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Research article

Weissella sp. SNUL2 as potential probiotics with broad-spectrum antimicrobial activities

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ABSTRACT

Probiotics have been applied to a wide range of bacteria, causing gastrointestinal and vaginal infections. However, probiotics generally possess limited antimicrobial spectra and are primarily utilized as dietary supplements. Recognizing the need for more versatile probiotics, this study focuses on isolating and characterizing strains suitable for antibiotic replacement. Among these strains, *Weissella* sp. SNUL2, derived from a traditional fermented food in Korea (i.e., Sikhae), emerged as a promising candidate. The correlation between optical density at 600 nm and colony-forming units was verified and applied in subsequent experiments. To assess the therapeutic potential of probiotics, antibacterial tests were conducted using a microplate reader to evaluate the inhibition of 60 bacterial strains (including common foodborne pathogens) induced by *Weissella* sp. SNUL2 cell-free supernatant (CFS). The results confirmed its broad-spectrum antibacterial properties compared to previously known probiotics. Furthermore, enzymatic treatment with proteinases (trypsin and pepsin) and a time-kill assay were conducted to elucidate the nature of the antibacterial substance in *Weissella* sp. SNUL2 CFS. Through sequential chromatography involving gel filtration and ion-exchange chromatography, specific fractions with enhanced antibacterial properties were identified. LC-MS/MS analysis of the secretome fraction revealed the presence of various proteins from the C39 family, peptidoglycan endopeptidases, and N-acetylmuramoyl-L-alanine amidase domain-containing protein precursors. Hence, the combined action of these proteins may contribute to *Weissella* sp. SNUL2's broad antimicrobial activity.

1. Introduction

Since the discovery of penicillin, antibiotics have played a crucial role in safeguarding humanity against bacterial infections.

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However, their excessive use worldwide has led to a considerable increase in antibiotic-resistant bacteria and related fatalities [1,2]. Based on the Centers for Disease Control and Prevention (CDC)'s 2019 Antibiotic Resistance Threats Report, antimicrobial resistance (AMR) is an urgent global public health threat, responsible for the death of 1.27 million people worldwide in 2019 and associated with nearly 5 million deaths. In the U.S., more than 2.8 million antimicrobial-resistant infections occur each year. More than 35,000 people die as a result [3]. Accordingly, antibiotic-resistant infections in healthcare settings have raised concerns among the scientific and medical communities [4]. Additionally, the UK Department of Health predicts that by 2050, AMR will be responsible for causing 10 million deaths annually, making it the leading cause of death [5].

Meanwhile, the continued use of antibiotics remains necessary for the treatment of numerous diseases. However, the growing demand for alternative treatments has prompted researchers worldwide to explore potential antibiotic replacements, including bacteriophages [6], antimicrobial peptides such as lysine [7], antibodies, efflux pump inhibitors, immunomodulatory agents [8], plant extracts, bee-derived products, prebiotics, synbiotics, postbiotics, and probiotics [9]. Each of these has advantages and disadvantages and is at different stages of clinical trials, depending on their intended therapeutic purposes [10]. Probiotics—living microorganisms designed to provide health benefits when consumed or applied—demonstrate antimicrobial activity through various mechanisms, including the production of specific proteins, such as bacteriocins. Bacteriocins are natural protein- or peptide-based antimicrobial agents released by microorganisms that function to eliminate bacterial competitors rather than merely inhibiting their growth [11]. Unlike antibiotics, which are secondary metabolites, these peptides are biodegradable and demonstrate effective antimicrobial effects even in small quantities [12]. Additionally, antimicrobial peptides have a relatively broader spectrum of activity [13]. However, the purification of these proteins during scale-up processes can be costly due to their low abundance. Additionally, lactic acid and other unidentified metabolites may contribute to the antimicrobial properties of probiotics [14]. The synergistic interaction between these proteins and metabolites may enhance the overall antimicrobial effectiveness of probiotics. The term "postbiotics", coined by the International Society for Probiotics and Prebiotics (ISAPP), refers to the preparation of inactive microorganisms and/or their components to provide health benefits [9].

Korean traditional fermented foods, including probiotics, serve as beneficial bacteria reservoirs [15]. Among the various Korean fermented foods, Jeotgal and Sikhae have specific storage protocols designed to prevent spoilage of seafood. Jeotgal achieves this by maintaining a high salt concentration (~20%), which effectively inhibits the growth of harmful bacteria [16]. In contrast, Sikhae contains a lower salt concentration [15]. Studies examining the food microbiome have demonstrated an altered bacterial composition in Sikhae [15]. Initially, Sikhae harbors a diverse range of bacteria, including opportunistic pathogens. However, as fermentation progresses, the population of harmful bacteria decreases, and lactic acid bacteria (LAB) including *Latilactobacillus* (formerly *Lactobacillus*) and *Weisella* spp. become dominant. This suggests that the beneficial bacteria exert a suppressive effect on harmful bacteria, highlighting their potential health benefits in Sikhae.

Recently, exploration into microbiomes has provided crucial insights, revealing the potential to manipulate the microbiome or intestinal environment as an alternative approach to conventional treatments for complex diseases. Studies have successfully established correlations between lean and obese phenotypes and the composition of gut microbiota in mice and humans [17–19]. This underscores the significance of such manipulation in addressing conditions like diabetes, inflammatory bowel diseases, liver disease, and chronic kidney disease [20–23]. Additionally, the administration of gut probiotic microbes, such as *Akkermansia muciniphila*, has proved effective in reducing the weight of patients with obesity [24,25]. Moreover, the supplementation of a healthy gut microbiome from unaffected individuals and probiotic strains effectively treats *Clostridium difficile* recurrence [26]. Similarly, studies have demonstrated that lactobacilli inhibit the growth of *C. difficile* in hamsters [27]. Various combinations of probiotics, including *L. acidophilus*, *L. casei*, mixed species, and *Saccharomyces boulardii* or *L. rhamnosus*, can reportedly control *C. difficile* infection [28]. Furthermore, the administration of *S. boulardii* combined with high-dose oral vancomycin reduces the likelihood of subsequent recurrence by nearly 50% in patients experiencing recurrent symptoms of *C. difficile*-related disease [29]. A previous study has also elucidated the relationship between diarrhea and the intestinal microbiome [30]. Specifically, a potential role for *Ruminococcus obeum* was established in mediating quorum sensing inhibition, associated with reduced *Vibrio cholerae* colonization and pathogenicity [30]. These collective findings underscore the potential of targeted administration of specific probiotics, including gut probiotics, in modulating the presence of pathogenic gut microbes.

In this study, we isolated a *Weissella* sp. SNUL2 strain from a traditional Korean fermented food that exhibited broad-spectrum antimicrobial activity, including against common bacteria that cause food poisoning. The main objective of this study is to investigate the potential of *Weissella* sp. SNUL2 as a probiotic to prevent diarrhea or food poisoning by selectively inhibiting harmful gut bacteria. Thus, we focus on the comprehensive exploration of postbiotics and investigate the antimicrobial properties of the cell-free supernatant (CFS) derived from our strain. To this end, we used size exclusion chromatography, specifically gel filtration and ion exchange chromatography, to partially purify the antibiotics present in the CFS of the bacterial strain. Subsequently, molecular weight analysis was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the components of the partially purified fractions were identified via liquid chromatography and mass spectrometry (LC-MS/MS). Taken together, the findings of this study provide useful insights to further expand our understanding of postbiotics.

2. Materials and methods

2.1. Strains and culture conditions

The strain *Weissella* sp. SNUL2 was isolated from the Korean traditional fermented food, Sikhae. Various fermented food samples were obtained from four regions of Gangwon-do (Yangyang, Gangneung, Jumunjin, and Samcheok) and one region of Gyeongsangbuk-

do (Daegu) (Table S1). After appropriate washing steps, all samples were cultured in 2% de Man, Rogosa, and Sharp (MRS, BD Difco™, Franklin lakes, NJ, USA) agar at 37 °C overnight. The colonies obtained were restreaked to isolate single colonies; each colony was sent to Macrogen (Seoul, Korea) for 16S rRNA sequencing. Based on the results, several thousand beneficial strains, including *Weissella* sp. SNUL2, were collected.

Weissella sp. SNUL2 was cultured in MRS broth for seed culture at 30 °C for 12 h in a shaking incubator at 100 rpm. The main culture was inoculated with a fresh 1% (v/v) inoculum and propagated in 100 mL of MRS medium at 30 °C for 24 h. Subsequently, the samples were collected every 4 h to measure the optical density of the culture broth at 600 nm (OD_{600}) using a UV-1280 spectrophotometer (Shimadzu, Kyoto, Japan). All OD_{600} values were converted to colony-forming unit (CFU)/mL based on the assumption that an OD_{600} of $1 = 9.9 \times 10^8$ *Weissella* sp. SNUL2 CFU/mL.

2.2. Antibacterial activity assessment

2.2.1. High-throughput antibacterial test

To assess the antibacterial properties of *Weissella* sp. SNUL2, we conducted an antibacterial test on 60 strains, including well-known foodborne pathogens and *Escherichia coli* strains (Table 1) [31–33]. After culturing *Weissella* sp. SNUL2 in fresh MRS broth and incubating at 30 °C overnight, the CFS was obtained via centrifugation and filtration through a sterile filter (pore size 0.2 μm; Sartorius,

Table 1
Antibacterial test against 60 bacterial strains.

Indicator strain	Growth inhibition		Indicator strain	Growth inhibition	
	+ *Weissella* sp. SNUL2 CFS	+*Pediococcus acidilactici* K10 CFS		+ *Weissella* sp. SNUL2 CFS	+ *Pediococcus acidilactici* K10 CFS
Listeria innuocua ATCC 33090	+	−	*Staphylococcus aureus* ATCC 25923	+	−
Listeria ivanovii subsp. *ivanovii* ATCC 19119	+	−	*Staphylococcus aureus* KCTC 1621	+	−
Listeria monocytogenes ATCC 19111	+	−	*Staphylococcus aureus* subsp. *aureus* ATCC 33591	+	−
Listeria seeligeri ATCC 35967	+	+	*Staphylococcus aureus* subsp. *aureus* ATCC 33593	+	−
Listeria welshimeri ATCC 35897	+	+	*Staphylococcus epidermidis* ATCC 12228	+	−
Salmonella enteritidis ATCC 13076	+	−	*Staphylococcus epidermidis* KACC 13234	+	−
Salmonella enteritidis KCCM 12021	+	+	*Staphylococcus hyicus* KTCC 13249	+	+
Salmonella enterica subsp. *enterica* ATCC 43971	+	−	*Streptococcus thermophilus* KCTC 5092	+	−
Salmonella typhimurium ATCC 19586	+	−	*Streptococcus thermophilus* KCTC 1621	+	−
Salmonella typhimurium ATCC 43174	+	−	*Streptococcus mutans*	+	−
Salmonella typhimurium DT104 killer cow	+	−	*Vibrio parahaemolyticus* ATCC 27969	+	+
Salmonella typhimurium ATCC 14028	+	+	*Vibrio parahaemolyticus* ATCC 17803	+	−
Staphylococcus aureus ATCC 6538	+	−	*Vibrio vulnificus* KCCM 13234	+	+
Staphylococcus intermedius KACC 13247	+	−	*Yersinia enterocolitica* KCCM 41657	+	+

Bacillus cereus ATCC 14579	+	−	*Bacillus thuringensis* ATCC 35646	+	−
Bacillus cereus ATCC 10987	+	−	*Bacillus thuringensis* ATCC 35866	+	−
Bacillus cereus DSM 102	+	−	*Bacillus thuringensis* KCTC 1510	+	−
Bacillus cereus ATCC 21768	+	−	*Bacillus thuringensis* ATCC 35679	+	−
Bacillus cereus KCTC 1094	+	−	*Bacillus mycoides* ATCC 6462	+	−
Bacillus cereus ATCC 10876	+	−	*Bacillus mycoides* ATCC 21929	+	−
Bacillus cereus ATCC 11778	+	−	*Bacteroides fragilis* KCTC 3688	+	−
Bacillus cereus ATCC 10702	+	−	*Escherichia coli* ATCC 10536	+	−
Bacillus subtilis ECE 22	+	−	*Escherichia coli* ATCC 9637	+	−
Bacillus subtilis DSM 102	+	−	*Escherichia coli* ATCC 11775	+	−
Bacillus subtilis ECE 139	+	+	*Escherichia coli* O157:H7 932	+	−
Bacillus pseudomycoides KCTC 3862	+	−	*Escherichia coli* O104 NCCP 13721	+	+
Enterococcus faecalis KCTC 3206	−	−	*Escherichia coli* O26 NCCP 13580	+	+
Enterococcus faecalis KFRI 354	+	−	*Escherichia coli* O121 NCCP 12551	+	+
Enterococcus faecium KFRI 132	+	−	*Escherichia coli* O111 NCCP 13581	+	−
Enterococcus faecium KFRI 131	+	−	*Listeria grayi* ATCC 19120	+	+

(+) shows growth inhibition of indicator strain, (−) shows no growth inhibition.

Goettingen, Germany). The CFS was cultured with 60 indicator bacteria at 37 °C or 30 °C for 24 h and media to confirm the range of inhibition of the growth of these indicator bacteria. A culture without *Weissella* sp. SNUL2 CFS served as the negative control, while a culture containing the CFS of *Pediococcus acidilactici* K10 was the positive control; *P. acidilactici* K10 CFS has high antimicrobial activity [34,35].

2.2.2. Kill-time assay

CFS and media were loaded at a 1:1 ratio in each well, and the OD_{600} was measured every 4 h. For cultivation with *E. coli* strains, 160 μL of fresh Luria–Bertani (LB) broth (BD Difco™, Franklin lakes, NJ, USA) containing 1×10^8 CFU/mL of *E. coli* inoculum and 40 μL of the *Weissella* sp. SNUL2 CFS were added per well in a 96-well plate. Notably, each CFS was adjusted to pH 4, 5, or 6 using 0.1 N NaOH; the sample without CFS treatment served as the negative control. The plates were incubated at 37 °C for 24 h, and OD_{600} was measured (VERSAmax, San Jose, CA, USA) every 4 h.

2.2.3. Pathogen-specific antibacterial activity assessment

To further determine whether the antibiotic substances in CFS exhibit antimicrobial activity, antibacterial tests were conducted using six pathogens: *Salmonella enterica* ATCC13076, *Salmonella enterica* ATCC43971, *Bacillus cereus* ATCC14579, *Staphylococcus aureus* ATCC25923, *Staphylococcus aureus* KCTC1917, and *Vibrio fluvialis* KCTC2473 [32]. The indicator pathogens were Biosafety level (BSL) grade 2 bacteria distributed from the Korean collection for type cultures (KCTC, Joengeup, Korea) and American type culture collection (ATCC, Manassas, VA, USA).

Seed (12 h) and main (overnight) pathogen cultures were prepared appropriately in MRS medium, from which samples were collected. The samples were categorized into five groups: Control (pH 6), Control (pH 4), *Weissella* sp. SNUL2 CFS-treated (pH 6), L2 CFS-treated (pH 4), and duramycin-treated groups. The samples were prepared using MRS media and the pH was adjusted with 1 N NaOH and 1 N HCl. Among the commercial bacteriocins, duramycin (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control due to its antimicrobial activity against Gram-negative bacteria. Duramycin solution was prepared by adding 10 mg of duramycin to 0.1 N HCl. Each group was incubated for 24 h in a shaking incubator at 100 rpm, and the OD_{600} value was measured every 4 h. The same experiment was conducted to assess if duramycin impacts the growth of four beneficial LABs. Four types of LABs (*Lactiplantibacillus plantarum* KCTC3108, *Latilactobacillus sakei* KCTC3598, *Lacticaseibacillus casei* ATCC393, and *Leuconostoc mesenteroides* KCTC3100) were used as an indicator of beneficial microbes. Nisin (Sigma-Aldrich, St. Louis, MO, USA)—a commercial bacteriocin—served as the positive control due to its excellent antimicrobial properties against Gram-positive bacteria. Nisin was added to 0.02 N HCl at a final concentration of 1 mg/mL to prepare a solution with a 1000 U/mL activity. Notably, nisin was added at the same concentration as CFS.

2.2.4. Minimum inhibitory concentration (MIC) assay

We determined the minimum inhibitory concentration (MIC) of *Weissella* sp. SNUL2 required to inhibit the indicator bacteria. MIC is the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism after overnight incubation. The MIC test was performed using the aforementioned six pathogens. Ampicillin was used as the control, and its MIC against the six indicator strains was compared with that of *Weissella* sp. SNUL2. The initial concentrations of *Weissella* sp. SNUL2 CFS and ampicillin were set at 0.2 mg/mL, and two-fold dilution was repeated six times to dilute the initial concentration to 1.5%. Additionally, the OD_{600} was measured for 24 h using a microplate; each well contained 100 μL of medium and indicator bacteria and 100 μL of *Weissella* sp. SNUL2 CFS (or ampicillin). The indicator bacteria were inoculated at 5×10^6 CFU/mL per well.

2.2.5. Enzymatic sensitivity testing of the antibiotic active component of the CFS

To confirm whether the antibiotic active component of *Weissella* sp. SNUL2 CFS was a peptide-based material, its sensitivity to enzymes was evaluated using the CFS of *Weiseslla* sp. SNUL2 treated with trypsin and pepsin (Sigma-Aldrich, St. Louis, MO, USA for both). Specifically, a solution comprising 1 mg/mL trypsin in 50 mM potassium phosphate buffer was mixed with *Weissella* sp. SNUL2 CFS at a 1:1 ratio and activated at 37 °C for 4 h at pH 7.8. Heat treatment was applied at 100 °C for 10 min to prevent additional enzymatic activity, and the pH was adjusted to 6. Additionally, a solution comprising 1 mg/mL pepsin and *Weissella* sp. SNUL2 CFS in 50 mM glycine-HCl buffer at a 1:1 ratio was activated at 37 °C for 4 h at pH 2.2. Heat treatment was applied at 100 °C for 10 min, and the pH was adjusted to 6. *E. coli* cultured for 24 h served as the indicator strain. Each well contained 100 μL of LB medium with 1×10^8 CFU/mL of *E. coli* and 100 μL of the enzyme solution adjusted to pH 6. Two controls were used: *E. coli* alone and *E. coli* with *Weissella* sp. SNUL2 CFS. The growth of *E. coli* was determined by measuring the OD_{600} 4 h intervals for 24 h.

2.2.6. Kirby–Bauer disk diffusion assay

The Kirby–Bauer disk diffusion assay is used to determine the susceptibility of antibiotic substances against particular strains [36]. In this study, the assay was modified slightly to determine how antimicrobial substances, including CFS, affect specific strains in agar conditions. Specifically, purified the antibiotic active components of the CFS were assessed to observe whether a small amount of CFS from *Weissella* sp. SNUL2 had a visible inhibitory effect against the aforementioned six types of pathogens. Three different media were used: MRS, nutrient broth (NB), and tryptic soy broth containing 2% (v/v) agar. Next, each pathogen was incubated for 24 h at different optimum conditions and diluted to 1×10^8 CFU/mL. Subsequently, the diluted strain was spread using the spot-on-lawn method with a sterile swab. After inoculation, the CFS loading position was punched using a sterile cork borer (5 mm). Without concentration or pretreatment, 40 μL of CFS was loaded in each spot and sealed. Every plate was cultured overnight in a stationary incubator at 37 °C.

2.3. Biochemical experiments

The Bradford assay was utilized for protein quantification. We used bovine serum albumin (BSA) in sterile PBS buffer for the calibration curve employing Bio-Rad Bradford dye reagent. The protein samples were loaded in the cuvette, and OD_{595} was measured in a UV-1280 spectrophotometer.

2.4. Purification and analysis for unknown antimicrobial substances

We performed liquid–liquid extraction using ethyl acetate (Duksan Pure Chemical, Ansan, Korea). Specifically, CFS from *Weissella* sp. SNUL2 and ethyl acetate were poured at a 1:1 ratio into a separatory funnel with the stopcock submerged. Next, the mixture was mixed by gentle agitation of the separatory funnel for 20 s. The cock was briefly opened thrice to provide ventilation, and the separatory funnel was placed upright in the ring clamp to allow the layers to separate. After separation, the bottom layer was drained into a clean Erlenmeyer flask, and the top layer was poured out to minimize the re-mixing of the solution. Subsequently, each layer was concentrated using a vacuum rotary evaporator at 78 °C to evaporate ethyl acetate.

Gel filtration was conducted to further refine the antibiotic substances in *Weissella* sp. SNUL2 CFS. Sephadex G-50 fine (Cytiva, Marlborough, MA, USA) was used as a gel filtration resin. First, the Sephadex G-50 column was swollen in 30 mM Tris-HCl buffer (pH 8.0) for 3 h, and the gel slurry equilibrated in 30 mM Tris-HCl buffer (pH 8.0) was loaded on an 87 × 300 (ø × mm) glass column. The ten times concentrated *Weissella* sp. SNUL2 CFS was loaded onto the top of the gel bed. Next, the elution buffer, 30 mM Tris-HCl buffer (pH 8.0), was passed through the column at a flow rate of 0.5 mL/min, and 4 mL fractions were collected. For each fraction, absorbance was measured at 280 nm to determine the protein concentration. The Kirby–Bauer disk diffusion assay was conducted for each fraction using *S. enterica* ATCC 13076. A spot was created using a sterile cork borer before addition of 40 μL of gel filtrated CFS (2 ×) to the nutrient agar plate.

After gel filtration was complete, ion exchange chromatography was performed using an Amberlite CG50 column (Sigma-Aldrich, St. Louis, MO, USA). The resin was swollen in 5 mM sodium phosphate buffer (pH 6.0) prepared with NaH_2PO_4 and Na_2HPO_4 at an 87.7:12.3 ratio for 24 h. Subsequently, the resin was packed in an Econo-Pac Disposable chromatography column (Bio-rad, Hercules, CA, USA) (1.5 × 12 cm) and equilibrated for another 3 h. When the gel bed was ready, the sample was loaded. The sample comprised the fraction exhibiting antimicrobial activity after the gel filtration and concentrated to four times the initial concentration using a SpeedVac. When the loaded sample was passed through the column, 5 × column volume (CV) of 5 mM sodium phosphate buffer (pH 6.0) was added to the column to clear proteins that were not bound to the column. Next, to elute the desired fraction, the six types of elution buffer with appropriate NaCl concentrations and 5 mM sodium phosphate buffer were loaded. Each fraction was collected by flowing the elution buffer (5 × CV) through the column. The Kirby–Bauer disk diffusion analysis using ion-exchanged CFS was performed similarly to CFS separated with gel filtration. Subsequently, the molecular weight of the antimicrobial substance was determined via SDS-PAGE according to the method reported by Laemmli [37]. Color Marker Ultra-low Range (Sigma-Aldrich, St. Louis, MO, USA) was used as a protein marker.

In-gel digestion was performed to obtain the proteins from each protein band after SDS-PAGE. The destaining procedure was performed for 3 h using a solution comprising 20% (v/v) ethanol and 5% (v/v) acetic acid. Next, the bands were cut using a razor blade and washed with 500 μL of 50% (v/v) acetonitrile (ACN) and 0.1% (v/v) trifluoroacetic acid solution (TFA). Subsequently, 100 mM ammonium bicarbonate (NH_4HCO_3) and 50% (v/v) ACN were added to the bands; destaining was repeated twice at 37 °C for 45 min. Dehydration was then performed with 100 μL of 100% (v/v) ACN for 5 min at 25 °C. Next, the bands were dried using a SpeedVac at room temperature for 10–15 min. For in-gel digestion, trypsin was dissolved in 50 mM acetic acid to obtain a 1 mg/mL solution that was diluted to 0.02 mg/mL with 40 mM NH_4HCO_3 and 10% (v/v) ACN solution. The gel was incubated at room temperature for 1 h in 20 μL of trypsin solution. Next, the re-hydrated gel was incubated at 37 °C for 24 h in a digestion buffer comprising 40 mM ammonium bicarbonate and 10% (v/v) ACN. The cultured gel was then extracted by vortexing in distilled water, placed in 50 μL of 50% ACN and 5% TFA, incubated for 1 h, and extracted again. Finally, the extracted liquid was stored in different tubes and dried for 2–4 h using a SpeedVac.

2.5. Mass spectrometry

LC-MS/MS analysis was performed using nano ACQUITY UPLC and a linear trap quadrupole (LTQ)-orbitrap-mass spectrometer (Thermo Electron, San Jose, CA). The BEH C-18 1.7 μm, 100 μm × 100 mm column (Waters, Milford, MA, USA) was used. Additionally, the mobile phase A for the LC separation was 0.1% formic acid in deionized water, and the mobile phase B was 0.1% formic acid in ACN. The chromatography gradient was set to achieve a linear increase from 10% B to 40% B for 21 min and 40% B to 95% B for 10 min at a flow rate of 0.5 μL/min. Mass spectra were collected using data-dependent acquisition with a complete mass scan (300–2000 *m/z*) followed by MS/MS scans for tandem mass spectrometry. Each MS/MS scan acquired was an average of one microscan on the LTQ. Furthermore, the temperature of the ion transfer tube was controlled at 275 °C, and the spray was controlled at 2.0 kV. For MS/MS, the normalized collision energy was set at 35%. Individual MS/MS spectra were processed using the SEQUEST software (Thermo Quest, San Jose, CA, USA), and the resulting peaks were used to query an internal database with the MASCOT program (Matrix Science Ltd., London, UK). For MS analysis, we applied carbamidomethyl (C), deamidated (NQ), and oxidation (M) modifications with a peptide mass tolerance of 10 ppm. Additionally, the MS/MS ion mass tolerance was 0.8 Da, the allowance of missed cleavage was 2, and charge states (+2, +3) were considered for data analysis. Finally, we considered only significant hits as defined by MASCOT probability analysis.

3. Results and discussion

3.1. Strains from Korean fermentation foods

We conducted 16S rRNA sequencing on samples from various fermented foods obtained from four regions in Gangwon Province, identifying and collecting beneficial bacteria from several genera, including *Pediococcus*, *Weissella*, *Lactobacillus*, *Enterococcus*, and *Bacillus*. Among these, we focused on *Weissella* sp. strains based on the results of a prior study that highlighted the prevalence of *Weissella* sp. strains in the fermentation of Alaskan pollock Sikhae, showcasing their inhibitory effects against harmful species [15]. Subsequently, we determined that *Weissella* sp. strains exhibited superior inhibitory effects on *E. coli*, confirmed through fermentation comparisons and involving thousands of beneficial bacteria collected from various fermented foods.

3.2. Microplate reader antibacterial assessment and flask culture confirmation

Antibacterial testing was performed to assess the degree of inhibition elicited by *Weissella* sp. SNUL2 CFS—obtained by filtration after cultivating *Weissella* sp. SNUL2 for 24 h—following treatment of 60 Gram-negative and Gram-positive bacteria (Table 1). Fifty-nine out of 60 indicator strains were inhibited by *Weissella* sp. SNUL2 CFS, while 13 were inhibited by *P. acidilactici* K10 CFS. Therefore, *Weissella* sp. SNUL2 CFS exhibited superior broad-spectrum antimicrobial properties compared with the positive control *P. acidilactici* K10 CFS. These results were validated in flasks by cultivating selected strains with prominent outcomes. The CFS from *Weissella* sp. SNUL2 was used, and the indicator strain was *E. coli* top 10. Tests were performed under three different pH conditions (Fig. 1A–C). At pH 4, the control group exhibited a log phase followed by a stationary phase, registering an OD_{600} value of 0.77 (Fig. 1A). In contrast, the *Weissella* sp. SNUL2 CFS-treated group showed no growth, with an OD_{600} value of 0.12, indicating complete inhibition. Similarly, at pH 5 and 6, the CFS-treated group exhibited superior growth inhibition compared with the control group (Fig. 1B and C). However, the inhibitory effect was more pronounced at lower pH values, suggesting that the acidic condition may contribute to the suppression of *E. coli* growth.

Prior research suggests that antimicrobial proteins exhibit maximum activity under acidic conditions [38]. Importantly, *Weissella*

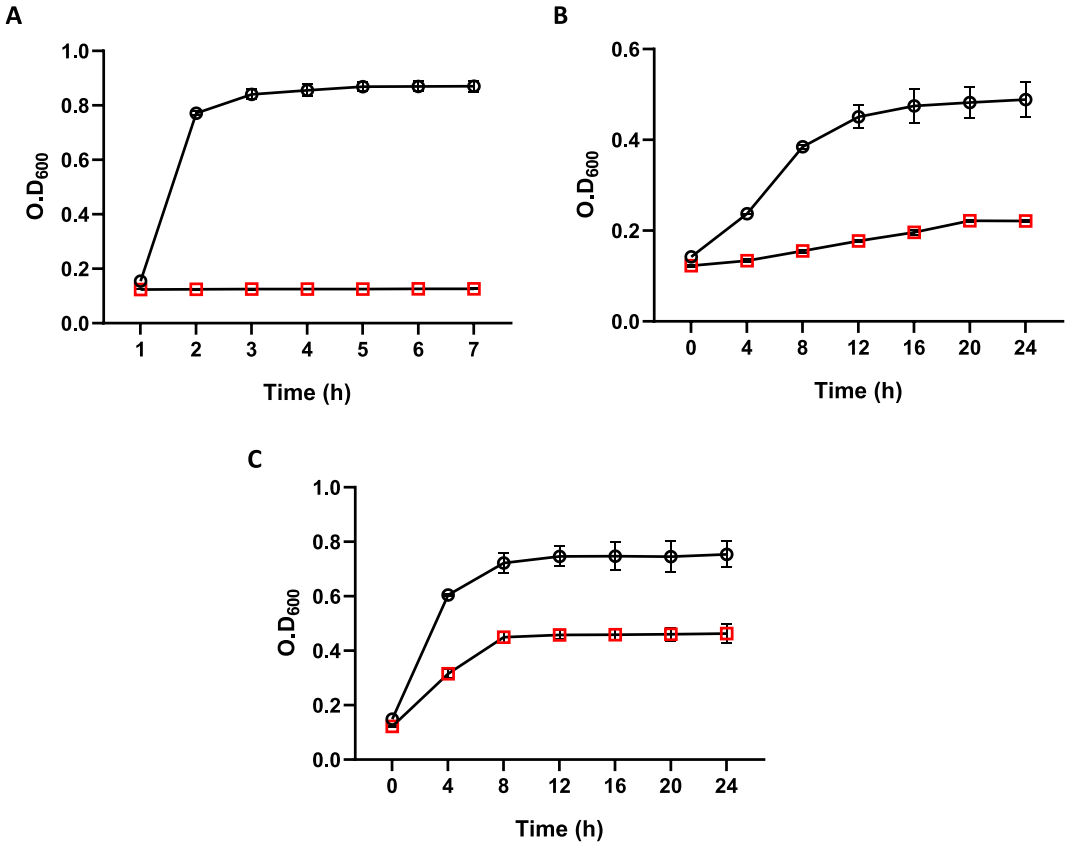

Fig. 1. Growth curves of *E. coli* cultured with the cell-free supernatant (CFS) of *Weissella* sp. SNUL2 at pH 4 (A), pH 5 (B), and pH 6 (C) are represented by open red squares, while the control group, *E. coli* cultured without CFS treatment, is depicted by open black circles. Each value represents the average of individual fermentations; error bars indicate standard deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

strains can create an acidic environment in the gut, suppressing competitor bacteria. Hence, the use of live probiotic *Weissella* strains may result in the localized production of antimicrobial substances within the gut, potentially enhancing long-term effectiveness. From an alternative perspective, this emphasizes the significance of antibacterial substances that can operate effectively without relying on an acidic environment, preventing the growth of *E. coli* strains to some extent even at neutral pH. This potential positive impact may broaden the application of *Weissella* sp. SNUL2 CFS as a postbiotic.

3.3. Analysis of Weissella sp. SNUL2 CFS for inhibitory agents against selected pathogens

Weissella sp. is classified as a LAB and is present in Korean traditional food, in particular, Kimchi, Jeotgal, and Sikhae [39–41]. The genus *Weissella* was proposed in 1993 by Collins and colleagues [42]. Compared with other LABs, minimal information is available regarding bacteriocins derived from the *Weissella* genus. Hence, an antimicrobial test was conducted with six BSL grade 2 pathogens. To eliminate the antimicrobial properties caused by lactic acid, in one group, the CFS from *Weissella* sp. SNUL2 was neutralized at pH 6, while that of another was adjusted to pH 4 (Fig. 2A–F). The growth of *S. enterica* ATCC 13076, *B. cereus* ATCC 14579, *S. epidermidis* KCTC 1917, *S. enterica* ATCC 43971, *S. aureus* ATCC 25923, and *V. fluvialis* KCTC 2473 treated with CFS at pH 6 was inhibited by 29.53%, 28%, 25.43%, 29.8%, 13.9%, and 91.16% compared with the respective controls, respectively (Fig. 2A–F). Similarly, the group treated with CFS at pH 4 showed clear inhibition compared with the control group that was not treated with CFS; the inhibition rates were 89.19%, 97.47%, 95.4%, 90.13%, 94.25%, and 80.96%, respectively (Fig. 2A–F). Duramycin elicited significant inhibitory activity against *S. enterica* ATCC 13076, *B. cereus* ATCC 14579, and *V. fluvialis* KCTC 2473, similar to the *Weissella* sp. SNUL2 CFS (pH 4) group (Fig. 2A, B, and F). However, *S. epidermidis* KCTC 1917, *S. enterica* ATCC 43971, and *S. aureus* ATCC 25923 were not suppressed by duramycin treatment, with similar growth as observed in the control group (Fig. 2C–E). This demonstrates the superior antimicrobial ability of the *Weissella* sp. SNUL2 CFS compared with duramycin. Specifically, supplementation with *Weissella* sp. SNUL2 CFS showed comparable antimicrobial activity with duramycin against *S. enterica* ATCC 13076, *B. cereus* ATCC 14579, and *V. fluvialis* KCTC 2473 strains and superior activity against the *S. epidermidis* KCTC 1917, *S. enterica* ATCC 43971 and *S. aureus* ATCC 25923 strains.

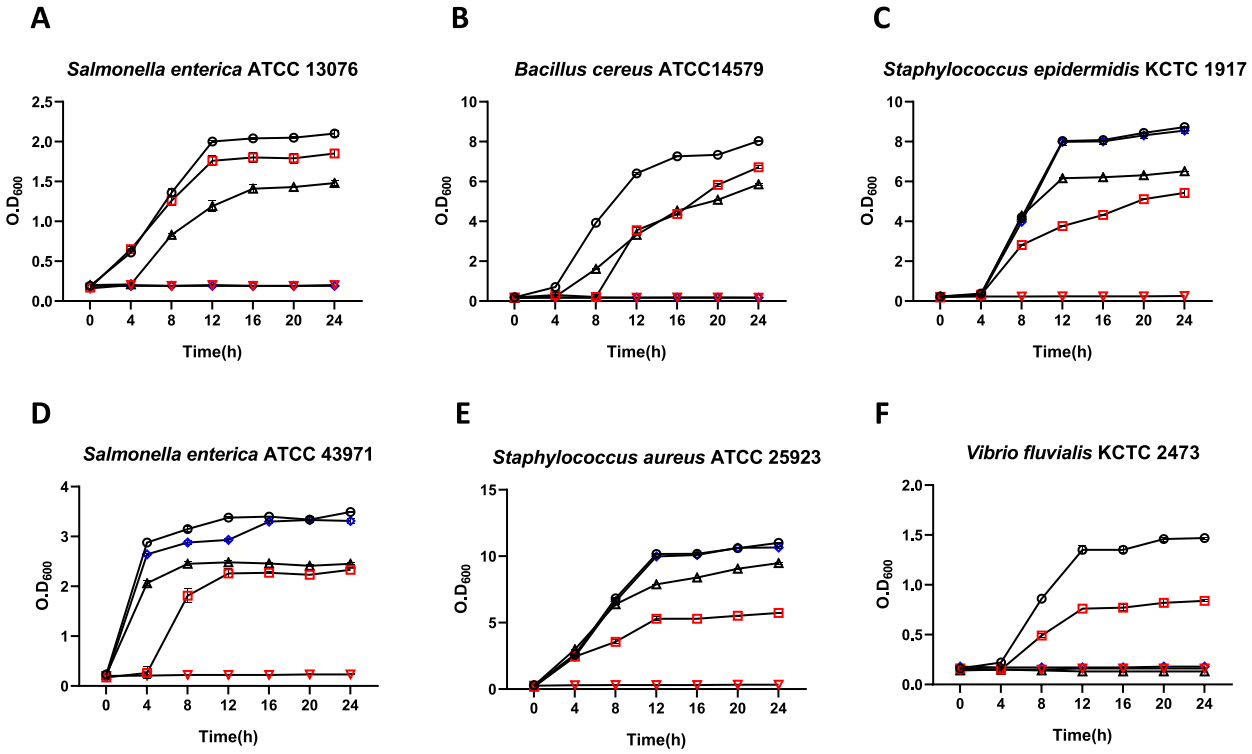

Fig. 2. Antibacterial activities of CFS obtained from *Weissella* sp. SNUL2 against (A) *Salmonella enterica* ATCC 13076, (B) *Bacillus cereus* ATCC14579, (C) *Staphylococcus epidermidis* KCTC 1917, (D) *Salmonella enterica* ATCC 43971, (E) *Staphylococcus aureus* ATCC 25923, and (F) *Vibrio fluvialis* KCTC2473. Open black circles: Control (pH 6), open black triangles: + L2 CFS (pH 6), open red squares: Control (pH 4), open inverted red triangles: + L2 CFS (pH 4), open blue diamonds: + duramycin. All values indicate the average of each fermentation, and error bars depict standard deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Influence of Weissella sp. SNUL2 CFS on beneficial microorganisms

Additionally, four beneficial microbes served as indicator strains (Fig. 3), and growth inhibition was assessed following *Weissella* sp. SNUL2 CFS treatment. Nisin, the most common commercial bacteriocin, is renowned for its inhibitory properties against a broad range of Gram-positive bacteria. The growth patterns of the four beneficial microbes were similar in the control and *Weissella* sp. SNUL2 CFS-treatment groups. In contrast, nisin treatment completely inhibited the growth of all beneficial bacteria (Fig. 3A–D). Meanwhile, treatment with the *Weissella* sp. SNUL2 CFS at pH 4 inhibited bacterial growth substantially less than nisin. These results indicate that the *Weissella* sp. SNUL2 CFS had an insignificant effect on the growth of the beneficial microbes, even under acidic media conditions (Fig. 3).

More notably, *Weissella* sp. SNUL2 CFS inhibited certain *Enterococcus* spp. despite their typical ability to thrive in acidic conditions (Table 1). This suggests that factors other than acidity likely contribute to the selective inhibition elicited by *Weissella* sp. SNUL2 CFS against *Enterococcus* strains. The potential inhibitory ability of *Weissella* sp. SNUL2 CFS against *Enterococcus* strains may prove useful, as *Enterococcus faecium* and *Enterococcus faecalis*, which are often beneficial gut microbes, can also evolve into multidrug-resistant strains, such as vancomycin-resistant enterococci (VRE) [43]. Thus, given its ability to selectively inhibit harmful microorganisms, *Weissella* sp. SNUL2 may be advantageous as a probiotic for modulating gut bacterial composition.

3.5. Antimicrobial activity and MIC evaluation of Weissella sp. SNUL2 CFS against selected pathogens

Six indicator strains (*S. enterica* ATCC 13076, *B. cereus* ATCC 14579, *S. epidermidis* KCTC 1917, *S. enterica* ATCC 43971, *S. aureus* ATCC 25923, and *V. fluvialis* KCTC 2473) were cultured overnight with two antimicrobial agents, *Weissella* sp. SNUL2 CFS and ampicillin, using the microdilution assay (Fig. 4A). Ampicillin exhibited good MIC against most indicator bacteria (Table S2). However, *Weissella* sp. SNUL2 CFS had markedly lower MICs than ampicillin against *Bacillus cereus* and recorded a similar MIC against *Vibrio fluvialis* (Table S2). Since *Weissella* sp. SNUL2 CFS is the extract of the cell fermentation, the antimicrobial activity of *Weissella* sp. SNUL2 could be improved through partial or complete purification of the antimicrobial agents from *Weissella* sp. SNUL2.

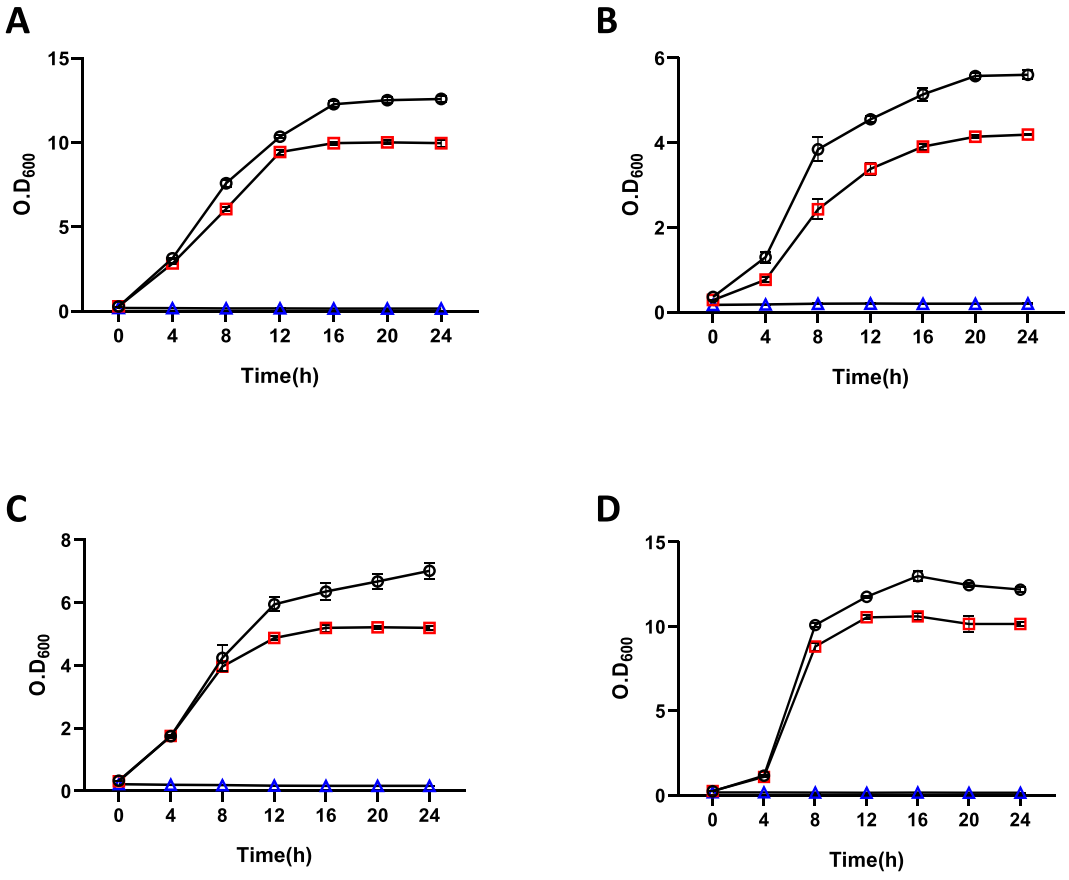

Fig. 3. Antibacterial activities of CFS obtained from *Weissella* sp. SNUL2 against beneficial microbes such as (A) *Lactiplantibacillus plantarum* KCTC3108, (B) *Leuconostoc mesenteroides* KCTC3100, (C) *Latilactobacillus sakei* KCTC3598, and (D) *Lacticaseibacillus casei* ATCC393. Open black circles: Control, open red squares: + L2 CFS, open blue triangles: + Nisin. All values indicate the average of each fermentation, and error bars depict standard deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.6. Time-kill assay and characterization of antibacterial substances in Weissella sp. SNUL2 CFS

We conducted a time-kill assay to evaluate the dynamics of antibiotic substance production during *Weissella* sp. SNUL2 CFS incubation. Six CFSs cultured for different durations (4, 8, 12, 16, 20, and 24 h) were employed. After 4 h of incubation, *Weissella* sp. SNUL2 CFS inhibited *E. coli* growth by 31.5% and 74% after 8 h, indicating nearly complete suppression (Fig. 4B). Notably, complete inhibition of *E. coli* growth was sustained beyond 8 h of incubation, suggesting that *Weissella* sp. SNUL2 initiates the synthesis and production of antibacterial substances after 4 h, reaching maximal activity after 8 h.

Given the observed increase and maximization of antimicrobial activity after 8 h of incubation, we hypothesized that the antibacterial substances might be proteins or metabolites synthesized during incubation. To explore the nature of the antibiotic substances, we treated the CFS with proteinases, namely trypsin and pepsin [44]. After 24 h of incubation, the control group had an absorbance of 0.623 at OD_{600}, while the *Weissella* sp. SNUL2 CFS group had an absorbance of 0.127, indicating complete inhibition of growth (Fig. 4C). Meanwhile, following supplementation with trypsin or pepsin, *E. coli* treated with *Weissella* sp. SNUL2 CFS exhibited complete growth restoration. This indicates that the proteolytic enzymes neutralized the antibacterial activity of *Weissella* sp. SNUL2 CFS, suggesting that the antibacterial substances produced by *Weissella* sp. SNUL2 are likely proteins.

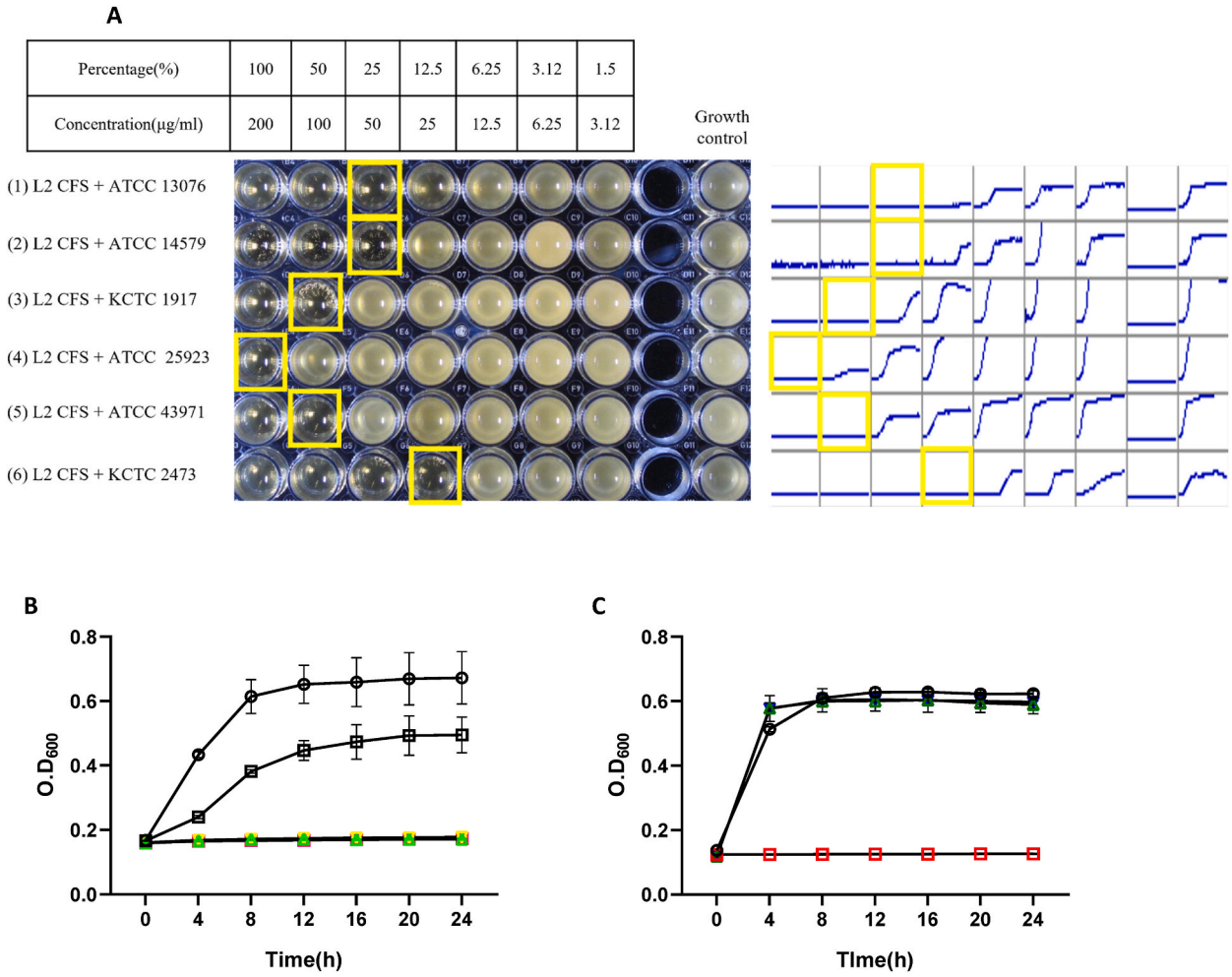

Fig. 4. Characterization of the antibacterial substances from *Weissella* sp. SNUL2 CFS. (A) Broth microdilution assay of six indicator strains added to *Weissella* sp. SNUL2 CFS and its growth curve. Yellow boxes represent the minimum inhibition concentration of *Weissella* sp. SNUL2 CFS. (B) Time-kill assay of *E. coli* using *Weissella* sp. SNUL2 CFS incubated at different times. Open black circles: Control, open black squares: *E. coli* with CFS incubated for 4 h, open green triangles: *E. coli* with CFS incubated for 8 h, open inverted yellow triangles: *E. coli* with CFS incubated for 12 h, closed blue diamonds: *E. coli* with CFS incubated for 16 h; black x symbols: *E. coli* with CFS incubated for 20 h; open red squares: *E. coli* with CFS incubated for 24 h. (C) Growth curve for *E. coli* co-cultured with CFS from *Weissella* sp. SNUL2 with two proteinases. Open black circles: Control, open red squares: *E. coli* with *Weissella* sp. SNUL2 CFS, open green triangles: *E. coli* with *Weissella* sp. SNUL2 CFS after trypsin treatment, open inverted blue triangles: *E. coli* with *Weissella* sp. SNUL2 CFS after pepsin treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.7. Inhibition zone analysis of selected pathogens treated with Weissella sp. SNUL2 CFS

We also examined the inhibition zone for pathogens treated with the *Weissella* sp. SNUL2 CFS using the Kirby–Bauer disk diffusion test (Fig. 5A–F and Table S3). Inhibition zones (mm) were observed for three indicator strains (*S. enterica* ATCC 13076, *B. cereus* ATCC 14579, and *V. fluvialis* KCTC 2473) treated with the CFS (Fig. 5A–C). Although the antibiotic substance in the CFS was not purified or concentrated, the inhibition zones (mm) of the three indicator strains were 14.00 ± 0.00, 13.00 ± 0.82, and 12.50 ± 0.70, respectively. However, no inhibition was observed for the remaining three indicator strains (*S. aureus* ATCC 25923, *S. enterica* ATCC 43971, and *S. epidermidis* KCTC 1917) (Fig. 5D and E). Moreover, the *S. enterica* ATCC 13076, *B. cereus* ATCC 14579, and *V. fluvialis* KCTC 2473 strains showed significant inhibition even with unpurified *Weissella* sp. SNUL2 CFS in the Kirby–Bauer test. This suggests that *Weissella* sp. SNUL2 CFS elicits a significant level of inhibitory effect against certain pathogenic strains.

3.8. Partial purification of the antibiotic substances from CFS

Our result indicated that the antibacterial activity of *Weissella* sp. SNUL2 CFS was due to proteins, thus, we sought to purify and identify the specific proteins via fractionation and LC-MS/MS analysis. Ethyl acetate extraction was used to extract antibiotic substances based on its selective separation of immiscible aqueous and organic solutions (Fig. 6A). Overall, 100 mL of the CFS was extracted and assessed using the Kirby–Bauer disk diffusion assay to confirm the antibacterial activity. Inhibition zones were not observed in the organic layer-loaded plate. However, an inhibition zone (17 ± 0.00 mm) was detected in the aqueous layer-loaded plate (Fig. 6B center); the inhibition zone on the plate loaded by a two-times concentrated aqueous layer was 25 ± 0.00 nm (Fig. 6B right). These results suggest that the antibiotic substances were in the aqueous layer.

Gel filtration was conducted to partially purify antibiotic substances from the CFS. Overall, 23 fractions were collected, of which two (No. 14 and 15) exhibited inhibition zones against the indicator strain (22 mm for both; Fig. 6C). According to the OD_{280}, fractions No. 7 and 11 had the highest protein concentrations yet did not elicit inhibitory activity.

Ion exchange chromatography was conducted sequentially to purify the obtained fractions based on ion strength characteristics. The fraction eluted using 5 mM sodium phosphate buffer +200 mM NaCl showed a 16.15 ± 0.21 mm inhibition zone, and the fraction eluted using 5 mM sodium phosphate buffer +400 mM NaCl showed a 17.50 ± 0.70 mm inhibition zone against the indicator strain (Fig. 6D). In the fractions eluted using other elution buffers, no inhibition zone (mm) was observed. These results indicate that the desired antibiotic substances were eluted under 200 mM and 400 mM NaCl.

3.9. LC-MS/MS analysis and identification of potential antibacterial proteins and implications

After a series of purification steps with gel filtration and ion-exchange chromatography, the partially purified *Weissella* sp. SNUL2 CFS was loaded onto the SDS-PAGE for further LC-MS/MS analysis [37]. Three clear bands were formed in lane 2 (whole cell extracts), among which one (red arrow pointed) formed in lane 3 (partially purified CFS; Fig. S1). We excised the band from lane 3 for LC-MS/MS analysis with a nano ACQUITY UPLC and LTQ-orbitrap-mass spectrometer. The individual MS/MS spectra were analyzed using the

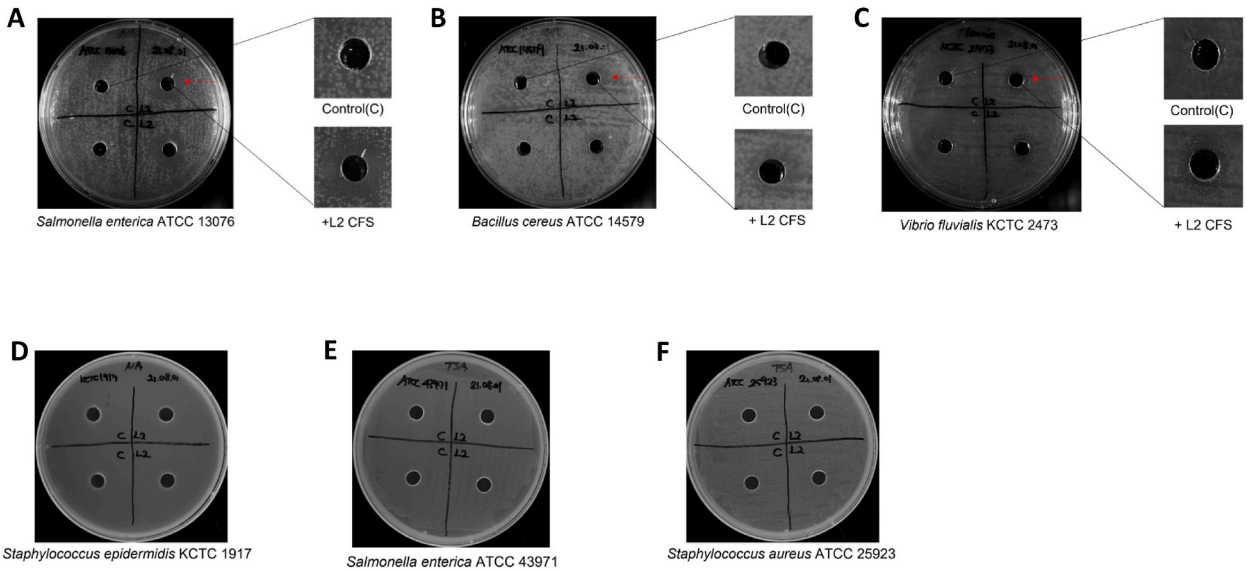

Fig. 5. Inhibition zones of pathogens treated with CFS from *Weissella* sp. SNUL2 using Kirby–Bauer disk diffusion test. Inhibition zones are observed for (A) *Salmonella enterica* ATCC 13076, (B) *Bacillus cereus* ATCC 14579, (C) *Vibrio fluvialis* KCTC 2473, (D) *Staphylococcus epidermidis* KCTC 1917, (E) *Salmonella enterica* ATCC 43971, and (F) *Staphylococcus aureus* ATCC 25923 when treated with CFS from *Weissella* sp. SNUL2. Control (C): Pathogens cultured without CFS; + L2 CFS: Pathogens cultured with CFS from *Weissella* sp. SNUL2.

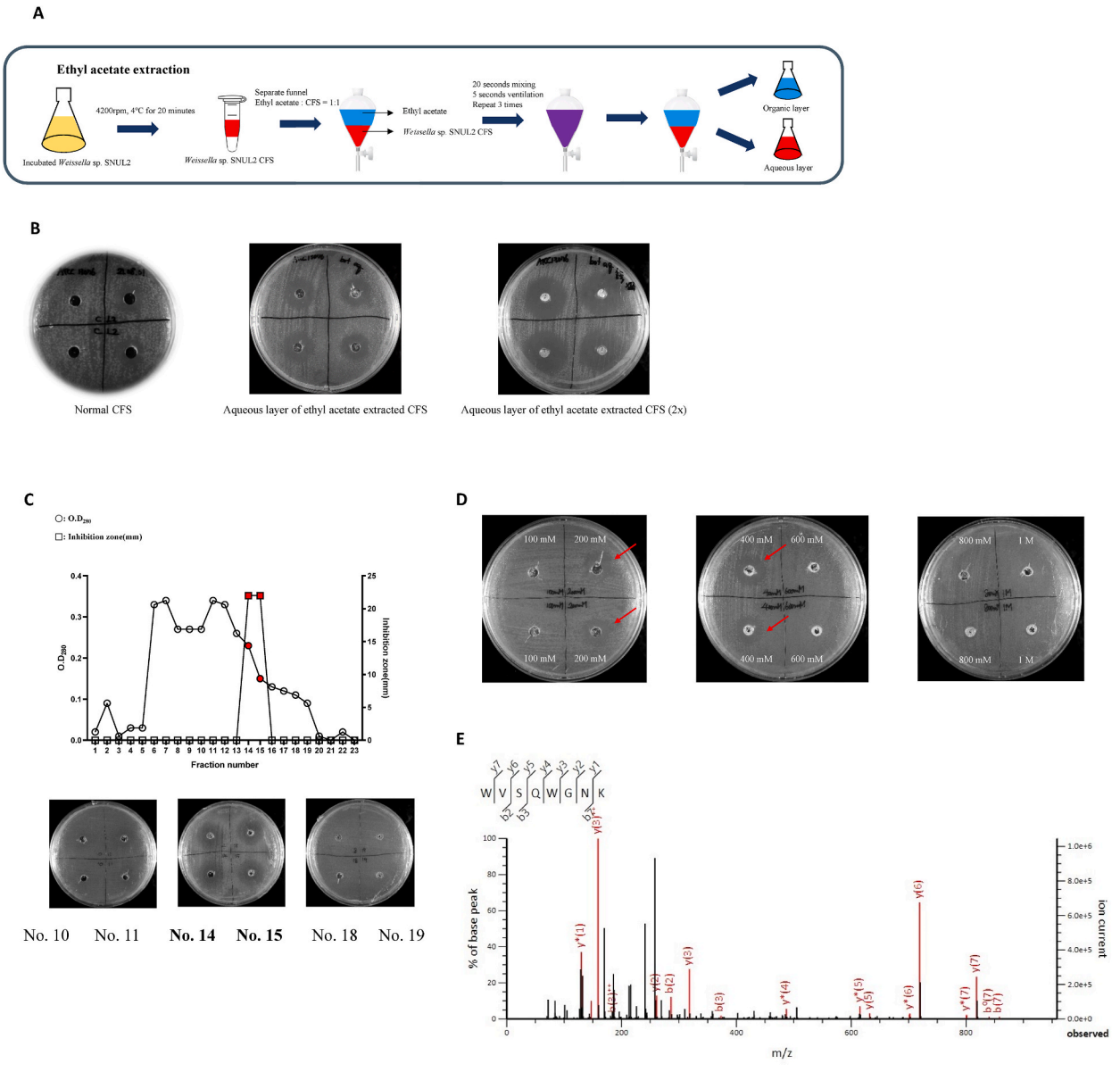

Fig. 6. Purification and identification of the antibiotic substances from *Weissella* sp. SNUL2 CFS (A) Experimental design for the ethyl acetate extraction of *Weissella* sp. SNUL2 CFS. (B) Inhibition zone (mm) created by three different CFSs from *Weissella* sp. SNUL2 following treatment of indicator pathogens. Normal CFS (left), aqueous layer of ethyl acetate extracted CFS (center), aqueous layer of ethyl acetate extracted CFS (2 ×, right). (C) Upper panel: gel filtration elution profile of partially purified antibiotic substances from a Sephadex G-50 column equilibrated with 30 mM Tris-HCl buffer (pH 8.0). Lower panel: inhibition zone (mm) against pathogens treated fraction from partially purified antimicrobial substances from *Weissella* sp. SNUL2 CFS by Kirby-Bauer disk diffusion test. (D) Inhibition zone (mm) of ion-exchanged CFS from *Weissella* sp. SNUL2 eluted according to the concentration gradient of the elution buffer by ion-exchange chromatography. To elute the desired fraction, six types of elution buffers were used according to the concentration gradient of NaCl: 5 mM sodium phosphate buffer +100 mM NaCl, 5 mM sodium phosphate buffer +200 mM NaCl, 5 mM sodium phosphate buffer +400 mM NaCl, 5 mM sodium phosphate buffer +600 mM NaCl, 5 mM sodium phosphate buffer +800 mM NaCl, and 5 mM sodium phosphate buffer +1 M NaCl. The red arrow indicates elution buffers with the strong inhibition zone (mm) of each agar plate. (E) An example of MS/MS fragmentation of WVSQWGNK found in L2_1_01888. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

SEQUEST software (Fig. 6E), and the generated peak list was used to query the in-house database using the MASCOT program. According to the MASCOT score histogram, scores >14 indicate identity or extensive homology ($P < 0.05$). Sixty-seven peptides were identified, with 21 scoring >100 (Table 2). L2_1_01888, which recorded the highest score, is a C39 family protein containing bacteriocin-processing endopeptidases. Notably, the double glycine bond of conserved leader peptides from the precursors of various bacteriocins is cleaved by the C39 family peptidase [45]. Five C39 family proteins were identified: L2_1_1888, L2_1_1932, L2_1_1872,

Table 2
Peptide summary report after LC-MS/MS analysis interpreted by MASCOT score histogram.

Locus_Tag	NCBI BLAST	Score	Mass	Sequence	*m/z*
L2_1_01888	C39 family protein	10138	72119	K.WVSQWGNK.Y	502.7474
				K.WYDFGSNK.T	508.7249
				K.RVTADPQFK.N	531.2953
				K.TYDLTNNLK.R	541.7686
				K.SFGIPVLDWR.E	595.3190
				K.TYDLTNNLKR.V	619.8198
				R.EGGYKVVTDANR.Q	436.8877
				K.LSENEHDLQVK.F	656.8187
				R.QYATNNALASGFK.N	682.8424
				R.WFENGKPYTGFR.L	751.8536
				R.QENKWVSQWGNK.Y	502.2417
				K.LVNGKWYDFGSNK.T	764.3759
				R.FYMGTYYWFEK.G	775.8341
				K.FTSNGWQTDESGNR.Q	800.3328
				K.TYNFGTNHTFFLR.-	809.8786
				K.TYYVATDGTTQEGIK.L	823.8926
				K.WYDFGSNKTYYAR.N	557.5883
				R.TAWNLKYYVDSEGR.A	567.9425
				K.SFGIPVLDWREGGYK.	575.2963
				R.MYMGAYYYFINGVR.Q	890.8861
				R.NFTQSGYLNTDQGWR.W	893.9062
				K.WVSQWGNKYYVGSDGR.S	634.6332
				K.NINGQGYFFNPSNNGLLK.G	999.4841
				K.IDGKTYNFGTNHTFFLR.-	508.5054
				K.GQAGYTFNQLADALTEVYK.S	1045.0070
				K.ASGYVSTPDGWLWIENGKR.Y	712.6840
				K.TYYVATDGTTQEGIKLVNGK.W	1080.0415
				K.LVNGKWYDFGSNKTYYAR.N	546.2710
				K.LVIYGVLPIPSYMGGDNLNVK.G	1139.6065
				R.GKASGYVSTPDGWLWIENGKR.Y	581.2922
				R.QENKWVSQWGNKYYVGSDGR.S	601.0353
				K.GKNQLAMAGGKTYYVATDGTTQEGIK.L	680.3351
				R.AVQGDGYNVDGTYYNFSHDGTFFLR.G	948.4228
				K.FTSNGWQTDESGNRQYATNNALASGFK.N	989.7750
				K.NAESLVLEIGVNDLNYSDNNLGYVQQR.L	1013.1620
				R.QQTLGDHEVHPTQETYANMAELMAQWMVDNSK.L	934.4135
				K. QNYPTLLASIIKPESMHAGYASSGSQISGNQNGPGDK.T	959.4570
L2_1_00882	Iron Transport-associated domain protein	1038	95518	R.FFNPTAK.L	412.7163
				R.FVTFEIPTK.D	541.2975
				K.NDITFNIPTK.D	581.8068
				K.IASFTSVPMSPSK.G	684.3440
				K.LVNNYYESTYR.M	711.3340
				K.TGTNNASAMNNYLK.T	749.8461
				K.LAVKGDTTDVTLSFK.D	532.2913
				K.SVTLASFTSVPMSPTK.G	834.9249
				K.TGTQEDSAMMSYVESK.G	890.3676
				K.NADGSYYGYLTTHTPK.L	894.4111
				K.GQSQMDMMTGFTVAGHTATK.S	716.3057
				R.FVTFEIPTKDLSPDDTNVAK.I	746.3785
L2_1_01908	Peptidoglycan DL-endopeptidase CwlO precursor	898	59707	R.NSWSIAAR.Q	453.2245
				R.YLFRNGVK.Q	499.2742
				R.QFDCSSFVR.W	573.2527
				K.FQGTYYMFNLR.D	728.3381
				R.WGTWYMYDETGR.C	782.8265
				R.TYLKHTNVYEPAR.W	531.2760
				R.AYAVPWYDIVDGVVR.R	861.9419
				R.EVTPSAQATHQSAAVVK.K	862.4467
				K.FQGTYYMFNLRDGR.M	595.2756
				K.GVQKFQGTYYMFNLR.D	623.3070
				R.VLTDVQPWAGGYYTFDAR.T	1029.9970
				K.ATNEAVWENGNEYWADGGGVVR.T	1198.0302
L2_1_01894	Chaperone protein DnaK	522	65296	R.GVPQIEVK.F	435.2559
				K.IAGLEVER.I	443.7509
				R.TTPSAVSFK.N	469.2510
				R.IPAVQESVK.G	485.7793
				R.FQLTDIPAAPR.G	614.8344
				K.DAGKIAGLEVER.I	419.8960

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Table 2 (*continued*)

Locus_Tag	NCBI BLAST	Score	Mass	Sequence	*m/z*
				R.AQFNQLTADLVK.R	674.3655
				K.LIDRNTTIPTSK.S	453.5898
				K.YAEDYLGETVDK.A	701.8188
				R.QAITNPDTILSIK.S	707.3985
				K.KYAEDYLGETVDK.A	510.9122
				R.TTPSAVSFKNGETQVGDTAK.R	680.3336
				R.TTPSAVSFKNGETQVGDTAKR.Q	549.5264
				K.SQVFSTAADNQPAVDIHVLQGER.S	828.0805
L2_1_01871	Peptidoglycan endopeptidase LytF precursor	398	37152	K.WENAWGMK.Y	511.2280
				K.MLGLPYAWGGK.T	596.8085
				R.QDSGWREAWGMK.Y	484.2209
				R.GKTSGYTDAGEGWK.W	486.2260
				R.QQNKWENAWGMK.Y	507.2390
				K.TWSGVDCAGYVALVR.S	551.9384
				R.EAWGMKYYTDNQGR.A	578.9197
				K.NVSGGTHYFGNDGTFFER.S	668.9615
				K.TSGYTDAGEGWKWYEGGQK.F	707.3107
L2_1_00448	Internalin-A precursor	336	86562	K.LTSLNFSK.D	455.2526
				K.TVGVATLMTTK.F	561.3127
				K.LTNLTTLSIGNNR.S	708.8891
				K.VGSNQITDFTPIAK.L	745.8961
				R.QALQSALGADVPLTK.S	756.4193
				K.VTSVPELGNLHQLR.E	521.6246
L2_1_01907	Murein DD-endopeptidase MepS/Murein LD-carboxypeptidase precursor	298	38865	K.YYVDNVGR.A	493.2377
				R.TTYEQAAYLR.A	608.3013
				K.TSGYTDAGQGWK.W	636.2780
				R.QDSGWREAWGMK.Y	484.2209
				K.WYENGQQFTGFR.F	766.8429
				K.SYYFGNDGTFFLR.D	793.8644
L2_1_01932	C39 family protein	240	40773	R.AVQGLQTINNK.K	593.3298
				K.DAYVPVNNSGWR.W	689.3275
				K.YYFGNDGTFYSR.A	745.3209
				K.VKDAYVPVNNSGWR.W	535.6069
				R.AVPGKPTYFSQWDGR.W	570.2835
L2_1_01872	C39 family protein	215	50074	K.YGVTGVIVK.L	468.2795
				K.TGNVTNNALAFR.N	639.8231
				K.SVVMVDDLEDSSTK.T	762.8557
				K.LTEHTTYVNPYAR.Q	522.2599
L2_1_00644	Xylulose-5-phosphate phosphoketolase	212	91993	K.ISNPTIFSR.M	517.7824
				R.YVNVVELHR.L	376.8749
				K.AEDLALPDWK.S	579.2914
				R.IFGPDETMSNR.L	641.7868
				M.AVDFDSKEYLAK.V	462.5662
				K.VMDQAIEDIQAIQK.D	539.9418
				K.GWGGPTHDQSGMPIEDSFR.A	697.3016
L2_1_00917	Methionine-tRNA ligase	203	74784	K.GEPIFPR.V	408.2212
				R.TADALAEVWK.L	552.2883
				R.VAAILLQPALTR.A	633.3950
				K.SHPEFIQPESR.M	442.8838
				R.VAEILEVTEVEK.S	679.8719
				R.VAEILEVTEVEKSNK.L	563.3043
L2_1_00297	Hypothetical protein	175	84321	K.LADAGALDATALK.T	615.3351
				K.ALDAIQLTSDGR.T	630.3304
				K.GNGAALNADVLATVAK.T	743.3972
L2_1_01489	Membrane lipoprotein TmpC precursor	164	39532	K.GGYDYFLSK.T	525.2485
				K.YALVDAQANPK.L	595.3119
				K.TQADFDTNFQQAQAAK.F	892.4127
L2_1_01501	Elongation factor G	141	78049	K.LAEEDPSFR.A	532.2539
				K.TAGAVILEPIMK.V	621.8578
				R.VYTGTLESGSYVLNTSK.G	909.9556
L2_1_02024	C39 family protein	134	36105	K.TYYLGADGR.S	508.2424
				R.QTNTWATEWGK.T	661.3101
				R.AVQGWQTINGIR.Y	671.8610
L2_1_00373	N-acetylmuramoyl-L-alanine amidase domain-containing protein precursor	132	28374	K.AVVLSAGTEYK.V	569.3082
				R.TISAAGIQFVH.-	572.3076
				K.LTPADKQDNNSASDALK.Q	596.6272
L2_1_00626	Neutral endopeptidase	132	70278	R.LQTADWLQPATR.E	700.3677
				K.VVLSQEESAEYAK.L	726.8619
				R.DALGTDPVKPLIAR.Y	489.2822

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Table 2 (*continued*)

Locus_Tag	NCBI BLAST	Score	Mass	Sequence	*m/z*
L2_1_02151	GTP-binding protein TypA/BipA	113	68463	R.MEWLVPSR.G	509.2620
				K.TTLVNELLK.Q	515.8097
				R.IDEPTLQMTFR.T	683.8334
				K.AFEQNLTPIVVVNK.V	786.4402
L2_1_00758	Hypothetical protein	106	68947	R.GLLSAAEYTK.A	526.7820
				-.MNIVLVGAGPR.N	563.8214

L2_1_2024, and L2_1_1847; the scores were 10138, 240, 215, 134, and 44, respectively. Each protein was identified based on the sequences presented in Table 2, and the sum of the MASCOT scores of the peptide sequence served as the corresponding protein score.

The C39 family protein identified in our analysis may not be the direct antibacterial protein. Previous studies have repeatedly reported C39 family proteins' involvement in self-immunity against antibacterial peptides [45,46]. While this protein may contribute to protecting *Weissella* sp. SNUL2 from self-produced antibacterial peptides, it is not likely the primary antibacterial peptide. Additionally, our analysis identified peptidoglycan endopeptidase-related precursors, such as L2_1_01908 and L2_1_01871, and murein endopeptidase-related precursors, such as L2_1_01907 and L2_1_00062. Peptidoglycan—a crucial cell wall polymer—is formed by glycan chains of β-(1–4)-linked-N-acetylglucosamine and N-acetylmuramic acid, cross-linked by short peptide stems [47]. Peptidoglycan maintains cell shape, protects bacteria from turgor pressure, and its endopeptidases regulate peptidoglycan degradation and synthesis [48,49]. Inhibition of this regulation, analogous to the reaction of penicillin against bacterial peptidoglycan, can be crucial for bacterial survival, potentially inhibiting competitors' growth by degrading their peptidoglycan. However, evidence supporting this mechanism is limited.

Among the identified peptides, the N-acetylmuramoyl-L-alanine amidase domain-containing protein precursor (L2_1_00373) displayed a high MASCOT score. Animal homologs of the gene encoding this protein participate in antibacterial immunity by hydrolyzing the lactyl bond between MurNAc and L-Ala in peptidoglycan [50]. The function of this protein aligns with that of the peptidoglycan endopeptidases mentioned earlier. Although further research is necessary, these results suggest that peptidoglycan hydrolysis by peptidases, particularly N-acetylmuramoyl-L-alanine amidase, may be responsible for the broad-spectrum antimicrobial activity of *Weissella* sp. SNUL2. The collective findings underscore the complexity of the antibacterial mechanisms at play, implicating a combination of enzymes. The potential inhibition of competitors' growth by a synergistic combination of enzymes appears to contribute to the broad antimicrobial properties of *Weissella* sp. SNUL2. As multiple enzymes participate in the antibacterial activity, the likelihood of harmful bacteria acquiring resistance to *Weissella* sp. SNUL2 CFS appears challenging.

4. Conclusions

Weissella sp. SNUL2, isolated from traditional Korean fermented food, demonstrates broad-spectrum antimicrobial activity surpassing known probiotics, positioning it as a potential candidate for targeted therapeutic probiotics against gut-related diseases, such as diarrhea and food poisoning. The strain demonstrates heightened antimicrobial activity at low pH and retains some efficacy at neutral pH. Notably, it displays relatively low antimicrobial activity against beneficial bacteria, suggesting potential selectivity in controlling harmful bacteria. Enzymatic tests affirm the proteinaceous nature of the antimicrobial substance, and subsequent partial purification and LC-MS/MS analysis identify proteins, including C39 family proteins, peptidoglycan endopeptidases, and N-acetylmuramoyl-L-alanine amidase domain-containing protein precursor within the antimicrobial active fraction. These findings imply that *Weissella* sp. SNUL2 secretes multiple proteins that collectively contribute to its potent and broad-spectrum antimicrobial properties. The combined action of these proteins is anticipated to hinder the development of resistance by harmful bacteria to *Weissella* sp. SNUL2 CFS. Beyond its antimicrobial prowess, insights from this study lay the groundwork for future research exploring the probiotic and postbiotic potential of *Weissella* sp. SNUL2 in modulating gut bacterial composition. However, it is important to recognize the limitations of this study, which mainly focused on antimicrobial activity experiments, and to emphasize that further development, including human trials, is required for industrialization. Future research should prioritize the purification of antimicrobial peptides and the comprehensive elucidation of their antimicrobial mechanisms.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Jae Won Han: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Nari Lee:** Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Hea Joon Kim:** Visualization, Validation, Software, Investigation. **Sung Jin Moon:** Resources, Conceptualization. **Soo Chan Lee:** Writing – review & editing, Formal analysis, Conceptualization. **Hyo Jin Kim:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

AMR	antimicrobial resistance
LAB	lactic acid bacteria
CFS	cell-free supernatant
SDS-PAGE	sulfate-polyacrylamide gel electrophoresis
MRS	de Man, Rogosa, and Sharp
CFU	colony-forming unit
MIC	minimum inhibitory concentration
CV	column-volume
ACN	acetonitrile
TFA	trifluoroacetic acid
LTQ	linear trap quadrupole

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28481.

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