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Pathogenic *Bacillus cereus* isolated from bovine - Analysis of Biofilm

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ABSTRACT

***Background*:** *Bacillus cereus* is a gram-positive bacterium, which is widely distributed and has certain pathogenicity. *Bacillus cereus* can cause food poisoning in animals and humans, and occasionally causes subcutaneous abscess, eye infection, bacteremia and other diseases. Our team has identified a strain of *Bacillus cereus* containing hemolysin BL (HBL), non-hemolysin enterotoxin (Nhe), cytotoxin K (CytK) and enterotoxin FM (entFM), and successfully constructed the detection method of Nhe gene RPA-LF. *Bacillus cereus*, can adhere to living and non-living surfaces and aggregate to form biofilms. This paper aims to provide data support for the elimination and control of *Bacillus cereus* biofilm.

***Materials, Methods & Results*:** In study, crystal violet staining and XTT detracting method were used to determine the forming ability, meta-bolic activity and some biological characteristics of biofilms, and microdilution method was used to determine the minimum inhibitory concentration of 5 kinds of Traditional Chinese medicine extracts and 5 kinds of antibiotics on biofilms. The effects of *Bacillus cereus* biofilm formation on the pathogenicity of different organs of animals were observed by inoculation test and pathological section. The results showed that the bacteria had strong ability to form biofilms and com-pact structure. The optimal temperature was 37 °C, and the optimal pH was 7, 0.5% NaCl, 0.5%-3% glucose and 200% TSB medium could promote the formation of biofilms. The minimum inhibi-tory concentration of baicalin, berberine, panicholide, emodin and sodium houttuynitin on biofilms were 128 μg/mL, 256 μg/mL, 256 μg/mL, 256 μg/mL and 512 μg/mL, respectively. The minimum inhibitory membrane concentrations of azithromycin, ampicillin, gentamicin, en-rofloxacin and flufenicol for biofilm were 32 μg/mL, 64 μg/mL, 64 μg/mL, 64 μg/mL and 128 μg/mL, respectively. The bacterium can make healthy mice infected and die, and cause different degrees of organ damage. The changes of nutrients and growth environment will lead to changes in the growth of biofilm. Different drugs had different inhibitory effects on biofilms, among which azithromycin and baicalin were the best.

***Discussion*:** *Bacillus cereus*, as a toxin-producing conditioned pathogen, has attracted great attention in various industries, because the bacterium can produce spores and biofilms in adverse environments, improving its ability to survive high temperature, drying and antibiotics. The formation of *Bacillus cereus* biofilms is currently a topic of great concern in medicine, the environment and food microbiology as it can cause serious public health problems, so infections caused by biofilms need to be treated at an early stage. At present, there is no best way to detect and treat biofilms in the body, so early diagnosis and treatment become particularly difficult, and there is a need to develop more effective methods to remove biofilms at the root to reduce the harm caused by biofilms. In this study, the biofilm forming ability of bovines lethal *Bacillus cereus* was identified, and its growth characteristics under different culture conditions were studied, and the inhibition and clearance effects of Chinese medicine extracts and antibiotics on *Bacillus cereus* were respectively explored, in order to lay a theoretical foundation for the subsequent study of its formation mechanism and comprehensive prevention and control of the disease.

Keywords: *Bacillus cereus*, biological membrane, biological characteristics, suppression and clearance.

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INTRODUCTION

Bacillus cereus is a Gram-positive endospore with periflagellar bacillus, which is highly similar to *Bacillus thuringiensis* and *Bacillus anthracis* [26]. As one of the important foodborne opportunistic pathogens causing diseases worldwide, this bacterium has been identified as a food contamination bacterium and has been isolated from dairy products, grains, meat products and fruits and vegetables [14,22,30]. Biofilms are complex bacterial communities embedded in substrates composed of extracellular polymers and attached to surfaces, making them much less susceptible to antimicrobial agents than free bacteria and thus difficult to remove [20]. In the past few decades, the generation of biofilms has received extensive attention, mainly focusing on potential risks such as drug resistance, production of pathogenic factors and persistence [13]. In recent years, the research focus of biofilm has been shifted to the aspects of biofilm clearance inhibition, complexity and interaction between biofilms.

In this study, a strain of highly pathogenic *Bacillus cereus* isolated from sudden bovine death in Tongliao area of Inner Mongolia Autonomous Region of China was selected as the research object. The biofilm formation ability of the strain was tested by changing some growth conditions and drug action, in order to lay a theoretical foundation for the prevention and control of highly pathogenic *B. cereus* in Tongliao area of Inner Mongolia Autonomous region of China.

MATERIALS AND METHODS

Strains and experimental animals

The tissue organs of dead cattle from a beef cattle farm in Tongliao City were isolated, identified and preserved by our laboratory. The serial number of this strain is OP028902 registered on Geenbank, and the number of the Treasure Center of China Microbial Culture Treasure Control Committee is CGMCC NO.17626.

Experimental animals: Kunming mice were purchased from Liaoning Changsheng Biotechnology[1]. The animal study was reviewed and approved by the Inner Mongolia Minzu University Institutional Animal Care and Use Committee.

Identification and morphological observation of biofilm formation ability

The activated bacterial solution was diluted to OD590=0.05 using TSB medium in 96-well plates with a final volume of 200 μL per well. Three repetitions were made and the medium was used as the control well. After completion, the 96-well plate was placed in a 37°C thermostatic box, and the OD590 was determined every 4h by crystal violet staining method. Before the determination, the remaining medium was discarded, washed with PBS for 3 times, and then fixed with 95% methanol for 30 min. The methanol was sucked out and washed with PBS for 3 times. After drying, 1% crystal violet was added to each well for 15 min staining. After that, the 24 h biofilm was placed on the slide for crystal violet staining and microscopic examination [25].

Determination of metabolic activity of biofilm

The metabolic activity of biofilm was determined by XTT decrement method. According to experiment 1.3.1, 2-24 h biofilm was cultured, XTT/Menadione solution was prepared for staining, and the OD490 value was determined after standing at 37°C for 3 h away from light [25].

Influence of different pH gradients on total biofilm formation

Add HCl and NaOH into TSB medium to adjust pH value to 5, 6, 7, 8, 9. The activated bacterial solution was diluted to OD600=0.05, and inoculated in medium containing different pH gradients, incubated at 37°C for 4 h to 36 h, crystal purple staining was performed, and the OD590 value was determined by enzyme marker with 3 repeats in each group, while TSB medium was used in control group.

Effects of different concentrations of NaCl on total biofilm formation

The NaCl concentration of TSB medium was adjusted to 0.5%, 1%, 1.5%, 2% and 2.5%. The bacterial solution was activated and diluted to OD600=0.05, and the medium containing different concentrations of NaCl was inoculated on 96-well plates successively, and cultured at 37°C for 4-36 h, crystal purple staining was carried out, and the OD590 value was determined by enzyme marker. The results were repeated three times in each group, and the conventional TSB medium was used as the control group.

Effects of different concentrations of glucose on total biofilm formation

The glucose concentration was adjusted to 1%, 3%, 5%, 7% and 10% by adding glucose in the medium successively. The activated bacterial solu-

tion was diluted to OD600=0.05 in 96-well plates, and the medium with different glucose concentrations was added successively. After incubating at 37°C for 4 h to 36 h in an incubator, crystal violet staining was carried out, and the OD590 value was determined by enzyme marker. Each group was repeated for 3 times, and normal TSB medium was used as control group.

Influence of TSB medium with different concentration on total biofilm formation

TSB medium concentration was adjusted to 10%, 20%, 50%, 100% and 200%, the bacteria solution was activated and diluted on 96-well plates. TSB medium of different concentrations was added successively, and cultured at 37°C in an incubator for 4-36 h. Crystal violet staining was performed, and OD590 value was determined by enzyme marker. TSB medium without any treatment was used as the control group.

Influence of different temperature gradients on total biofilm formation

The activated bacterial solution was thoroughly mixed, diluted to OD600=0.05 in a 96-well plate, and incubated at 27°C, 37°C, 47°C, 57°C and 67°C for 4 h to 36 h. After crystal purple staining, the OD590 value was determined. Three repeats were performed for each well. The OD590 value was measured every 4 h by crystal violet staining, and the amount of *Bacillus cereus* biofilm formation was determined.

Effects of different pH gradients on metabolic activity of biofilms

According to the specific method, biofilms of 4 h to 36 h were cultured in incubators with different pH gradients. XTT/Menadione working solution was used for staining, and the OD490 value was determined after standing at 37°C for 3 h away from light.

Effects of different concentrations of NaCl on metabolic activity of biofilms

According to the specific method, TSB medium containing different NaCl concentrations was used to culture biofilms for 4-36 h at 37°C. XTT/Menadione working solution was used for staining, and the OD490 value was determined after standing at 37°C for 3 h away from light.

Effects of different concentrations of glucose on metabolic activity of biofilms

According to the specific method, biofilms of 4 h to 3 6h were cultured in an incubator with TSB medium containing different concentrations of glucose. The biofilm growing for 4 h to 36 h was stained with XTT/Menadione working solution, and the OD490 value was determined after standing at 37°C for 3 h away from light.

Effects of different concentration culture-medium on metabolic activity of biofilm

According to the specific method, biofilms of 4-36 h were cultured at 37°C using TSB medium with different concentrations. XTT/Menadione working solution was used to stain the biofilm grown for 4 h to 36 h, and the OD490 value was determined after standing at 37 °C for 3 h away from light [25].

Effects of different temperature gradients on metabolic activity of biofilms

According to the specific method, biofilms were cultured at 27°C, 37°C, 47°C, 57°C and 67°C for 4 h to 36 h. After staining with XTT/Menadione working solution, the biofilms were placed at 37°C for 3 h, and the OD490 values were determined.

Determination of minimum inhibitory concentration (MBIC) and minimum inhibitory concentration (MIC) of biofilm by Chinese and Western medicines

Five extracts of traditional Chinese medicine (Berberine, emodin, baicalin, sodium Hdata and andrographolide) and five antibiotics[2] (gentamicin, azithromycin, enrofloxacin, flufenicol and ampicillin). They were used to investigate the inhibitory effects of Chinese and Western drugs on biofilms. Under aseptic conditions, the OD600 of bacterial solution was adjusted to 0.5 and 100 μL bacterial solution was added into the 96-well plate successively. The concentration of the pharmaceutical solution was 1 024 μg/mL in the first well. After mixing, 100 μL was absorbed into the second well, and then mixed again, 100 μL was absorbed into the third well, and the above operation was repeated to the 11th well. The mixture of 100 μL in the 11th well was discarded, and the mixture in the 12th well was used as the control group. Each drug was repeated three times and cultured at 37°C for 24 h. The results were observed with enzyme markers after crystal violet staining.

Artificial infection test of biofilm

According to the results of MBIC, one extract of traditional Chinese medicine and one antibiotic were selected to have the best inhibition effect on biofilm. The drug concentration was adjusted to 1/2 MBIC and 1 MBIC and acted on the bacterial solution to make the bacterial solution concentration 1×10^6 CFU/mL. Sixty 25-day-old male and female mice with body weight of 25-30 g were randomly divided into 6 groups with 10 mice in each group. 4 groups were intraperitoneally injected with 1 mL bacterial solution, 1 group was intraperitoneally injected with 1 mL pure bacterial solution, and the other group was intraperitoneally injected with 1 mL normal saline. The clinical manifestations of mice were checked every 30 min, and the dead mice were separated in time, and the heart, liver, spleen, lung and kidney of the dead mice were made pathological tissue sections to observe the pathological changes.

Histopathological observation of infected mice

The mice that died after injection were dissected, and the heart, kidney, liver, spleen and lungs were taken for pathological tissue sections. Meanwhile, the organs of healthy mice were taken as the control group for pathological section observation.

RESULTS

Measurement of total biofilm formation and morphological observation results

In this experiment, the total amount of *B. cereus* biofilm was quantified at 7 time points under normal conditions, and the growth curve and metabolic activity curve of the total amount of *B. cereus* membrane were drawn from 4 h to 36 h (Figure 1). As can be seen from the figure, biofilm grows rapidly and has strong metabolic activity from 4 h to 12 h. After 12 h, biofilm keeps growing but slows down, while metabolic activity begins to decline. At the same time, crystal violet staining was also performed on the biofilm and observed under an optical microscope. Under the microscope, purple dense membrane was visible (Figure 2).

Effects of different pH gradients on the total amount of biofilm formation and metabolic activity

At pH 5 and pH 9, the total amount of biofilm formation and metabolic activity of biofilm were the lowest. Under the condition of pH 6 and pH 8, the total amount of biofilm formation and metabolic activity increased slightly. Under the neutral condition of pH 7, the total amount of biofilm formation and metabolic activity were at a relatively high level (Figure 3).

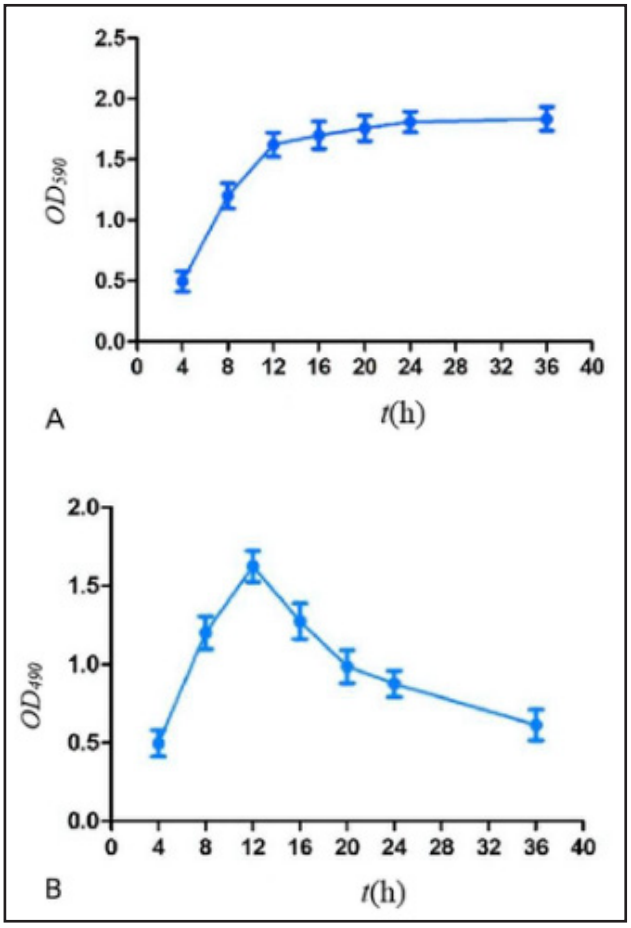

Figure 1. Curve of biofilm formation and metabolism of *Bacillus cereus*. A- Biofilm formation curve; B- Change curve of biofilm metabolic activity.

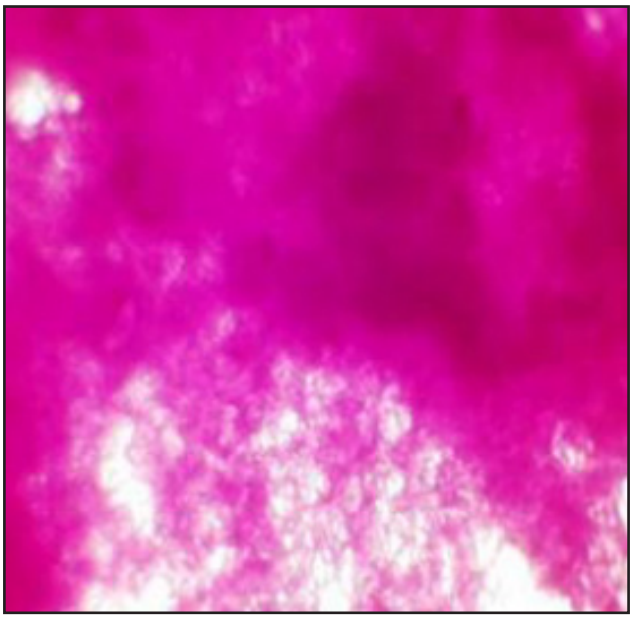

Figure 2. Microscopy with biofilm staining *Bacillus cereus*.

Effects of different concentrations of NaCl on total amount of biofilm formation and metabolic activity

When NaCl concentration was 0.5%, the growth rate and metabolic activity of biofilm were stronger than those of other groups, but still lower than that of the control group. With the increase of NaCl concentration, the growth rate and metabolic activity of biofilm decreased, especially the metabolic activity of the membrane decreased to below 0.5 after 20 h. This indicates that high concentration of NaCl may inhibit the formation of biofilms (Figure 4).

Effects of glucose concentration on total biofilm formation and membrane metabolic activity

From 4 h to 12 h, the growth rate of biofilm was faster under the condition of 1% and 5% glucose concentration, and the metabolic rate was the highest at 1% and 3%. After 12 h, the growth rate slowed down, and the metabolic activity of all experimental groups showed a downward trend. When the concentration is 3%, the growth rate increases rapidly after 12 h, and slows down after 24 h. When the concentration is 7% and 10%, the growth rate of the two is almost the same before 16 h, and after 16 h, the growth of the latter shows a downward trend (Figure 5).

Influence of different concentration medium on total biofilm formation and metabolic activity

The growth rate and metabolic rate of biofilm were the strongest when the medium concentration was 200%, and the total amount of biofilm formation and metabolic activity decreased gradually with the decrease of the medium concentration (Figure 6).

Effects of different temperature gradients on the total amount of biofilm formation and metabolic activity

At 37°C, both the total amount of biofilm formation and metabolic activity were the strongest, and with the increase and decrease of temperature, the total amount of biofilm formation and metabolic activity decreased gradually (Figure 7).

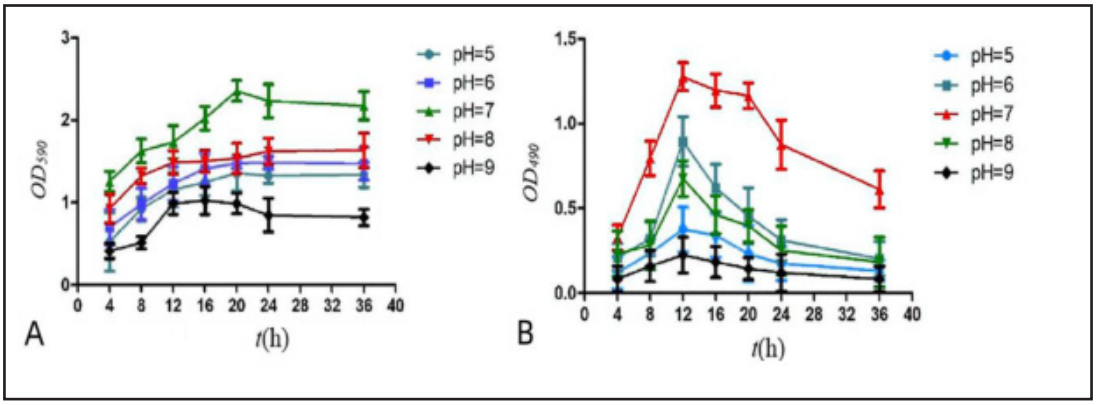

Figure 3. Effect of pH on biofilm formation and total metabolism. A- The effect of pH on the total amount of biofilm formation; B- The effect of pH on the metabolic activity of biofilms.

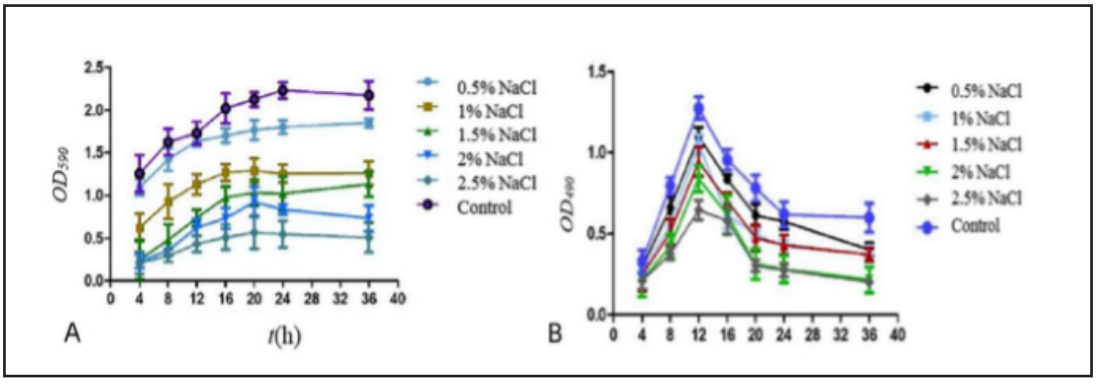

Figure 4. Effects of NaCl on biofilm formation and total metabolism. A- The effect of NaCl on the total amount of biofilm formation; B- The effect of NaCl on the metabolic activity of biofilms.

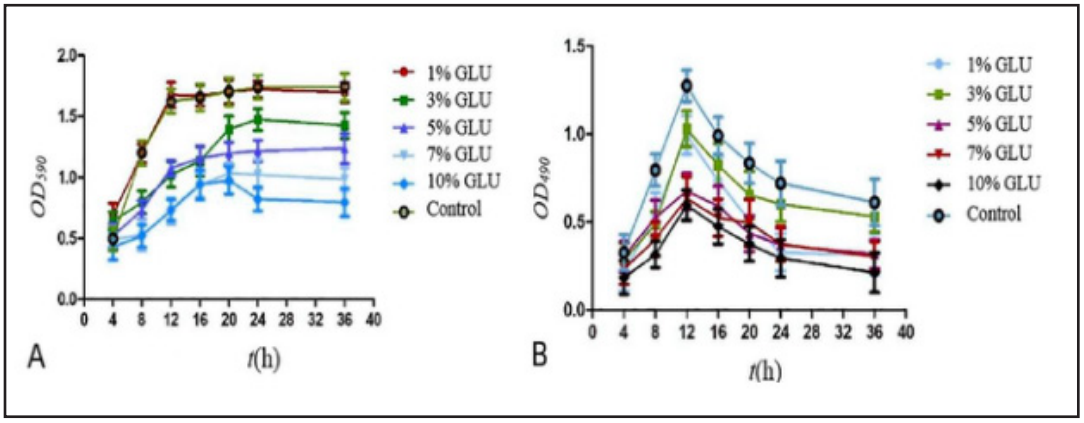

Figure 5. Influence of glucose on biofilm formation and total metabolism. A- The effect of glucose on the total amount of biofilm formation; B- The effect of glucose on the metabolic activity of biofilms.

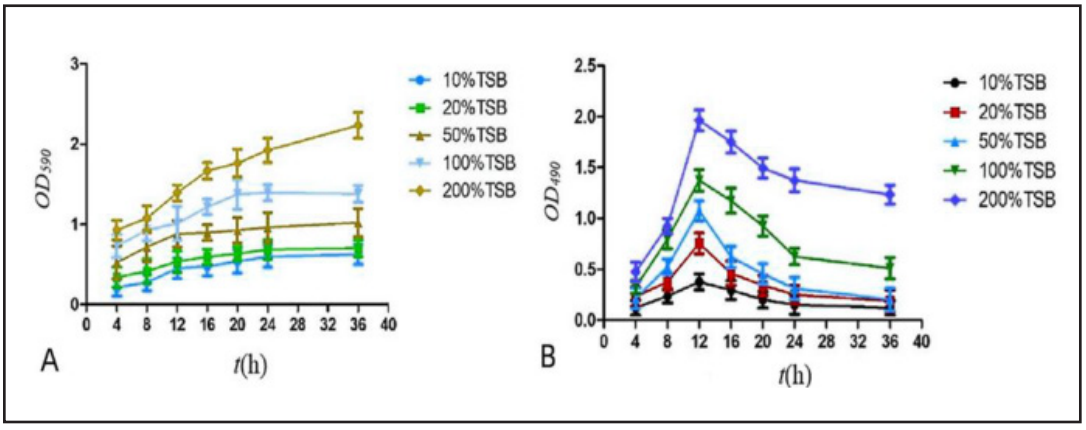

Figure 6. Effect of TSB concentration on biofilm formation and total metabolism. A- The effect of TSB concentration on the total amount of biofilm formation; B- The effect of TSB concentration on metabolic activity of biofilms.

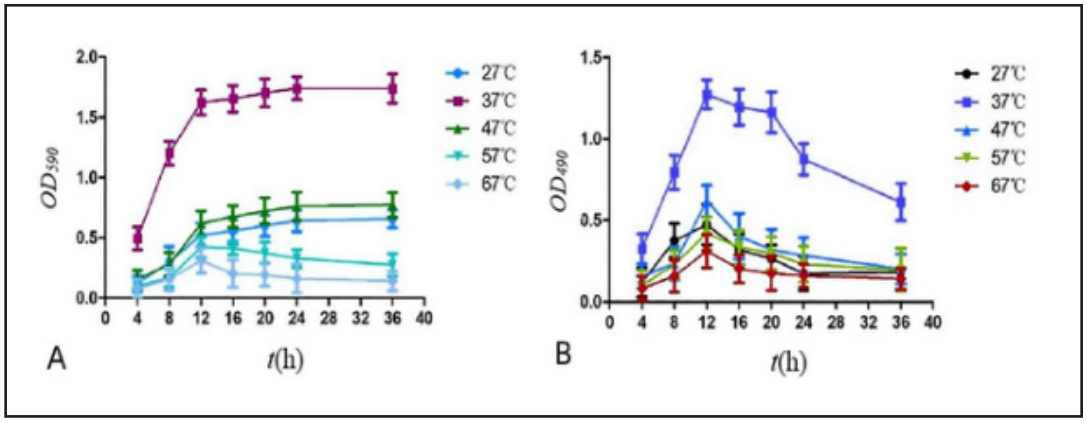

Figure 7. Effect of temperature on biofilm formation and total metabolism. A- The effect of temperature on the total amount of biofilm formation; B- The effect of temperature on the metabolic activity of biofilms.

Inhibitory effects of Chinese and Western medicines on biofilms

In traditional Chinese medicine, baicalin had the best inhibition effect on *Bacillus cereus* biofilm, MBIC was 128 μg/mL, berberine, androholide and baicalin had the best antibacterial effect, and MIC was 512 μg/mL. Among western medicines, azithromycin had the best inhibition effect on *Bacillus cereus* biofilm, with MBIC of 32 μg/mL, and azithromycin, ampicillin and Enrofloxacin had the best inhibition effect, with MIC of 128 μg/mL (Table 1).

Table 1. Minimum bacteriostatic concentration and the minimum film inhibitory concentration of the drug on biofilms.

Antibacterial drug	MBIC/μg/mL	MIC/μg/mL
Baicalin	128	512
Berberine	256	512
Andrographolide	256	512
Emodin	256	1024
Sodium Houttuyfonate	512	1024
Azithromycin	32	128
Ampicillin	64	128
Gentamicin	64	256
Enrofloxacin	64	128
Florfenicol	128	512

Artificial infection test of biofilm

During the experiment, the hair of mice in azithromycin group and baicalin group with drug concentration of 1 MBIC was reversed and did not like to move after 10 h injection. One of the mice in the baicalin group with a concentration of 1/2 MBIC died 1 day after injection, while the mice in the azithromycin group with a concentration of 1/2 MBIC showed shortness of breath. After 4 days, 3 mice died in the 1/2 MBIC azithromycin group and 4 mice in the 1/2 MBIC baicalin group. In the pure bacterial solution group, 1 mouse died 5 h later, and the other mice showed dyspnea and reduced feed intake, and all died 20 h later. Mice in the control group ate normally without any abnormal condition.

Histopathological observation of infected mice

The results of pathological tissue sections are shown in Figure 8. The above experiments indicate that the pathological damage of the bacterial injection group was more serious: myocardial fibers were swollen and broken, and myocardial nucleus disappeared. The hepatic blood sinuses widened, the nuclei of a large number of hepatocytes shrank, and some hepatocytes necrosis and dissolution disappeared. Lymphocyte necrosis and disappearance in the red pulp of spleen; Alveolar wall telangiectasia, a large number of leukocyte hyperplasia, respiratory bronchiolar epithelial cells shed, there is a serous substance in the lumen; The mesangial cells of the glomeruli proliferated and the epithelial cells of the renal tubules were exfoliated and necrotic. The order of pathological damage from serious to light was as follows: bacterial solution group, 1/2 MBIC baicalin group, 1 MBIC baicalin group, 1/2 MBIC azithromycin group and 1 MBIC azithromycin group.

DISCUSSION

Bacillus cereus is commonly associated with spices and foods such as cardamom, rice, grains and dairy products [5,8]. *Bacillus cereus* promotes transmission by producing highly stress-resistant resting spores that germinate under nutrient-rich conditions through the interaction (combination) of germination agents and various nutrient germination receptors [26]. Germination of *B. cereus* spores in food will lead to food deterioration and safety problems [29,32]. The bacterium's vegetative cells produce toxins before food intake or in the small intestine, usually resulting in mild self-limiting symptoms, with life-threatening conditions occurring only in rare cases. *Bacillus cereus* can also cause wound infection, eye infection and bacteremia [1]. More seriously, it can cause food poisoning and local and systemic infection, and is associated with the syndrome related to food poisoning: diarrhea syndrome and vomiting syndrome [4,18,27]. In addition to several exogenous proteins, such as HBL, Nhe, CytK, and hemolysin I (CLO), phospholipase, protease, and EntD proteins may also cause diarrheal syndrome [22]. So far, cases of *B. cereus* infection have been reported in different kinds of animals such as alpine vulture, cattle, yellow sand turtle, fish, deer and pig [6,7,12,15,17,28,31].

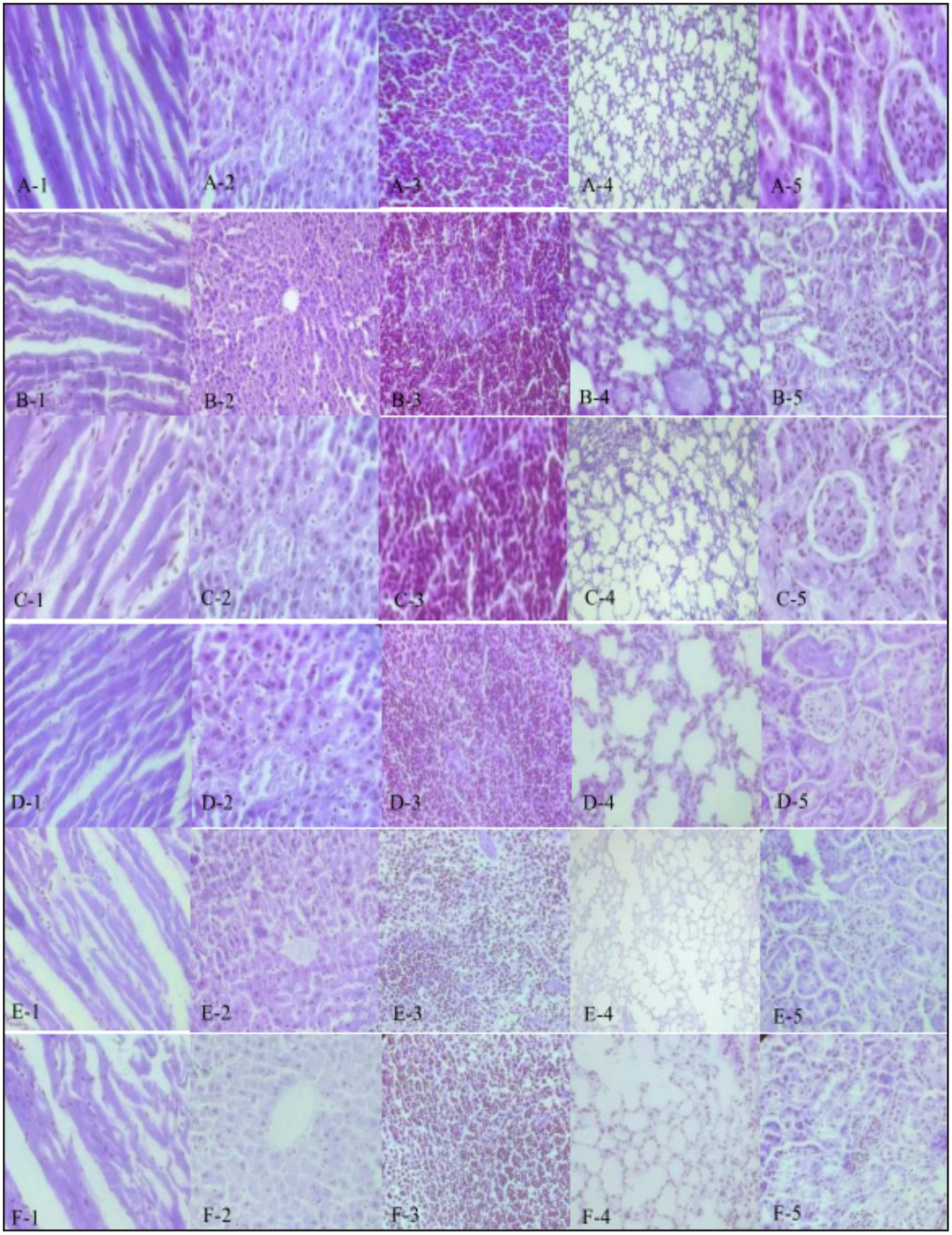

Figure 8. Histological section (HE staining). A- Microbial group; B- 1/2 MBIC Baicalin group; C- 1 MBIC Baicalin group; D- 1/2 MBIC Azithromycin group; E- 1 MBIC Azithromycin group; F- The control group.1: Heart; 2: liver; 3: spleen; 4: kidney; 5: lung.

Biofilm formation is a dynamic process that is divided into three distinct phases: (1) the initial attachment phase (plankton cells attach to the surface), (2) the maturity phase (the beginning of the production of adhesion proteins and other extracellular polymers), (3) the separation and diffusion phase (small portions of bacteria or biofilms separate and re-attach to new surfaces) [19]. Biofilm formation is an important step in the life cycle of most bacteria and is associated with disease outbreaks, bacterial resistance or contamination of medical and industrial equipment [3]. According to statistics, about 65% of bacterial diseases are associated with bacterial biofilms, and the proportion of chronic infections caused by bacterial biofilms has risen to 80%. Therefore, Biofilm is also considered as a potential target against bacterial diseases [16].

The *B. cereus* biofilm in this study had strong forming ability and dense structure. The growth of biofilm was best when pH was 7, and the growth of biofilm was inhibited under strong acid and base conditions. NaCl at 0.5% can promote biofilm metabolism, while NaCl at higher than 0.5% can inhibit biofilm metabolism. TSB medium with concentration less than 100% inhibited the formation of biofilm, and high concentration of TSB medium promoted the formation of biofilm. When the glucose concentration was 0.5% to 3%, it promoted the formation of biofilm, and when the glucose concentration was higher than 5%, it inhibited the formation of biofilm to a certain extent. The results showed that the changes of nutrients and growth environment would lead to changes in the growth of biofilm. Different drugs had different inhibitory effects on biofilms, among which azithromycin and baicalin were the best. Studies showed that *B. cereus* from different sources had different sensitivity to the culture environment. Gao T *et al*. [10] isolated a *B. cereus* from the root of wheat, and *B. cereus* had the strongest biofilm formation ability when the pH of the medium was adjusted to 6, while *B. cereus* could not form biofilm when pH was ≥ 7. Only when 1% glucose was added into TSB medium, the subaqueous biofilm could be formed. In addition, the biofilm-forming ability of *B. cereus* was also related to individual differences of strains. Fei P *et al*. [8] tested the biofilm-forming ability of 42 strains of *B. cereus*, and 64.3% of *B. cereus* belonged to strong membrane forming strain and 35.7% of *B. cereus* belonged to weak membrane forming strain. However, only 53.7% of the 41 strains of *B. cereus* studied by Radmehr B *et al*. [23] had the ability of biofilm formation, and only 4.9% of the strains had strong membrane formation. In addition, the same drug has different effects on different strains of biofilm. In this study, the sensitivity of B. cereus biofilm to azithromycin and baicalin were 32 μg/mL and 128 μg/mL respectively.

B. cereus, *B. thuringiensis*, and *B. Anthracis* all produce biofilms. The biofilms produced by these bacteria can either float as thin films, adhere to non-living surfaces, or remain in living tissues. This may be a key factor in the colonization of *B. cereus* in different environments [21]. Like spores, Biofilm gives bacteria high resistance to a variety of adverse conditions and high adhesion ability on a variety of substrates, including the formation of Biofilm on stainless steel surfaces and conveyor belt surfaces during food processing. In these devices, *B. cereus* can survive for a long time and even be sterilized. However, Biofilm formation is affected by many factors, including nutrient utilization, carbon source and cleaning process [8]. As for *B. cereus*, it can metabolize a variety of carbon sources (such as glucose), and NaCl is widely used in the food and medical industries. Different products have different concentrations of NaCl, and controlling the concentration of NaCl can affect the formation of *B. cereus* biofilm. Therefore, It is important to control the potential influence of related conditions on the formation of *B. cereus* biofilms.

As a toxin-producing opportunistic pathogen, *B. cereus* has attracted high attention in various industries. Because it can produce spores in adverse environments, it has improved its ability to withstand high temperature, drying and antibiotics [24]. *B. cereus* biofilm formation is currently a topic of great concern in medicine, environmental and food microbiology because of the serious public health problems it can cause. Therefore, infections caused by biofilms need to be treated at an early stage, but currently there is no best way to detect and treat biofilms *in vivo*, so early diagnosis and treatment becomes particularly difficult. Therefore, more effective methods need to be developed to fundamentally remove biofilms, so as to reduce the harm caused by biofilms.

At present, the removal of biofilms at home and abroad is mainly focused on the food processing industry and the medical industry, and most of the research content is focused on the use of single or dif-

ferent methods at a certain stage of biofilms, and the research work on the characteristics of each stage in the formation process, different maturity levels and the combined use of various methods needs to be further carried out [2,9,11].

CONCLUSION

Crystal violet staining method and XTT subtractive method were used to investigate the biofilm formation ability of Bacillus cereus and its characteristics at different growth stages: the optimal biofilm formation temperature was 37℃, and the optimal pH was 7, 0.5% NaCl, 1% and 3% glucose and 200% TSB medium could promote the biofilm formation. It can make healthy mice infected and die, and cause different degrees of damage to the organs.

The minimum inhibition concentration of 5 commonly used Chinese medicines and chemical drugs on biofilm showed that the best drugs were azithromycin and baicalin.

MANUFACTURERS

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***Ethical approval*.** The animal study was reviewed and approved by the Inner Mongolia Minzu University Institutional Animal Care and Use Committee.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper..

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