. The stem of the nail-shaped silicone implant (14 mm long) was inserted into the intramedullary canal of the tibia, such that the implant head (15x5 mm) replaced the tibial plateau. The deep fascia and skin were finally closed to complete the procedure.

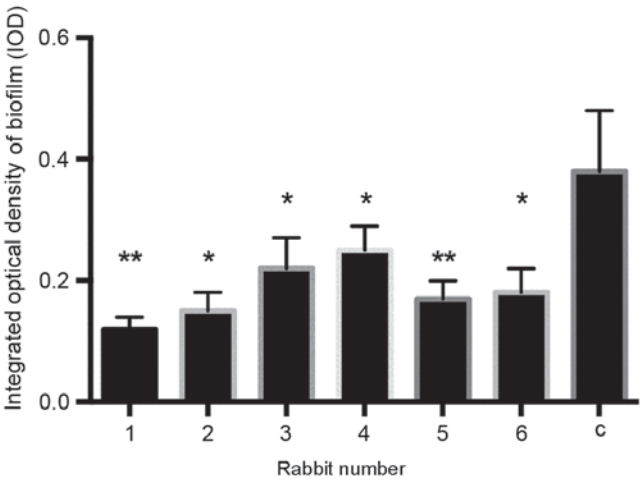

Figure 1. Bacteriocin decreases the biofilm formation of *Staphylococcus aureus* in rabbits after knee replacement surgery and infection. Biofilm formation was measured in each of the bacteriocin-treated rabbits (1-6) and the saline-treated treated rabbits 5 days after surgery and infection. $^{*}P<0.05$ and $^{**}P<0.01$ vs. the control group. IOD, integrated optical density; c, control.

Immediately after the surgical wound was closed, the 12 rabbits were infected by the intra-articular injection of 0 5 ml inoculum containing $3x10^5$ CFU/ml *S. aureus*. Then, 6 rabbits were injected with 1 ml of the bacteriocin suspension 4 h after the injection of *S. aureus*, and the other 6 rabbits were injected with 1 ml sterile saline solution.

Detection of biofilm formation on the implants. To test for biofilm formation in the rabbit model used in the present study, the implants were harvested from euthanized rabbits 5 days after surgery. The specimens were fixed with 2.5% glutaraldehyde at 4˚C for 1 h. Following fixation, the tissues were stained with 0.01% acridine orange solution at 4˚C for 2 h (Sigma-Aldrich; Merck KGaA), as described previously (13), and washed three times with PBS. The 50-μm-thick sections were then observed under an Eclipse 80i microscope (Nikon Corporation, Tokyo, Japan) equipped with an argon laser at an excitation wavelength of 488 nm. All images were quantified using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA). The fluorescence intensities of the biofilms were expressed as integrated optical density (IOD) values, which were calculated by the multiplications of the area and the density of the biofilm (14).

Determination of the serum levels of inflammatory cytokines. Blood samples were collected at 1, 2, 3, 4 and 5 days after

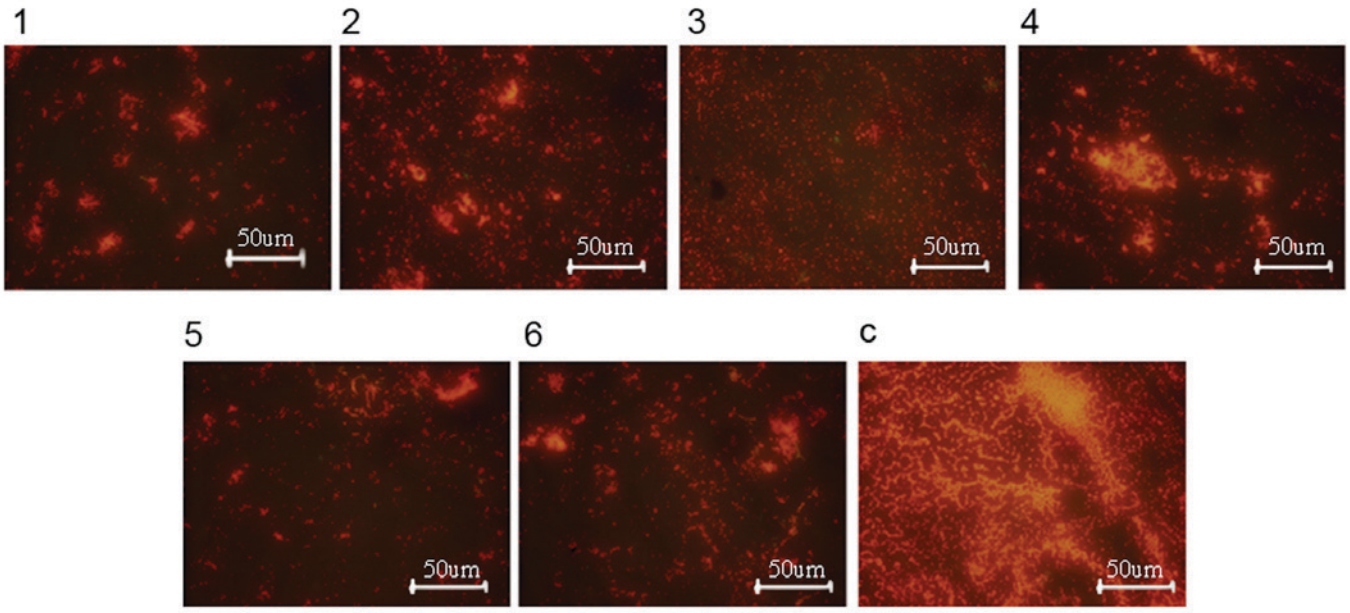

Figure 2. Fluorescent images of the *Staphyloccocus aureus* biofilm in bacteriocins-treated or saline-treated rabbits after knee replacement surgery and infection. The biofilm was stained with acridine orange and then visualized under a fluorescence microscope. The results for each of the bacteriocin-treated rabbits (1-6) and the PBS-treated treated rabbits 5 days after surgery and infection are shown. c, control.

the injection of bacteriocin or saline. The samples were centrifuged at 7,000 x g at 4˚C for 5 min and then stored at -70˚C until required. CRP and IL-6 levels were measured using commercial ELISA kits for CRP (cat. no. E-EL-RB0005) and IL-6 (cat. no. E-EL-RB0014; both Elabscience Biotechnology Co., Ltd., Wuhan, China), respectively, according to the manufacturer's protocol All measurements were performed in triplicate. The levels of CRP and IL-6 were expressed as mg/ml protein.

Statistical analysis. All tests were repeated three times. The results are presented as the mean ± standard deviation. Data analysis was performed using SPSS software (version 14.0; SPSS, Inc., Chicago, IL, USA). The results between two groups were compared using a Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Biofilm formation is decreased on implants after bacteriocin treatment. The IOD values of the biofilms the 6 infected rabbits revealed that there was a significant decrease in biofilm formation compared with the control group 5 days after surgery and infection ($P<0.01$; Fig. 1). Fluorescence microscopy of the biofilms revealed that the density of bacteria in the rabbits injected with the bacteriocin suspension was markedly lower than that of the control group (Fig. 2). The Tricine-SDS-PAGE of the bacteriocin suspension is presented in Fig. 3. The molecular weight of the bacteriocin was found to be 10-15 kDa.

Bacteriocin treatment attenuates S. aureus infection-induced inflammation. Due to the infection with *S. aureus* following the surgery, the levels of CRP and IL-6 in the control group increased, reaching maximum levels at 5 days post-surgery (Fig. 4). However, the levels of CRP and IL-6 in the rabbits treated with bacteriocin remained at a steady low level, and were significantly lower than the levels in the control group at days 3, 4 and 4 post-surgery (all $P<0.05$; Fig. 4).

Discussion

S. aureus is a pathogenic bacterium that is the cause of numerous infections (15). As one of the most important pathogens in orthopaedic infections (16), the treatment of *S. aureus* is of the utmost importance. However, the treatment of *S. aureus* infections may be complicated due to antibiotic resistance and/or biofilm formation (17). Traditional antimicrobial agents frequently lead to drug resistance and are not effective in removing biofilms (18). Therefore, there is an urgent requirement to identfy for novel antibacterial agents.

The ability of lactic acid bacteria to adjust the balance of intestinal microflora, enhance immunity and resistance, and promote the growth and development of the gut has been widely applied in clinical settings (19). Bacteriocins are a class of antibacterial peptides secreted by lactic acid bacteria (20), the majority of which are water-soluble. Bacteriocins exhibit marked antibacterial effects against numerous gram-positive bacteria (21). In the present study, bacteriocins were identified to possess significant inhibitory effects on *S. aureus* biofilm formation, which is consistent with previous results from similar studies (22).

CRP is an acute phase reaction protein that is indicative of septicemia (23). CRP levels are raised during acute infection, tissue damage, the presence or a malignant tumor and myocardial infarction (23). CRP levels can quickly drop when the patient recovers. CRP is thus a sensitive index in the acute phase of disease and facilitates the early diagnosis of infection. The liver synthesizes increased levels of CRP upon inflammation and tissue damage, which are alleviated

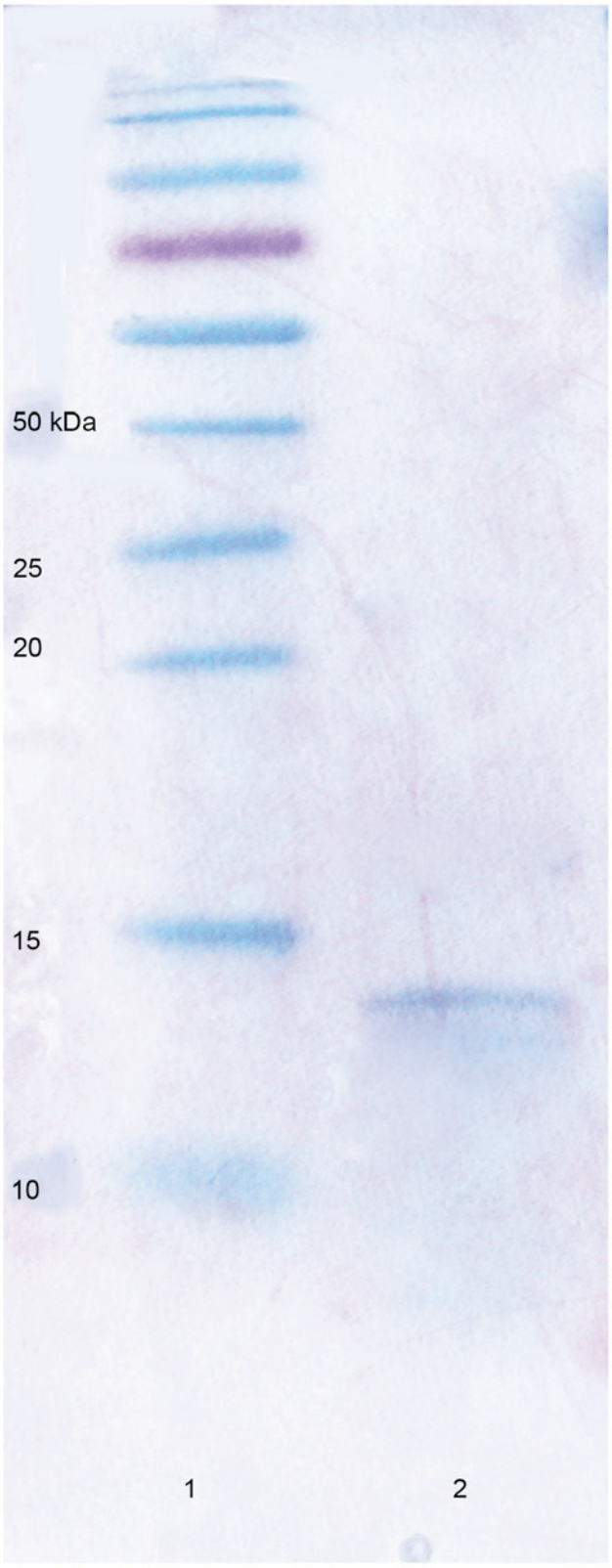

Figure 3. Tricine SDS-PAGE of the *Lactobacillus rhamnosus* protein supernatant following purification. Lane 1, protein ladder; lane 2, purified protein supernatant.

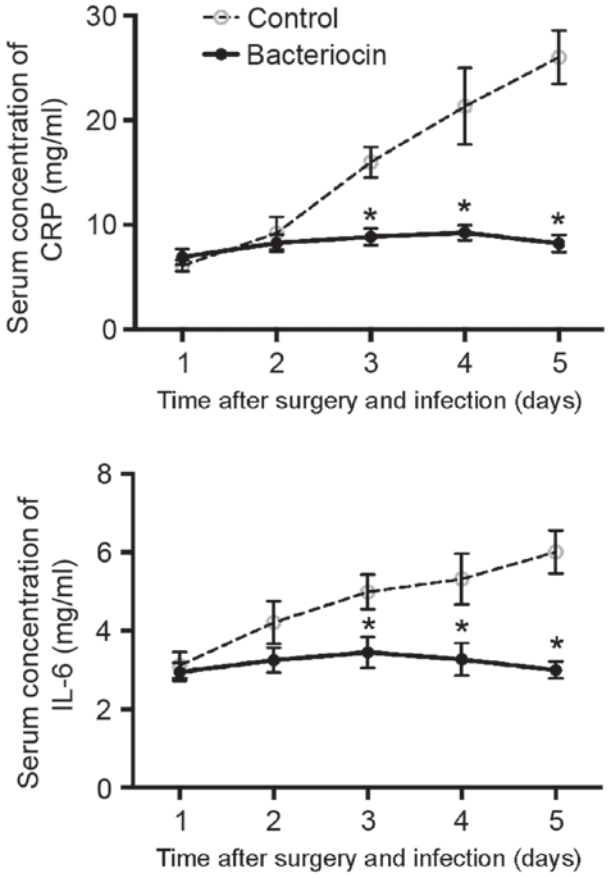

Figure 4. Serum concentrations of CRP and IL-6 in bacteriocins-treated or saline-treated rabbits after knee replacement surgery and infection. *P<0.05 vs. the control group. CRP, C-reactive protein; IL, interleukin.

by the inflammatory response (24). Therefore, the detection of serum CRP is clinically important in order to understand and treat inflammation. IL-6 is a proinflammatory factor that is produced by several types of cells in the body (25). IL-6 has a variety of biological activities, including regulation of the immune response under normal conditions and causing immune damage in pathological conditions (26). A previous *in vivo* study indicated that CRP levels are positively correlated with IL-6 levels, and that CRP can increase IL-6 production (27). The present study identified that bacteriocins attenuated the typical increase in CRP and IL-6 levels following infection, indicating that bacteriocins are a potential agent for the control of infection following orthopedic surgery.

In the present study, the standard laboratory strain of *S. aureus* was used. However, in clinical settings infections following orthopedic surgery tend to be caused by drug-resistant *S. aureus* (28). The infection produced in the rabbit model may be different to human infections. The results of the present study may therefore have a number of limitations and further research is required in order to investigate the effects of bacteriocins on drug-resistant *S. aureus*.

In conclusion, the present study demonstrated the antibacterial effect of bacteriocins in the rabbit model of *S. aureus* infection following knee replacement. This results indicates that bacteriocins may be a potentially agent to control infection following orthopedic surgery.

Competing interests

The authors declare that they have no competing interests.

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