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Comparison of Antibacterial Activities of Six *Bacillus amyloliquefaciens* Strains

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Abstract: In this study, six bacterial strains were isolated from the sediment, probiotic fermentation products, and lake sediments, they were identified as *Bacillus amyloliquefaciens* using genetic evolution analysis, which were named B3, B4, B5, XD3, YF6, and YF8. The comparison of the antibacterial activity, hemolytic activity, and antibiotic sensitivity of six *Bacillus amyloliquefaciens* strains laid a foundation for the development and application of antimicrobial peptide products. A surface activity assay was used to determine the production of biosurfactants in six *Bacillus amyloliquefaciens* strains. With *Staphylococcus aureus* and *Escherichia coli* as indicator bacteria, their antibacterial activity was determined using the agar diffusion method; the same diffusion method was used to determine the antibiotic susceptibility of *Bacillus amyloliquefaciens*. The results showed that the six *Bacillus amyloliquefaciens* strains had obvious biosurface activity, and the bacteria inhibited *Staphylococcus aureus* and *Escherichia coli*, from strong to weak: YF8, XD3, B3, B4, YF6, and B5. Strain YF8 had the best broad-spectrum bacteriostatic effect, followed by strain XD3. All *Bacillus amyloliquefaciens* strains were susceptible to 16 common drugs, except for *Bacillus amyloliquefaciens* strain YF8, which was intermediate to neomycin. The study shows that *Bacillus amyloliquefaciens* and secondary metabolites have the ability to produce a variety of active peptides, exert a certain inhibitory effect on common pathogens, and have the potential to develop as animal probiotics.

Keywords: *Bacillus amyloliquefaciens*; Identification; Biosurface activity; Antibacterial activity; Drug sensitivity test

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1. Introduction

Bacillus amyloliquefaciens is widely distributed in nature and has no toxic effect on humans and animals. It is a microbial that is beneficial to the host [1]. *Bacillus amyloliquefaciens* is a Gram-positive, aerobic bacterium with an internal, oval-shaped spore that is typically located at the middle or at the ends of the cell. It features

flagella that encircle the cell, providing motility. *Bacillus amyloliquefaciens* is a recently identified species of *Bacillus* that serves as a novel source of microbial agents [2]. It exhibits antagonistic activity against pathogens, clinical multidrug-resistant strains, and plant pathogenic fungi [3]. Its applications extend beyond food and feed to include medicine, agriculture, environmental management, industry, and bioengineering [4], garnering significant attention for its diverse utility. During its growth, *Bacillus amyloliquefaciens* produces secondary metabolites such as surfactin, various enzymes, and extracellular polysaccharides. These compounds can enhance crop growth [5] or disrupt the cellular structures of pathogens and produce volatile substances [6] that inhibit the growth and development of pathogenic bacteria. Given these biological characteristics, *Bacillus amyloliquefaciens* shows promise as a candidate for screening and development as probiotic additives in animal feed.

In this study, the six strains of *Bacillus amyloliquefaciens* were selected from the natural environment and probiotics fermentation products to compare the biosurface activity characteristics, the antibacterial activity, hemolytic properties, and antibiotic susceptibility test of each strain, in order to provide a reference for the development of *Bacillus amyloliquefaciens* as animal probiotic species.

2. Materials and methods

2.1. Materials

2.1.1. Strain of origin

All six *Bacillus amyloliquefaciens* (*B. amyloliquefaciens*) strains were isolated from the natural environment and probiotic products, as shown in **Table 1**.

Table 1. Source of strains

Strain names	Sources
B. amyloliquefaciens B3	Turtle pool bottom mud
B. amyloliquefaciens YF6	Probiotic bacterial fermentation products
B. amyloliquefaciens YF8	Probiotic bacterial fermentation products
B. amyloliquefaciens B5	Fish pond sediment
B. amyloliquefaciens XD3	Xianxi Lake bottom mud
B. amyloliquefaciens B4	Turtle pool bottom mud

2.1.2. Bacterial indicator

Escherichia coli (K12D31) and *Staphylococcus aureus* (ATCC 29213) were purchased from China MicCollection Management Center.

2.1.3. Culture medium and reagents

Blood agar and nutrient agar media: Guangdong Huankai Microbiology Technology Co., Ltd.; Drug susceptibility test paper: Thermo Fisher Technology Co., Ltd. (China); 20% Tween-80 (chemical purity): Guangdong Canton Test Reagent Technology Co., Ltd.; Glucose (analytical purity): Shanghai McBiochemical Technology Co., Ltd.; Yeast powder (biochemical reagent): Beijing Hongrun Baoshun Technology Co., Ltd.; Beef paste (biochemical reagent), protein (biochemical reagent): Beijing Aboxing Biotechnology Co., Ltd.

2.2. Methods

2.2.1. Preparation of the culture medium

(1) Luria–Bertani (LB) broth (g/L): 10 g of glucose, 5 g of yeast powder, 10 g of NaCl, and 10 g of protein were weighed and dissolved in 1L of distilled water, adjusted pH to 7.0 and stored at 4℃ after sterilization. LB solid medium was made by adding 17 g of nutrient agar medium to 1 liter of LB liquid medium.

(2) De Man–Rogosa–Sharpe liquid medium (g/L): 10 g of protein, 5 g of beef paste powder, 4 g of yeast powder, 20 g of glucose, 2 g of K_2HPO_4, 1 mL of Tween-80, 5 g of sodium acetate, 2 g of triammonium citrate, 0.2 g of $MgSO_4.7H_2O$, 0.05 g of $MnSO_4.4H_2O$, 17 g of agar were weighed and dissolved in 1L of distilled water, adjusted pH to 6.2 and stored at 4℃ after sterilization.

2.2.2. Strain reactivation

Six strains of *Bacillus amyloliquefaciens* were inoculated into LB solid medium and incubated at 37℃ for 24 hours. Typical single colonies were picked, inoculated in LB liquid medium, and incubated in a 37℃ 120 rpm/min shaker, stored for 24 hours at 4℃ and set aside.

2.2.3. Activation of the indicator bacteria

Staphylococcus aureus (ATCC 29213) and *Escherichia coli* (K12D31) were inoculated in LB liquid medium and incubated at 37℃ 120 rpm/min for activation. After activation, the bacterial solution was streaked on LB solid medium and cultured at 37℃ for 24 hours. A typical single colony was picked and inoculated in LB medium, cultured at 37℃ 120 rpm/min for 16 to 24 hours, and the bacterial concentration was adjusted to an optical density of 600 nm of 1.0 and stored at 4℃.

2.2.4. Analysis of genetic evolution

The cultures of six *Bacillus amyloliquefaciens* strains were subjected to sequencing analysis by Guangzhou Ai Gene Biotechnology Co., Ltd. to obtain the gene sequence of the strains. In the similarity analysis of the sequencing results on NCBI, the sequence with the highest homology was selected as the reference object, and the phylogenetic tree of each strain was constructed by using the MEGA 7.0 software.

2.2.5. Biosurface activity detection

(1) Emulsification activity (EA): A 2.5 mL liquid culture of sterile bacteria was incubated for 24 hours, followed by centrifugation at 10,000 rpm/min to remove the supernatant. An equal volume of petroleum ether was then added to the supernatant and mixed for 2 minutes. After allowing the mixture to stand for 1 minute, the height of the emulsion layer was measured. The emulsification capacity was calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying by 100.

(2) Emulsification index (E24): After detecting the emulsification activity, the tube was stored at 4°C for 24 hours. The emulsification index was calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying the result by 100.

2.2.6. Antibacterial activity

Using the agar diffusion method, the indicator bacteria *Escherichia coli* (K12D31) and *Staphylococcus aureus*

(ATCC 29213) were added to the respective LB agar medium. After the LB agar medium solidified, wells were made on top. The cultures of six *Bacillus amyloliquefaciens* strains were added to the wells (60 μL each). The diameter of the inhibition zone was measured after 18 to 24 hours. *Escherichia coli* indicator plates had neomycin as blank control and *Staphylococcus aureus* indicator plates had medium as blank control.

2.2.7. Hemolytic activity

Using the filter paper inoculation method (with 20% Tween 80 as a positive control), 10 μL of culture was applied to each filter paper disc and incubated at 37°C for 24 hours. The hemolytic halo around the filter paper was then observed to assess the production intensity of antimicrobial peptides.

2.2.8. Drug susceptibility test

According to the requirements and standards of drug susceptibility testing issued by NCCLS, the antibiotic susceptibility of the strains was determined by the agar diffusion method (Kirby–Bauer) [7]. Six strains of *Bacillus amyloliquefaciens* were inoculated on LB agar medium using the lawn culture method. Drugs including amikacin, penicillin, deoxytetracycline, enrofloxacin, sulfamethoxazole/methyl pyrimidine, gentamicin, ciprofloxacin, florfenicol, neomycin, spectinomycin, cefotaxime, ofloxacin, cefradine, and other antibiotic discs were put on the plate, the diameter of the inhibition zone was determined after incubation at 37℃ for 24 hours.

3. Results

3.1. Phylogenetic tree analysis

A phylogenetic tree of six *Bacillus amyloliquefaciens* strains was established by sequencing the 16S rDNA gene fragment and comparing sequence homology with the GenBank database. The analysis of genetic evolutionary relationships showed that *Bacillus amyloliquefaciens* YF6, XD3, YF8, and B4 are 66% identical; *Bacillus amyloliquefaciens* B3 and B5 share 59% similarity, the results are shown in **Figure 1**.

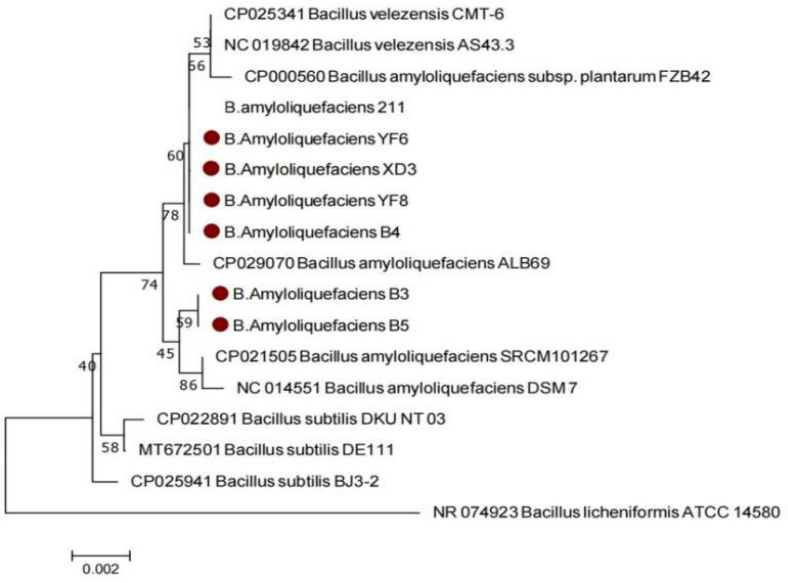

Figure 1. A phylogenetic tree analysis of *Bacillus amyloliquefaciens* strains

3.2. Biosurface activity detection

According to the biosurface activity detection results, the emulsification activity of *Bacillus amyloliquefaciens* was 65% for B3, 70% for YF6, 68% for YF8, 68% for B5, 58% for XD3, and 63% for B4, as shown in **Figure 2**. The emulsification indexes of *Bacillus amyloliquefaciens* were 63% for B3, 68% for YF6, 63% for YF8, 63% for B5, 55% for XD3, and 60% for B4, as presented in **Figure 3**.

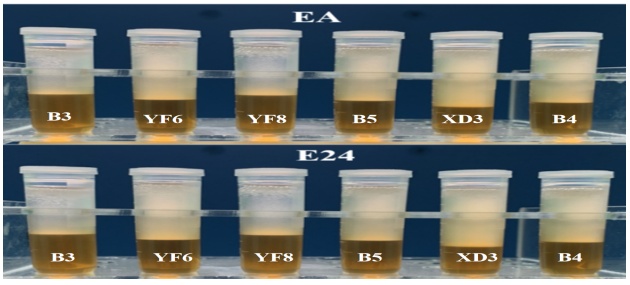

Figure 2. Results of the emulsification activity (EA)

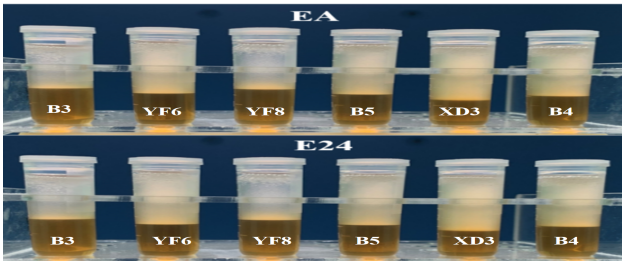

Figure 3. Results of the emulsification index (E24)

3.3. Antibacterial activity

Using agar diffusion method, the results showed that the six strains of *Bacillus amyloliquefaciens* had obvious biosurface activity, and their bacterial solution inhibited the indicator bacteria *Staphylococcus aureus* and *Escherichia coli* to varying degrees, and positively correlated with the intensity of biosurface activity. The results are shown in **Figure 4**.

According to **Table 2**, the antibacterial activities of strains XD3, YF8, B4, and B3 against *Staphylococcus aureus* were judged as "+++," and the diameter of the inhibition zone was 20 mm, 18 mm, 18 mm, and 15 mm respectively. The antibacterial activities of strains YF6 and B5 against *Staphylococcus aureus* were determined as "++." The antibacterial activity of strain YF8 against *Escherichia coli* was determined as "++++," and the diameter of the inhibition zone was 21 mm. The antibacterial activity of strains B3, XD3, and YF 6 against *Escherichia coli* was determined as "+++," and the diameter of the inhibition zone was 20 mm, 19 mm, and 15 mm, respectively. The strain YF8 showed the best broad-spectrum antibacterial effect, followed by strain XD3, and the results are shown in **Table 2**. In short, the six strains have obvious antibacterial activity, from strong to weak: YF8, XD3, B3, B4, YF6, and B5.

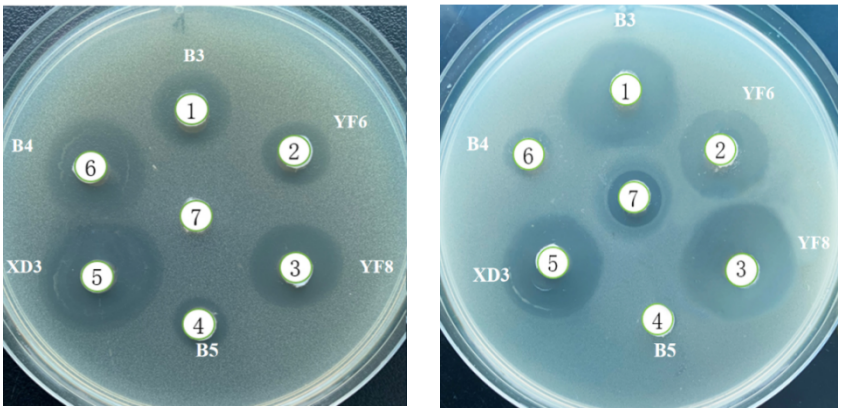

Figure 4. The antibacterial activity of *Bacillus amyloliquefaciens*. Number 7 is a blank control: medium for *Staphylococcus aureus* (ATCC 29213) (left); neomycin for *Escherichia coli* (K12D31) (right).

Table 2. Comparison of six *Bacillus amyloliquefaciens* strains

Names of strains	*Staphylococcus aureus*		*Escherichia coli*	
	Diameter (mm)	Strength	Diameter (mm)	Strength
B. amyloliquefaciens YF6	12	++	15	+++
B. amyloliquefaciens XD3	20	+++	19	+++
B. amyloliquefaciens B4	18	+++	10	++
B. amyloliquefaciens B5	10	++	8	+
B. amyloliquefaciens B3	15	+++	20	+++
B. amyloliquefaciens YF8	18	+++	21	++++

Note: The diameter of the inhibition zone of 0 mm was judged as "-"; below 10 mm, judged as "+"; between 10–14 mm, judged as "++"; between 15–20 mm, judged as "+++"; above 20 mm, judged as "++++."

3.4. Drug susceptibility test

The antibiotic sensitivity of the six *Bacillus amyloliquefaciens* strains was determined by the agar diffusion method. According to the drug susceptibility test results, the diameter of the inhibition zone of *Bacillus amyloliquefaciens* YF8 on neomycin was 14 mm, which is intermediate, and the inhibition zone of the other strains was highly sensitive. The results are shown in **Table 3**.

3.5. Hemolytic activity

Based on **Figure 5**, the six *Bacillus amyloliquefaciens* strains could secrete surfactin. The surrounding area presents different degrees of β-hemolysis ring, and the surrounding boundary is clear and completely transparent. The YF8 strain also formed a larger diameter α-hemolytic ring, suggesting that strains B3, YF6, YF8, B5, XD3, and B4 had biosurface activity produced by antimicrobial peptides.

Table 3. Drug susceptibility test results (diameter in mm)

Drug classes	Drugs	XD3		B3		B4		B5		F6		YF8	
		Diameter	Strength	Diameter	Strength	Diameter	Strength	Diameter	Strength	Diameter	Strength	Diameter	Strength
β-endoamide	Penicillin	26	++++	25	++++	28	++++	28	++++	25	++++	26	++++
	Amoxicillin	22	++++	23	++++	22	++++	24	++++	16	+++	22	++++
	Cefradine	24	++++	26	++++	26	++++	28	++++	24	++++	18	+++
	Ceftriaxone	24	++++	24	++++	26	++++	23	++++	24	++++	26	++++
	Cefotaxime	24	++++	26	++++	27	++++	25	++++	22	++++	30	++++
Macrolides	Tilmicosin	23	++++	17	+++	18	+++	18	+++	18	+++	19	+++
Aminoglycoside	Amikacin	23	++++	22	++++	21	++++	22	++++	22	++++	22	++++
	Gentamicin	22	++++	22	++++	21	++++	23	++++	24	++++	19	+++
	Neomycin	16	+++	15	+++	16	+++	18	+++	16	+++	14	++
	Spectinomycin	19	+++	18	+++	18	+++	18	+++	20	++++	18	+++
Acetamine alcohols	Florfenicol	20	+++	24	++++	26	++++	25	++++	24	++++	28	++++
Tetracyclines	Deoxytetracycline	20	+++	24	++++	22	++++	23	++++	18	+++	30	++++
Sulfanilamide	Sulfamethoxazole/ Methylpyrimidine	20	+++	24	++++	26	++++	24	++++	19	+++	24	++++
Fluoroquinolones	Ciprofloxacin	27	++++	28	++++	30	++++	27	++++	26	++++	28	++++
	Enrofloxacin	23	++++	28	++++	27	++++	28	++++	28	++++	30	++++
	Ofloxacin	27	++++	26	++++	26	++++	25	++++	26	++++	24	++++

Note: The diameter of the inhibition zone of 0 mm was judged as "-"; below 10 mm, judged as "+"; between 10–14 mm, judged as "++"; between 15–20 mm, judged as "+++"; above 20 mm, judged as "++++."

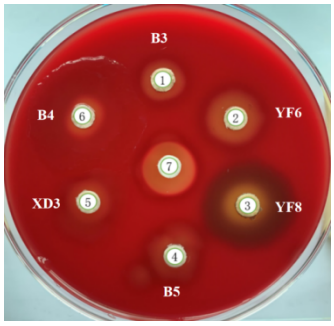

Figure 5. Results of hemolysis test (number 7 is positive control)

4. Discussion

As a green feed additive, microecosystems have effectively addressed many of the negative effects associated with antibiotics, making them viable alternatives. This has great significance for the development and application of microecosystems [8]. Commonly used microecological preparations both domestically and internationally include *Bacillus*, *Actinobacteria*, *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, photosynthetic bacteria, and yeast. The primary types of microecosystems consist of probiotics, prebiotics, and symbiotics. The study of dietary probiotics and the development of bacterial strains are key factors, with *Bacillus* accounting for approximately 70% of probiotics [9]. Domestic research on antibacterial substances mainly focuses on the screening of target strains, as well as the isolation, purification, identification, characterization, and action mechanism of antibacterial components. Although there are many antibacterial substances produced by *Bacillus*, the yield of a single antibacterial component is low and the purification process is complex, which are difficult to meet the growing demand for antibacterial substances in agriculture, industry, food, and other industries. Searching for improving the yield and use efficiency of antimicrobial substances is an important research direction [10].

4.1. Antibacterial study

Bacillus sp. is a class of bacteria widely found in natural environments and organisms, which can synthesize lipopeptide antibacterial activity [11] under the action of a non-ribosomal peptide synthase line. In the genus *Bacillus*, *Bacillus amyloliquefaciens* is the lipopeptide-producing bacterium next to *Bacillus subtilis* [12,13]. A number of genomic studies have shown that *Bacillus amyloliquefaciens* contains the gene cluster encoding various lipopeptide substances such as iturin, fengycin, and surfactin [14,15]. Bacillomycin D belongs to the iturin family, and has a strong inhibitory effect on conidia as well as antifungal activity [16]. Lv *et al*. found that after electron microscopic scanning of *Clostridium difficile* ATCC 9689, the lipopeptides (surfactin, fengycin, iturin, etc.) produced by *Bacillus amyloliquefaciens* C-1 were damaged, the exudate surrounded the bacteria, the cell wall and cell membrane were interrupted and blurred, while the untreated cells were smooth and uninterrupted. Furthermore, with increasing lipopeptide concentrations, the bacteria are surrounded by exudates, which may be the cytoplasm extruded from the cell [17].

Wang *et al.* found that *Bacillus amyloliquefaciens* DH8030, at four times the minimum inhibitory concentration of amylocyclicin W5, can act on the cell wall of *Bacillus cereus* LMGT2805. This action disrupts the structure, forms holes, and causes leakage of cellular contents. As a result, the bacteria cannot carry out

normal metabolism during their growth or reproductive stages, leading to complete inhibition of growth and eventual cell death [18].

4.2. Antimicrobial peptide

The antimicrobial peptide has antibacterial, antiviral, and anti-tumor cells, and can improve animal immunity, showing good results in replacing antibiotics [19]. Antimicrobial peptides are widely found in nature and are an important part of the host's innate immune system against pathogens, including antibacterial, antifungal, antiviral, anti-parasitic, immune regulation, and neutralizing endotoxin [20,21]. Antimicrobial peptides, also known as host defense peptides, are small molecule polypeptides encoded by host genes. They are mostly cationic and amphiphilic, and their secondary structures include α-helix, β-folding, irregular coil, etc. Antimicrobial peptides such as iturin, surfactin, and fengycin, with antifungal properties mainly based on their ability to disrupt the cell wall [22], are currently of great interest.

Microbial-derived antimicrobial peptides are mainly bacteriocins or peptide antibiotics derived from bacteria [23]. Antimicrobial peptides kill pathogens by disrupting cell membrane structure or inhibiting viral assembly [24]. Antibacterial peptides can inhibit both Gram-positive and Gram-negative bacteria. As the microbial membrane structure is highly conserved, all are composed of phospholipids, phosphatidylglycerol, cardiolipins, and phosphatidylethanolamine [25], the biological activity of antimicrobial peptides is based on the interaction with the pathogen cell membrane [26]. The cationic residues in the antimicrobial peptides bind to the negatively charged bacterial membrane surface, the hydrophilic group is inserted into the lipid molecule, and the hydrophobic group points to the outer membrane environment, forming an ion channel, leading to the rupture of the cell membrane or the leakage of endoplasmic substances, thus killing the bacteria [27].

Bacillus amyloliquefaciens is classified under the FDA's GRAS (Generally Recognized as Safe) category [28]. As a novel source of microbial agents [2], it can produce antibacterial proteins, lipopeptides, and other active substances through its metabolic processes, which are effective in inhibiting pathogens, fungi, viruses, and more. Its antibacterial agents or extracts possess several advantages, including being non-toxic, harmless, residue-free, and providing long-lasting antibacterial effects [29]. Therefore, *Bacillus amyloliquefaciens* and its lipopeptide metabolites show great potential for use in the biological control of crops, fruit and vegetable preservation, and post-harvest microbial control, making them highly promising for future development and application [30]. In this study, six *Bacillus amyloliquefaciens* strains obtained from natural environments and probiotic fermentation products all demonstrated the ability to produce biosurfactants. The biosurface activity of these strains, ranked from strongest to weakest, was: YF8, XD3, B3, B4, YF6, and B5. Analysis of the results indicated that strain YF8 exhibited the best broad-spectrum antibacterial effect, followed by strain XD3. These findings suggest that strains YF8 and XD3 have potential as probiotic animal feed additives. Further research on the antibacterial and antifungal properties of the six *Bacillus amyloliquefaciens* strains is necessary, providing valuable reference material for the development of their secondary metabolites as lipopeptide additives or therapeutic agents.

4.3. Drug resistance

In order to avoid the transmission of drug resistance in strains derived from probiotics and the poor induction of endogenous bacteria, probiotic strains should not be drug-resistant. One of the test methods for drug resistance of probiotics is to analyze the resistance to broad-spectrum antibiotics. Therefore, the disc diffusion method was

used to study the resistance of six *Bacillus amyloliquefaciens* strains to 16 antimicrobial drugs, and found that none of them was drug-resistant, so it met the requirements of probiotics.

5. Conclusion

In this study, the biosurfactant properties produced by six *Bacillus amyloliquefaciens* strains were compared and their bacteriostatic activity was analyzed. The results showed that the solution of each strain had a significant antibacterial effect on *Staphylococcus aureus* and *Escherichia coli*, and was positively correlated with the activity intensity of biosurfactant. The results lay the foundation for the selection of *Bacillus amyloliquefaciens* as a candidate probiotic strain.

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Disclosure statement

The authors declare no conflict of interest.

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