Lim *Fisheries and Aquatic Sciences* (2016) 19:42
DOI 10.1186/s41240-016-0040-x

Fisheries and Aquatic Sciences

RESEARCH ARTICLE Open Access

Inhibitory effect of bacteriocin-producing lactic acid bacteria against histamine-forming bacteria isolated from *Myeolchi-jeot*

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Abstract

The objectives of this study were to identify the histamine-forming bacteria and bacteriocin- producing lactic acid bacteria (LAB) isolated from *Myeolchi-jeot* according to sequence analysis of the 16S rRNA gene, to evaluate the inhibitory effects of the bacteriocin on the growth and histamine accumulation of histamine-forming bacteria, and to assess the physico-chemical properties of the bacteriocin. Based on 16S rRNA gene sequences, histamine-forming bacteria were identified as *Bacillus licheniformis* MCH01, *Serratia marcescens* MCH02, *Staphylococcus xylosus* MCH03, *Aeromonas hydrophila* MCH04, and *Morganella morganii* MCH05. The five LAB strains identified as *Pediococcus acidilactici* MCL11, *Leuconostoc mesenteroides* MCL12, *Enterococcus faecium* MCL13, *Lactobacillus sakei* MCL14, and *Lactobacillus acidophilus* MCL15 were found to produce an antibacterial compound with inhibitory activity against the tested histamine-producing bacteria. The inhibitory activity of these bacteriocins obtained from the five LAB remained stable after incubation at pH 4.0–8.0 and heating for 10 min at 80 °C; however, the bacteriocin activity was destroyed after treatment with papain, pepsin, proteinase K, α-chymotrypsin, or trypsin. Meanwhile, these bacteriocins produced by the tested LAB strains also exhibited histamine-degradation ability. Therefore, these antimicrobial substances may play a role in inhibiting histamine formation in the fermented fish products and preventing seafood-related food-borne disease caused by bacterially generated histamine.

Keywords: Bacteriocin, Histamine, Lactic acid bacteria

Background

Myeolchi-jeot, a traditional Korean salted and fermented seafood, is made of anchovies (*Engraulis japonicas*) and is mostly used as an ingredient in Kimchi. Since proteins are broken down into precursor amino acids of biogenic amines through the action of digestive enzymes and microbes during the fermentation process, it contains relatively high concentrations of biogenic amines (Mah et al., 2002). According to their chemical structure, biogenic amines are classified into aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenlethylamine), and heterocyclic (histamine, tryptamine) (Santos, 1996). High doses of biogenic amines such as histamine (>500 mg/kg) and tyramine (100–800 mg/kg) contained mainly in fermented foods can cause adverse health effects to consumers (Joosten and Nuńez, 1996).

Histamine is the causative agent of scombroid poisoning which is often manifested by a wide variety of symptoms such as rash, urticarial, nausea, vomiting, diarrhea, abdominal cramp, hypotension, localized inflammation, headache, palpitation, and severe respiratory distress (Taylor, 1986). Histamine is a basic nitrogenous compound formed mainly through the decarboxylation of histidine by exogenous decarboxylases released from the many different bacterial species associated with salted and fermented seafoods including *Myeolchi-jeot* (An and Ben-Gigirey, 1998). In *Myeolchi-jeot*, *Bacillus licheniformis* has been known to raise levels of histamine in retail canned anchovies during the storage at an ambient temperature Mah and Hwang (2003). The presence of histidine decarboxylase activity has been described in different microbial groups such as seafood-borne bacteria,

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spoilage microorganisms, and lactic acid bacteria (López-Sabater et al., 1994).

Fortunately, in recent years, some lactic acid bacteria (LAB) have been reported to degrade biogenic amine through the production of amine oxidase enzymes or antimicrobial substances (Garciz-Ruiz et al., 2011; Joosten and Nuńez, 1996). LAB that are usually designated as generally recognized as safe (GRAS) status in foods can also exert a biopreservative effect against other microorganisms as a result of competition for nutrients and/or of the production of antagonistic compounds such as organic acids, diacetyl, acetoin, hydrogen peroxide, antibiotics, and bacteriocins (Schillinger et al., 1996). Among anti-microbial metabolites, bacteriocins are ribosomally synthesized and defined as extracelluarly released peptides or protein molecules produced by specific bacteria during the primary phase of growth, though antibiotics are usually secondary metabolites (Zacharof and Lovitt, 2012). Brillet et al. (2005) has shown that bacteriocin produced by *Carnobacterium divergens* V41 can be used as a biopreservative to inhibit the growth *Listeria monocytogenes* in cold smoked salmon; therefore, bacteriocins can be applied in the seafood industry for extension of shelf life as natural preservatives against pathogens and food spoilage.

In particular, outgrowth of histamine producer *Lactobacillus buchneri* St2A was almost completely inhibited by treatment of bacteriocin-producing enterococci and *Lactococcus lactis* strain as fermentation starters (Joosten and Nuńez, 1996). Tabanelli et al. (2014) reported that bacteriocin forming lactococci strains were able to reduce the growth extent and histamine accumulation of *Streptococcus thermophilus* PRI60. The studies reported previously indicate that the applications of bacteriocins in food industries can extend shelf life of foods, inhibit growth of foodborne pathogens during the manufacture of food, prevent formation of toxic substances by harmful bacteria, ameliorate the economic losses due to food spoilage, and reduce the application of chemical preservatives (Gálvez et al., 2007).

The objectives of this study were to (1) identify the histamine-forming bacteria and bacteriocin-producing LAB isolated from *Myeolchi-jeot* according to sequence analysis of the 16S rRNA gene, (2) evaluate the inhibitory effects of the bacteriocin on the growth and histamine accumulation of histamine-forming bacteria, and (3) assess the physico-chemical properties of the bacteriocin.

Methods

Isolation and identification of histamine-forming bacteria and LAB

Five samples of salted-fermented anchovy were obtained from retail stores in Busan and aseptically collected for these experiments. Each sample (50 g) was diluted with sterile peptone water (450 mL) and homogenized at high speed for 2 min in stomacher. The decimal serial dilutions of the homogenates were then subsequently prepared using a sterile peptone water, and 1.0-mL aliquots of the dilutes were inoculated into brain heart infusion (BHI) agar (BD Difco Co., Sparks, MD, USA) and incubated for 48 h at 37 °C. To isolate histamine-forming bacteria, each strain collected from the plates was subcultured for 48 h at 35 °C in decarboxylating broth contained L-histidine monohydrochloride monohydrate (Sigma-Aldrich, St Louis, MO, USA, 1 g/L) and pyridoxal-5′-phosphate (Sigma-Aldrich, 1 mg/L) according to the method of Bover-Cid and Holzapfel (1999) with minor modifications. The cell culture (0.1 mL) was spread on trypticase soy agar (TSA, BD Difco Co.) supplemented with 2.0% (*w*/*v*) L-histidine hydrochloride monohydrate. After incubation under anaerobic conditions for 4 days at 35 °C (Anoxomat system, MART Co., Netherland), the colonies with blue or purple color on the plates were considered as histamine-forming bacteria. Meanwhile, to distinguish lactic acid-producing bacteria from salted-fermented anchovy, the homogenized sample was spread directly onto the surface of MRS (BD Difco Co.) agar plates containing 1% $CaCO_3$. After incubation under aerobic conditions for 48 h at 37 °C, the colonies surrounded by a clear zone were randomly selected from the plates, purified on MRS agar, and examined histamine production as the abovementioned. The identity of histamine-forming isolates was confirmed by using 16S rRNA gene sequence analysis (Chen et al., 2008). Amplification of the isolates was performed with the universal primers UNI-L (5′-AGAGTTT GATCATGGCTCAG-3′) and UNI-R (5′-GTGTGACGG GCGGTGTGTAC-3′). Bacterial cells were cultivated in BHI broth at 37 °C with shaking overnight and centrifuged at 7000×*g* for 10 min. After washing, the cell pellets were resuspended in PBS (pH 7.0) and lysed by 20% sodium dodecyl sulfate (SDS). After the boiling process for 20 min at 85 °C, the cell debris was removed by centrifugation (13,000×*g*, 5 min, 4 °C). DNA in the supernatant was precipitated by addition of 70% ethanol and used as template DNA for polymerase chain reaction (PCR). PCR amplification was carried out with a reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 pmol of each primer, 0.2 mM deoxynucleotide triphosphates, 0.5 U of Taq DNA polymerase (Applied Biosystem, Foster City, CA, USA), and template DNA (10 ng). Cycle conditions were an initial denaturation for 4 min at 94 °C, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C, 1 min primer extension at 72 °C, followed by a final extension for 7 min at 72 °C in a PCR Thermal Cycler (Bio-Rad Laboratories Ltd., Canada). To identify the LAB isolates which do not produce histamine,

the universal primer used for identification of LAB was 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492RF (5′-GGTTACCTTGTTACGACTT-3′). Thermocycling was carried out using denaturation step at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, for a total of 30 cycles. Amplicons were visualized on a 1.5% agarose gel staining with ethidium bromide to confirm successful amplification and then PCR product was purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). The amplified DNA was directly sequenced with a DNA sequencer (ABI Prism® 3730 Avant Genetic Analyzer, Applied Biosystem) and sequence similarity searching was performed using the National Center for Biotechnology Information (NCBI) Basic Local Align Search Tool (BLAST).

Preparation of bacteriocin solution and determination of bacteriocin activity

To prepare the bacteriocin solution of the LAB strains, the culture extracts of the isolated LAB were obtained after 24-h incubation at 37 °C in MRS broth. The cultures were centrifuged at 7000×*g* for 10 min at 4 °C. The cell-free supernatant (CFS) was then adjusted with 1 N of NaOH to pH 6.5 to avoid effect of acid, treated with catalase (200 U/mL) for 30 min at 25 °C to remove hydrogen peroxide, and filtered through a 0.22-μm membrane filter (Millipore Corp., USA). Crude bacteriocin was precipitated from the CFS by 50% (*w*/*v*) ammonium sulfate and the precipitates were kept overnight at 4 °C with gentle stirring. After centrifugation (at 12,000×*g* for 30 min at 4 °C), the resulting pellets were dissolved in 20 mM sodium phosphate buffer (pH 6.5) and dialyzed overnight at 4 °C against distilled water using dialysis membrane (molecular weight cutoff, 1,000; Spectrum Labs., Gardena, CA, USA). The antimicrobial activity of the bacteriocin was assayed by microtiter plate assay (Holo et al., 1991), using histamine-forming bacteria as an indicator strain. In brief, each well of the microtiter plate (BD Falcon, Franklin Lakes, NJ, USA) was filled with 800 μL of BHI broth, 100 μL of a twofold serial dilution of the crude bacteriocin solution or PBS (pH 7.0), and 100 μL of cell cultures (1.0×10^5 CFU/mL) of indicator organism grown in BHI broth. The plates were then placed in an aerobic incubator for 12 h at 37 °C. The extent of growth inhibition was measured spectrophotometrically at 600 nm using microplate reader (Bioteck, Inc., Korea). One bacteriocin unit (BU/mL) was arbitrarily defined as the reciprocal of the highest dilution inhibiting the 50% growth of the indicator strain compared to the control.

Inhibitory effect of the bacteriocin on histamine accumulation

Histamine-forming bacteria isolated from the samples were seeded in TSB enriches with L-histidine hydrochloride monohydrate (0.5%) and pyridoxal-HCl (0.0005%) and incubated at 35 °C for 24 h. The cultures (1 mL) were transferred to test tubes containing the same broth (9 mL) and bacteriocin solution (100 and 200 BU/mL) from the LAB isolates and incubated for 24 h at 35 °C. These broth cultures were centrifuged (7000×*g*, 10 min, 4 °C) and filtered through a 0.22-μm membrane filter. The histamine content in the cultures was performed according to the procedure modified by Eerola et al. (1993) and Mah and Hwang (2003) using high-pressure liquid chromatography (HPLC, Hitachi, Tokyo, Japan). Briefly, 1 mL of the cell cultures or standard histamine solutions was added to 9 mL of 0.4 M perchloric acid (Merck, Darmstadt, Germany) and vigorously mixed. The mixture was then centrifuged at 3000×*g* for 10 min and the supernatant was filtered through Whatman paper No. 1. The samples (1 mL) were mixed with 2 M sodium hydroxide (200 μL) and saturated sodium bicarbonate solution (300 μL). Subsequently, the samples were added with 2 mL of 10 mg/mL dansyl chloride (Sigma-Aldrich) solution prepared in acetone and the mixture solution was incubated at 40 °C for 45 min. Residual dansyl chloride was removed by addition of 25% ammonium hydroxide (100 μL). After incubation for 30 min at room temperature, the volume of mixture was adjusted to 5 mL with acetonitrile. Finally, the dansyl derivatives were centrifuged at 2500×*g* for 5 min, and the supernatant was filtered through a 0.22-μm filter. A Nova-Pak C_{18} column (150 mm × 3.9 mm, Waters, Milford, MA, USA) was used for the separation of histamine, and acetonitrile (solvent B) as the mobile phases at the flow rate of 1 mL/min. The sample volume injected was 20 μL, and the eluted sample was monitored at 254 nm. All experiments were performed in triplicate. Data are expressed as means ± standard deviation (SD) and analyzed with SPSS program (ver. 12.0, SPSS Inc., Chicago, IL, USA). Then data comparisons were performed with paired *t* test and differences were considered statistically significant when P values were <0.05.

Physical and chemical properties of the bacteriocn

The effect of pH on antimicrobial activity of the crude bacteriocin was determined by incubating the crude bacteriocin in various buffers [0.1 M acetate buffer (pH 2.0–5.0), 0.1 M phosphate buffer (pH 6.0–7.0), and Tris HCl (pH 8.0–10.0)] with pH ranging from 2 to 10 for 24 h at 37 °C. To evaluate the heat stability, the crude bacteriocin was heated for 10 min at 80, 100, and 120 °C. Sensitivity of the crude bacteriocin to proteolytic enzymes was examined by incubation of the sample for 1 h at 37 °C after treatment with the following enzymes (1 mg/mL): proteinase K in 50 mM Tris-HCl (pH 7.5), trypsin in 50 mM Tris-HCl (pH 8.0), pepsin in 100 mM

Trish-HCl (pH 3.0), papain in 50 sodium phosphate acetate (pH 5.0), and α-chymotrypsin in 20 mM Tris-HCl (pH 8.0). Following incubation, the enzymes were heat inactivated for 3 min at 100 °C. The residual bacteriocin activity was determined by the microtiter plate assay as described earlier. In addition, the bacteriocin treated under each physical or chemical condition were added to TSB containing L-histidine hydrochloride monohydrate (0.5%) and pyridoxal-HCl (0.0005%) and inoculated with initial population of approximately 1.0×10^5 CFU/mL of histamine-forming bacteria. After incubation for 24 h at 35 °C, histamine levels in the cultures were analyzed using HPLC described above. All the experiments were done in triplicate.

Results and discussion

Isolation and identification of histamine-forming bacteria and bacteriocin-producing LAB

The results of the identification of histamine-forming bacteria and bacteriocin-producing LAB found in *Myeolchi-jeot* are presented in Table 1. Based on 16S rRNA gene sequences, histamine-forming bacteria were identified as *B. licheniformis* MCH01, *Serratia marcescens* MCH02, *Staphylococcus xylosus* MCH03, *Aeromonas hydrophila* MCH04, and *Morganella morganii* MCH05, with 98.0–99.9% similarity to the GenBank database. Meanwhile, 53 LAB strains isolated from *Myeolchi-jeot* were screened for the inhibition activity against histamine-forming bacteria, using a microtiter plate assay. Out of these strains, five strains (9.4%) presented 98.3–100.0% similarity with the 16S rRNA sequences reported for *Pediococcus acidilactici* MCL11, *Leuconostoc mesenteroides* MCL12, *Enterococcus faecium* MCL13, *Lactobacillus sakei* MCL14, and *Lactobacillus acidophilus* MCL15 in the GenBank database.

Several fish species including tuna, mackerel, sardines, and anchovy contain high levels of free histidine in their tissues. These fishes subjected to temperature abuse after the catch and before consumption can cause formation of histamine from histidine by bacterial histidine decarboylases (Visciano et al., 2012). Histamine-rich foods may cause food intolerance in sensitive individuals and histamine poisoning that is a common seafood-borne disease causing various symptoms such as rash, nausea, vomiting, fever, diarrhea, headache, itching, flushing, and abdominal cramps (Taylor, 1986). *Proteus vulgaris*, *Proteus mirabilis*, *Clostridium perfringens*, *Enterobacter aerogenes*, *Klebsiella pneumonia*, *Hafnia alvei*, and *Vibrio alginolyticus* and enterobacteriaceae have been isolated from fish and described as the predominant histamine-forming bacteria (Shalaby, 1996; Kung et al., 2009). Moreover, there have been several reports describing amino acid decarboxylation activity of different genera, such as *Acinetobacter*, *Aeromonas*, *Bacillus*, *Cedecea*, *Citrobacter*, *Clostridium*, *Escherichia*, *Klebsiella*, *Plesiomonas*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, and *Vibrio*, and of some lactic acid bacteria (Kim et al., 2009). Our data are partially consistent with results reported by Guan et al. (2011) and Kung et al. (2009). The commonly isolated genera from *Myeolchi-jeot* were *Microbacterium* sp., *Kocuria* sp., *Vibrio* sp., *Psychrobacter* sp., *Halomonas* sp., *Brevibacillus* sp., *Bacillus* sp., *Enterococcus* sp., *Tetragenococcus* sp., *Weissella* sp., *Lactobacillus* sp., and *Staphylococcus* sp. (Guan et al., 2011). Among bacterial communities in Jeotgal, *M. morganii*, *K. pneumoniae*, and *H. alvei* have been known as the strains causing scombroid poisoning (often called "histamine poisoning") (Kung et al., 2009). In addition, *Staphylococcus epidermidis* obtained during the ripening of anchovies showed a powerful histamine-forming activity (Hernández-Herrero et al. 1999).

Bacteriocin activity of the isolates against histamine-forming bacteria

As shown in Table 2, the five LAB strains isolated from *Myeolchi-jeot* were found to produce an antibacterial compound with inhibitory activity against histamine-producing bacteria such as *B. licheniformis* MCH01, *S. marcescens* MCH02, *S. xylosus* MCH03, *A. hydrophila* MCH04, or *M. moarganii* MCH05. The crude bacteriocin of *P. acidilactici* MCL11 inhibited the growth of *B.*

Table 1 Identification of histamine-forming bacteria and lactic acid bacteria isolated from *Myeolchi-jeot* using 16S rRNA gene sequence analysis

Histamine-forming bacteria				LAB			
Strain	Accession no. related strain in NCBI	Similarity (%)	Identification	Strain	Accession no. related strain in NCBI	Similarity (%)	Identification
MCH01	EF433410	98.6	*Bacillus licheniformis*	MCL11	EF059986	99.9	*Pediococcus acidilactici*
MCH02	EF194094	99.7	*Serratia marcescens*	MCL12	KF673541	100.0	*Leuconostoc mesenteroides*
MCH03	EU266748	99.9	*Staphylococcus xylosus*	MCL13	EU887814	99.2	*Enterococcus faecium*
MCH04	AY538658	99.0	*Aeromonas hydrophila*	MCL14	AB650590	98.3	*Lactobacillus sakei*
MCH05	AB680150	98.0	*Morganella morganii*	MCL15	KT222158	99.5	*Lactobacillus acidophilus*

Table 2 Antibacterial activity of the bacteriocin produced by lactic acid bacteria against histamine-forming bacteria isolated from *Myeolchi-jeot*

Indicator organism	Bacteriocin activity (BU/mL)				
	LAB				
	Pediococcus acidilactici MCL11	*Leuconostoc mesenteroides* MCL12	*Enterococcus faecium* MCL13	*Lactobacillus sakei* MCL14	*Lactobacillus acidophilus* MCL15
Bacillus licheniformis MCH01	256	ND	ND	ND	ND
Serratia marcescens MCH02	ND	128	ND	256	ND
Staphylococcus xylosus MCH03	512	ND	64	ND	ND
Aeromonas hydrophila MCH04	ND	ND	256	ND	64
Morganella morganii MCH05	ND	ND	ND	128	ND

ND not detected

licheniformis MCH01 and *S. xylosus* MCH03. In particular, *P. acidilactici* MCL11 showed the strongest antimicrobial activity (512 BU/mL) against *S. xylosus* MCH03. The antimicrobial compound produced by *L. mesenteroides* MCL12 (128 BU/mL) and *L. sakei* MCL14 (256 BU/mL) strains showed activity against *S. marcescens* MCH02. The crude bacteriocin produced by *E. faecium* MCL13 showed an antimicrobial activity of 64 and 256 BU/mL against *S. xylosus* MCH03 and *A. hydrophila* MCH04, respectively. *L. acidophilus* MCL15 was also found to have a bacteriocin activity against *A. hydrophila* MCH04, which was weaker than that of *E. faecium* MCL13.

Joosten and Nuñez (1996) reported that the bacteriocin-producing enterococci and *L. lactis* strains completely inhibited the outgrowth of histamine producer *L. buchneri* St2A; therefore, no histamine formation was detected in the cheeses made with bacteriocin-producing starters. Our results are in agreement with the findings of previous studies. Gómez-Sala et al. (2015) demonstrated that analysis of 1245 LAB isolates obtained from fish, seafood, and fish products showed that 197 exerted direct antimicrobial activity against 20 spoilage and food-borne pathogenic microorganisms. Furthermore, LAB isolates selected on the basis of their direct antimicrobial activity were identified as *E. faecium*, *E. faecalis*, *Pediococcus pentosaceus*, *Weissella cibaria*, *L. sakei* subsp. *carnosus*, *L. sakei* subsp. *sakei*, *Lactobacillus curvatus*, and *L. mesenteroides* subsp. *cremoris* based on 16S rDNA sequences. *L. mesenteroides* HK4, HK5, and HK11 and *Streptococcus salivarius* HK8 strains isolated from Jeotgal were also chosen by preliminary bacteriocin activity test (Cho and Do, 2006). *L. lactis* subsp. *lactis* VR84 produced nisin Z induced the death of the histamine producing strain *S. thermophilus* PRI60. However, *L. lactis* subsp. *lactis* EG46-produced lacticin 481 did not show a lethal action against PRI60 strain, but were able to reduce its growth extent and histamine accumulation (Tabanelli et al., 2014). Furthermore, the bacteriocin produced by *L. casei* was able to inhibit the activity of the histamine-forming bacteria such as *Pseudomonas* sp., *Proteus morganii*, and *Micrococcus* sp. (Nugrahani et al., 2016). *S. xylosus* no. 0538 obtained from a salted and fermented anchovy (*Myeolchi-jeot*) possessed not only the greater capability to degrade histamine but a detectable ability to degrade tyramine as well. In addition, this strain was also found to produce the bacteriocin-like inhibitory substance(s) and have the highest antimicrobial activity against *B. licheniformis* strains defined as amine producers. *S. xylosus* no. 0538 exhibited significantly greater ability to degrade histamine, degrading histamine to about 62–68% of its initial concentration within 24 h (Mah and Hwang, 2009).

Reduction of histamine accumulation by the bacteriocin of LAB

As shown in Table 3, the five histamine-producing bacteria isolated from *Myeolchi-jeot* had strong ability to produce histamine. Among the tested strains, the highest level of histamine (2869.4 ± 49.0 mg/L) formation was observed for the *M. morganii* MCH05 strain. In addition, the concentration of histamine produced by *S. xylosus* MCH03 (2257 ± 30.7 mg/L) strain was higher than that produced by *B. licheniformis* MCH01 (1699.3 ± 35.6 mg/L), *S. marcescens* MCH02 (1987.2 ± 27.8 mg/L), and *A. hydrophila* MCH04 (1655.5 ± 41.2 mg/L). Meanwhile, the treatment with 100 and 200 BU/mL of the bacteriocin obtained from the tested LAB significantly reduced the histamine content of the five histamine-producing bacteria. After 24 h of incubation in the presence of the bacteriocin (200 BU/mL) of *P. acidilactici* MCL11, histamine contents of *B. licheniformis* MCL01 and *S. xylosus* MCH03 were reduced by 49 and 27%, respectively, as compared to the control (without bacteriocin). The histamine accumulation of *S. xylosus* MCH03 and *A. hydrophilia* MCH04 at 24 h of incubation in the presence of the bacteriocin of *E. faecium* MCL13 was significantly inhibited compared with the control group. The bacteriocin obtained from *L. sakei* MCL14 was effective in degrading histamine produced by *S. marcescens* MCH02 and *M. morganii* MCH05 strains. These bacteriocins produced by *L. mesenteroides* MCL12

Table 3 Inhibitory effect of the bacteriocin from the tested LAB on histamine accumulation of histamine-forming bacteria isolated from *Myeolchi-jeot*

Histamine-forming bacteria	Histamine content (mg/L)										
	Control	Bacteriocin-producing LAB									
		MCL11		MCL12		MCL13		MCL14		MCL15	
		Bacteriocin concentration (BU/mL)									
		100	200	100	200	100	200	100	200	100	200
Bacillus licheniformis MCH01	1699.3 ± 35.6	1086.9 ± 31.2*	863.2 ± 22.3*	1668.4 ± 45.4	1681.5 ± 24.8	1679.5 ± 28.1	1673.2 ± 15.9	1667.3 ± 40.0	1702.5 ± 12.4	1671.5 ± 23.6	1715.2 ± 39.1
Serratia marcescens MCH02	1987.2 ± 27.8	1979.1 ± 16.3	1995.8 ± 20.5	1423.5 ± 14.1*	1016.3 ± 10.7*	1964.3 ± 30.5	1990.5 ± 9.9	1619.1 ± 9.8*	1426.3 ± 20.4*	1964.0 ± 13.8	1980.5 ± 34.1
Staphylococcus xylosus MCH03	2257.4 ± 30.7	1805.8 ± 21.7*	1653.7 ± 10.1*	2234.1 ± 13.2	2294.0 ± 17.5	754.3 ± 12.5*	518.5 ± 16.1*	2219.8 ± 29.4	2273.4 ± 26.0	2281.3 ± 41.2	2270.3 ± 11.6
Aeromonas hydrophila MCH04	1655.5 ± 41.2	1603.8 ± 30.8	1682.3 ± 25.5	1690.3 ± 33.4	1682.5 ± 26.1	1518.2 ± 17.6*	1305.8 ± 20.4*	1633.9 ± 8.7	1690.5 ± 22.8	909.7 ± 11.4*	825.5 ± 18.8*
Morganella morganii MCH05	2869.4 ± 49.0	2805.3 ± 10.2	2811.5 ± 20.7	2897.0 ± 36.1	2815.9 ± 7.5	2900.5 ± 16.4	2822.4 ± 13.8	1915.4 ± 30.7*	1688.5 ± 21.2*	2809.9 ± 18.4	2894.6 ± 33.8

Data are presented as mean ± standard deviation (SD) from three independent experiments
*Significantly differ ($P < 0.05$) from the control group by paired *t* test

and *L. acidophilus* MCL15 strains also exhibited excellent histamine degradation ability. The histamine degradation ability of these bacteriocins increased in a concentration-dependent manner. The reduction of histamine content by treatment with these bacteriocins may be related to antagonistic activity of these antibacterial substances against histamine-producing bacteria.

Prolific histamine formers in Indian anchovy were identified as *M. morganii*, *P. vulgaris*, and *E. aerogenes*, and produced high histamine content of 104.1–203.0 mg/100 mL (Rodtong et al., 2005). Kim et al. (2009) noted that the histamine contents in fish, squid, and shellfish samples remarkably increased up to 36.6–2123.9 mg/kg after 24 h of storage at 25 °C, while the contents began to gradually increase after 2–3 days of storage at 4–10 °C. The dominant microbial group in these samples was enterobacteria throughout the storage period. In our results, the histamine-producing isolates from *Myeolchi-jeot* were identified as *B. licheniformis*, *S. marcescens*, *S. xylosus*, *A. hydrophila*, and *M. morganii* and these strains were capable of producing histamine in the range of 1655.5 to 2869.4 mg/L. Consequently, in raw fish, histamine content is linked to the type of histamine-forming bacteria, the type of seafood, and temperature/time storage conditions (Visciano et al., 2012).

Regarding to the inhibition of histamine formation, Zaman et al. (2011) observed that *Staphylococcus carnosus* FS19 and *Bacillus amyloiquefaciens* FS05 isolated from fish sauce which possess amine oxidase activity were found to be effective in reducing biogenic amine accumulation, and histamine concentration was reduced by 27.7 and 15.4% by FS19 and FS05, respectively, which is in disagreement with our observations that the LAB isolates tested in our study showed histamine degradation capacity by production of the antimicrobial substance such as bacteriocin. The histamine levels produced by *B. licheniformis* MCH01, *S. marcescens* MCH02, *S. xylosus* MCH03, *A. hydrophila* MCH04, and *M. morganii* MCH05 were reduced by 21–77% in the presence of the bacteriocin (200 BU/mL) obtained from the tested LAB.

Effect of enzymes, pH, and temperature on antimicrobial activity of the bacteriocin

These bacteriocins obtained from *P. acidilactici* MCL11 and *L. sakei* MCL14 remained stable after incubation at pH 4.0–8.0. However, no the bacteriocin activity was recorded under extremely acidic condition (pH 2.0). The bacteriocin of *L. mesenteroides* MCL12 and *E. faecium* MCL13 remained stable after incubation for 24 h at pH from 4.0 to 10.0, but not when kept at pH 2.0. The bacteriocin of *L. acidophilus* MCL15 remained active after 24 h of exposure to pH values ranging from 3.0 to 9.0 at 37 °C. The activity of the bacteriocin produced by *P. acidilactici* MCL11, *L. mesenteroides* MCL12, and *E. faecium* MCL13 remained almost completely after heating for 10 min at 80 °C; however, the bacteriocin activity of these strains was partially destroyed after 10 min at 100 °C. In particular, the bacteriocin produced by *E. faecium* MCL13 was inactivated after 10 min at 120 °C, whereas the inhibitory activity of *L. sakei* MCL14 was 100% stable to heat treatment at 100 °C for up to 10 min, and the antimicrobial activity of *L. acidophilus* MCL15 was not affected by the heat treatment for 10 min at 120 °C. The activity of the bacteriocin produced by *P. acidilactici* MCL11 was destroyed after treatment with papain, pepsin, and proteinase K, but not when treated with α-chymotrypsin and trypsin. However, the treatment with papain and proteinase K had no effect on the activity of the bacteriocin of *L. mesenteroides* MCL12. Treatment of the bacteriocin produced by *E. faecium* MCL13 with papain and trypsin did not result in any activity loss, but the bacteriocin activity partially inactivated by treatment with pepsin, proteinase K, and α-chymotrypsin. Meanwhile, the bacteriocin of *L. sakei* MCL14 was destroyed by all the proteolytic enzymes tested such as papain, pepsin, proteinase K, α-chymotrypsin, and trypsin. Complete inactivation in antimicrobial activity of the bacteriocin produced by *L. acidophilus* MC15 was observed after treatment with α-chymotrypsin and trypsin. The histamine-degrading ability of the bacteriocin inactivated by some physico-chemical treatments was significantly reduced compared to the control group (Table 4).

The bacteriocin activity of *P. acidilactici* MCL11 was not affected by treatment with α-chymotrypsin and trypsin, but was lost after incubation with proteolytic enzymes such as papain, pepsin, and proteinase K. This bacteriocin was stable at up to 80 °C for 10 min and was in the pH range of 4.0–8.0. This is in disagreement with results recorded for pediocin SA-1. Pediocin SA-1 from *P. acidilactici* NRRLB5627 was inhibitory to several food spoilage bacteria and food-borne pathogens such as *Listeria* spp. and found to be very effective against the anaerobic *Clostridium sporogenes* and *Clostridium thiaminolyticum* (Papagianni and Anastasiadou, 2009). This bacteriocin was heat stable for up to 60 min at 121 °C, not impaired even following incubation at 30 °C for 1 week at pH values ranging between 3.0 and 12.0, and found to be resistant to treatment with trypsin, α-chymotrypsin, pepsin, and papain, but not to proteinase K (Anastasiadou et al., 2008).

The bacteriocin produced by *L. mesenteroides* MCL12 showed slight resistance to proteolytic enzymes such as α-chymotrypsin and trypsin. Treatment of this bacteriocin with papain and proteinase K had no effect on activity. The activity of this bacteriocin did not decrease after heat treatment at 80 °C for 10 min and the stability of the antimicrobial activity was observed at pH range of 4.0 to 10.0. Characteristics of the bacteriocin produced by *L. mesenteroides* MCL12 were widely different from

Table 4 Effects of pH, temperature, and proteolytic enzymes on the antibacterial activity and histamine-degrading ability of the bacteriocin from the tested LAB

Treatment		Bacteriocin-producing LAB																	
		MCL11[a]				MCL12[b]				MCL13[c]				MCL14[d]				MCL15[e]			
		BA (BU/mL)		HC (mg/L)		BA (BU/mL)		HC (mg/L)		BA (BU/mL)		HC (mg/L)		BA (BU/mL)		HC (mg/L)		BA (BU/mL)		HC (mg/L)	
		C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
pH	2.0	512	ND	1156.4 ± 22.1	2251.4 ± 16.9	128	16	1217.5 ± 30.5	1898.2 ± 16.9	256	128	1236.3 ± 15.4	1476.2 ± 24.2	256	ND	1367.5 ± 11.8	1972.5 ± 13.5	64	32	926.5 ± 37.8	1325.2 ± 11.3
	3.0		128		1715.2 ± 26.5		32		1853.6 ± 15.2		256		1204.3 ± 18.2		64		1707.2 ± 21.3		64		1161.4 ± 19.2
	4.0–8.0		512		1208.2 ± 30.2		128		1198.5 ± 47.1		256		1247.0 ± 40.3		256		1388.2 ± 16.7		64		1185.7 ± 21.2
	9.0		256		1619.5 ± 22.5		128		1219.3 ± 38.2		256		1203.4 ± 32.1		128		1571.4 ± 22.4		64		1201.2 ± 34.2
	10.0		64		1906.1 ± 30.7		128		1199.6 ± 30.8		256		1216.4 ± 25.4		128		1586.1 ± 13.7		32		1334.0 ± 40.2
Heating	80 °C, 10 min		512		1178.9 ± 42.2		128		1203.5 ± 13.2		256		1207.3 ± 19.3		256		1375.1 ± 48.8		64		1194.4 ± 27.1
	100 °C, 10 min		128		1690.5 ± 30.4		64		1688.3 ± 14.5		32		1598.3 ± 15.4		256		1390.1 ± 50.2		64		1203.8 ± 13.6
	120 °C, 10 min		128		1709.2 ± 24.7		64		1674.2 ± 15.8		ND		1708.5 ± 33.6		64		1698.2 ± 20.2		64		1208.9 ± 22.5
Enzyme	Papain		ND		2268.1 ± 22.4		128		1228.5 ± 16.7		256		1200.3 ± 14.2		64		1671.3 ± 16.2		16		1526.3 ± 40.0
	Pepsin		32		1998.1 ± 13.2		ND		2015.0 ± 20.7		32		1602.2 ± 27.1		32		1776.2 ± 13.5		16		1533.7 ± 9.0
	Proteinase K		64		1922.2 ± 25.8		128		1200.4 ± 41.7		64		1579.2 ± 30.5		64		1726.3 ± 40.0		32		1329.2 ± 27.5
	α-Chymotrypsin		512		1047.1 ± 24.1		32		1844.6 ± 23.1		128		1399.5 ± 23.0		64		1715.3 ± 33.0		ND		1676.0 ± 48.2
	Trypsin		512		1188.2 ± 12.6		64		1680.5 ± 22.7		256		1214.1 ± 20.1		128		1599.2 ± 10.7		ND		1673.0 ± 33.1

BA bacteriocin activity, *HC* histamine content, *C* bacteriocin before physico-chemical treatment, *T* bacteriocin after physico-chemical treatment

[a]Activity of *P. acidilactici* MCL11 against *S. xylosus* MCH03

[b]Activity of *L. mesenteroides* MCL12 against *S. marcescens* MCH02

[c]Activity of *E. faecium* MCL13 against *S. xylosus* MCH03

[d]Activity of *L. sakei* MCL14 against *A. hydrophila* MCH04

[e]Activity of *L. acidophilus* MCL15 against *M. morganii* MCH05

those of the mesentericin Y105. Mesentericin Y105, the bacteriocin from *L. mesenteroides*, had a narrow inhibitory spectrum limited to *Listeria* genus. Neither the Gram-negative and other Gram-positive indicator bacteria nor the related LAB species were inhibited when tested by the well-diffusion assay. This bacteriocin exhibited excellent stability under heating and acidic conditions. However, all of the proteolytic enzymes such as pronase, proteinase K, trypsin, chymotrypsin, and pepsin totally inhibited the antimicrobial activity of this bacteriocin (Héchard et al., 1992).

The bacteriocin activity of *E. faecium* MCL13 was stable the pH range between 3.0 and 10.0. However, a reduction in the activity was observed when the bacteriocin was exposed to 100 °C for 10 min. The bacteriocin activity was not affected by the presence of papain and trypsin. Unlike our results, the bacteriocins produced by the *E. faecium* strain showed a broader spectrum of activity against indicator strains of *Enterococcus* spp., *Listeria* spp., *Clostridium* spp., and *Propionibacterium* spp. This bactercioin was inactivated by α-chymotrypsin, proteinase K, trypsin, pronase, pepsin, and papain, but not by lipase, lysozyme, and catalase. The bacteriocin was heat stable and displayed highest activity at neutral pH (Toit et al., 2000).

Jiang et al. (2012) reported that sakacin LSJ618 produced by the strain *L. sakei* LSJ618 exhibited inhibitory activity against food-spoiling bacteria and food-borne pathogens, including the Gram-positive *L. monocytogenes*, *Staphylococcus aureus*, *Sarcina* spp., *Micrococcus luteus*, and the Gram-negative *Proteus* spp. and *Escherichia coli*, but not against most of the LAB tested. This bacteriocin was completely inactivated by pepsin, papain, trypsin, and lipase, was stable between pH 2.0 and 8.0, and was heat resistant (30 min at 121 °C), which is partially in agreement with our observations. The bacteriocin of *L. sakei* MCL14 retained their activity at pH 4.0–8.0 and was thermally stable over a wide temperature range up to 100 °C for 10 min. Also, the bacteriocin activity was unstable after treatment with all the proteolytic enzymes like papain, pepsin, proteinase K, α-chymotrypsin, and trypsin.

The activity of the bacteriocin produced by *L. acidophilus* MCL15 was stable a pH range between 2.0 and 10.0, and remained constant after heating at 80, 100, and 120 °C for 10 min. However, the bacteriocin activity was destroyed or diminished after treatment with proteolytic enzymes such as papain, pepsin, proteinase K, α-chymotrypsin, and trypsin. The same results were recorded for the bacteriocin of *L. acidophilus* IBB 801. Acidophilin 801 obtained from *L. acidophilus* IBB 801 strain displayed a narrow inhibitory spectrum, being active particularly towards closely related lactobacilli and two Gram-negative pathogenic bacteria including *E. coli* Row and *Salmonella panama* 1467, whereas acidolin and acidophilin produced by *L. acidophilus* strains showed a wide inhibitory spectrum against Gram-positive and Gram-negative bacteria. The antimicrobial activity of acidophilin 801 was insensitive to catalase but sensitive to proteolytic enzymes such as trypsin, proteinase K, and pronase, heat-stable (30 min at 121 °C), and maintained in a wide pH range (pH 3.0–10.0) (Zamfir et al., 1999).

Conclusion

In conclusion, these bacteriocins produced from the LAB isolates (P. *acidilactici* MCL11, *L. mesenteroides* MCL12, *E. faecium* MCL13, *L. sakei* MCL14, and *L. acidophilus* MCL15) may be a useful as a food biopreservative for controlling microbial deterioration, enhancing the hygienic quality, and extending the shelf-life of fish and seafood products. Notably, these antimicrobial substances may play a role in inhibiting histamine formation in the fermented fish products and preventing seafood-related food-borne disease caused by bacterially-generated histamine.

Abbreviations

LAB: lactic acid bacteria; *B. licheniformis*: *Bacillus licheniformis*; *S. marcescens*: *Serratia marcescens*; *S. xylosus*: *Staphylococcus xylosus*; *A. hydrophila*: *Aeromonas hydrophila*; *M. morganii*: *Morganella morganii*; *P. acidilactici*: *Pediococcus acidilactici*; *L. mesenteroides*: *Leuconostoc mesenteroides*; *E. faecium*: *Enterococcus faecium*; *L. sakei*: *Lactobacillus sakei*; *L. acidophilus*: *Lactobacillus acidophilus*; GRAS: generally recognized as safe; CFS: cell-free supernatant; BU: bacteriocin unit; *K. pneumonia*: *Klebsiella pneumonia*; *H. alvei*: *Hafnia alvei*; *P. vulgaris*: *Proteus vulgaris*; *E. aerogenes*: *Enterobacter aerogenes*; *L. buchneri*: *Lactobacillus buchneri*; *L. sakei*: *Lactobacillus sakei*; *L. lactis*: *Lactococcus lactis*; *S. thermophilus*: *Streptococcus thermophilus*; *L. monocytogenes*: *Listeria monocytogenes*; *E. coli*: *Escherichia coli*

Acknowledgements

We acknowledge the technical assistance provided by Lee Jong-Gab Professor.

Funding

This Research was supported by the Tongmyong University Research.

Availability of data and materials

Not applicable.

Competing interests

The author declares that he/she has no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Received: 6 September 2016 Accepted: 5 November 2016
Published online: 09 December 2016

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