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Antibacterial and Antibiofilm Activities of *Weissella Cibaria* CHK903 Against *Proteus mirabilis*

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Abstract

This study aimed to identify and characterize lactic acid bacteria strains with antimicrobial activity against *Proteus mirabilis*, a gram-negative bacterium associated with Parkinson's disease, Crohn's disease, and nosocomial infections. Among the 987 lactic acid bacteria strains isolated from various sources, strain no. CHK903 showed high antimicrobial activity against *P. mirabilis*. Phylogenetic tree analysis based on the 16S rRNA gene and scanning electron microscope analysis identified the selected strain as the rod-shaped *Weissella cibaria*. The culture supernatants of *W. cibaria* CHK903 showed antimicrobial activity against some pathogens. Two antimicrobial compounds with molecular weights 189 and 365 Da were partially purified from the culture supernatants of *W. cibaria* CHK903 using Biogel P2 gel permeation column chromatography. The culture supernatants of *W. cibaria* CHK903 also showed significant antibiofilm properties, inhibiting biofilm formation by 90% and removing pre-formed biofilms by 60%. These findings suggest the potential therapeutic use of *W. cibaria* CHK903 as a natural antimicrobial against *P. mirabilis*-related infections.

Keywords: lactic acid bacteria, *Weissella cibaria*, antimicrobial activity, antibiofilm activity, natural antimicrobial

Introduction

Proteus mirabilis, often isolated from the gastrointestinal tract, is a member of *Enterobacteriaceae* (Daly et al., 2016) and a pathogenic species related to human diseases that can cause nosocomial infections. *P. mirabilis* can cause various infections in humans, such as in the skin, wounds, eyes, ears, nose, respiratory tract, and gastrointestinal tract and, in particular, has been reported as a urinary tract infection-causing bacterium (Jacobsen & Shirtliff, 2011).

Recently, interest in the relationship between intestinal imbalance and Parkinson's disease (PD) has been increasing. PD is a progressive neurological disorder that exhibits symptoms such as rigidity, resting tremors, slowness of movements, and postural instability. As a neuropathological characteristic of PD, dopaminergic neurons can be lost in the substantia nigra pars compacta (Hirsch et al., 2005). Reactive nitrogen species play a pathogenic role in the brains of patients with PD by modifying α-synuclein and parkin. Protein tyrosine residues or free tyrosine react with nitrating oxidants and produce 3-nitrotyrosine, a marker for reactive nitrogen species. Reactive nitrogen species induce dopaminergic neuron death (Blanchard-Fillion et al., 2006). Thus, the accumulation of α-synuclein and 3-nitrotyrosine may induce apoptotic cell death. Furthermore, increasing intestinal penetrability, lipopolysaccharide-binding protein in plasma, colonic inflammation, and pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β, were reported as causes of PD (Devos et al., 2013; Kelly et al., 2014; Pellegrini et al., 2016). *P. mirabilis* is also related to Crohn's disease (CD) and can cause inflammation in cells and animal models of colitis in the gut (Manichanh et al., 2006). Commensal flora has various abilities to induce inflammation and protective immune responses (Sartor, 2001). Changes in the composition of intestinal bacteria and the abundance and diversity of reduced microorganisms were observed in patients with CD (Manichanh et al., 2006).

Lactic acid bacteria (LAB) are classified as Gram-positive, spore non-forming, anaerobic, non-motile, and rod- or coccus-shaped bacteria that can be found in various sources. LAB are important in food fermentation as they can ferment carbohydrates and produce lactic acid if supplied into the medium. *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* are representative genera found in a variety of fermented foods (Nettles & Barefoot, 1993). Probiotic LAB have interesting therapeutic properties, such as improving the gut immune system and biological availability of nutrients and reducing lactose intolerance and

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formation of pathogenic biofilms from pathogenic bacteria (Gómez et al., 2016). LAB produce various metabolites from glucose fermentation. Some LAB produce antimicrobial compounds as metabolites, including low-molecular-mass compounds such as organic acids (lactic, acetic, formic, propionic, and butyric acids), ethanol, fatty acids, acetoin, hydrogen peroxide, carbon dioxide, diacetyl, antifungal compound, bacteriocins, and bacteriocin-like high-molecular-mass compounds (Reis et al., 2012; Yang, 2000).

Antibiotics are chemicals that prevent bacterial growth by stopping the cell from dividing or killing bacterial cells. Antibiotic-resistant bacterial infections, such as respiratory infections and skin and tissue infections, are some of the most serious challenges and are now widespread in both healthcare settings and the wider community (Lushniak, 2014). Antibiotic-resistant infections increase patient morbidity and mortality as antibiotic treatments are not effective against them (Weiner et al., 2016). As the incidence of drug-resistant pathogens increases, natural antibacterial agents are attracting attention as an alternative strategy to prevent infectious diseases (Savoia, 2012).

In this study, a *Weissella cibaria* strain with high antimicrobial and antibiofilm activities against *P. mirabilis* was isolated from kimchi. The antimicrobial compounds were partially purified from the culture supernatant of the selected strain, and their antimicrobial activity was evaluated.

Materials and Methods

Bacterial strains and culture media

A total of 987 LAB strains from our laboratory were used. *Proteus mirabilis* KCCM 11381P provided by the Korean Culture Center of Microorganisms (KCCM; Seoul, South Korea) was also used. The LAB strains were cultured in lactobacilli de Man, Rogosa, and Sharpe broth (MRS; BD, Franklin Lakes, NJ, USA), while *P. mirabilis* was cultured in nutrient broth (BD) and brain heart infusion broth (BHI; BD).

Pathogenic strains and LAB strains obtained from our laboratory stock (Korean Culture Collection of Probiotics, KCCP), KCCM, Korean Collection for Type Cultures (KCTC; Jeongup, South Korea), and the American Type Culture Collection (ATCC; Manassas, VA, USA) were also used. *Escherichia coli* ATCC 10536 was cultured in Luria-Bertani broth (BD), *Bacillus cereus* KCCM 11204, *Staphylococcus aureus* KCCP 60043, *Salmonella* Enteritidis ATCC 13076, *Pseudomonas aeruginosa* ATCC 10145, *Salmonella* Typhimurium ATCC 14028, and *Listeria monocytogenes* KCCM 40307 were cultured in nutrient broth, *Weissella paramesenteroides* KCCP 11717 and *Leuconostoc lactis* KCCP 11247 were cultured in MRS broth, and *Candida albicans* KCTC 7270 was cultured in yeast malt broth (BD). All strains were incubated at 37°C.

Screening of lactic acid bacteria with growth inhibition activity against *Proteus mirabilis*

Bacterial culture and supernatant preparation

The LAB strains were cultured on MRS agar plates and used to prepare the culture supernatant. After incubating a single colony in 1 mL of MRS at 37°C for 20 h, the culture supernatant was collected by centrifugation at 14,000×*g* for 5 min to obtain the 1× supernatant. The prepared culture supernatant was used to examine the growth inhibition activity against *P. mirabilis*.

Measurement of antimicrobial activity

Antimicrobial activity was measured using a modified version of the paper disc test, also known as the disc diffusion method (Pulusani et al., 1979). A paper disc (diameter 6 mm; ADVANTEC CO., LTD., Tokyo, Japan) loaded with 15 μL of the culture supernatant of LAB was placed onto an agar plate overlaid with *P. mirabilis* (1.5×10^8 CFU/mL). After incubation at 37°C for 24 h, the diameter of the inhibition zone surrounding the paper disc was measured to evaluate the antibacterial activity of the supernatant of the LAB strain. Any strain that exhibited a large inhibition zone was considered as having high antibacterial activity.

Identification of selected strains of lactic acid bacteria

As described by Cho et al. (2023), 16S rRNA sequencing and phylogenetic analyses were carried out. Genomic DNA was extracted using the AccuPrep® Genomic DNA Extraction Kit (Bioneer, Daejeon, South Korea), and the 16S rRNA gene was amplified through polymerase chain reaction (PCR) using AccuPower® PCR PreMix (Bioneer) and primers. The nucleotide sequences of the 16S rRNA gene were determined by Bionics Co., Ltd. (Seoul, South Korea) and analyzed using BLAST from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were constructed using ClustalX (Larkin et al., 2007) and MEGA (Saitou & Nei, 1987) software.

Gram staining and catalase test were carried out as described by Cappuccino & Sherman (1983). The selected LAB strains were observed under a scanning electron microscope (SEM; H-7600, Hitachi, Tokyo, Japan) installed at Eulji University (Seongnam, South Korea). The selected sample was prepared

by fixing it with glutaraldehyde and osmium tetroxide, dehydrating it with ethanol, and coating it with platinum, following the methods of Cho et al. (2023) and Kang et al. (2023). ImageJ software (Schneider et al., 2012) was used for the size analysis of scanning electron micrographs. The utilization pattern of carbohydrates was determined using the API 50 CHL kit (API System, bioMérieux, Montalieu Vercie, France) according to the manufacturer's instructions.

Characterization of antimicrobial compounds

Preparation of antimicrobial compounds

To concentrate the culture supernatant, 1 mL of 1× supernatant was processed using a speed vac (VC2127, LaboGene A/S, Lynge, Denmark) to obtain 100 μL of 10× supernatant. Freeze-dried supernatant was also concentrated using a freeze dryer (FDU-1110, EYELA, Tokyo, Japan). The freeze-dried supernatant was dissolved in 20 mM sodium phosphate buffer (pH 7.0) to a final concentration of 1 g/mL.

Determination of antimicrobial activity according to incubation time

To determine the optimal incubation time for the production of antimicrobial compounds, 1× supernatant was collected at specific time points (0, 3, 6, 9, 12, and 24 h) during incubation. The antimicrobial activity of each sample was measured using the disc diffusion method.

pH stability of antimicrobial activity

To determine the stability of the antimicrobial compound at different pH levels, the pH of the 1× supernatant was adjusted to 2, 3, 4, 5, 6, 7, 8, 9, and 10 using 1 N NaOH (Samchun Chemical Co., Ltd., Seoul, South Korea) and 1 N HCl (Samchun Chemical Co., Ltd.) Each supernatant was incubated at 30°C for 3 h, and then the pH was adjusted to 6.5 with 1 N NaOH or 1 N HCl. The antimicrobial activity of each supernatant was evaluated using the disc diffusion method.

Temperature stability of antimicrobial activity

A 1× supernatant of the strains was exposed to a range of temperatures: 40, 50, 60, 70, 80, 90, and 100°C for 10 min. The antimicrobial activity of the supernatant after exposure to different temperatures was evaluated using the disc diffusion method.

Effect of proteolytic enzyme on antimicrobial activity

Proteinase K (Sigma-Aldrich, Burlington, MA, USA), trypsin (Roche, Basel, Switzerland), and α-chymotrypsin (Sigma-Aldrich) were used. Each enzyme was added to 1× supernatant at a final concentration of 1 mg/mL and incubated at 37°C for 3 h. The effect of the enzymes on the antimicrobial activity of the supernatant was measured using the disc diffusion method.

Determination of antimicrobial spectrum

To determine the antimicrobial spectrum, 10× supernatants from selected strains were tested against the bacterial strains and culture media as described above. The antimicrobial activity of the 10× supernatant was evaluated using the disc diffusion method.

Gel permeation column chromatography

The antimicrobial compounds were partially purified using gel permeation column chromatography as described by Huttunen et al. (1995) with slight modification. Glass Econo-Column (1.5 × 120 cm) (Bio-Rad, Hercules, CA, USA) was packed with Bio-Gel P-2 polyacrylamide gel (Bio-Rad) and pre-equilibrated with distilled water. Freeze-dried supernatant was loaded onto the column and eluted at a rate of 0.3 mL/min. Eluents were fractionated with a volume of 3 mL using a fraction collector (Bio-Rad) and 25-fold concentrated using a speed vac. The antimicrobial activity of each fraction was evaluated using the disc diffusion method, and the active fractions, which showed antimicrobial activity, were pooled.

Thin-layer chromatography

The antimicrobial activity of the selected fractions was confirmed through thin-layer chromatography (TLC). Each fraction was spotted onto TLC plates (silica gel 60F254, Merck, Darmstadt, Germany). The plates were then developed twice with a mobile phase consisting of nitromethane, 1-propanol, and water in a ratio of 2:5:1.5 (v/v/v) (Seo et al., 2004). The developed plate was dried, and spots of antimicrobial compounds were visualized by spraying with a solution of 0.3% (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride (Sigma-Aldrich) in methanol and 5% (v/v) H_2SO_4 (Duksan, Seoul, South Korea), followed by heating at 120°C for 3 min. The glucose polymers (G1-G8, Carbosynth Co., Compton, UK) were used as standards.

Quantification of reducing sugar and protein content

The concentration of reducing sugar and protein in a sample was determined using the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952) and Bradford protein assay (Bradford, 1976), respectively.

Size exclusion HPLC

The molecular size of antimicrobial compounds was determined using size exclusion high-performance liquid chromatography (HPLC, UltiMate™ 3000 RSSC nanosystem, Thermo Fisher Scientific Inc., Waltham, MA, USA). A Shodex

OHpak SB-802.5 column (8.0 × 300 mm, Resonac Co., Tokyo, Japan) was used. HPLC-grade distilled water was used as the mobile phase, and the flow rate was 0.4 mL/min. The column oven temperature was set at 35°C, and a refractive index detector (Shodex RI-101, Resonac Co.) was used. Glucose polymers (G1, G2, G3, G5, and G7) (Carbosynth Co.) were used as standards.

Determination of antibiofilm activity

The antibiofilm activity of a set of antimicrobial compounds was evaluated using a modified crystal violet assay (described by Lee et al., 2021). Briefly, *W. cibaria* strains were cultured in MRS at 37°C for 20 h, and their culture supernatant was diluted 2-fold (1/2, 1/4, and 1/8) with phosphate-buffered saline.

To assess the effect of the culture supernatant of *W. cibaria* strains on the biofilm formation, *P. mirabilis* was cultured in BHI broth at 37°C for 20 h. The BHI broth, which was inoculated with *P. mirabilis* culture at a concentration of 1% (v/v), was then mixed with the diluted culture supernatant of *W. cibaria* strains at a 1:1 ratio (v/v) and incubated in a 96-well plate at 37°C for 48 h to allow for biofilm formation. Fresh MRS broth was used as a control instead of the diluted culture supernatant of *W. cibaria* strains.

To assess the effect of the culture supernatant of *W. cibaria* strains on the removal of preformed biofilm, *P. mirabilis* was cultured in BHI broth in a 96-well plate at 37°C for 48 h to allow for biofilm formation. The 96-well plate was carefully washed twice with phosphate-buffered saline to remove any planktonic bacteria. The preformed biofilm was then incubated with the culture supernatant of the *W. cibaria* strain at 37°C for 24 h. The pre-formed biofilm incubated with fresh MRS broth was used as a control instead of being incubated with the culture supernatant of the *W. cibaria* strain.

After the biofilm was stained with crystal violet, as described by Yoo (2022), the absorbance of the sample was measured at a wavelength of 595 nm using a microplate reader (Epoch microplate reader, BioTek Instruments Inc., Winooski, VT, USA). The inhibition rate of biofilm formation and removal rate of preformed biofilm of the sample were calculated using the following formula:

$$\text{antibiofilm activity (\%)} = [1 - (A_c/A_0)] \times 100,$$

where A_c represents the absorbance of the well treated with the culture supernatant of *W. cibaria* strain, and A_0 represents the absorbance of the control.

Statistical analysis

The experiments were conducted in triplicate, and the data are presented as mean±standard deviation. Statistical analysis was performed using SPSS 23 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p<0.05$) was used to determine the significance between groups.

Results and Discussion

Screening of lactic acid bacteria with growth inhibition activity against *Proteus mirabilis*

A total of 987 LAB strains from our laboratory were used to screen for strains with high growth inhibitory activity against *Proteus mirabilis.* Among 987 LAB examined, 32 strains had growth inhibition zone of 10 mm or more in diameter, and only one strain, CHK 903, had a growth inhibition zone of over 15 mm in diameter. Strain no. CHK903, which was isolated from kimchi, showed an inhibition zone of 16 mm and, therefore, was selected as the strain with the highest antimicrobial activity against *P. mirabilis* for this study.

Bartkiene et al. (2019) reported that *Lactobacillus brevis* strain no. 173 exhibited an inhibition zone of 15.3 mm against *P. mirabilis*. Additionally, LAB (*Leuconostoc*, *Lactobacillus*, *Enterococcus*, and *Pediococcus* species) isolated from sourdough have also been found to possess antibacterial activity against *P. mirabilis*. In another study, *W. confusa* isolated from a vaginal swab from Indian women showed antimicrobial activity against *P. mirabilis* (15 mm) (Purkayastha et al., 2017). These reports and our results indicate that various types of LAB have high antimicrobial activity against *P. mirabilis* and may serve as potential antibacterial agents against *P. mirabilis*.

Identification of selected lactic acid bacteria strain

When the nucleotide sequence of the 16S rRNA gene of stain no. CHK903 was analyzed by BLAST, it was 100% identical to that of 87 strains of *W. cibaria* and to that of four strains of *W. confusa* registered in the NCBI database, which confirms the genus *Weissella* (data not shown). The selected strain was identified as *W. cibaria* through phylogenetic tree analysis (Fig. 1a), and SEM analysis showed that the length of the cells ranged from 1.59 to 1.93 μm, while the width ranged from 0.68 to 0.80 μm (Fig. 1b). The strain was rod-shaped with rounded ends and was Gram-positive and catalase-negative. These characteristics are consistent with the profile of LAB, which are typically cocci- or bacilli-shaped, Gram-positive, and catalase-negative. The carbohydrate utilization

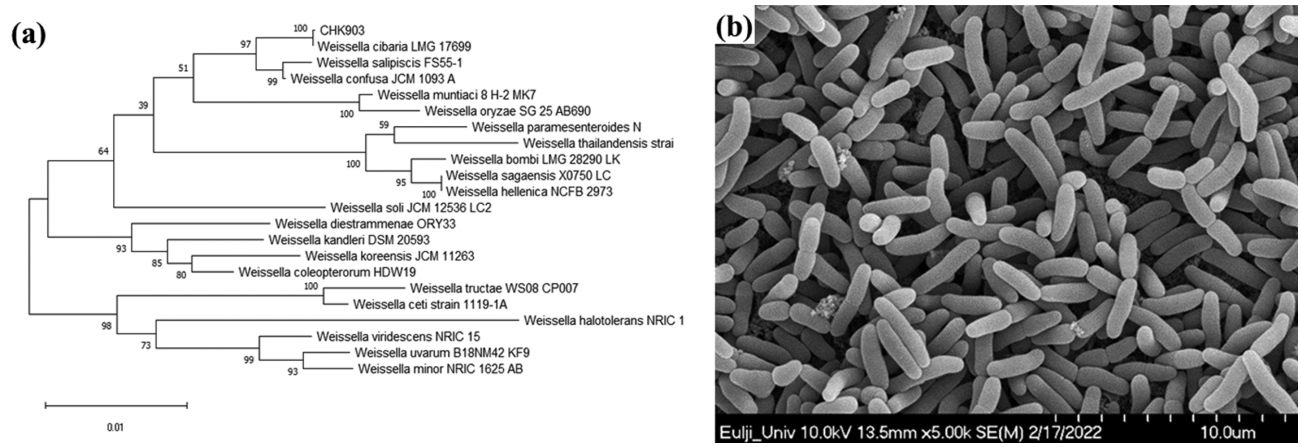

Fig. 1. Phylogenetic tree generated by the neighbor-joining method based on the sequence of the 16S rRNA gene and scanning electron microscope (SEM) image of strain no. CHK903. (a) Phylogenetic tree and (b) SEM image. The scale bar represents 10 μm (×5,000).

Table 1. Carbohydrate utilization pattern of strain no. CHK903

Characteristics	*Weissella cibaria* KCCM 41287	Strain no. CHK903	Characteristics	*Weissella cibaria* KCCM 41287	Strain no. CHK903
Glycerol	–*	–	Salicin	+	+
Erythritol	–	–	D-Cellobiose	+	+
D-Arabinose	–	–	D-Maltose	+	+
L-Arabinose	–	+	D-Lactose (bovine origin)	–	–
D-Ribose	–	–	D-Melibiose	–	–
D-Xylose	+	+	D-Saccharose (sucrose)	+	+
L-Xylose	–	–	D-Trehalose	–	–
D-Adonitol	–	–	Inulin	–	–
Methyl-β-D-xylopyranoside	–	–	D-Melezitose	–	–
D-Galactose	+	–	D-Raffinose	–	–
D-Glucose	+	+	Amidon (starch)	–	–
D-Fructose	+	+	Glycogen	–	–
D-Mannose	+	+	Xylitol	–	–
L-Sorbose	–	–	Gentiobiose	+	+
L-Rhanmose	–	–	D-Turanose	–	–
Ducitol	–	–	D-Lyxose	–	–
Inositol	–	–	D-Tagatose	–	–
D-Mannitol	–	–	D-Fucose	–	–
D-Sorbitol	–	–	L-Fucose	–	–
Methyl-α-D-mannopyranoside	–	–	D-Arabitol	–	–
Methyl-α-D-glucopyranoside	–	–	L-Arabitol	–	–
N-Acetyl glucosamine	+	+	Potassium gluconate	+	+
Amygdalin	+	+	Potassium 2-ketogluconate	+	–
Arbutin	+	+	Potassium 5-ketogluconate	–	–
Esculin ferric citrate	+	+			

*+ : Positive reaction; – : Negative reaction

pattern of *W. cibaria* CHK903 was determined and compared with that of the type strain *W. cibaria* KCCM 41287. *W. cibaria* CHK903 was confirmed to be able to ferment various carbohydrates (Table 1), such as L-arabinose, D-xylose, D-glucose, D-fructose, D-mannose, *N*-acetyl glucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-saccharose, gentiobiose, and potassium gluconate. However, this strain could not utilize D-galactose, unlike the type strain. The results of the phenotypic and biochemical tests were consistent with the identification of the selected strain as *W. cibaria*, which was previously reported by Rao & Goyal (2013).

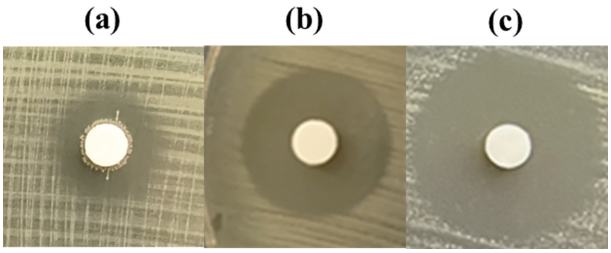

Fig. 2. Antimicrobial activity of the culture supernatant of *W. cibaria* CHK903. (a) 1× supernatant, (b) 10× supernatant, and (c) freeze-dried supernatant.

Characterization of antimicrobial compounds

Antimicrobial activity

The inhibition zone diameter for *W. cibaria* CHK903 at 1× supernatant concentration was 15.3 mm (Fig. 2). When the supernatant was concentrated to 10×, the diameter of the inhibition zone increased by 1.5-fold, and when freeze-dried, it increased by 1.7-fold. This result indicates that the concentrated and freeze-dried supernatants had higher antimicrobial activity than the 1× supernatant. The pH of the supernatant from the selected strain was 4.6. At this pH, the diameter of the inhibition zone was 14.7 mm. After adjusting the pH to 6.5, the diameter of the inhibition zone slightly decreased (data not shown).

Antimicrobial activity according to incubation time

When the antimicrobial activity of the culture supernatant of *W. cibaria* CHK903 was determined at different time points (3, 6, 9, 12, and 24 h), the maximum antimicrobial activity of the strain was observed at 12 h after incubation (Fig. 3a). The antimicrobial activity of the supernatant between 3 and 12 h was similar but slightly decreased after 12 h of incubation.

The antimicrobial activity of the bacteriocin produced by *B. cereus* GN105 was found to be dependent on the growth phase. The antimicrobial activity was not observed during the exponential phase and decreased after 3 h during the stationary phase. Specifically, the antimicrobial activity was observed after 1.5 h and decreased after 7 h of incubation (Naclerio et al., 1993).

Stability of antimicrobial activity at different pH and temperature

The stability of the antimicrobial activity of the culture supernatant of *W. cibaria* CHK903 at different pH levels is presented in Fig. 3b. The culture supernatant maintained its activity at pH 4 to 8 but completely lost its antimicrobial activity below pH 3 and above pH 9. Previous studies have shown that bacteriocins produced by *L. brevis* OG1 and *Lactiplantibacillus plantarum* F1 were stable at pH 2 to 8 and pH 2 to 6, respectively (Ogunbanwo et al., 2003). Compared to the stability of bacteriocins and antimicrobial compounds from other sources, the antimicrobial activity of *W. cibaria* CHK903 was found to be stable within a range of pH values.

Regarding temperature, the antimicrobial activity of *W. cibaria* CHK903 was maintained at 100% until 40°C but decreased to 70% at 50°C (Fig. 3c). Bacteriocin produced by *B. cereus* GN105 maintained its activity at temperatures up to 75°C but lost activity after 15 min of exposure to 90°C (Naclerio et al., 1993).

Effect of proteolytic enzymes on antimicrobial activity

The culture supernatant of *W. cibaria* CHK903 was treated with proteolytic enzymes, such as proteinase K, trypsin, and α-chymotrypsin, and its antimicrobial activity was checked to determine whether the antimicrobial compounds are proteinaceous substances. As shown in Table 2, the antimicrobial activity of this strain was not affected by treatment with the proteolytic enzymes, which indicated that the antimicrobial compounds are not proteinaceous. LAB produce various proteins that have antimicrobial activity against pathogenic bacteria, such as bacteriocins. Bacteriocins are antimicrobial peptides or proteins produced by bacteria. They are typically small in size and have specific antimicrobial

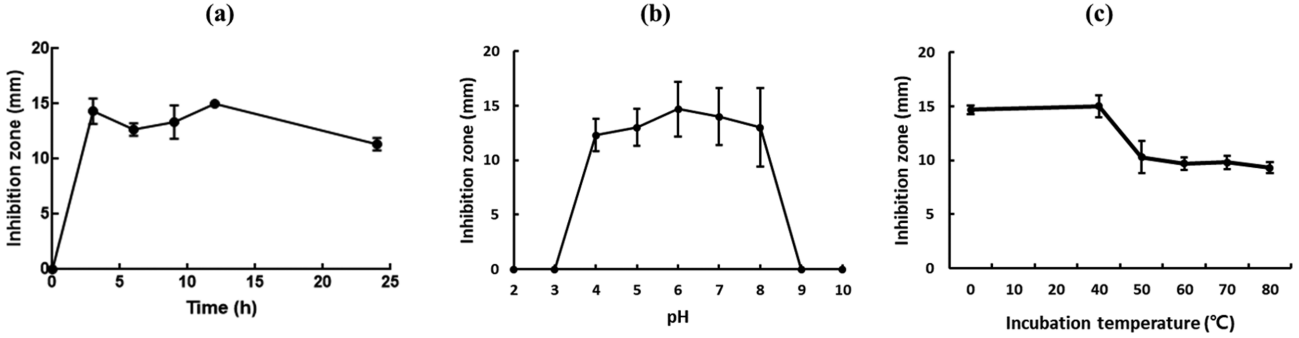

Fig. 3. Antimicrobial activity of *W. cibaria* CHK903 against *P. mirabilis*. Antimicrobial activity according to (a) time, (b) pH, and (c) temperature.

Table 2. Effect of enzymes on antimicrobial activity of supernatants from *W. cibaria* CHK903

Treatment	Distilled water	Proteinase K	Trypsin	α-Chymotrypsin
Inhibition zone (mm)	13.3±3.7	12.8±3.8	12.6±3.9	12.8±4.1

Diameter of filter paper disc: 6 mm. The data are presented as mean±standard deviation (n = 3).

Table 3. Antimicrobial spectrum of the freeze-dried supernatant of *W. cibaria* CHK903

Microorganism	Strain	Inhibition zone (mm)
Gram-negative bacteria		
Proteus mirabilis	KCCM 11381P	25.3±0.6
Escherichia coli	ATCC 10536	12.7±0.6
Pseudomonas aeruginosa	ATCC 10145	–*
Salmonella Enteritidis	ATCC 13076	12.7±1.2
Salmonella Typhimurium	ATCC 14028	10.7±2.1
Gram-positive bacteria		
Weissella cibaria CHK903	KCCP 11638	ND**
Weissella cibaria SGW054	KCCP 11767	–
Weissella cibaria SPW2014	KCCP 12062	–
Weissella paramesenteroides	KCCP 11717	–
Bacillus cereus	KCCM 11204	13.7±0.6
Listeria monocytogenes	KCCM 40307	12.3±1.5
Staphylococcus aureus	ATCC 14028	–
Leuconostoc lactis	KCCP 11247	–
Yeast		
Candida albicans	KCTC 7270	–

*– : no inhibition zone; **ND : not determined. The data are presented as mean±standard deviation (n = 3). Diameter of paper disc: 6 mm.

activity against related or unrelated bacteria (Ahmad et al., 2017). The results of the present study imply that the antimicrobial compounds produced by *W. cibaria* CHK903 are unlikely to be bacteriocin. Besides bacteriocins, the antimicrobial components of LAB are diverse and include organic acids, hydrogen peroxide, diacetyl, and other volatile organic compounds (Yang, 2000). Organic acids such as lactic, acetic, propionic, and formic acids are commonly produced by LAB during fermentation. These organic acids lower the pH of the environment and disrupt the proton motive force of bacteria, leading to cell death. Hydrogen peroxide (H_2O_2) is produced by some strains of LAB and generates reactive oxygen species that can damage bacterial DNA and other cellular components, leading to cell death (Reis et al., 2012). While there are some reports that lactic acid bacteria inhibit *P. mirabilis* growth, there is no report identifying the novel substances produced by lactic acid bacteria that inhibit *P. mirabilis* growth.

Antimicrobial spectrum

The antimicrobial activity of *W. cibaria* CHK903 against some pathogenic microorganisms and LAB was checked to evaluate the antimicrobial spectrum. As shown in Table 3, the strain inhibited the growth of *E. coli*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *B. cereus*, and *L. monocytogenes*. However, the strain could not inhibit the growth of *W. cibaria*, *W. paramesenteroides*, and *Leuconostoc lactis.* This result indicates that *W. cibaria* CHK903 selectively inhibits the growth of pathogenic bacteria and does not show antimicrobial activity against LAB. Previous studies have also reported significant inhibitory effects of *W. cibaria* against various bacterial strains, such as *Acinetobacter baumannii*, *E. coli*, *L. monocytogenes*, *P. mirabilis*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Staphylococcus epidermidis*, and *Streptococcus pyogenes* (Yu et al., 2018; Jha et al., 2022). These findings suggest that *W. cibaria* CHK903 has the potential to serve as a natural food preservative in the food industry.

Partial purification of antimicrobial compounds

In this study, we revealed that the culture supernatant of *W. cibaria* CHK903 has high antimicrobial activity against *P. mirabilis* and some other pathogenic bacteria, including foodborne pathogens. Thus, the antimicrobial compounds from *W. cibaria* CHK903 were partially purified using gel permeation column chromatography. Among the fractions obtained from the eluent, samples with fraction numbers 37 to

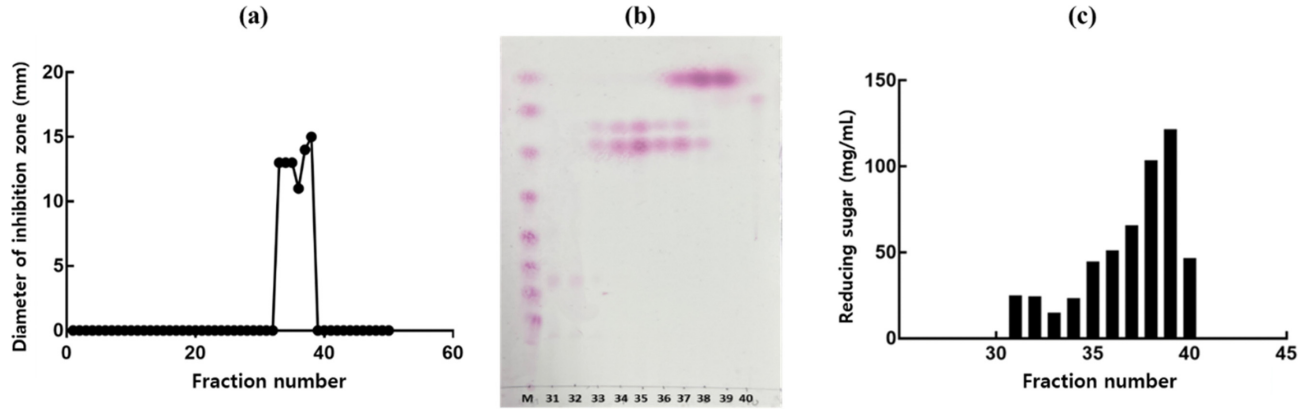

Fig. 4. Antimicrobial activity, TLC chromatogram, and reducing sugar content of fractions eluted from gel permeation column chromatography. (a) Antimicrobial activity, (b) TLC chromatogram, M: glucose polymers, G1-G8, and (c) reducing sugar content.

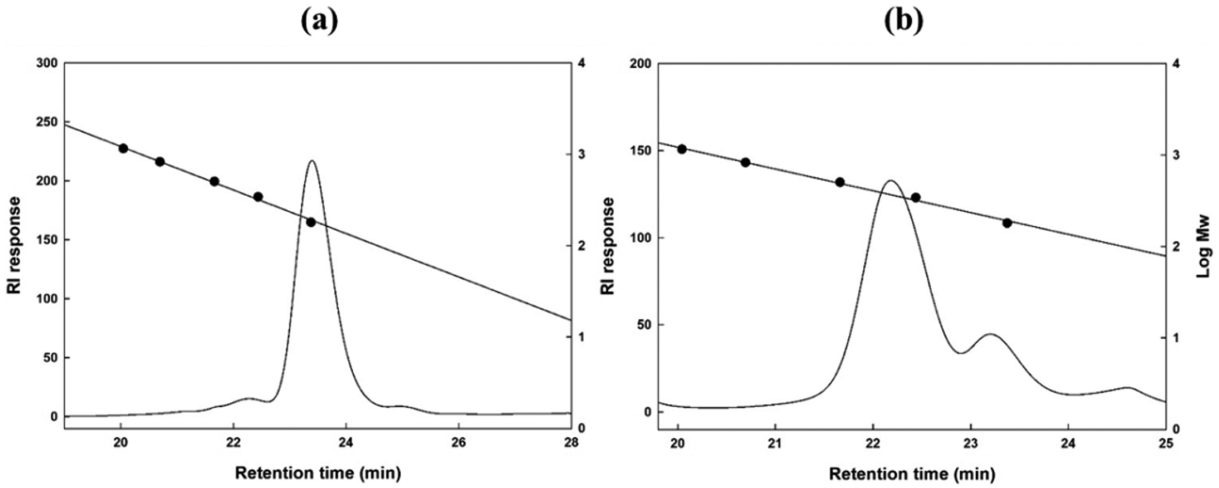

Fig. 5. Size exclusion HPLC chromatogram. (a) Antimicrobial compound 1, molecular size was 189 Da; (b) antimicrobial compound 2, molecular size was 365 Da.

38 and 33 to 36 showed antimicrobial activity with the average diameter of the inhibition zone being 14.5 and 12.5 mm, respectively. These results revealed that there were two antimicrobial compounds in the partially purified supernatant, which was also confirmed by TLC (Fig. 4a, b).

When the content of protein and carbohydrate of the partially purified antimicrobial compounds was determined to identify the antimicrobial compounds, it was confirmed that there was no protein in the sample, whereas reducing sugar was present, ranging from 14.2 to 24.3 mg/mL for fractions 31 to 34, 44.1 to 64.9 mg/mL for fractions 35 to 37, 102.9 and 120.9 mg/mL for fractions 38 and 39, and 46.1 mg/mL for fraction 40 (Fig. 4c). Based on these results, the active fractions were considered to contain carbohydrates and not protein.

TLC analysis revealed two separate spots related to the antimicrobial compounds between glucose polymer G2 and G3 (Fig. 4b). The molecular weight of the two antimicrobial compounds was determined to be 189 and 365 Da (Fig. 5).

Inhibition of biofilm formation and reduction of pre-formed biofilm

Besides the antimicrobial activity of *W. cibaria* CHK903 against *P. mirabilis*, the antibiofilm activity of the strain against *P. mirabilis* was evaluated. The antibiofilm activity can be divided into the inhibitory activity of biofilm formation and the removal activity of pre-formed biofilm. The supernatant of *W. cibaria* CHK903 showed dose-dependent inhibitory activity against the biofilm formation of *P. mirabilis* with an inhibition rate of 90% (Fig. 6a). The strain also exhibited removal activity against the pre-formed biofilm of *P. mirabilis* with a removal rate of over 60% (Fig. 6b). Biofilms are

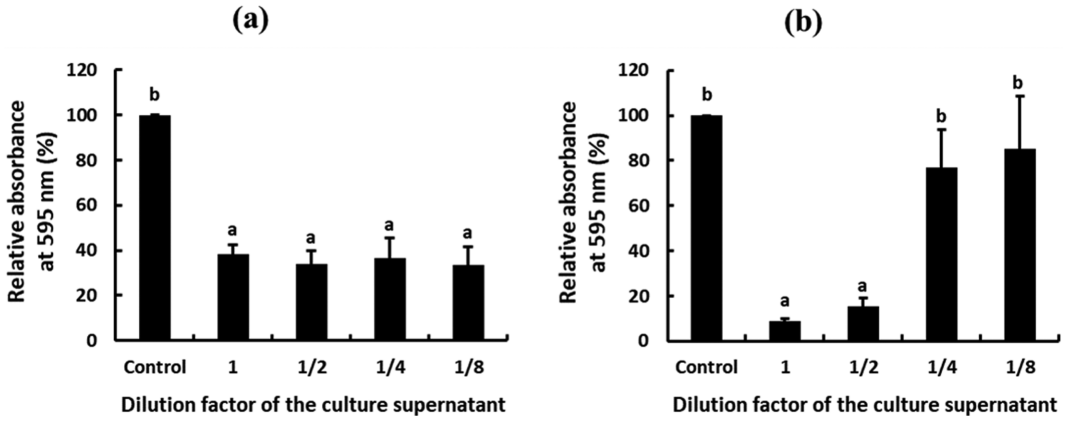

Fig. 6. Effect of culture supernatant of *W. cibaria* CHK903 on the inhibition of biofilm formation and removal of the pre-formed biofilm of *P. mirabilis*. (a) Inhibition rate of biofilm formation, (b) removal rate of pre-formed biofilm. Control: treated with fresh MRS broth. Different letters show significant differences among groups at $p<0.05$ ($n \geq 3$).

communities of microorganisms that adhere to biopolymers on surfaces, such as medical devices, and are composed of a self-produced extracellular polymeric matrix. The matrix can reduce sensitivity to host defense systems, antibiotics, and other drugs, making biofilms a major contributor to chronic infections (Yan et al., 2019).

The results of our study suggest that the supernatant of *W. cibaria* CHK903 has significant potential and can be used as a natural antimicrobial and anti-biofilm agent against *P. mirabilis*. These properties make this strain valuable to various industries, including the food and medical fields, as its application could potentially prevent chronic infections and antibiotic resistance.

In conclusion, in this study, we evaluated the antimicrobial and antibiofilm activities of *W. cibaria* CHK903 against *P. mirabilis*. The selected strain exhibited potent antibacterial activity against *P. mirabilis*. The strains also showed broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria, including major foodborne pathogens. The partially purified antimicrobial compounds from *W. cibaria* CHK903 were identified as two compounds containing carbohydrates with molecular weights of 189 and 365 Da. However, the antimicrobial compounds partially purified in this study are heat-labile unlike carbohydrates, and it can be inferred that these compounds have additional residues bound to carbohydrates. This study also demonstrated the antibiofilm properties of *W. cibaria* CHK903, that is, its ability to inhibit biofilm formation and remove pre-formed biofilms of *P. mirabilis*. Given that *P. mirabilis* is associated with diseases such as urinary tract infections, pneumonia, otitis media, and PD, the findings in this study suggest that *W. cibaria* CHK903 has significant potential as a natural antimicrobial and antibiofilm agent for use in the prevention and treatment of diseases related to *P. mirabilis*.

Overall, this study provides valuable insights into the potential applications of *W. cibaria* CHK903 for developing natural antimicrobial agents and functional foods. Further research is needed to identify the antimicrobial compounds, elucidate their definite structure, explore the mechanism of antimicrobial activity, and develop effective formulations of these antimicrobial compounds for practical applications.

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