

# Extracts containing CLPs of *Bacillus amyloliquefaciens* JN68 isolated from chicken intestines exert antimicrobial effects, particularly on methicillin-resistant *Staphylococcus aureus* and *Listeria monocytogenes*

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**Abstract.** *Bacillus amyloliquefaciens* JN68, which has been discussed with regards to its antimicrobial activities, was successfully isolated from healthy chicken intestines in the present study. Using the spot-on-the-lawn antagonism method, the preliminary study indicated that a suspension culture of the *B. amyloliquefaciens* JN68 strain can inhibit the growth of *Aspergillus niger* and *Penicillium pinophilum*. Furthermore, the cyclic lipopeptides (CLPs) produced by the *B. amyloliquefaciens* JN68 strain were further purified through acid precipitation and Bond Elut®C18 chromatography, and their structures were identified using the liquid chromatography-electrospray ionization-mass spectrometry (MS)/MS method. Purified CLPs exerted broad spectrum antimicrobial activities on various pathogenic and foodborne bacteria and fungi, as determined using the agar well diffusion method. *Listeria monocytogenes* can induce listeriosis, which is associated with a high mortality rate. Methicillin-resistant

*Staphylococcus aureus* (MRSA) is a major pathogenic bacteria that causes nosocomial infections. Therefore, *L. monocytogenes* and MRSA are currently of great concern. The present study aimed to determine whether *B. amyloliquefaciens* JN68 extracts could inhibit *L. monocytogenes* and MRSA. The results indicated that extracts of *B. amyloliquefaciens* JN68 have CLP components, and can successfully inhibit the growth of *L. monocytogenes* and MRSA.

## Introduction

*B. amyloliquefaciens* is widely present in soil, plants and some animals (1-6); therefore, up to now, several strains of *B. amyloliquefaciens* have been isolated. Although different strains of *B. amyloliquefaciens* have a similar genome sequence, they have marked differences in the variable part of the genome (7); therefore, various strains of *B. amyloliquefaciens* may possess different abilities. Previous studies have demonstrated that numerous strains of *B. amyloliquefaciens* exert antifungal (8-10) and antibacterial activities (6,11-13). However, these studies have also indicated that various strains exert different antimicrobial activities. At present, several studies have suggested that *B. amyloliquefaciens* may be used as a potential therapeutic strategy that targets pathogens.

The major antimicrobial components of *B. amyloliquefaciens* are cyclic lipopeptides (CLPs) (14-16). At present, the three major CLPs that are known to possess antimicrobial activities are surfactin, iturin and fengycin A. Previous studies have demonstrated that surfactin, iturin and fengycin A possess antifungal and antibacterial

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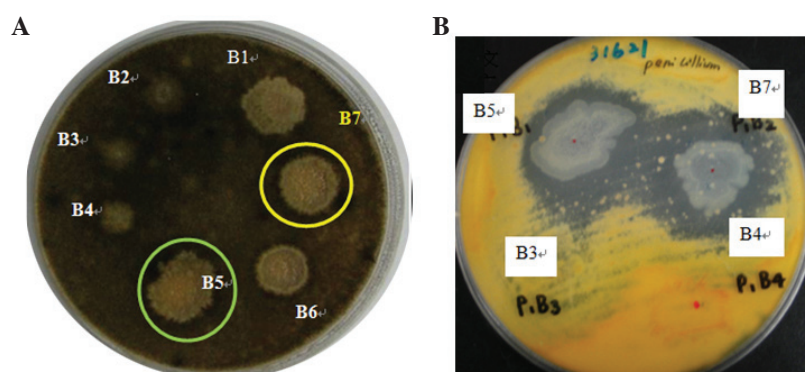


Figure 1. Screening of potential antimicrobial strains against (A) *Aspergillus niger* and (B) *Penicillium pinophilum*, as demonstrated from the zones of inhibition produced using the spot-on-the-lawn method.

activities (6,17-19). Notably, CLPs have several advantages, including low toxicity, high biodegradability and environmentally positive characteristics (20-23). These studies indicated that CLPs may be suitable for the treatment of pathogenic infections. In addition, other non-CLP antimicrobial peptides exist, such as amylolysin, which is a type of bacteriocin that is produced by *B. amyloliquefaciens* (24,25). The majority of bacteriocins only inhibit gram-positive bacteria, and certain non-CLPs are produced using the ribosomal synthesis method with gene modification, which is not the same as in the *Bacillus* genus (25). Therefore, CLPs isolated from *B. amyloliquefaciens* may be considered a better choice for the treatment of pathogenic infections, compared with non-CLPs.

*Listeria monocytogenes* is a foodborne pathogen, which induces listeriosis (26,27). *L. monocytogenes* is present in several ready-to-eat foods, including vegetables, poultry and beef, which have been found to cause human listeriosis (28,29). Due to clinical severity and the high mortality rates of *L. monocytogenes*-induced listeriosis, the control of *L. monocytogenes* food contamination is an important issue (30,31). Previous studies have reported that bacteriocins produced by *B. amyloliquefaciens* can inhibit the growth of *L. monocytogenes* (32,33). These studies indicated that products of *B. amyloliquefaciens* may be useful to control growth of *L. monocytogenes*; however, whether CLPs produced by *B. amyloliquefaciens* may target *L. monocytogenes* remains unclear.

*Staphylococcus aureus* can cause clinical acute hematogenous, osteomyelitis, endocarditis, pulmonary abscesses and spondylodiscitis (34-36). Methicillin-resistant *S. aureus* (MRSA) is not inhibited by treatment with several antibiotics (37,38). MRSA is a major and serious pathogen, which is particularly associated with nosocomial infection (39,40). Since numerous antibiotics can not effectively inhibit MRSA, the development of a novel anti-MRSA drug is important. A previous study indicated that *B. amyloliquefaciens* produced amylolysin, a ribosomally synthesized peptide, which can inhibit MRSA (25). However, to the best of our knowledge, whether CLPs produced by *B. amyloliquefaciens* can target MRSA has not yet been reported.

Based on the findings of previous studies, the present study aimed to determine whether CLPs isolated from

*B. amyloliquefaciens* purified from chicken intestines can exert antibacterial effects on MRSA and *L. monocytogenes*.

## Materials and methods

**Materials.** Difco™ agar and Luria broth (LB) culture medium were obtained from BD Biosciences (Franklin Lakes, NJ, USA). The polymerase chain reaction (PCR) primers for 16S ribosomal (rRNA and DNA gyrase, subunit B (gyrB) genes were synthesized by MD Bio (Taipei, Taiwan). The API® 50 CHB system assay kit was obtained from bioMérieux (Marcy-l'Étoile, France). Bond Elut® C18 was purchased from Agilent Technologies (Santa Clara, CA, USA). The polytetrafluoroethylene membrane (JP020) was obtained from Advantec Co., Ltd. (Tokyo, Japan). The indicator strains *Staphylococcus epidermidis* and *Bacillus cereus* were obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). The indicator strains *Streptococcus pyogenes*, *Listeria monocytogenes*, *Clostridium tyrobutyricum*, *Escherichia coli*, *Helicobacter pylori*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Aspergillus flavus* var. *flavus*, *Aspergillus niger* and *Penicillium pinophilum* were obtained from American Type Culture Collection (Manassas, VA, USA). MRSA HCT20 was kindly provided by the Department of Pathology and Laboratory Medicine, Taichung Veterans General Hospital (Taichung, Taiwan). The iturin A and surfactin standards were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany).

**Selection of potential bacterial strains from chicken intestines.** Potential bacterial strains with antimicrobial activities isolated from chicken intestines (obtained from a local market, Taichung, Taiwan) using microscopic examination and streak plate methods were selected and determined using the modified spot-on-the-lawn method, as previously described (41,42). Briefly, 0.7% top agar containing  $10^5$  colony-forming units (CFU)/ml indicator strains (*A. niger* or *P. pinophilum*) was overlaid onto a potato dextrose agar (PDA) plate, dried for 30 min, and subsequently spotted with  $2 \mu\text{l}$  ( $10^8$  CFU/ml) of an overnight culture of the candidate strains. Subsequently, the PDA plates were incubated at  $25^\circ\text{C}$  for 5 days. Zones of inhibition are found in the sites containing potential strains with antimicrobial activities.

**Identification of potential bacterial strain.** The *B. amyloliquefaciens* JN68 strain isolated from chicken intestines was identified by analyzing data from the API® 50 CHB system (43,44), as well as the results of a 16S rRNA and gyrB sequence detection (45-47). The results were analyzed on the National Center for Biotechnology Information BLAST tool (blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE\_TYPE=BlastSearch&LINK\_LOC=blasthome). According to morphological and physiological characteristics, biological testing and comparison of the 16S rRNA sequence, the strain was identified as *B. amyloliquefaciens*. Briefly, phenotypic characterization and sugar fermentation pattern of the candidate strain was analyzed according to standard methods, using the API® 50 CHB system assay kit. The obtained sugar fermentation pattern was analyzed using APILAB Plus software version 3.2.2 (bioMerieux). Further strain identification was confirmed by PCR detection of 16S rRNA and gyrB. Briefly, the candidate strain was cultured in LB culture medium (30°C, 200 x g, 8-12 h) and the genomic DNA was extracted using the Blood and Tissue Genomic DNA Extraction Miniprep (Viogene BioTek Corporation, New Taipei City). The amplification of the 16S rRNA and gyrB gene region of candidate strain was conducted in a 50 µl reaction mixture containing 1 µl genomic DNA template, 5 µl PCR reaction buffer [10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl and 0.1% Triton X-100] containing 200 µM of each deoxynucleoside triphosphate (GE Healthcare Life Sciences, Chalfont, UK), 4 µl MgCl<sub>2</sub> (52 mM), 1 µM each primer (Table I), 1 unit of Taq polymerase (GE Healthcare Life Sciences) and 39 µl autoclaved Milli-Q water (Merck Millipore). The amplification was performed with thermocycling conditions as follows: Denaturation for 10 min at 95°C; 35 cycles (as presented in Table I); termination at 72°C for 7 min. The PCR was conducted in a Robocycler® temperature thermal cycler (Agilent Technologies, Inc., Santa Clara, CA, USA). The PCR products were sequenced using an automatic sequencer (ABI PRISM® 3730; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Databases were screened for similarities using Basic Local Alignment Search Tool (48) and the alignment of overlapping fragments was performed using Vector NTI Advance 10 Contig Express software (Thermo Fisher Scientific, Inc.).

**Culture conditions and isolation of the lipopeptide fraction.** The *B. amyloliquefaciens* JN68 strain was cultured and activated with 5 ml LB culture medium at 30°C. Subsequently, 5% *B. amyloliquefaciens* JN68 was seeded into 200 ml number 3 medium (49). After incubation under an agitation rate of ~200 rpm at 30°C for 4 days, the fermentation medium was centrifuged at 11,000 x g for 20 min at 4°C. The supernatant was collected and filtered through a polytetrafluoroethylene membrane (0.22 µM pore size). The filtered supernatant was then treated with 6 N HCl (pH 2.0) at 4°C overnight, and was further centrifuged at 11,000 x g for 20 min at 4°C. The sediment was washed with PBS and extracted three times with methanol. The methanolic extracts were concentrated and dissolved in methanol. Finally, the purified CLPs of the *B. amyloliquefaciens* JN68 strain were obtained from the methanolic extracts using the Bond Elut® C18 (5 g) mini column as previously described (50).

Table I. Primer sequences and PCR conditions used in the present study.

First author, year	Target gene and primers	Sequence (5' to 3')	PCR conditions	Product size	Refs.
Wang, 2007	gyrB degenerate primers UP-1S UP-2Sr	CGTAGAGCCACTTGAGCG CTGCCGTTACAGTTCCTT	94°C x1 min/55°C x30 sec/72°C x1 min	898 bp	(47)
Perea Vélez, 2007	16S universal primer for bacteria Pro26 Pro27	AGAGTTTGATCCTGGGCTCAG AAGGAGGTGATCCAGCCGCA	94°C x1 min/45°C x30 sec/72°C x1 min	~1.5 kb	(51)

Total number of cycles conducted was 35, initial denaturation step was 10 min at 95°C and final termination step was 7 min at 72°C. PCR, polymerase chain reaction; gyrB, DNA gyrase, subunit B.



**Identification of CLP structure by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS)/MS.** Purified CLPs produced by *B. amyloliquefaciens* JN68 were obtained from methanolic extracts as aforementioned. Subsequently, the structure of the CLPs was detected using LC and MS. For LC, an Agilent 1200 series system (Agilent Technologies), including a binary pump and an autosampler, was used for the chromatographic separation. The separation was conducted using an Atlantis C18 column (150x2.1 mm, 3.5  $\mu$ m; Waters UK, Elstree, UK) at 30°C with a flow rate of 200  $\mu$ l/min using the following gradient: i) 50% B for 2 min; ii) a gradient of 50-100% B for 10 min; iii) 100% B for 10 min; iv) re-equilibration with 50% B for 10 min. Mobile phase A consisted of 10% methanol and mobile phase B consisted of 100% methanol. The injection volume was set to 10  $\mu$ l. For MS analysis an LTQ XL<sup>TM</sup> Linear Ion Trap MS was used (Thermo Fisher Scientific, Inc.), which was equipped with an ESI source. MS was operated in positive-ion mode with a spray voltage of 5.0 KV, and a capillary voltage of 40 V. Capillary temperature and sheath gas ( $N_2$ ) were set at 275°C and 20 arbitrary units, respectively. The precursor ion width was set at 3 Da and the normalized collision energy was set at 35%. For LC-ESI-MS/MS, the MS was operated in the selected target precursor ions as follows:  $m/z$  1,036, surfactin; 1,043, iturin A; 1464, fengycin A, using the Linear Ion Trap MS. The three major CLP components containing surfactin, iturin A and fengycin A were analyzed in the present study as previously described (52-54).

**Antimicrobial profile assay.** The antimicrobial activities of the purified CLPs produced by *B. amyloliquefaciens* JN68 were determined using an agar well diffusion assay, as previously described (41,55). Briefly, small holes were dug into agar plates using a 200  $\mu$ l pipette tip. Each indicator microorganism ( $\sim 10^6$  CFU/ml bacterium or  $10^5$  CFU/ml fungus) was spread onto an agar plate. Subsequently, 50  $\mu$ l *B. amyloliquefaciens* JN68 extracts were seeded into the holes on the agar plates for 2 h at 4°C. The agar plates were then incubated for 16-18 h at 37°C (indicator bacteria) or at 30°C (indicator fungi). After a 16-18 h incubation, the appearance of the zones of inhibition was determined.

## Results

**Screening of potential antimicrobial strains.** Several bacterial strains were obtained from the chicken intestines. In order to identify potential strains with antimicrobial activities, the spot-on-the-lawn method was used (41). In the present study, bacterial strains isolated from chicken intestines were spotted onto top agar plates containing indicator strains (*A. niger* or *P. pinophilum*). Zones of inhibition are found in the sites containing potential strains with antimicrobial activities. The results indicated that marked zones of inhibition appeared at the B5 and B7 sites; these sites therefore contained potential strains that can effectively inhibit the growth of *A. niger* (Fig. 1A) and *P. pinophilum* (Fig. 1B). Therefore, the strains at the B5 and B7 sites were considered to possess antimicrobial activities. In the present study, the potential bacterium at the B7 site was selected and analyzed (identical to B5).

**Identification of the bacterial strain.** The potential bacteria at the B7 site was primarily identified using the API<sup>®</sup> 50 CHB system (Table II). Subsequently, the potential bacteria at the B7 site was further confirmed by 16S rRNA and gyrB sequence detection. The PCR primer sequences of 16S rRNA and gyrB used in the present study are presented in Table I. Analysis of the API<sup>®</sup> 50 CHB system and gene sequence detection indicated that the potential bacteria at the B7 site belonged to the *Bacillus amyloliquefaciens* family. In the present study, this potential bacterial strain was named *Bacillus amyloliquefaciens* JN68.

**Determination of CLP extract components.** LC-ESI-MS/MS analysis of the CLP extracts is presented in Figs. 2-4. As shown in Fig. 2C, the main peak of surfactin lipopeptide was revealed at retention time ( $R_t$ ) 17.93 min corresponding to protonated molecules  $[M+H]^+$  at  $m/z$  1,036 in positive modality. The representative MS/MS spectrum is shown in Fig. 2D. These results were similar to those observed during surfactin standard LC-ESI-MS/MS analysis (Fig. 2A and B). This analysis confirmed the presence of one main surfactin in the extracted CLPs. As shown in Fig. 3C, the one main peak of iturin A lipopeptide was revealed at  $R_t$  13.16 min corresponding to the protonated molecules  $[M+H]^+$  at  $m/z$  1,043 in positive modality. The representative MS/MS spectrum is shown in Fig. 3D. The  $R_t$  and product ions were similar to those obtained during iturin A standard LC-ESI-MS/MS analysis (Fig. 3A and B). The results of the LC-ESI-MS/MS analysis of fengycin A lipopeptide are presented in Fig. 4. The one main peak of fengycin A was observed at  $R_t$  13.90 min corresponding to the protonated molecules  $[M+H]^+$  at  $m/z$  1,464 in positive modality (Fig. 4A). The representative MS/MS spectrum is shown in Fig. 4B. Although no fengycin A standard was used in the present study, the product ions were consistent with those from previous studies, which reported that product ions at  $m/z$  1,080 and 966 were representative ions present in the fengycin A MS/MS spectrum at  $m/z$  1,464 (56,57). Taking into account the results obtained by LC-MS/MS analysis of CLPs, there are at least three lipopeptide groups within the whole *B. amyloliquefaciens* extract, which included surfactant, iturin A and fengycin A.

**Antimicrobial spectrum of the potential strain.** Previous studies have demonstrated that CLPs isolated from various *B. amyloliquefaciens* strains possess distinct antifungal (9,17,58,59) and antibacterial activities (60-63). However, to the best of our knowledge, no previous experiment has demonstrated that CLPs isolated from *B. amyloliquefaciens* can inhibit MRSA and *L. monocytogenes*. The present study indicated that CLPs containing surfactin, iturin and fengycin A, isolated from *B. amyloliquefaciens* JN68, can inhibit several pathogenic fungal and bacterial strains (Table III). Notably, the present study is the first, to the best of our knowledge, to indicate that CLPs isolated from *B. amyloliquefaciens* JN68 can inhibit the growth of MRSA and *L. monocytogenes* (Table III).

## Discussion

At present, various *B. amyloliquefaciens* strains have been isolated from plants and soil; in addition, some

Table II. Sugar fermentation pattern of the candidate strain was determined using the API 50 CHB system.

Carbohydrate fermentation	
Active ingredient	Response
Control	-
Glycerol	+
Erythritol	-
D-arabinose	-
L-arabinose	+
D-ribose	+
D-cylose	w
L-cylose	-
D-adonitol	-
β-methyl-D-xylopyranoside	-
D-galactose	-
D-glucose	+
D-fructose	+
D-mannose	+
L-sorbose	-
L-rhamnose	-
Dulcitol	-
Inositol	+
D-mannitol	+
D-sorbitol	+
Methyl α-D-mannopyranoside	-
Methyl α-D-glucopyranoside	+
N-acetyl glucosamine	-
Amygdalin	-
Arbutin	+
Esculin (ferric citrate)	+
Salicin	+
D-cellobiose	+
D-maltose	+
D-lactose (bovine origin)	w
D-melibiose	-
D-saccharose (sucrose)	+
D-trehalose	+
Inulin	-
D-melezitose	-
D-raffinose	-
Amidon (starch)	w
Glycogen	±
Xylitol	-
Gentiobiose	w
D-turanose	-
D-lyxose	-
D-tagatose	-
D-fucose	-
L-fucose	-
D-Arabitol	-
L-arabitol	-
Potassium giuconate	-
Potassium 2-ketogluconate	-

Table II. Continued.

Carbohydrate fermentation	
Active ingredient	Response
Potassium 5-ketogluconate	-
Control is the culture medium alone. +, positive reaction; -, negative reaction; w, weak reaction.	

*B. amyloliquefaciens* strains have been found to be present in animals (1-6). Several studies have reported that extracts of various *B. amyloliquefaciens* strains exert antimicrobial activities (12,64,65). However, to the best of our knowledge, no previous studies have reported that *B. amyloliquefaciens* strains isolated from chickens possess antimicrobial activities. The present study isolated the *B. amyloliquefaciens* JN68 strain from chicken intestines, and confirmed it possessed antimicrobial activities. At present, several components have been identified that exert antimicrobial activities, including CLPs (15,66,67), antifungal enzymes (5,68) and non-CLPs (24,25). Among these components, CLPs exert more broad-spectrum antifungal and antibacterial activities, compared with antifungal enzymes and non-CLPs (17,64). In general, CLPs can exert antifungal and antibacterial activities (against both gram-positive and gram-negative bacteria) (64,67), whereas antifungal enzymes only exert antifungal activities (5,68) and non-CLPs exert only antibacterial activities against gram-positive bacteria (24,25). In the present study, the CLP extracts purified from the *B. amyloliquefaciens* JN68 strain exerted antifungal and antibacterial activities on gram-positive and gram-negative bacteria.

Since CLPs produced by *B. amyloliquefaciens* possess several advantages, including broad-spectrum antimicrobial activities, low toxicity, high biodegradability and environmentally positive characteristics (20-23,64), CLPs may therefore be considered potential antimicrobials for food and clinical application. Previous studies have reported that surfactin, iturin and fengycin A are major CLP components that exert antimicrobial activities (6,17-19). In the present study, the extracts purified from the *B. amyloliquefaciens* JN68 strain were confirmed to be surfactin, iturin and fengycin A. Since various *B. amyloliquefaciens* strains have similar genome sequences, with the exception of differences in the variable region of the genome (7), there are several types and expression levels of surfactin, iturin and fengycin A in the different strains (69). This may be why CLPs produced by different strains possess distinct antimicrobial activities.

Previous studies have reported that CLPs produced by some *B. amyloliquefaciens* strains possess antifungal activities, including the CGMCC5569, NJN-6, Q-426 and PPCB004 strains (9,17,58,59). Furthermore, CLPs produced by some *B. amyloliquefaciens* strains possess antibacterial activities, including FZB42, HR62, NJN-6 and B9601-Y2 strains (60-63). In addition, CLPs produced by some *B. amyloliquefaciens* strains possess antifungal and antibacterial activities, such as the NJN-6 and B9601-Y2 strains (9,62,63).

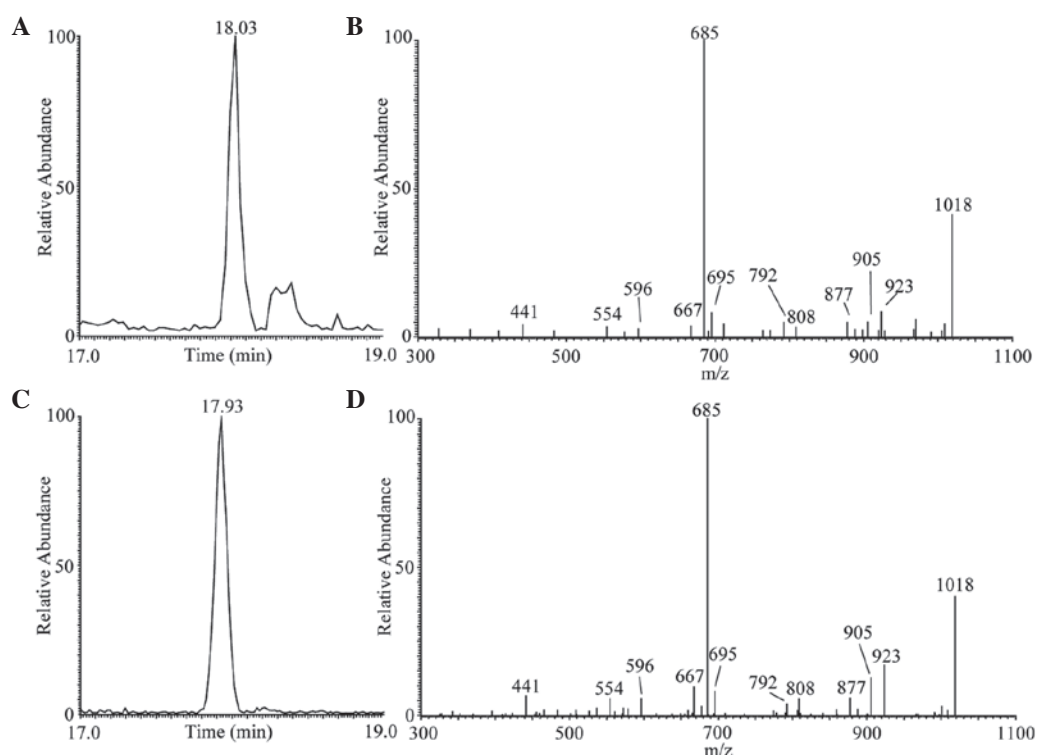


Figure 2. LC-ESI-MS/MS analysis of (A and B) surfactin standard and (C and D) biosurfactant lipopeptide extract. (A and C) Exact ion current chromatogram of surfactin ( $m/z$  1,036). (B and D) Product ion spectra of the protonated molecules  $[M+H]^+$  of surfactin at  $m/z$  1,036. LC-ESI-MS/MS, liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry.

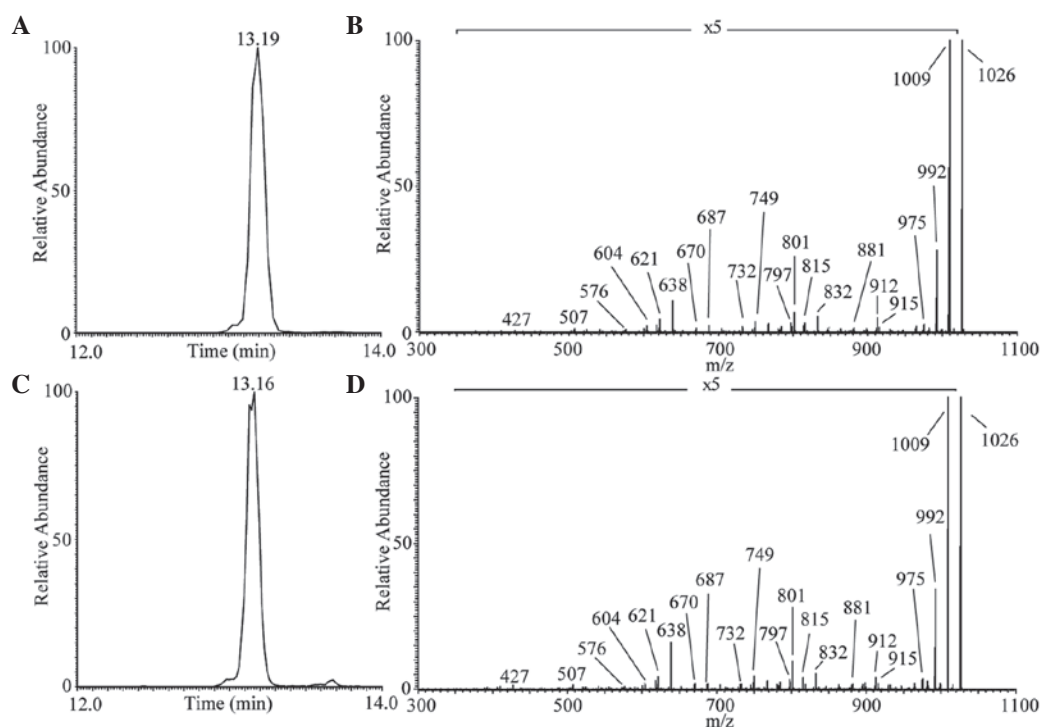


Figure 3. LC-ESI-MS/MS analysis of (A and B) iturin A standard and (C and D) biosurfactant lipopeptide extract. (A and C) Exact ion current chromatogram of iturin A ( $m/z$  1,043). (B and D) Product ion spectra of the protonated molecules  $[M+H]^+$  of iturin A at  $m/z$  1,043. LC-ESI-MS/MS, liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry.

Compared with previous studies, antifungal and antibacterial activities were detected in the *B. amyloliquefaciens* JN68 strain isolated from chicken intestines in the present study. It is well

known that MRSA is an important bacterial species that causes nosocomial infections (39,40), whereas *L. monocytogenes* induces listeriosis, which has a high mortality rate (30,31). No

Table III. Antimicrobial spectrum of CLPs produced by *Bacillus amyloliquefaciens* JN68 using the agar well diffusion method.

Indicator strain	Antimicrobial activity <sup>a</sup>	
	Growth medium	Unpurified CLP
Gram-positive		
HCT20 MRSA	TSA	+++
<i>Staphylococcus epidermidis</i> BCRC15245	NA	++
<i>Streptococcus pyogenes</i> ATCC12344	TSA with 5% blood	+++
<i>Listeria monocytogenes</i> ATCC15313	BHI	+++
<i>Clostridium tyrobutyricum</i> ATCC25755	NA	+
<i>Bacillus cereus</i> BCRC10250	NA	+
Gram-negative		
<i>Escherichia coli</i> ATCC11775 (10675)	NA	++
<i>Helicobacter pylori</i> ATCC43526	Brucella with 3% blood	+
<i>Salmonella typhimurium</i> ATCC13311	TSA	+++
<i>Pseudomonas aeruginosa</i> ATCC9027 (11633)	NA	++
Mold		
<i>Aspergillus flavus</i> var. <i>flavus</i> <sup>b</sup> ATCC26770	PDA	+++
<i>Aspergillus niger</i> ATCC16404	PDA	+
<i>Penicillium pinophilum</i> ATCC9644	PDA	+++

<sup>a</sup>Interpretation of zone of inhibition diameter: +, 5-10 mm (weak inhibition); ++, 10-15 mm (moderate inhibition); +++, >15 mm (strong inhibition). <sup>b</sup>Production of  $\alpha$ -toxins, B1, B2, G1 and G2. BCRC, Bioresource Collection and Research Center; ATCC, American Type Culture Collection; MRSA, methicillin-resistant *Staphylococcus aureus*; TSA, tryptone soya agar; NA, nutrient agar; BHI, brain-heart infusion medium; PDA, potato dextrose agar.

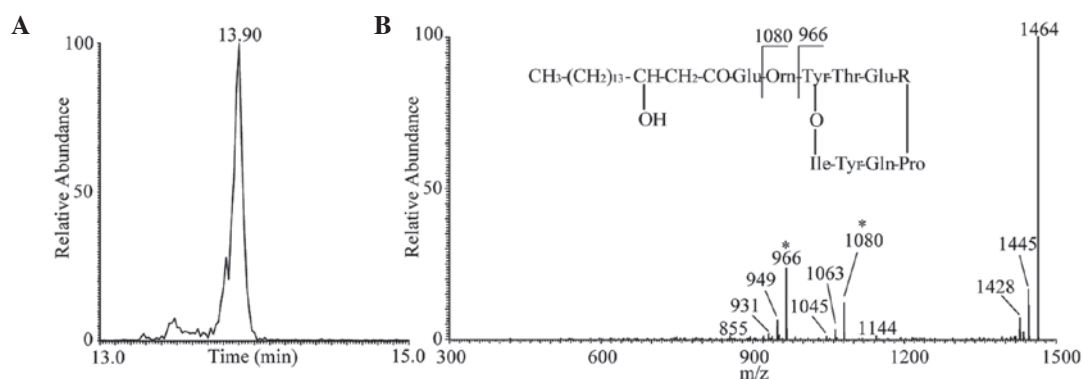


Figure 4. LC-ESI-MS/MS analysis of biosurfactant lipopeptide extract. (A) Exact ion current chromatogram of fengycin A ( $m/z$  1,464). (B) Product ion spectra of the protonated molecules  $[M+H]^+$  of fengycin A at  $m/z$  1,464. Significant fragment ions of fengycin A were marked with an asterisk. LC-ESI-MS/MS, liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry.

previous studies, to the best of our knowledge, have demonstrated that CLPs produced by *B. amyloliquefaciens* can inhibit the growth of MRSA and *L. monocytogenes*. Therefore, the present study is the first to indicate that the *B. amyloliquefaciens* JN68 strain can inhibit MRSA and *L. monocytogenes*.

In conclusion, the present study isolated *B. amyloliquefaciens* JN68 from chicken intestines, and confirmed it possessed antifungal and antibacterial activities. In particular, *B. amyloliquefaciens* JN68 was able to inhibit the growth of MRSA and *L. monocytogenes*. These results suggested that the *B. amyloliquefaciens* JN68 strain may be applied to prevent the occurrence of foodborne diseases and nosocomial infections.

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