Isolation of Antifungal Activity of *Leuconostoc mesenteroides* TA from Kimchi and Characterization of Its Antifungal Compounds

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Abstract Strain TA harboring antifungal activity was isolated from kimchi and identified based on Gram-staining, biochemical properties using an API 50 CHL, determination of rRNA gene sequences, and RAPD analysis. The determined gene sequences and RAPD pattern showed 100% homology with those of *Leuconostoc mesenteroides* ATCC 8293^T. However, their properties were slightly different from each other. Especially, TA could not utilize lactose while strain ATCC 8293 could use lactose. *Leu. mesenteroides* ATCC 8293 showed higher antibacterial activity against *Bacillus cereus* than *Leu. mesenteroides* TA, whereas the antifungal activities of these 2 LAB against *Aspergillus fumigatus* were the same. *Leu. mesenteroides* TA showed broad antimicrobial activities against Gram-positive and -negative bacteria as well as molds. We determined that the responsible antifungal compounds from *Leu. mesenteroides* TA are lactic acid, acetic acid and unidentified hydrophobic compound(s). Additionally, synergistic interactions involving acetic acid, lactic acid, phenyllactic acid, and unidentified hydrophobic compound(s) contribute to the overall inhibitory activity of *Leu. mesenteroides* TA.

Keywords: kimchi, *Leuconostoc mesenteroides*, antifungal activity, hydrophobic compounds

Introduction

Spoilage of foods and feeds due to mold growth and further contamination by mycotoxin is a worldwide problem impacting public health, food security, and economy (1–3). Various additives to prevent food spoilage have been investigated and commercialized, including chemical products, animal-derived products, plant-derived products, and microbial-derived products (4). Moreover, physical food preservation techniques such as low temperature, low water activity, acidification, irradiation, and others have been used (2). However, use of such additives or techniques has frequently resulted in undesirable sensory food characteristics (5).

As fresh food and minimized-processed food markets have gained recent popularity, there is now a need for food-compatible antimicrobials produced by microorganisms for the purpose of biopreservation (1,6). Lactic acid bacteria (LAB) have a long history as biopreservatives for food and feed storage, suggesting that LAB and their metabolites are promising alternatives as biopreservatives. There have been numerous investigations of antibacterial compounds from LAB (7), but only few reports characterized antifungal compounds from LAB (8). Moreover, studies carried out to identify effective antifungal compounds from LAB have mostly reported on *Lactobacillus* species, and identified antifungal compounds from *Lactobacillus* species are mostly organic acids and proteinaceous compounds as well as low molecular mass compounds (less than 1,000 Da) such as

reuterin, hydroxylated fatty acids, methylhydantoin, mevalonolactone, and several cyclic dipeptides (5,7,9).

On the other hand, studies have found that the antifungal activities of *Leuconostoc* species are lower compared to *Lactobacillus* species (10–14). Compared to homo-fermentative LAB (e.g. *Lactobacillus* species), hetero-fermentative LAB such as *Lactococcus* sp. or *Leu. mesenteroides* are known to produce an improved aroma profile (15). Some hetero-fermentative LAB strains have already been proven to be suitable as LAB starter cultures and were found to produce metabolites with antimicrobial activity (16). Thus, application of their metabolites as biopreservatives in both fermented and non-fermented foods has become an important trend in food preservation (17).

In this study, we isolated a LAB, *Leuconostoc* sp., harboring antifungal activity from kimchi. We identified the isolated LAB strain and tried to characterize antifungal compounds from the LAB strain using partially purified antifungal compounds.

Materials and Methods

Cultures and media The microorganisms used in this study and their culture media with culture conditions are listed in Table 1. ATCC strains were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). KCTC strain was purchased from the

Table 1. Microbial strains used in this study

Strain	Medium ¹⁾	Culture condition
LAB		
<i>Leuconostoc mesenteroides</i> TA	MRS	30°C, 24 h
<i>Leuconostoc mesenteroides</i> ATCC 8293	MRS	30°C, 24 h
Mold		
<i>Aspergillus flavus</i> ATCC 22546	MEA	30°C, 48 h
<i>Aspergillus fumigatus</i> ATCC 96918	MEA	30°C, 48 h
<i>Aspergillus ochraceus</i> PF-2	PDA	30°C, 48 h
<i>Aspergillus nidulans</i> PF-3	MEA	30°C, 48 h
<i>Penicillium roqueforti</i> ATCC 10110	PDA	25°C, 72 h
Bacteria		
<i>Bacillus cereus</i> KCTC 3624	LB	37°C, 24 h
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> ATCC 29213	TSB	37°C, 24 h
<i>Escherichia coli</i> O157:H7 ATCC 43895	LB	37°C, 24 h
<i>Salmonella enterica</i> serovar. Typhi ATCC 19430	LB	37°C, 24 h
<i>Micrococcus luteus</i> ATCC 15307	TSB	30°C, 24 h
<i>Pseudomonas aeruginosa</i> ATCC 27853	LB	37°C, 24 h

¹⁾MRS, de Man Rogosa Sharpe (Difco); MEA, malt extract agar (Difco); PDA, potato dextrose agar (Difco); LB, 1% bacto-tryptone, 0.5% yeast extract, 1% NaCl; TSB, tryptic soy broth (Difco)

Korean Culture Type Collection (Daejeon, Korea), and PF-2 and PF-3 fungal strains were isolated by our laboratory (18).

Isolation of LAB from kimchi Freshly made kimchi samples were purchased from kimchi companies in Korea. The purchased kimchi was incubated at 6.5°C (HB 103S; HanBaek Scientific, Bucheon, Korea) until the acidity of kimchi reached 0.6–0.7% (v/w). At that time, LAB were isolated from the fermented kimchi samples according to a previously described method (1,18). Briefly, kimchi samples were macerated (hand blender, HHM-600; Hanil, Seoul, Korea) and filtered through a thin sterile cloth, after which the filtrate was serially diluted and then spread onto MRS and MRS+2% CaCO₃ agar. The plates were incubated at 30°C for 2 days, after which the colonies with a clear zone on MRS+2% CaCO₃ agar were selected.

Identification of the isolate Isolate was identified based on its morphological properties under a microscope, biochemical characteristics using an API 50 CHL (BioMérieux, Marcy-l'Étoile, France), and determination of 16S, ITS, and 23S rRNA gene sequences using an ABI prism 3730 DNA analyzer (Applied Biosystems, Foster city, CA, USA) according to the method described by Yang and Chang (18). The determined rRNA gene sequences were compared with sequences available in the GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the BLASTN program.

Random amplified polymorphic DNA (RAPD)-PCR analysis was performed using 2 different primers, 239 (5'-CTGAAGCGGA-3') and M13 (5'-GAGGTGGCGTTCT-3'), which are known to specific detection primers for *Leu. mesenteroides* subsp. *mesenteroides* (19,20). The 30 µL PCR mixture contained 3 µL of 40 ng template DNA, 0.3 µL of 100 pmol primer, and 26.7 µL of G-tag mastermix (Cosmo Genetech,

Seoul, Korea). PCR reactions were performed in a PCR system (ProFlex PCR system; Applied Biosystems) using annealing temperatures of 30, 35, 40, 45, 50, and 55°C, respectively. Amplified products were resolved by electrophoresis on a 1.5% (w/v) agarose-TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) and 1 kb Labo DNA ladder (Cosmo Genetech) was used as a size marker.

Antimicrobial activity assay LAB strain was incubated in MRS (Difco) broth for 24–48 h, after which the culture was centrifuged (9,950×g, 15 min, 4°C, SUPRA 22K; Hanil) and then filter-sterilized (0.45 µm pore size, Sartorius, Göttingen, Germany). The cell-free culture filtrate was concentrated and used for antimicrobial activity assay.

Spot-on-the-lawn assay (21) was used to detect antimicrobial activity. Plates were prepared by adding the mold (6.0 log spores per 20 mL of MEA or PDA) to 1.5% bacto agar (Duchefa, Harlem, The Netherlands) for antifungal activity assay or by spreading the bacterium (6.0 log CFU/mL) onto LB or TSB agar for antibacterial activity assay. Spore solution was prepared as previously reported (9). Antimicrobial activity, expressed as arbitrary units (AU) per milliliter, was defined as the reciprocal of the highest dilution at which microbial growth was inhibited. Antimicrobial titer was calculated as (1000/*d*) D, where *d* is dose (amount of antimicrobial samples pipetted onto each spot) and D is the dilution factor. The above experiment was run in triplicate.

Analysis of organic acids Organic acids produced by *Leu. mesenteroides* TA were analyzed as previously described (9). Briefly, overnight grown cell culture was centrifuged (9,950×g, 15 min, 4°C, Hanil), membrane filtered (Sartorius) and then analyzed by Dionex-500 ion chromatography (IC) system (Dionex, Sunnyvale, CA, USA) equipped with an electro conductivity detector. Dionex IonPac ICE-AS6 column (9×253 mm) and a mobile phase (0.4 mM heptafluorobutyrate) with a flow rate of 1.0 mL/min at room temperature was used. Organic acids were detected using an anion-ICE micro membrane suppressor (Dionex). Eight acids were used as standard acids; acetic acid (9515-03; JT Baker, Center Valley, PA, USA), formic acid (94318; Fluka, Sao Paulo, Brazil), lactic acid (69775; Fluka), citric acid (C3476; Showa Chem, Beijing, China), malic acid (25044-00; Kanto Chem, Tokyo, Japan), succinic acid (398055; Sigma-Aldrich, St. Louis, MO, USA), oxalic acid (1224045329; Showa Chem), and fumaric acid (47910; Showa Chem).

Solid phase extraction (SPE) of *Leu. mesenteroides* TA culture Antifungal compound from *Leu. mesenteroides* TA was partially purified using a SPE (ISOLUTE® C18, 10 g; International Sorbent Technology, Hengoed, UK) as previously described (1,9). Cell-free culture filtrate (2.5 L) of *Leu. mesenteroides* TA was loaded onto the activated SPE column, after which the column was washed with 100 mL of 5% (v/v) aqueous acetonitrile and eluted with 30 mL of 95% (v/v) aqueous acetonitrile. The eluted sample was vacuum

evaporated using a SpeedVac (VS-802; Vision, Seoul, Korea), dissolved in 125 μ L of 10 mM sodium acetate buffer (pH 4.4), and then used for further experiments.

Characterization of SPE sample containing active substance (SPE-AS) Effects of pH, temperature, and different enzymes on partially purified antifungal compound (SPE-AS) were investigated. Control experiment was carried out with the cell-free culture filtrate of *Leu. mesenteroides* TA. After pH, temperature, and enzyme treatments to SPE-AS, residual antifungal activity was assayed using *A. fumigatus* ATCC 96918 as a sensitive mold.

Effect of pH: SPE-AS was equilibrated to pH 3.0 (50 mM glycine-HCl), pH 4.0-6.0 (50 mM sodium acetate), and pH 7.0-8.0 (50 mM Tris-HCl) for 2 h at 4°C.

Effect of temperature: SPE-AS was treated at different temperatures of 4, 25, 30, 37, 50, and 70°C for 24 h, 100°C for 30 min, and 121°C for 15 min.

Effects of different enzymes: SPE-AS was treated separately with enzymes, including proteinase K (EC 3.4.21.64; Sigma-Aldrich), protease (type I; Sigma-Aldrich), trypsin (EC 3.4.21.4; Sigma-Aldrich), carboxypeptidase A (EC 3.4.17.1; Sigma-Aldrich) in 50 mM Tris-HCl/10 mM CaCl_2 buffer (pH 7.5), aminopeptidase I (EC 3.4.11.22; Sigma-Aldrich) in 50 mM Tris-HCl/10 mM CaCl_2 buffer (pH 8.0), lipase (EC 3.1.1.3, type VII; Sigma-Aldrich) in 50 mM Tris-HCl/10 mM CaCl_2 buffer (pH 7.0), pepsin (EC 3.4.23.1; Sigma-Aldrich) in 10 mM citrate buffer (pH 2.0), and α -amylase (EC 3.2.1.1, type VII; Sigma-Aldrich) in 50 mM sodium phosphate/10 mM NaCl buffer (pH 7.0). All enzyme reactions were performed at a final enzyme concentration of 2 mg/mL for 6 h at 37°C. The pH levels of the SPE-AS and enzyme mixtures were then re-equilibrated to pH 4.4, which was the final pH of *Leu. mesenteroides* TA culture after 24 h of cultivation.

High performance liquid chromatography (HPLC) for analysis of phenyllactic acid Cell-free culture filtrate and prepared SPE-AS from *Leu. mesenteroides* TA were analyzed by HPLC. Phenyllactic acid (P7251; Sigma-Aldrich) was used as a standard with 1,000 ppm concentration. Each sample (10 μ L) was injected into HPLC. We used an Aminex 87 H column (300x7.8 mm, Bio rad, Hercules, CA, USA) with Dionex ultimate 3000 (Dionex) fitted with a refractive index detector (Refractor MAX 520; Dionex). The flow rate of the mobile phase (0.01 N H_2SO_4) was 0.5 mL/min and monitored the absorbance at 210 nm.

Results and Discussion

Isolation and identification of LAB harboring antifungal activity

LAB strain TA showing antifungal activity (Fig. 1) was isolated from kimchi sample purchased from Taebak Kimchi Company (Hwasung, Korea) on 19th March, 2014. Isolate TA was determined to be Gram-positive and catalase-negative. When the 16S rRNA gene sequences

Fig. 1. Isolation of antifungal lactic acid bacterium.

of strain TA were determined and compared with those of LAB type strains in GenBank, the full sequences (1,479 bp) of strain TA showed 100% homology with those of *Leu. mesenteroides* ATCC 8293^T. *Leu. mesenteroides* ATCC 8293^T has been isolated from fermenting olives by Vaughn in 1941 (22). Thus, we further determined ITS rRNA (425 bp) and 23S rRNA (3,243 bp) gene sequences of isolate TA and compared them with those of *Leu. mesenteroides* ATCC 8293^T. The ITS and 23S rRNA sequences of isolate TA also showed 100% homology with those of *Leu. mesenteroides* ATCC 8293^T. Three subspecies are known for *Leu. mesenteroides* such as *Leu. mesenteroides* subsp. *mesenteroides*, *Leu. mesenteroides* subsp. *cremoris*, and *Leu. mesenteroides* subsp. *dextranicum* (19). *Leu. mesenteroides* ATCC 8293 was identified as *Leu. mesenteroides* subsp. *mesenteroides* (20, 22). RAPD-PCR using strain- and species-specific primers allows identification of strains belonging to subspecies of the same species (20). Thus, genotypic differences among closely related strains (isolate TA and ATCC 8293) were analyzed by RAPD-PCR. As shown in Fig. 2, two LAB strains (isolate TA and ATCC 8293) displayed exactly same patterns of RAPD using 239 and M13 primers under different annealing temperatures (30-55°C). These results demonstrated that the isolated strain TA is not a new strain and is instead the same strain already registered *Leu. mesenteroides* subsp. *mesenteroides* ATCC 8293^T. Thus, this isolate TA was finally identified as *Leu. mesenteroides* subsp. *mesenteroides*, and was designated as *Leu. mesenteroides* subsp. *mesenteroides* TA in the study.

Assessment of carbohydrate utilization In assessment of carbohydrate utilization using the API CHL system, the isolate TA showed 99.9% homology with *Leu. mesenteroides*, whereas *Leu. mesenteroides* ATCC 8293 showed 99.1% homology with *Leu. mesenteroides*. LAB strains TA and ATCC 8293 were shown to be exactly the same strain based on molecular biological identification (16S+ITS+23S rRNA gene sequences and RAPD analysis), however they showed different carbohydrate utilization profiles of gentiobiose, amygdalin, cellobiose, lactose, and gluconate (Table 2). As shown in

Fig. 2. RAPD patterns of lactic acid bacteria.

Table 2, *Leu. mesenteroides* TA could not utilize lactose, whereas *Leu. mesenteroides* ATCC 8293 utilized lactose well. Although it is well

Table 2. Carbohydrate utilization profile of the isolate TA

Characteristic	Strain		Characteristic	Strain	
	TA	8293		TA	8293
Glycerol	-	-	Mannitol	+	+
Erythritol	-	-	Sorbitol	-	-
D-Arabinose	-	-	α -Methyl-D-mannoside	-	-
L-Arabinose	+	+	α -Methyl-D-Glucoside	+	+
Ribose	+	+	N-Acetyl glucosamine	-	-
D-Xylose	+	+	Amygdalin	-	+
L-Xylose	-	-	Arbutin	-	-
Adonitol	-	-	Esculine	+	+
β -Methyl-xyloside	-	-	Salicin	+	+
Galactose	+	+	Cellobiose	-	+
D-Glucose	+	+	Maltose	+	+
D-Fructose	+	+	Lactose	-	+
D-Mannose	+	+	Melibiose	+	+
L-Sorbose	-	-	Sucrose	+	+
Rhamnose	-	-	Trehalose	+	+
Dulcitol	-	-	Inuline	-	-
D-Raffinose	+	+	D-Fucose	-	-
Starch	-	-	L-Fucose	-	-
Glycogen	-	-	D-Arabitol	-	-
Xylitol	-	-	L-Arabitol	-	-
Gentiobiose	-	+	Gluconate	+	-
D-Turanose	+	+	2 keto-gluconate	-	-
D-Lyxose	-	-	5 keto-gluconate	-	-
Inositol	-	-	Melezitose	-	-

+, positive; -, negative

Characteristics were determined using API 50 CHL system.

known that LAB can utilize lactose, it is more common in dairy-originated LAB than kimchi-originated LAB. As kimchi does not contain lactose, LAB in kimchi do not require any lactose utilizing pathway. We have previously observed identical results (lactose-negative metabolism) during isolation of LAB from kimchi (1,18,23). Interestingly, we isolated *Leu. mesenteroides* TA in Korea, which is the same LAB strain as *Leu. mesenteroides* ATCC 8293 isolated nearly 73 years ago in the USA from fermenting olives by Vaughn (22). Moreover, molecular biological identification (16S+ITS+23S rRNA gene sequences and RAPD analysis) determined that these 2 strains are exactly the same, but their metabolic properties are slightly different (Table 2) due to microbial evolutionary adaption to their environment.

Spectrum of antimicrobial activity and identification of responsible agents showing antifungal activity Ever since *Leu. mesenteroides* ATCC 8293 was isolated in 1941, several investigations have reported that *Leu. mesenteroides* ATCC 8293 harbored weak antifungal activity (16,24,25), and its antifungal compound was reported as phenyllactic acid (12,13).

In this study, the antimicrobial activities of *Leu. mesenteroides* ATCC 8293 and *Leu. mesenteroides* TA were determined against *A. fumigatus* ATCC 96918 and *B. cereus* KCTC 3624, respectively. They showed slightly different patterns; *Leu. mesenteroides* ATCC 8293 showed antifungal activity of 40 AU/mL and antibacterial activity of 56 AU/mL, whereas *Leu. mesenteroides* TA showed antifungal and antibacterial activities of 40 AU/mL, respectively (data not shown). Furthermore, antimicrobial activities of *Leu. mesenteroides* TA against various food-borne pathogens and putrefactive microorganisms

Table 3. Antimicrobial activity of *Leu. mesenteroides* TA against bacteria and molds

Micro-organism	Indicator	Antimicrobial activity (AU/mL)				
		Culture filtrate ¹⁾	Mixture of detected organic acids ²⁾	Acetic acid ³⁾	Lactic acid ⁴⁾	SPE-AS ⁵⁾
Bacteria	<i>Bacillus cereus</i> KCTC 3624	40	40	4	32	800
	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> ATCC 29213	0	0	0	0	0
	<i>Escherichia coli</i> O157:H7 ATCC 43895	8	8	0	4	0
	<i>Salmonella enterica</i> serovar. Typhi ATCC 19430	16	16	0	8	0
	<i>Micrococcus luteus</i> ATCC 15307	8	8	0	4	0
	<i>Pseudomonas aeruginosa</i> ATCC 27853	16	16	0	8	0
Molds	<i>Penicillium roqueforti</i> ATCC 10110	8	8	4	4	400
	<i>Aspergillus nidulans</i> PF-3	24	16	0	16	1,600
	<i>Aspergillus ochraceus</i> PF-2	28	24	4	20	800
	<i>Aspergillus fumigatus</i> ATCC 96918	40	32	12	16	800
	<i>Aspergillus flavus</i> ATCC 22546	12	12	4	8	400

¹⁾Cell-free culture filtrate of *Leu. mesenteroides* TA.

^{2), 3), 4)}Concentration of each organic acid from *Leu. mesenteroides* TA culture filtrate was quantified by IC analysis.

⁵⁾SPE-AS, Solid phase extraction sample containing active substance from *Leu. mesenteroides* TA culture.

were determined. *Leu. mesenteroides* TA showed antibacterial as well as antifungal activities (8-40 AU/mL) against all tested micro-organisms except *Staphylococcus aureus* ATCC 29213 (Table 3).

There have been a large number of reports on antibacterial compounds from LAB, but only few in investigations were available on antifungal compounds from LAB (7,8). Thus we investigated the antifungal characteristics of *Leu. mesenteroides* TA in this study. First, to determine whether or not the antifungal activity can be attributed to organic acids from LAB, analysis of organic acids was performed using IC and HPLC in *Leu. mesenteroides* TA culture. Seven organic acids were detected; lactic acid was produced in the highest quantity (8,450.904 mg/L), followed by acetic acid (6,606.233 mg/L), citric acid (1,798.515 mg/L), succinic acid (71.478 mg/L), formic acid (50.475 mg/L), malic acid (32.071 mg/L), and phenyllactic acid (13.313 mg/L).

Each organic acid and organic acid mixture in *Leu. mesenteroides* TA culture filtrate quantified by IC and HPLC analysis were examined for their activities (Table 3). When antimicrobial activities of the 7 detected organic acids in *Leu. mesenteroides* TA were individually determined, lactic acid and acetic acid showed antimicrobial activity, whereas the 5 other organic acids showed no antimicrobial activity against any of the tested microorganisms (data not shown). In the antimicrobial spectra (antifungal and antibacterial) of the cell-free culture filtrate, lactic acid, and mixture of 7 detected organic acids from *Leu. mesenteroides* TA were identical, whereas their activities were slightly different. On the other hand, acetic acid showed mainly antifungal activity rather than antibacterial activity.

SPE-AS prepared by 95% acetonitrile elution showed high hydrophobicity. SPE-AS showed the same antifungal spectra as the culture filtrate, however, SPE-AS did not show antibacterial activity against the tested bacteria, except *B. cereus* KCTC 3624 (Table 3). This result suggests that SPE-AS contains antifungal compounds rather

than antibacterial compounds. It was previously reported that the antifungal compound produced by *Leu. mesenteroides* ATCC 8293 is phenyllactic acid with 0.1 mM productivity (12). In the organic acid analysis, *Leu. mesenteroides* TA produced also almost the same concentration of phenyllactic acid (0.08-0.1 mM) as *Leu. mesenteroides* ATCC 8293. Thus, we examined if SPE-AS contains phenyllactic acid using HPLC analysis, as phenyllactic acid has hydrophobicity with established antibacterial (26) and antifungal activities (2,7). As shown in Fig. 3C, phenyllactic acid was not detected from SPE-AS, whereas the injected SPE-AS into HPLC (10 µL) displayed antifungal activity of 800 AU/mL (Fig. 3D). On the other hand, injection of 1,000 ppm (10 µL) of phenyllactic acid as an authentic standard showed a sharp and distinct peak but no antifungal activity (Fig. 3A and 3B). The tested 1,000 ppm concentration in Fig. 3A is much higher (60x) than the concentration (0.08-0.1 mM) produced by *Leu. mesenteroides* TA and ATCC 8293. The result suggests that SPE-AS contains certain unidentified antifungal compound(s) that is not phenyllactic acid.

Characteristics of antifungal compounds from *Leu. mesenteroides* TA

As shown in Table 4, heat treatment at 4-121°C as well as different enzyme treatments did not affect antifungal activities of the cell-free culture filtrate and SPE-AS from *Leu. mesenteroides* TA, and pH levels ranging from 3.0-4.0 did not affect antifungal activity either. The antifungal activity of the culture filtrate decreased sharply at pH 5.0, after which activity was completely eliminated over pH 6.0. However, the antifungal activity of SPE-AS was not eliminated at pH 5.0-8.0, even though its activity decreased to 25% of its maximum activity at pH 3.0-4.0. The function of phenyllactic acid is known to be pH-dependent (27). However, SPE-AS prepared in this study did not show an absolute pH-labile property.

It has been reported that *Leu. mesenteroides* ATCC 8293 exclusively produces the D-form of phenyllactic acid (13). D-Phenyllactic acid was

Fig. 3. HPLC analysis of PLA and its antifungal activity. (A) PLA standard; (C) SPE-AS from *Leu. mesenteroides* TA. Antifungal activities of PLA standard (B) and SPE-AS (D) from *Leu. mesenteroides* TA were determined against *A. fumigatus* ATCC 96918 using spot-on-the-lawn assay.

reported to display antibacterial activity (26) and L-phenyllactic acid has more potent antifungal activity than D-phenyllactic acid (27). LAB producing phenyllactic acid with high antifungal activity are mainly *Lactobacillus* species (28-35). Even though phenyllactic acid was previously reported as an antifungal compound from *Leu. mesenteroides* ATCC 8293, only weak antifungal activity of *Leu. mesenteroides* ATCC 8293 was observed with 0.1 mM phenyllactic acid detected in culture of *Leu. mesenteroides* ATCC 8293 (12,13). However, antifungal activity of 0.1 mM phenyllactic acid was not verified in their studies (12,13).

There have been several investigations about antifungal activity of *Leu. mesenteroides* (10-12,14,16,24,36,37), however, only a small number of investigations successfully identified antifungal compound from *Leu. mesenteroides*, such as lactic acid, acetic acid, phenyllactic acid, and peptides (10-12,14). In this study, we demonstrated lactic acid, acetic acid, and unidentified hydrophobic compounds in SPE-AS as the responsible antifungal compounds from *Leu. mesenteroides* TA. Moreover, 0.1 mM phenyllactic acid from *Leu. mesenteroides* TA did not present antibacterial or antifungal activity. Despite of this fact, a mixture of organic acids produced by *Leu. mesenteroides* TA, including lactic, acetic, and phenyllactic acids showed increased antibacterial activity compared to individual organic acids, but

Table 4. Stability of antifungal compounds from *Leu. mesenteroides* TA

	Treatment	Culture filtrate ¹⁾	SPE-AS ²⁾
pH	Control (pH 4.4)	40	800
	3.0	40	800
	4.0	40	800
	5.0	12	200
	6.0	0	200
	7.0	0	200
	8.0	0	200
Heating	4°C, 24 h	40	800
	25°C, 24 h	40	800
	30°C, 24 h	40	800
	50°C, 24 h	40	800
	70°C, 24 h	40	800
	100°C, 30 min	40	800
	121°C, 15 min	40	800
Enzyme	proteinase K	40	800
	protease	40	800
	pepsin	40	800
	trypsin	40	800
	aminopeptidase I	40	800
	carboxypeptidase A	40	800
	α-amylase	40	800
	lipase	40	800

A. fumigatus ATCC 96918 was used as an indicator for antifungal activity assay.

¹⁾Cell-free culture filtrate of *Leu. mesenteroides* TA.

²⁾Solid phase extraction (SPE) of the culture filtrate of *Leu. mesenteroides* TA containing the active substance was prepared according to materials and methods.

unaltered antifungal activity. Only a few studies have demonstrated wide spectrum antimicrobial activity, especially having antifungal activity on *Leuconostoc* isolates (35). In this study, we isolated a broad spectrum antifungal LAB, *Leu. mesenteroides* TA. The use of *Leu. mesenteroides* TA may lead to the development of useful biopreservative systems in food industries. The unidentified novel antifungal compound(s) in SPE-AS, which is hydrophobic and non-proteinaceous compound, will further be purified and identified in terms of structure in the following investigation, despite its low productivity.

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Disclosure The authors declare no conflict of interest.

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