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Identification and Antibacterial Activity Study of a Terrestrial Strain of *Brevibacterium aureus* 431

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Abstract: This study isolated and purified strain 431 from an animal probiotic product. Through staining and microscopic examination, colony morphology analysis, biochemical reaction tests, and 16S rDNA sequence alignment, the strain was identified and named *Brevibacterium aureus* 431. The study focused on the production of biosurfactants by strain 431, and antibacterial activity tests were conducted on the strain and its secondary metabolites. The results showed that strain 431 exhibited no resistance to 10 commonly used drugs, and its concentrated secondary metabolites were highly sensitive to the indicator bacterium *Escherichia coli*. Oral administration of strain 431 to BALB/c mice resulted in normal mental state, diet, and bowel movements, with no signs of illness or death, indicating that strain 431 is highly safe and non-pathogenic to mice. The study suggests that *Brevibacterium aureus* 431 has significant research value as a new source of actinomycetes and that its secondary metabolites have potential application value in the development of antibacterial drugs.

Keywords: *Brevibacterium aureus* 431; Isolation and identification; Genetic evolution analysis; Antibacterial activity; Drug susceptibility test; Safety test

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1. Introduction

The influence of animal food drug residue and animal source bacterial drug resistance on food safety and public health safety has attracted much attention. Following the implementation of the prohibition measures in Sweden, Denmark, the United Kingdom and other countries, the Ministry of Agriculture and Rural Affairs of the People's Republic of China Announcement No.194 clearly stipulates that: from July 1, 2020, Chinese feed manufacturers will stop the production of commercial feed containing feed additives for growth-promoting drugs [1]. Feed "resistance" not only increases the incidence and fatality rate of livestock and

poultry but also leads to slow production, decreased resistance, and frequent gastrointestinal diseases [1]. It is inevitable to improve the production performance of livestock and poultry, enhance the quality of livestock and poultry products, and accelerate the research and development of safe and drug-free microecological agents that can replace antibiotics [2]. Probiotics can regulate the balance of intestinal flora, enhance the digestion and absorption of nutrients, and boost the immune system of animals. Numerous studies have shown [3-5] that common probiotics used domestically and internationally include actinomycetes, lactic acid bacteria, bifidobacteria, streptococci, bacilli, photosynthetic bacteria, and yeasts [6]. These probiotics have gained attention in the livestock and poultry industry as ideal alternatives to antibiotics [7].

Actinomycetes are a prokaryotic group belonging to the bacterial kingdom phylum Actinobacteria, Gram-positive bacteria that are susceptible to antibiotics. *Actinomycetes* are widely distributed in nature and are closely related to human production and life. About 70% of the widely used antibiotics are produced by various actinomycetes [8]. Some species of actinomycetes can also produce various enzymes such as protease, amylase and cellulase, vitamin B_{12}, and organic acids. In addition, actinomycetes can also be used for steroid transformation, hydrocarbon fermentation, petroleum dewaxing, and sewage treatment. Therefore, actinomycetes are closely related to human beings and have great significance in the pharmaceutical industry [8].

Marine actinomycetes that can produce active natural products are more favored by researchers [9]. Among marine microorganisms, the secondary metabolites of marine actinomycetes are diverse, rich, and complex, covering almost all compound types. Marine microorganisms have received attention due to their complex biosynthetic pathways and their potential implications for the development of anticancer and anti-infective agents in response to multidrug-resistant strains [10]. Natural products have become one of the important sources of many clinical drugs and new drug lead compounds because of their novel structure and unique active role. Studies have shown that the ocean provides more than 30,000 natural products with novel structures or good active activities, and it is increasing year by year [11]. *Brevibacterium aureum* is a marine actinobacteria, which is one of the source strains for the research and development of marine actinobacteria. In recent years, the emergence of new diseases and drug-resistant pathogenic bacteria has continuously weakened the clinical efficacy of existing antibiotic drugs, and it is of great significance to develop drugs with novel action mechanism or significant activity. In this study, terrestrial strain *Brevibacterium aureum* 431 were isolated from animal probiotic products, and their characteristics were studied, analyzed, and evaluated, laying a foundation for the development and application of animal probiotics and the research and application of antibacterial metabolites.

2. Materials and methods

2.1. Materials

Luria-Bertani (LB) culture medium, blood agar culture medium, bacterial biochemical reaction reagent, hexadecyltrimethylammonium bromide, and susceptibility test paper disks were purchased from Guangdong Huankai Microbiology Technology Co., LTD. De Man–Rogosa–Sharpe (MRS) medium and cetyltrimethylammonium bromide agar were purchased from Qingdao High-tech Industrial Park Haibo Biotechnology Co. The indicator bacteria *Escherichia coli* (K12D31) and *Staphylococcus aureus* (ATCC 29213) were obtained from China. BALB/c mice were purchased from the Guangdong Provincial Medical Laboratory Animal Center.

2.2. Methods

2.2.1. Bacterial culture and filtrate preparation

The indicator bacteria *Escherichia coli* (K12D31) and *Staphylococcus aureus* (ATCC 29213) were inoculated into an LB liquid culture medium and were cultivated in a shaking incubator (37°C, 120 r/min, for 16 to 24 hours). The bacterial concentration was measured at a wavelength of 600 nm; when the OD value reached 1.0, the culture was stored at 4°C for later use.

The 431 strain was inoculated onto LB agar plates and was incubated at 37°C for 24 hours. Typical colonies were picked and inoculated into an MRS liquid culture medium for shaking incubation (37°C, 120 r/min, for 48 hours). After incubation, the bacterial liquid (named original bacterial liquid A) was taken and stored at 4°C. After shaking, a small amount of the 431 bacterial liquid was taken, centrifuged at 10,000 r/min for 10 minutes, and the supernatant was collected. The supernatant was filtered using a 0.45 μm filter, and the filtrate (named extraction liquid B) was collected and stored at 4°C.

2.2.2. Extraction of the biosurfactant

The supernatant was removed from 25 mL of the 431 bacterial strain medium by centrifugation at 10,000 r/min for 10 min. 85% phosphoric acid was added to the supernatant, so that the final concentration of phosphate was 1% (v/v) of the supernatant, and the pH value of the supernatant was adjusted to be 2–3. It was mixed well with ethyl acetate at 1:1.25 and extracted twice. The superlayer fluid was transferred to a centrifuge tube twice, ethyl acetate was removed by evaporation at room temperature, and an appropriate amount of deionized water was added to dissolve the sediment (named concentrate C), and stored at 4℃.

2.2.3. Biological identification

The 431 bacterial strain was inoculated into MRS agar plates and typical colonies were selected for Gram stain microscopy, colony morphology, and biochemical tests.

2.2.4. 16S rDNA sequencing and genetic evolution analysis

Gene sequencing of the 431 strain was completed by Guangzhou Eki Biotechnology Co., Ltd. A genetic evolution tree was constructed using MEGA 7.0 based on 16S rRNA gene fragment sequencing of isolate 431 against 29 reference sequences in the GenBank database.

2.2.5. Biosurface activity detection

The medium of the 431 bacterial strain was centrifuged at 10,000 r/min for 10 min, and 2 mL of the supernatant was taken and mixed with an equal amount of petroleum ether. The mixture was vortexed for 2 minutes and then allowed to stand for 1 minute. The height of the emulsion layer was measured. The emulsion layer height was divided by the total height of the mixture and multiplied by 100 to calculate the emulsification activity (EA). The tube containing the emulsion was stored at 4°C for 24 hours, after which the height of the emulsion layer was measured again. This height was divided by the total height of the mixture and multiplied by 100 to calculate the emulsification index (E24).

For hemolytic activity, bacterial isolation culture and the filter paper method were used. The hemolysis ring around the colony or filter paper was observed to determine the intensity of biosurfactant (BS) production. The 431 strain was added to filter paper on blood agar medium at 8 μL per paper. A 20% Tween-80 solution was

used as a positive control, and the plates were incubated at 37°C for 48 hours.

For cetyltrimethylammonium bromide (CTAB) detection, the bacterial liquid (10 μL per paper) was applied using the same method as for hemolytic activity. A 20% Tween-80 solution was used as the positive control, and the plates were incubated at 37°C for 24 to 48 hours. The CTAB agar plate was observed for a dark blue halo around the colonies, indicating a positive result, while the absence of a halo indicated a negative result.

2.2.6. Drug susceptibility test

The following ten commonly used antibiotics were selected for the antimicrobial susceptibility test: amoxicillin, amikacin, gentamicin, enrofloxacin, ciprofloxacin, florfenicol, neomycin, ceftriaxone, doxycycline, and trimethoprim-sulfamethoxazole. The disk diffusion method was used, and after 24 hours of incubation at 37°C, the diameter of the inhibition zones was measured to determine the sensitivity of the *Brevibacterium aureum* strain 431 to the antibiotics. *Escherichia coli* (K12D31) was used as a control. The criteria for interpretation were as follows: inhibition zones larger than 20 mm were classified as extremely sensitive, 15–20 mm as highly sensitive, 10–15 mm as moderately sensitive, and less than 10 mm as resistant.

2.2.7. Bacteriostatic test

The indicator bacteria *Escherichia coli* (K12D31) and *Staphylococcus aureus* (ATCC 29213) were coated on LB solid medium, and the filter paper was put on the surface of the indicator bacteria plate, and the bacterial solution (liquid A), filtrate (liquid B), and concentrated extract (liquid C) were added to the filter paper (10 μL / piece) for the antibacterial test. The *Escherichia coli* indicator plate was used with neomycin antibiotic sensitivity disks as the control, while the *Staphylococcus aureus* indicator plate used LB agar as the control. Both plates were incubated at 37°C for 24–48 hours, and the diameter of the inhibition zones was measured to assess antimicrobial activity.

2.2.8. Safety test

BALB/c mice were fed and adapted for two days and divided into an experimental group and a control group (seven mice in each group). On the first day of the experiment, each mouse in the experimental group was orally administered 0.1 mL of the bacterial suspension. From the second day onwards, 0.1 mL of the bacterial suspension per mouse per day was added to their drinking water, allowing the mice to drink freely until the 7th day. In the control group, a liquid culture medium was used as a substitute. Daily observations were recorded, including the mice's behavior, diet, feces, signs of illness, or mortality. On the 3rd and 7th days, both the experimental and control groups were weighed, and the body weight results were recorded.

3. Results

3.1. Gram stain and colony characteristics

The 431 bacterial strain was inoculated in MRS medium for 37°C and cultured for 48 hours. Typical colonies were selected for Gram staining and observed under a light microscope. The bacteria were Gram-positive, thicker and shorter bacilli, as shown in **Figure 1**. The colonies are round, about 1–2 mm in diameter, with a moist surface, raised, and well-edged, as shown in **Figure 2**.

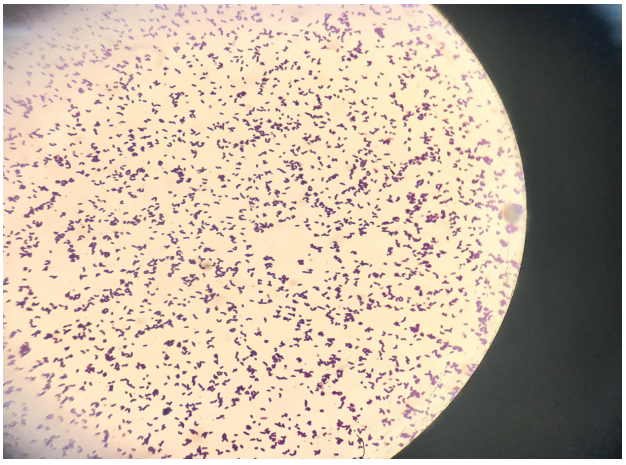

Figure 1. Gram staining microscopy (1000x)

Figure 2. Colonial morphology

3.2. Biochemical test results

The 431 strain was inoculated into bacterial microreaction tubes and incubated at 37°C for 24 hours, with results shown in **Table 1**. The results indicated that the 431 strain could grow in 6.5% high-salt broth. The 431 strain tested positive for glucose, fructose, maltose, sucrose, cellobiose, myo-inositol, starch hydrolysis, arginine hydrolysis, and N-acetylglucosamine reactions, while it tested negative for lactose, mannitol, trehalose, malonate, citrate, nitrate reduction, phenylalanine, and hydrogen sulfide reactions. The biochemical reaction results align with the biochemical characteristics of *Brevibacterium*.

Table 1. Biochemical test results of *Brevibacterium aureum* 431

Tests	*Brevibacterium aureum* 431	Tests	*Brevibacterium aureum* 431
Glucose	+	6.5% high-salt broth	Grow
Lactose	−	Malonate	−
Fructose	+	Citrate	−
Maltose	+	Nitrate reduction	−
Mannitol	−	Starch hydrolysis	+
Sucrose	+	Phe (F)	−
Cellobiose	+	Arginine hydrolysis	+
Trehalose	−	H_2S	−
Myo-inositol	+	Motility	+
Rhamnose	−	N-acetylglucosamine	+

Note: "+" positive reaction; "–" negative reaction.

3.3. Analysis of genetic evolution

Through sequencing of the 16S rDNA gene fragment of the 431 bacterial strain, and sequence homology comparison with the GenBank database of 29 strains from different countries and regions, the genetic developmental trees of the 431 bacterial strain are shown in **Figure 3**. The genetic distance between strain

431 and other known *Brevibacterium* strains was relatively large, confirming that the 431 isolate is a strain of *Brevibacterium aureum* and forms a distinct new branch.

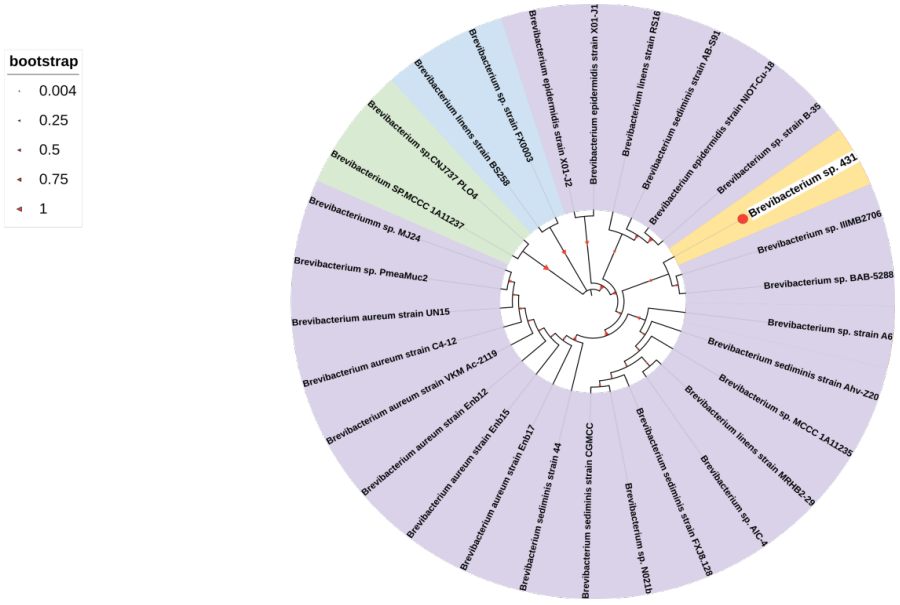

Figure 3. Genetic development tree of strain *Brevibacterium aureum* 431

3.4. Biosurfactant test

Biosurface activity of the 431 bacterial strain was detected according to **2.2.5**. The results showed that the emulsification activity of 431 bacterial strains was 57% and the emulsification index was 33%, shown in **Figure 4A**. The CTAB agar emulsification test results showed that *Brevibacterium aureum* strain 431 with numbers 1, 3, and 5 formed blue halos around the bacterial lawn, indicating a positive result, as shown in **Figure 4B**.

On the blood agar plate, *Brevibacterium aureum* strain 431 was streaked and incubated at 37°C for 24 hours. The colonies appeared as moist, raised, circular colonies with smooth edges, golden yellow in color, and a diameter of approximately 1–2 mm. Mild α-hemolysis was observed around the colonies, and the bacterial lawn also exhibited α-hemolysis, as shown in **Figure 4C**. The hemolysis phenomenon observed by the filter paper disk method indicated that strains 1, 3, and 5 were *Brevibacterium aureum* strain 431, showing α-hemolysis around the bacterial lawn, while strain 7 served as the 20% Tween-80 positive control, as shown in **Figure 4D**.

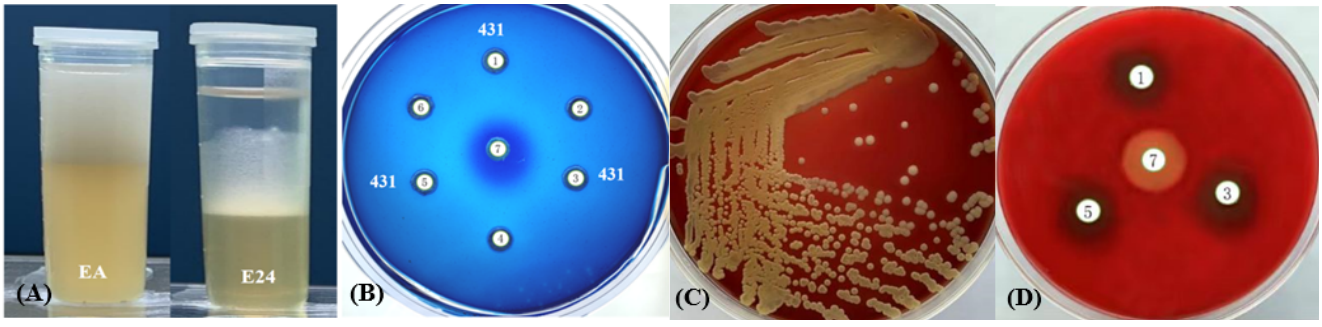

Figure 4. Detection of biosurface activity of *Brevibacterium aureus* 431. (A) Emulsification test; (B) CTAB test; (C) Colony morphology of blood agar plates; (D) Blood agar plate-based hemolysis test.

3.5. Drug susceptibility test

Drug tests were conducted on *Brevibacterium aureum* strain 431 and the indicator bacterium *Escherichia coli* (K12D31) using 10 commonly used drugs. The results showed that strain 431 had an inhibition zone diameter greater than or equal to 20 mm for amoxicillin, gentamicin, enrofloxacin, ciprofloxacin, florfenicol, and ceftriaxone, indicating extreme sensitivity. For amikacin, neomycin, doxycycline, and trimethoprim-sulfamethoxazole, the inhibition zone diameters were between 15–16 mm, indicating high sensitivity. In short, *Brevibacterium aureum* strain 431 does not exhibit resistance to the commonly used antibacterial drugs. The results are shown in **Table 2**.

3.6. Antibacterial activity test

Using the methods from sections **2.2.2.** and **2.2.7.**, qualitative antibacterial tests were conducted on the original culture fluid (A), the extracted solution (B), and the concentrated solution (C) of *Brevibacterium aureum* strain 431. The results showed that the concentrated solution of strain 431 exhibited inhibitory effects against the indicator bacterium *Escherichia coli*, with inhibition zones ranging from 21 to 23 mm, indicating extreme sensitivity. However, the inhibition against *Staphylococcus aureus* was weaker, with inhibition zones of 12 to 14 mm, indicating moderate sensitivity.

The results indicate that the concentrated extract of strain 431 had the strongest antibacterial activity, followed by the extracted supernatant, while the original culture fluid showed no antibacterial effect. These results are illustrated in **Figure 5**.

Table 2. Results of the drug sensitivity test of *Brevibacterium aureum* strain 431 (diameter of antibacterial circle/ mm)

Drugs	***Brevibacterium aureum* 431**	***Escherichia coli* K12D31**
Amoxicillin	20	10
Amikacin	15	20
Gentamicin	20	18
Enrofloxacin	22	28
Ciprofloxacin	26	28
Florfenicol	26	27
Neomycin	15	13
Ceftriaxone	21	26
Doxycycline	16	26
Trimethoprim-sulfamethoxazole	15	28

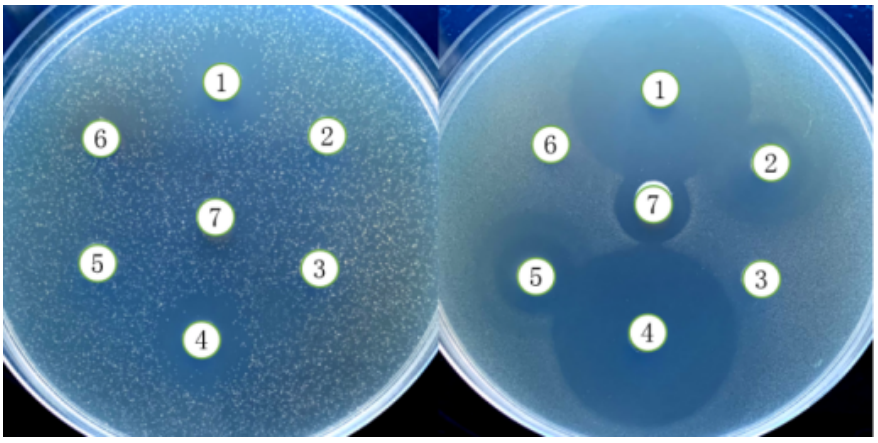

Figure 5. Antibacterial activity test of *Brevibacterium aureum* strain 431. Left: Escherichia coli (ATCC29213); Right: *Staphylococcus aureus* (K12D31); Note: 1 and 4 are concentrate C, 2 and 5 are extract B, 3 and 6 are raw solution A, 7 is a blank control (medium for left, neomycin for right)

3.7. Safety test

BALB/c mouse had good mental status, a normal diet, normal feces, and no morbidity and death during the feeding test. The results showed that oral administration of *Brevibacterium aureum* 431 was safe and not pathogenic for mice; there was no significant difference in weight gain between the experimental and control groups.

4. Discussion

4.1. Actinomyces

Marine bacteria and fungi are the "reservoirs" for the development of new sources of bioactive products, with the potential of a large number of secondary metabolites in ecological restoration, biomedicine and drug development. Marine *Actinomycetes* is one of the microbial provenances [8,10], which is an important producer of natural products from marine sources. The natural products of microorganisms are potential bioactive sources and have been widely used by pharmaceutical companies to develop a new generation of anti-infective drugs. Marine microorganisms have received attention due to their complex biosynthetic pathways and their potential implications for the development of anticancer and anti-infective agents in response to multidrug-resistant strains [11]. Natural products have become one of the important sources of many clinical drugs and new drug lead compounds because of their novel structure and unique active role.

The genus *Brevibacterium*, classified under the family Brevibacteriaceae and the class Actinobacteria, was first proposed by Breed in 1953, with *Brevibacterium linens* as the type species [12]. The description of this genus was later confirmed by Collins *et al.* in 1980 [13]. Since then, many species belonging to the genus *Brevibacterium* have been isolated and studied [14]. Currently, there are 36 published species of *Brevibacterium*, which have been isolated from various habitats [15-19], including rice [15], human skin [16], and deep-sea sediments [13]. Members of this genus have also been reported to be degraders of organic pollutants such as phenol and 4-chlorophenol [14].

Brevibacterium aureum is classified under Actinobacteria, and most strains of *Brevibacterium aureum* abroad have been isolated and studied from marine environments [13,16]. In China, a new strain of *Brevibacterium aureum* (DG-12) was isolated from activated sludge by a research team at South China Agricultural University in 2013 [20]. This strain can degrade and utilize cypermethrin as a growth substrate in mineral media. Ibrahim *et al.* [10] isolated 76 strains from soil, one of which was *Brevibacterium aureum*. Their team studied marine

strains of *Brevibacterium aureum* and its main component, naphthoic acid (also known as transparent acid), a derivative of 1-phenylcarboxylic acid, which showed anti-inflammatory activity by inducing pro-inflammatory cytokines in lipopolysaccharide-stimulated macrophages. The *Brevibacterium aureum* 431 strain was accidentally isolated from probiotic fermentation products. From the perspective of isolation sources, international research suggests that the biodiversity of bioactive compounds in *Brevibacterium aureum* is a result of stress from marine environments and biological symbiosis. Regarding the strains of *Brevibacterium aureum*, there is both an element of chance in isolation and an inevitability in evolution.

4.2. Antibacterial activity

Biosurfactants are secreted by microorganisms such as bacteria, yeasts, and fungi [21]. These biosurfactants produced by microorganisms can either be extracellular or cell-associated, linked to the cell membrane [22]. Bacteria are the primary group that produces biosurfactants, with different types of bacteria, including *Pseudomonas*, *Acinetobacter*, *Bacillus*, and *Rhodococcus*, being the most studied in scientific research [23]. Due to their low or non-toxicity, high biodegradability, ecological acceptability, high foaming capacity, and stability in extreme environments, microbial biosurfactants have vast potential applications in industries such as petroleum, cosmetics, specialty chemicals, food, agriculture, and pharmaceuticals [22]. Another potential application of biosurfactants is in biological control, as they possess antibacterial properties that can help remove residual oil from coastlines, prevent marine pollution, and effectively protect marine ecosystems [24].

Given the impact of probiotics on human health, there has been increasing attention in recent years on using probiotics as non-pathogenic and safe microorganisms in biosurfactant production. Most biosurfactants derived from probiotics have been reported to be associated with various lactic acid bacteria. These types of biosurfactants can be categorized into protein-carbohydrate complexes, lipids, or fatty acids [23]. Antibiotic resistance is an urgent public health issue, and the antibacterial activity of biosurfactants driven by probiotics presents an innovative approach that can be applied in various industries, including food and medicine [25]. The antibacterial activity of probiotic biosurfactants has been documented, with Rodrigues *et al.* [21] studying two probiotic lactic acid bacteria (*Lactobacillus* and *Streptococcus thermophilus* A) for their antibacterial activity against multiple bacterial and yeast strains. The results indicated that these biosurfactants exhibit high antibacterial activity against *Clostridium tropicalis* (GB9/9), even at low concentrations.

Brevibacterium aureum is known for producing secondary metabolites, primarily biosurfactants, which explains their widespread application in environmental settings. In 2010, Seghal *et al.* [26] detected biosurfactant production using droplet collapse and oil displacement tests, measuring lipase activity on triphenylmethane agar plates. They isolated marine actinomycetes from marine sponges, identifying 57 purified strains, with MSA04 and MSA13 recognized as new sources of glycolipid and lipopeptide biosurfactants. The isolate MSA13 was identified as *Brevibacterium aureum* MSA13. This study first confirmed the new lipopeptide biosurfactant produced by the strain *Brevibacterium aureum* MSA13, named “brevifactin.” This research guides us in studying the antibacterial properties of *Brevibacterium aureum* 431, which produces biosurfactants. The results show that the concentrated extracts and supernatants from *Brevibacterium aureum* 431 have inhibitory effects on the indicator bacterium, the Gram-negative *Escherichia coli*. This antibacterial property is closely related to the biosurfactant-producing nature of the 431 strain. Further research is needed to determine whether the antibacterial substances produced by *Brevibacterium aureum* 431 are lipopeptides or other antimicrobial compounds. The secondary metabolites of *Brevibacterium aureum* 431 exhibit antibacterial activity, indicating

that this strain is worth studying for bioremediation and biovalorization, as its secondary metabolites hold potential significant value and meaning for the development of antimicrobial agents.

4.3. Safety

Currently, there are no reported studies regarding the pathogenicity or conditional pathogenicity of *Brevibacterium aureum*. In terms of biosafety, the antimicrobial susceptibility testing of *Brevibacterium aureum* 431 indicates that this strain is sensitive to commonly used antibacterial agents, indirectly suggesting the absence of related drug resistance genes or drug resistance itself. Safety experiments conducted by feeding mice demonstrated that *Brevibacterium aureum* 431 is safe for consumption and shows no pathogenicity, making it a promising candidate strain of actinomycetes for further research and application as an animal probiotic.

5. Conclusion

Brevibacterium aureum 431 exhibits excellent antibacterial activity and is not resistant to conventional drugs, confirming its safety and reliability. This provides a substantial basis for the development of this actinomycete strain as a candidate for animal probiotics, establishing both practical and theoretical foundations for its application in this area.

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Disclosure statement

The authors declare no conflict of interest.

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