Postprint: High-Performance Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry Analysis of Lipopeptide Antimicrobial Components from Probiotic Bacillus

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Abstract

This study aimed to elucidate the antimicrobial effects of a Bacillus subtilis N2-10 strain with probiotic potential against intestinal bacteria and its active components, thereby laying a foundation for in-depth evaluation of the probiotic function and mechanism of this strain. Using common intestinal pathogenic bacteria and beneficial bacteria as indicator strains, the antimicrobial activity of fermentation broth components was determined by the agar diffusion method, and the active components were analyzed by High Performance Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry (HPLC-ESI-MS/MS). The results showed that chloroform and n-butanol extracts of Bacillus subtilis N2-10 fermentation broth had no significant inhibitory effect on the tested bacteria; lipopeptides exhibited significant antimicrobial activity against intestinal pathogens such as Escherichia coli, Shigella, and Salmonella, while showing very weak inhibition against the beneficial intestinal bacterium Lactobacillus bulgaricus and no inhibition against Streptococcus thermophilus. HPLC-ESI-MS/MS analysis of the crude lipopeptide extract revealed that the lipopeptide antibiotics were homologs of C14-C17 Mycosubtilin from the iturin family. Therefore, it is inferred that the inhibitory effect of Bacillus subtilis N2-10 on intestinal pathogens depends on its production of the lipopeptide antibiotic Mycosubtilin.

Full Text

High-Performance Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry Analysis of Lipopeptide Antibacterial Components from Probiotic Bacillus

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Abstract

This study aimed to investigate the antibacterial effects and active constituents of *Bacillus subtilis* strain N2-10, a strain with probiotic potential, against intestinal bacteria, thereby establishing a foundation for comprehensive evaluation of this strain' s probiotic functions and mechanisms. Using common intestinal pathogens and beneficial bacteria as indicator strains, the antibacterial activity of fermentation broth components was determined via the filter paper disc diffusion method, and the active constituents were analyzed by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS). The results demonstrated that chloroform and n-butanol extracts of the fermentation broth exhibited no significant inhibitory effects on the tested strains. In contrast, the crude lipopeptide extract showed pronounced antibacterial activity against intestinal pathogens such as *Escherichia coli*, *Shigella flexneri*, and *Salmonella enterica*, while displaying only weak inhibition against the beneficial *Lactobacillus bulgaricus* and no inhibition against *Streptococcus thermophilus*. HPLC-ESI-MS/MS analysis of the crude lipopeptide extract revealed that the lipopeptide antibiotics belonged to the iturin family as C14~C17 mycosubtilin homologues. Therefore, it is concluded that the inhibitory effect of *B. subtilis* N2-10 on intestinal pathogens depends on its production of the lipopeptide antibiotic mycosubtilin.

Keywords: *Bacillus subtilis*; lipopeptides; antibacterial activity; HPLC-ESI-MS/MS; mycosubtilin

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Introduction

Bacillus species represent an important probiotic resource with multiple functions including inhibition of intestinal pathogens, regulation of intestinal flora balance, prevention and treatment of gastrointestinal diseases, and enhancement of host immunity, playing significant roles in food, pharmaceutical, and feed additive applications [1-2]. The antimicrobial substances produced during *Bacillus* growth and metabolism constitute the essential material basis for their probiotic effects [3]. Therefore, elucidating the antibacterial activity and components of

a strain represents a crucial aspect of investigating its probiotic mechanisms. Since Johnson et al. [4] first reported in 1945 that *Bacillus subtilis* produces antibacterial substances, numerous antimicrobial components have been isolated from this species, demonstrating broad-spectrum inhibition against pathogenic microorganisms [5-7]. Non-ribosomally synthesized lipopeptide antimicrobial substances are among the most common types. These compounds generally refer to cyclic peptides composed of a β-hydroxy fatty acid linked via amide bonds to 7-10 amino acids [8], primarily including three families: surfactin, iturin, and fengycin [9-12]. Different *Bacillus* lipopeptides exhibit varying antimicrobial properties: iturin primarily inhibits fungi and some bacteria [13], surfactin demonstrates significant inhibitory effects against diverse bacteria and fungi including Gram-positive bacteria, Gram-negative bacteria, and molds, as well as notable activity against viruses, mycoplasmas, and protozoa [14], while fengycin mainly exhibits strong inhibition against filamentous fungi [15].

To date, research on the antimicrobial spectrum and applications of *Bacillus* lipopeptides has primarily focused on fungi, particularly plant pathogenic fungi, with relatively in-depth investigations into their antimicrobial mechanisms [16]. In contrast, studies on their antibacterial effects remain limited. Reported *Bacillus* lipopeptides with antibacterial activity have been largely restricted to bacillomycin L (iturin family) [17] and surfactin [18], with few reports on other *Bacillus* lipopeptides exhibiting antibacterial activity. Moreover, research on the antimicrobial spectra of bacillomycin L and surfactin has primarily emphasized pathogens such as *Staphylococcus aureus*, *Salmonella enterica*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Escherichia coli*, while neglecting their effects on beneficial bacteria, thereby precluding objective evaluation of *Bacillus* effects on intestinal flora.

Strain N2-10 is a *B. subtilis* isolate obtained from fresh Simmental cattle feces by our research group. Previous studies have confirmed that this strain possesses excellent probiotic potential, including tolerance to artificial gastric and intestinal fluids and bile salts, certain inhibitory effects against *E. coli*, and production of digestive enzymes such as protease and amylase. To comprehensively and objectively evaluate its probiotic functions, this study selected both intestinal pathogens and beneficial bacteria as indicator strains to determine the antibacterial activity of fermentation broth components from *B. subtilis* N2-10. Furthermore, HPLC-ESI-MS/MS was employed to analyze and identify the antibacterial constituents. The research aims to elucidate the antibacterial components of *B. subtilis* N2-10, providing a basis for investigating this strain' s probiotic functions and mechanisms.

Materials and Methods

1.1 Strains and Culture Media

Bacillus subtilis strain N2-10 was isolated and preserved by our research group. The tested pathogenic bacteria included *Escherichia coli* CICC-10004, *Shigella flexneri* CICC21678, *Salmonella enterica* CICC21490, *Staphylococcus aureus* CICC10306, *Streptococcus thermophilus* CICC20174, and *Lactobacillus bulgaricus* CICC20247, all maintained at the Pharmaceutical Engineering Laboratory, College of Life Sciences, Hebei Agricultural University.

Nutrient Broth (NB) medium (g/L): peptone 10 g, beef extract 5 g, sodium chloride 5 g, used for bacterial cultivation.

Fermentation medium (g/L): glucose 10 g, corn flour 13 g, soybean flour 13 g.

1.2 Main Instruments

Agilent LC-MSD-Trap-XCT ion trap liquid chromatography-mass spectrometer (Agilent, USA), Agilent C18 reverse-phase column (1.8 m, 2.1 mm$×$100 mm) (Agilent, USA), Adventurer electronic balance (Ohaus, USA), MLS-3020 autoclave (SANYO, Japan), SW-CJ-2FD clean bench (Suzhou Taian Air Technology Co., Ltd.), GL-21M high-speed refrigerated centrifuge (Shanghai Luxiangyi Centrifuge Instrument Co., Ltd.), RE-52AA rotary evaporator (Henan Yuhua Instrument Co., Ltd.), Nanopure ultrapure water system (Thermo Fisher Scientific, USA).

1.3 Fermentation Culture of Strain

The N2-10 strain was inoculated into NB medium and cultured at 37°C with shaking at 180 rpm for 16 h, then transferred to fermentation medium and cultured under the same conditions for 72 h to obtain fermentation broth.

1.4 Processing of Fermentation Broth

Two hundred milliliters of fermentation broth were centrifuged at 4°C and 5,000 rpm for 10 min. One hundred milliliters of the supernatant were sequentially extracted three times with three volumes of chloroform and n-butanol. The extracts were combined and concentrated. Another 100 mL of supernatant were placed in a sterile Erlenmeyer flask, adjusted to pH 2.0 with 6 mol/L concentrated hydrochloric acid, and stored overnight. The mixture was aliquoted into 50 mL centrifuge tubes and centrifuged at 10,000 rpm for 15 min, after which the supernatant was discarded. To the precipitate in each tube, 1.5 mL of neutral methanol was added, mixed, and extracted for 8 h, followed by centrifugation for 15 min. The precipitate was discarded, and the supernatant was retained and concentrated to obtain the crude lipopeptide extract. The chloroform extract, n-butanol extract, and crude lipopeptide extract were concentrated to dryness using a nitrogen evaporator. To each dried extract, 2 mL of ultrapure

water was added, vortexed to uniformity, and prepared as aqueous solutions of each extract.

1.5 Determination of in vitro Antibacterial Activity

Antibacterial activity was detected using the filter paper disc diffusion method. *E. coli*, *S. flexneri*, *S. enterica*, *S. thermophilus*, and *L. bulgaricus* were individually inoculated into NB medium and cultured at 37°C with shaking at 180 rpm for 18 h to prepare seed cultures.

In a clean bench, 4 mL of each seed culture was added to 100 mL of NB medium pre-warmed to approximately 45°C, mixed thoroughly, and poured into plates. After the pathogen plates solidified, sterile filter paper discs (8 mm diameter) saturated with aqueous solutions of each extract were placed on the plates at appropriate intervals, with three replicate plates per group. The plates were incubated at 35°C for 24 h, after which the presence and size of inhibition zones were observed and recorded.

1.6 Analysis of Antibacterial Components

One milliliter of the crude lipopeptide methanol extract was filtered through a 0.45 m membrane, and the filtrate was analyzed by HPLC-ESI-MS/MS. The HPLC-ESI-MS/MS system consisted of an Agilent LC-MSD-Trap-XCT ion trap mass spectrometer equipped with a C18 column (2.1 mm$×$100 mm, 3.5 m). Isocratic elution was performed using 0.1% formic acid aqueous solution:acetonitrile (60:40) at a flow rate of 0.8 mL/min. Detection wavelength was 230 nm, injection volume was 5 L. Electrospray ionization in positive ion mode was employed. The XCT ion trap mass detector was operated in the range of 100-1,700 u.

Results

2.1 Antibacterial Activity of Fermentation Broth Components

The antibacterial activities of different extracts obtained from *B. subtilis* N2-10 fermentation broth were evaluated using six test strains: *S. flexneri*, *E. coli*, *S. enterica*, *S. aureus*, *L. bulgaricus*, and *S. thermophilus* (Figure 1 [Figure 1: see original paper] and Table 1). The results indicated that chloroform and n-butanol extracts exhibited weak or negligible inhibitory effects on the tested strains, whereas the crude lipopeptide extract demonstrated pronounced antibacterial activity against pathogenic bacteria but weak or no activity against beneficial bacteria. Specifically, the chloroform extract showed weak inhibition only against *L. bulgaricus* and *S. flexneri*, with essentially no effect on other tested strains. The n-butanol extract displayed weak inhibition against *E. coli*, *S. flexneri*, and *S. aureus*, but no inhibitory effect on the remaining strains.

The crude lipopeptide extract exhibited significant inhibition against intestinal pathogens including *S. flexneri*, *E. coli*, *S. enterica*, and *S. aureus*, weak inhibition against the beneficial *L. bulgaricus*, and no inhibition against *S. thermophilus*. These results demonstrate that the primary antibacterial components produced by *B. subtilis* N2-10 are lipopeptides, which show marked inhibitory effects on intestinal pathogens while exerting minimal or no inhibitory effects on beneficial bacteria.

Figure 1 [Figure 1: see original paper]. Antibacterial activities of different fermentation supernatant extracts. A: *Escherichia coli*; B: *Salmonella enterica*; C: *Shigella flexneri*; D: *Staphylococcus aureus*; E: *Streptococcus thermophilus*; F: *Lactobacillus bulgaricus*; 1: Lipopeptide crude extract; 5: Chloroform extract; 6: n-butanol extract.

Table 1 . Inhibition zone diameter of different fermentation supernatant extracts (mm)

Strains	Chloroform extract	Lipopeptide crude extract	n-butanol extract
Escherichia coli	0.0$±0.0|21.5±0.27|9.1±0.12||∗ *Salmonellaenterica*∗ |0.0±0.0|24.6±0.18|10.1±0.24||∗ *Shigellaflexneri*∗ |9.1±0.21|23.7±0.46|9.1±0.36||∗ *Staphylococcusaureus*∗ |10.9±0.19|0.0±0.0|9.8±0.22||∗ *Streptococcusthermophilus*∗ |0.0±0.0|0.0±0.0|0.0±0.0||∗ *Lactobacillusbulgaricus*∗ |11.2±0.34|12.2±0.16|0.0±$0.0		

2.2 Analysis and Identification of Antibacterial Components

HPLC-ESI-MS/MS was employed to analyze the antibacterial components in the crude lipopeptide extract. The relative molecular mass information of target compounds was first obtained from the primary mass spectrometry (ESI-MS) chromatogram of HPLC-MS, followed by MS2 fragmentation analysis of each quasi-molecular ion to deduce compound structures based on mass spectrometry fragmentation patterns.

2.2.1 Primary Mass Spectrometry Results As shown in Figure 2 [Figure 2: see original paper], HPLC-ESI-MS analysis of the crude extract revealed four peaks in the total ion chromatogram (TIC) at retention times of 3.6, 4.4, 6.1, and 8.4 min, with mass-to-charge ratios (m/z) of 1,065.7, 1,079.7, 1,093.7, and 1,107.8, respectively. The molecular weights of these four compounds differed successively by 14 u, corresponding exactly to the length of fatty acid chains

(-CH2), and matched the [M+Na]+ ions of mycosubtilin in the iturin family. Therefore, these four compounds were preliminarily identified as C14~C17 mycosubtilin homologues.

2.2.2 Secondary Mass Spectrometry (ESI-MS2) Results Secondary mass spectrometry analysis was performed on the [M+H]+ ions corresponding to m/z 1,065.7, 1,079.7, 1,093.7, and 1,107.8. The resulting fragment ions are shown in Figure 3 [Figure 3: see original paper]. In the secondary mass spectrum of the compound with [M+H]+ at 1,043.8 u, two fragment ion peaks appeared at m/z 638.5 and 801.5. The secondary spectrum of [M+H]+ at 1,057.8 u showed three fragment ion peaks at m/z 652.6, 815.6, and 928.7. The secondary spectrum of [M+H]+ at 1,071.8 u displayed only one fragment ion peak at m/z 666.5. The secondary spectrum of [M+H]+ at 1,085.8 u exhibited two prominent fragment ion peaks at m/z 680.5 and 843.5. Among these fragment ion peaks, m/z 638.5, 652.6, 666.5, and 680.5 differed successively by 14 u, consistent with the b5 fragment ions in the b- and y-type characteristic fragments produced by CID fragmentation of mycosubtilin (Figure 4 [Figure 4: see original paper]). The fragment ion peaks at m/z 801.5, 815.6, and 843.5 corresponded to the b6 characteristic fragments of C14 mycosubtilin, C15 mycosubtilin, and C17 mycosubtilin, respectively. The m/z 928.7 fragment ion observed in the compound with [M+H]+ at 1,057.8 u was identified as the b7 characteristic fragment ion.

In summary, the lipopeptide components produced by *B. subtilis* N2-10 strain were identified as four homologues: C14 mycosubtilin, C15 mycosubtilin, C16 mycosubtilin, and C17 mycosubtilin.

Figure 2 [Figure 2: see original paper]. HPLC-ESI-MS chromatogram of the lipopeptide crude extract from strain N2-10 fermentation supernatant. A: Total ion chromatogram of crude extract; B-E: ESI-MS chromatogram peaks at retention times of 3.6, 4.4, 6.1, and 8.4 min.

Figure 3 [Figure 3: see original paper]. Two-stage mass chromatogram of the protonated substance at m/z 1,043.8, 1,057.8, 1,071.8, and 1,085.8.

Figure 4 [Figure 4: see original paper]. Possible b- and y-type fragments produced by CID from mycosubtilin.

Discussion

3.1 Antibacterial Effects of *Bacillus subtilis* N2-10 Fermentation Broth Components

With the increasing prevalence of antibiotic-resistant strains and antibiotic residue problems, the search for safe, environmentally friendly, non-resistance-inducing, and residue-free antibiotic alternatives has become a research priority. Probiotic *Bacillus* and their lipopeptide antimicrobial substances exhibit

broad-spectrum antibacterial activity without readily inducing resistance, representing potential ideal substitutes for conventional antibiotics. To further explore the probiotic potential of *B. subtilis* N2-10, a strain with preliminary probiotic effects obtained by our research group, this study evaluated the antibacterial activity of its fermentation broth components against common intestinal pathogens and beneficial bacteria. The findings revealed that *B. subtilis* N2-10 produces antibacterial substances during growth and metabolism, with lipopeptides demonstrating the most pronounced antimicrobial activity. Notably, these lipopeptides exhibited significant inhibition against common intestinal pathogens including *E. coli*, *S. flexneri*, and *S. enterica*, while showing essentially no inhibitory effects on beneficial bacteria such as *S. thermophilus* and *L. bulgaricus*. This antibacterial characteristic is advantageous for the strain' s probiotic functions in regulating animal intestinal balance and preventing intestinal diseases caused by pathogenic bacteria. These results align with Fukushima et al. [19], who reported that *Bacillus* can increase *Lactobacillus* populations in animal gastrointestinal tracts while significantly reducing coliform counts. This study establishes a foundation for further evaluation of the strain' s probiotic potential and subsequent research on its antibacterial substances.

3.2 HPLC-ESI-MS/MS Analysis of Lipopeptide Antibacterial Components

HPLC-ESI-MS/MS represents an effective tool for analyzing the molecular weights and structures of lipopeptide compounds [20]. In this study, we first identified four molecular ion peaks at 1,065.7, 1,079.7, 1,093.7, and 1,107.8 u from the primary mass spectrometry chromatogram, differing successively by 14 u. Comparison with existing literature [21] revealed consistency with the [M+Na]+ ions of C14~C17 mycosubtilin homologues in the iturin family. Subsequent MS2 fragmentation analysis of the [M+H]+ ions of these four target compounds revealed characteristic fragment ions consistent with the fragmentation patterns of C14~C17 mycosubtilin in all four compounds. We therefore concluded that the lipopeptide antibacterial substance produced by *B. subtilis* N2-10 is mycosubtilin from the iturin family.

This study elucidated the lipopeptide composition of *B. subtilis* N2-10 and its antibacterial spectrum against intestinal bacteria.

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