Comparative research on antibacterial peptides, bacteriocins, produced by two strains of lactic acid bacteria, *Leuconostoc mesenteroides* subsp. *mesenteroides* 406 and 213M0, isolated from Mongolian traditional fermented milk, airag

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I. GENERAL INTRODUCTION

Listeria monocytogenes is recognized as one of the most harmful foodborne pathogenic bacteria causing gastrointestinal diseases, septicemia, etc., leading to higher mortality in pregnant women, newborns, the elderly, and immunocompromised individuals (Buchanan *et al*., 2017). *Lis. monocytogenes* is the causative agent of multiple outbreaks of listeriosis worldwide. In the United States of America, outbreaks, or sporadic cases of the infection of *Listeria*, have been reported almost every year (Kaptchouang Tchatchouang *et al*., 2020). From 2008 to 2015, there was a statistically significant increase in listeriosis cases within the European Union (EU) (European Food Safety Authority and European Centre for Disease Prevention and Control, 2017). Although outbreaks of listeriosis have been reported in Asia less frequently than in the US and Europe in recent years, outbreaks continue to be observed in China, Japan, and Thailand. South Africa has had a terrible listeriosis outbreak, the worst in its history. There have been 820 instances of listeriosis, with 193 deaths. (Kaptchouang Tchatchouang et *al*., 2020). *Lis. monocytogenes* can survive in harsh conditions such as a wide pH range, cold temperatures, and high salt concentrations and has been identified in a variety of raw foods, such as uncooked meats and vegetables, as well as cooked or processed foods such as dairy products and fermented meats (Buchanan *et al*., 2017; Den Bakker *et al*., 2010; Osek *et al*., 2022).

In the food industry, canning, pasteurization, freezing, irradiation, and the addition of preservatives are common methods of preservation. However, because of the risks to human health associated with the use of chemical preservatives, people have become more aware of the importance of food safety in recent years, leading to an increase in demand for more naturally occurring and minimally processed foods (Possas *et al*., 2022; Umair *et al*., 2022). Meanwhile, due to their potential as natural substitutes for chemical preservatives, antibacterial active substances produced by lactic acid bacteria (LAB) have drawn more attention in recent decades (Chen *et al*.,

2017; Daba and Elkhateeb, 2020). LAB is a group of bacteria with morphological, metabolic, and physiological characteristics, as well as a relatively close phylogenetic relationship (Khalid, 2011). They are gram-positive, non-spore-forming, cocci or rods, catalase-negative, and fastidious organisms with a high tolerance for low pH that comprises many different genera of bacteria such as *Lactobacillus*, *Lactococcus*,*Leuconostoc*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Weissella,* and others (Wang *et al*., 2021) and widespread in nature such as milk (Chen *et al*., 2020; Kim *et al*., 2023; Taye *et al*., 2021), meat and plants (Hernández-Aquino *et al*., 2020; Phuengjayaem *et al*., 2021), the gastrointestinal and urogenital tracts of humans and animals (Pan *et al*., 2020), as well as soil and water (Nüesch-Inderbinen *et al*., 2021; Woo *et al*., 2021). Fermentation, one of the oldest biotechnology methods, has historically been used in the processing and preservation of food, in which lactic acid bacteria play an important role (Barcenilla *et al*., 2022). The bioactive molecules produced by LAB during fermentation include organic acids, hydrogen peroxide, amino acids, acetoin, polyols, and specialized flavor compounds (diacetyl, carboxylic acids, aldehydes, ketones, and esters), antibacterial and antifungal peptides, exopolysaccharides (EPS), some amylase, protease, and lipase enzymes, which improve the sensory characteristics of food and enhance flavor development (Wang *et al*., 2021). Consumption of LAB-processed food has indirect benefits for humans, such as enhancing lactose digestion to prevent diarrhea, altering the intestinal microbiome, decreasing cholesterol to prevent cardiovascular disease, activating the immune system, etc. (Abdul Hakim *et al*., 2023). Most LAB are known as "Generally Recognized as Safe" (GRAS) or "Qualified Presumption of Safety" (QPS) because to their nonpathogenic nature, utility in technical and industrial processes, and long-standing use by humans worldwide (Tang *et al*., 2023).

1. Inhibitory substances of lactic acid bacteria

Some strains of LAB synthesize compounds such as bacteriocins, exopolysaccharides, and antioxidants, but the majority of LAB produce conventional antibacterial chemicals such organic acids. All these compounds work synergistically to inhibit pathogens and spoilage microorganisms in food. Additionally, it will enhance the food's flavor and texture, offer extra nutrients, and have a variety of probiotic effects. They were widely used in agriculture, food, medicine, and the chemical industry. This section primarily describes the metabolic substances of LAB, apart from bacteriocins, which were the primary research topic of this study and will be introduced in the following part.

1.1 Organic acids

Specific metabolic features of LAB vary across genera. LAB's key distinctive metabolite is the conversion of sugar (glucose, fructose, mannose, or galactose) to lactic acid. *Lactococcus and Lactobacillus* use glucose as a carbon source to produce pyruvate and then produce lactic acid via the enzyme lactate dehydrogenase (LDH) in a process of fermentation during normal metabolism; this type is called homolactic fermentation, and while other genera such as *Leuconostoc, Weissella,* and *Oenococcus* decompose glucose into lactic acid with other byproducts such as ethanol, carbon dioxide, and acetic acid through the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway, it is called heterolactic fermentation (Wang *et al*., 2021). Both types of LAB fermentation produce lactic acid. In addition, it can also produce various other organic acids, such as formic acid, acetic acid, propionic acid, butyric acid, succinic acid, 3-hydroxypropionic acid, etc. (Kumar *et al*., 2013; Özcelik *et al*., 2016). They were mainly synthesized by the heterolactic fermentation pathway and amino acid metabolism. For example, pyruvate can be decomposed into acetate via acetyl-CoA and into propionate via the succinate pathway. Excess pyruvate can also be converted to α-acetolactate by α-acetolactate synthase (ALS) under aerobic conditions (Tang *et al*.,

2023). Amino acid metabolism leads to the production of 3-phenyllactic acid (PLA) and 2-hydroxyisocaproic acid (HICA). L-leucine is converted into 2-ketoisocaproic acid (KICA) by a branched-chain amino acid aminotransferase and then can be reduced to 2-hydroxyisocaproic acid (HICA) in some LABs such as *L. lactis*, *Leu. mesenteroides*, *L. plantarum, P. pentosaceus,* etc. 3-phenyllactic acid (PLA) is converted from L-phenylalanine to phenylpyruvic acid (PPA) by aminotransferase and finally reduced by dehydrogenases such as lactate dehydrogenase (Lee *et al*., 2021). These organic acids not only contribute to the flavor of food but also play many other roles, such as food preservatives. During fermentation, LAB yields organic acids to reduce the pH of the environment, create a microenvironment that is unsuitable for the growth of pathogens, inhibit the pathogenic microorganisms, and even kill them. Therefore, LAB fermentation is considered a natural method for extending shelf life and enhancing food safety. When beef cuts, turkey rolls, pork belly, and chicken skin were surface sprayed with lactic acid and acetic acid (2%), all inoculated pathogenic strains (*E. coli* O157:H7, *Lis. monocytogenes,* and *Salmonella*) were lowered compared to no-wash controls, and especially acetic acid prevented residual growth of *E. coli* and *Lis. monocytogenes*, and it reduced the numbers of *Salmonella* on chicken skin to below recoverable levels (Carpenter *et al*., 2011). Similar to this study, spraying PLA (1.5%) inactivated all *E. coli* O157:H7 and *S. Typhimurim* cells inoculated in beef cuts (Zheng *et al*., 2019). After comparing the inhibitory activity of six organic acids, it was found that acetic acid had the strongest effect on common meat spoilage bacteria, with propionic acid, lactic acid, and citric acid following (Ouattara *et al*., 1997). The LAB amino acid metabolism in sourdough produced antifungal carboxylic acids, such as 4-hydroxyphenyllactic acid from tyrosine, benzoic acid, and salicylic acid from phenylalanine, or 2-hydroxyisocaproic acid from leucine, which were linked to antifungal action (Axel *et al*., 2016).

1.2 Exopolysaccharides

Various lactic acid bacteria, such as *Str. thermophilus*, *Limosilactobacillus reuteri*, *L. casei*, *L. plantarum,* and so on, contain one or more clusters of genes for synthesizing different kinds of macromolecular substances produced by the polymerization of multiple monosaccharides or their derivatives, which are called exopolysaccharides (EPS) (Wang *et al*., 2021). LAB synthesizes and secretes extracellular polysaccharides, which exist on the cell surface or slime. Due to the sugar composition and chain length of the EPS, it can be classified into two types: homopolysaccharides (HoPS) and heteropolysaccharides (HePS). HoPS is a multi-molecule substance composed of a single monosaccharide polymerized by a polymerase. For example, dextran or fructan are polymerized from glucose or fructose via glycansucrase or fructansucrase, produced by some *Leuconostoc*, *Lactobacillus*, and *Streptococcus* species grown on sucrose-containing medium (Lee *et al*., 2021). Polysaccharides composed of repeating units of different monosaccharides, such as those produced by *Lactobacillus paracasei*, *L. lactis*, and *Lactococcus cremoris*, belong to this type called HePS. HePS biosynthesis and composition are more complex; in addition to having different proportions of monosaccharides, they may also contain N-acetyl-D-glucosamine, N-acetylgalactosamine, uronic acid, or some non-carbohydrate substituents, such as pyruvic acid, acetate, phosphate, or succinate (Tang *et al*., 2023). EPS are not only related to the adhesion of LAB but also have important effects on the organoleptic and functional properties of food. It is used as a thickener, stabilizer, and emulsifier to improve the rheological and organoleptic properties, sensory attributes, and texture of fermented foods (Singh and Saini, 2017). EPS-producing strain *L. mocosae* DPC 6426 increased the water retention of yogurt, reduced syneresis, and improved rheological properties (London *et al*., 2015). EPS-producing strains are used in half-fat cheddar cheese making, influencing the rheological properties and improving the texture of the product (Zhang *et al*., 2015). It exhibits many beneficial properties, such as immune modulation, antitumor, antioxidant, antibacterial, and antiviral activities. Dextran

synthesized by *L. sakei* MN1 and *Leu. mesenteroides* RTF10 has shown functional activity against salmonid viruses, infectious pancreatic necrosis virus (IPNV), and infectious hematopoietic necrosis virus (IHNV) (Nácher-Vázquez *et al*., 2015). EPS from *Lac. lactis* subsp. *lactis* displayed superoxide anion hydroxyl radical and DPPH scavenging activities and increased catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activity in mouse serum and liver (Guo *et al*., 2013). The EPS produced by *L.cereus* proved to be highly effective against a variety of bacteria, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E. coli*, *Lis. monocytogenes*, *Bacillus cereus*, *Proteus mirabilis*, *Acinetobacter baumannii*, *Enterobacter cloacae*, and *Candida albicans*, in addition to demonstrating good water and oil retention capabilities (Nehal *et al*., 2019).

1.3 Antioxidant Substances

Some LAB strains have antioxidant characteristics due to synthetic chemicals such as EPS (mentioned above), vitamins (Capozzi *et al*., 2012), hydrogen peroxide (Hertzberger *et al*., 2014), phenol metabolites (Ryu *et al*., 2019), chlorogenic acid glucoside (Nam *et al*., 2017), sulforaphane (Wu et *al*., 2018), and hydroxycinnamic acid(Rodríguez *et al*., 2008). Yamamoto et al. demonstrated that EPS and isoflavone aglycones are the antioxidant compounds produced during the fermentation of soy milk with LAB (Yamamoto *et al*., 2019). During blueberry fermentation, *L. plantarum* biotransformed blueberry polyphenols into active phenol metabolites with strong antioxidant activity (Ryu *et al*., 2019). Some LABs, such as *En. faecalis*, *L. casei*, *Str. thermophilus*, *L. plantarum, L. rhamnosus,* etc., produce glutathione, which is a natural antioxidant (Wang *et al*., 2021). The food industry has made extensive use of LAB, and the antioxidant compounds that are produced from them during the fermentation process increase the antioxidant activity of food (Zhao *et al*., 2021). LAB can synthesize several vitamins, including riboflavin, folates, vitamin B12, and other B-

group vitamins, during the fermentation process to enhance food nutrition (LeBlanc *et al*., 2011). Although certain vitamins, including riboflavin and folates, are strong antioxidants, the ability of LAB to produce vitamins is typically strain- or species-dependent. Most LABs are able to synthesize folate, such as *Lactococcus*, *Streptococcus*, and *Lactobacillus*; *Enterococcus* has been reported to synthesize folic acid; and *L. plantarum* is a high folic acid producer in *Lactobacillus* (Liu *et al*., 2022). Several studies have demonstrated the relationship between culture conditions, etc., and the capacity to synthesis folate. For instance, the synthesis of folate in *Lactobacillus* strains depends on the presence of para-aminobenzoic acid (PABA) in the growth medium (Wegkamp *et al*., 2007).

2. Bacteriocins

Bacteriocins are antibacterial peptides ribosomally biosynthesized by some strains of bacteria including LAB and inhibit the growth of bacteria related (narrow-spectrum) or unrelated (broad-spectrum) to the producers (Mokoena *et al*., 2021). The first bacteriocins were found in 1925 by Belgian scientist André Gratia, who demonstrated that *Escherichia coli* V produced a dialyzable and heat-stable substance that inhibited the growth of *Escherichia coli* S. This substance was subsequently named "colicin V." (Kaur and Kaur, 2015). Three years later, in England, Rogers and Whittier noted that some lactic streptococci produced an antimicrobial substance that inhibited the starter cheese cultures. Later, in 1947, Mattick and Hirsch successfully purified this substance and termed it "nisin" (Setiarto *et al*., 2023). In addition, Fredericq (1946) revealed that the proteinaceous nature of colicin and its inhibitory activity were due to the presence of specific surface receptors on sensitive cells (Gillor *et al*., 2008). By 1953, the more general term “bacteriocin” was coined by Jacob et al. to refer to protein-like substances produced by bacteria that inhibit the growth of other strains or species (Jack *et al*., 1995). Numerous bacteriocins from various strains have been found and characterized up to

this point. While LAB-produced bacteriocins have garnered greater attention regarding safety.

2.1 Bacteriocins produced by lactic acid bacteria

LAB-bacteriocins are active proteins synthesized in ribosomes and act against other bacteria without affecting their own growth. Early research considered that bacteriocins mainly have inhibitory effects on closely related bacteria, but as research continues, it has been found that several bacteriocins have a relatively broad-spectrum antibacterial ability; they not only inhibit closely related bacteria but also have certain inhibitory effects on distantly related bacteria, such as gram-negative bacteria, viruses, and fungi (Yu *et al*., 2023). Furthermore, LAB-bacteriocins can be degraded by digestive tract enzymes, cannot accumulate in the body, and are not associated with resistance strains, making them utilized in food production as biopreservative. It could restrict the growth of pathogenic bacteria during fermentation and maturation, increase product shelf life, as well as increase product safety (Mokoena *et al*., 2021).

2.2 Classification of bacteriocins

There is not a common, universally accepted classification of bacteriocins. Initially, Klaenhammer classified LAB bacteriocins into four classes based on their molecular weight, structure, biochemical and physical properties, and mode of action (Klaenhammer, 1993). Class I bacteriocins are post-translationally modified membrane-targeting peptides called lantibiotics, containing unusual amino acids or amino acid residues such as 2,3-didehydroalanine, D-alanine, and 2,3-didehydrobutyrine, as well as the eponymous lanthionine or β-methyllanthionine residues that form lanthionine rings, giving lantibiotics characteristic structure. They have an extremely low molecular weight (<5 kDa) and thermostable properties. Class II bacteriocins are membrane-targeting and heat-stable peptides without lanthionine residues; their molecular weight is up to 10 kDa. They are further divided into three subclasses,

including class IIa antilisterial peptides, class IIb bacteriocins, which require two peptides for their activity, and class IIc thiol-activated peptides, whose activity depends on the presence of a reduced cysteine residue. However, the classification of bacteriocins remains controversial, and different classification systems have been proposed over the years. The different classifications of bacteriocins are summarized in **Table 1**.

Cotter et al. (2005) modified Klaenhammer's classification scheme and divided the bacteriocins into two distinct categories: lantibiotics (class I) and non-lanthionine-containing bacteriocins (class II). Class II bacteriocins are further divided into four subclasses. Class IIa and Class IIb are similar to Klaenhammer's classification; Class IIc are circular bacteriocins whose N- and C-termini of the peptide are covalently linked to form a cyclic structure. The authors also proposed that such bacteriocins could be further divided based on the percentage of amino acid sequence identity as subclass c (i) and subclass c (ii). Class IId bacteriocins that have no significant sequence similarity with other Class II bacteriocins, such as “single peptide non-pediocin linear," are classified into this category. The authors excluded the large-molecular-weight thermolabile peptides (formerly class III bacteriocin) from the bacteriocin classes and designed them as “bacteriolysins" and additionally recommended that previous class IV should be extinguished (Cotter *et al*., 2005). However, LAB-bacteriocins were classified into three categories by Drider et al. (2006) based on their genetic and biochemical properties. Class I is further sub-categorized into two types (Type A and Type B) according to their structural and functional characteristics. Class II is divided into three subclasses (Drider *et al*., 2006). In 2016, Alvarez-Sieiro et al. suggested a slightly adjusted classification scheme that can accommodate the novel subclasses that are appearing, which categorizes LAB-bacteriocins into three main classes: Class I is further classified into six subcategories, and Class II consists of other low molecular weights (<10 kD) unmodified thermostable bacteriocins, which include 4 sub-classes. The descriptions of IIa, IIb, and IId are similar to the previous

classifications; class IIc was described as a unique bacteriocin that is synthesized without an N-terminal leader peptide required for secretion, modification, and maintaining the bacteriocin inactive inside the producer cell (Alvarez-Sieiro *et al*., 2016). Bacteriocin categorization is constantly changing as novel bacteriocins with diverse structures and characteristics appear.

Table 1 Different schemes of LAB-bacteriocin classification

Author	Class	Characteristic	Sub class	Examples	
				bacteriocins	Producer strains
Todd R. Klaenhammer (1993)	Class I	Lantibiotics MW<5 kDA	-	Nisin	*Lactococcus lactis* subsp. *lactis*
				Lacticin 481	*Lactococcus lactis* 481
	Class II	Non-lanthionine Thermostable Hydrophobicity MW < 10 kDa	IIa: Anti listeria	Pediocin PA-1	*Pediococcus acidilactici* PAC 1.0
				Sakacin A	*Lactobacillus sakei* 706
			IIb: Two proteina-ceous peptides	Lactococcin G	*Lactococcus lactis* LMG 208
				Lactacin F	*Lactobacillus johnsonii* VPI 11088
			IIc: Thiol-activated peptides requiring reduced cysteine residues for activity	Lactococcin B	*Lactococcus lactis* subsp. *cremoris* 9B4
	Class III	Thermolabile MW >30 kDa	-	Helveticin J	*Lactobacillus helveticus* 481
	Class IV	Complex bacteri-ocins, composed of enormous pep-tides(carbohy-drates or lipids) for their activity	-	Plantaricin S	*Lactobacillus plantarum*
				Leuconocin S	*Leuconostoc paramesenteroides* OX
				Lactocin 27	*Lactobacillus Helveticus*
Cotter *et al*. (2005)	Class I	Lantibiotic Small peptides	Single peptide	Nisin	*Lactococcus lactis* subsp. *lactis*
			Two peptides	Lacticin 3147	*Lactococcus lactis* subsp. *lactis* DPC3147
	Class II	Non-lanthionine, MW < 10 kDa	IIa: pediocin-like peptides	Pediocin PA-1	*Pediococcus acidilactici*

		Thermolabile	IIb: two-peptide bacteriocins	Lactacin F	*Lactobacillus johnsonii* VPI 11088
			IIc: circular bacteriocins	Enterocin AS48	*Enterococcus faecalis* S-48
			IId: no significant sequence similarity with other Class II bacteriocins	Lactococcin A	*Lactococcus lactis* subsp. *cremoris* LMG 2130
				Divergicin A	*Carnobacterium divergens* LV13
Drider *et al.*(2006)	Class I	Lantibiotics Lower weigh Thermostable	Type A: elongated molecules, MW <4 kDa	Nisin A Nisin Z	*Lactococcus lactis* *Lactococcus lactis*
			Type B: globular molecules, MW: 1.8 -2.1 kDa	Mersacidin Mutacin II	*Bacillus Mersacidin* *Streptococcus mutans* T8
	Class II	Unmodified Thermostable MW < 10 kDa	IIa: pediocin-like	Pediocin PA-1 Mesentericin Y105 Leucocin A	*Pediococcus acidilactici* PAC 1.0 *Leuconostoc mesenteroides* Y105 *Leuconostoc gelidum* UAL 187
			IIb: two peptides	Lactococcin G	*Lactococcus lactis* LMG 208
			IIc: one-peptide non pediocin-like	Lactocin B	*Lactobacillus acidophilus* N2
	Class III	Thermosensitive MW >30 kDa	-	Helveticin J Millericin B	*Lactobacillus helveticus* 481 *Streptococcus milleri* NMSCC 061
Alvarez-Sieiro *et al*.（2016）	Class I	Posttranslationally modified peptides MW < 10 kDa Thermostable	Ia: lanthipeptides	Nisin	*Lactococcus lactis*
			Ib:cyclized peptides	Enterocin AS48	*Enterococcus faecalis* S-48
			Ic: sactibiotics	Subtilosin A*	*Bacillus subtilis 168*

		Id: linear azol(in)e-containing peptides	Streptolysin S	*Streptococcus pyogenes*
		Ie: glycocins	Glycocin F	*Lactobacillus plantarum*
		If:lasso peptides	Microcin J25*	*Escherichia coli*
Class II	Unmodified Thermostable MW < 10 kDa	IIa: pediocin-like	Pediocin PA-1	*Pediococcus acidilactici* PAC 1.0
		IIb:Two peptides	Lactococcin G	*Lactococcus lactis* LMG 208
		IIc: Leaderless	Lcticin Q	*Lactococcus lactis* QU 5
		IId:Non pediocin-like single peptides	Lactococcin A	*Lactococcus lactis* subsp. *cremoris* LMG 2130
Class III	Thermo-labile MW >10 kDa	Bacteriolysin	Enterolysin A	*Enterococcus faecalis* LMG 2333
		Non-lytic	Dysgalacticin	*Streptococcus dysgalactiae* subsp. *equisimilis*

*Bacteriocins from non-lactic acid bacteria；

2.3 Antibacterial mechanisms of bacteriocins

2.3.1 Mechanism of cell wall targeting

A. Pores formation through electrostatic effects

Most bacteriocins are generally cationic and could be adsorbed to the cell membrane through electrostatic interactions with negatively charged components of the cell membrane of the target strain and further inserted into the cytoplasmic membrane to form ion-permeable channels or pores, leading to dissipation of the proton motive force (PMF), finally resulting in damage and cell lysis (Todorov, 2009).The PMF is generated by an electrochemical gradient across the cytoplasmic membrane, which is made up of two connected potential energies: an electrical membrane potential (Δψm) and a pH differential (ΔpH) (Berry and Kaeberlein, 2021). In the membrane, the

synthesis of ATP and the transport of metabolites such as ions are driven by a PMF-driven transport system (Wikström and Springett, 2020). Bacteriocins combine with the membrane to form pores, causing the effluxion of intracellular substances such as ions (K^+, PO_4^{2-}, H^+), amino acids, and ATP from cells. thereby collapsing the PMF affects ATP synthesis and collapsing pH gradients that regulate ion exchange between cell interior and exterior, blocking the ATP production pathway, stopping cell metabolism, and ultimately leading to cell death (Moll *et al*., 1999). Different classes of bacteriocins form pores in the cell membrane of the target cells, such as nisin, lactisin 3147, and streptomycin FF22, resulting in the release of low-molecular-weight intracellular compounds such as amino acids, ions, and ATP (Pérez-Ramos *et al*., 2021).

B. Receptor-mediated cell wall synthesis and pore formation

Bacteriocins have been demonstrated to bind to cells by electrostatic action and form non-specific and temporary pores, which frequently require high peptide concentrations. Furthermore, it was found that transporters, which are present in inhibited strains for uptake of nutrients or enzymes involved in critical processes, are directly targets of bacteriocins or indirectly related in their antibacterial activity (Davidson *et al*., 2008). The receptors present in the cytoplasm of sensitive bacteria specifically bind to bacteriocins, thereby forming pores on the cell membrane surface, resulting in the leakage of many intracellular components and cell death (Pérez-Ramos *et al*., 2021). In addition, binding of bacteriocins to receptors can inhibit cell membrane biosynthesis and cause the death of sensitive bacteria (Darbandi *et al*., 2022).Membrane-bound peptidoglycan precursor lipid II is a more common receptor, which is a vital precursor in the biosynthesis of bacterial cell walls and an intermediate in the biosynthesis of peptidoglycan (Dickman *et al*., 2019). Peptidoglycan is the main component of the bacterial cell wall and is essential for the integrity and survival of bacteria. Bacteriocins inhibit bacterial growth by docking lipid II to create pores in the cell

membrane or interrupting cell wall biosynthesis (Prince *et al*., 2016). Nisin has been demonstrated in detail to specifically bind to the cell wall precursor molecule lipid II. The adsorption of nisin takes place across the cell membrane and binds to lipid II used as a docking molecule via the characteristic lantibiotic ring structures in the N-terminal part of the peptide. The poration complex is formed and stabilized, finally causing rapid killing of the cell (Pérez-Ramos *et al*., 2021). In addition to pore formation, it has also been proposed that lipid II binding with bacteriocins inhibits target cell formation by blocking cell wall formation. When bacteriocins combine with lipid II, they form a complex that blocks the trans glycosylation reaction during peptidoglycan biosynthesis, interfering with the transport of peptidoglycan subunits from the cytoplasm to the cell wall, thereby inhibiting cell wall formation. Furthermore, it leads to fast dissipation of transmembrane electrostatic potential, which accelerates cell death (Pérez-Ramos *et al*., 2021). Several lantibiotics have been demonstrated to bind to lipid II and interfere with the transfer of peptidoglycan subunits from the cytoplasm into the cell wall, therefore limiting cell wall synthesis and eventually leading to the death of sensitive strains (Surati, 2021). Non-lantibiotics such as lactococcin 972 also kill target strains by inhibiting cell wall synthesis, excluding pore formation (Martınez *et al*., 2008). The mannose phosphotransferase system (man-PTS) and glucose phosphotransferase system (glu-PTS) have been shown to be targets for both gram-positive and gram-negative bacteriocins. In the case of pediocin, which belongs to subclass IIa, binding takes place between the IIAB, IIC, and IID subunits that are part of man-PTS. Moreover, IIC and IID subunits are recognized by the bacteriocins, and IIC behaves like a receptor. Furthermore, the bacteria infuse themselves within the cell membrane, leading to pore formation and finally resulting in the efflux of ions and molecules. Lactococcin A employs the proteins IIC and IID of the man-PTS as a receptor on target cells, thereby promoting its insertion, oligomerization, and pore formation. In addition to lactococcin A, the man-PTS components IIC and IID are also targeted by different class II peptide-bacteriocins, such as lactococcin B, and

pediocin-like (class IIa) bacteriocins, such mesentericin Y105 *(Dalet et al*., 2001; Diep *et al*., 2007). Glu-PTS is required for the glycosylated bacteriocin sublancin activity (Biswas *et al*., 2021). Receptors for virous bacteriocin have been found, such as the maltose ABC transporter for circular bacteriocin garvicin ML (Gabrielsen *et al*., 2012), zinc-dependent metallopeptidase YvjB for bacteriocin LsbB (Uzelac *et al*., 2013) and undecaprenyl pyrophosphate phosphatase (UppP) for lactococcin G (Kjos *et al*., 2014).

2.3.2 Inhibit gene expression and protein synthesis

Some bacteriocins pass through the cell membrane and accumulate in cells, interfering with the normal metabolism of cells, causing DNA damage, or acting on DNA-related enzymes to affect the formation of DNA structures, interfering with the normal replication of DNA, thereby inhibiting the growth of target strains. This type of bacteriocin can be collectively referred to as nuclease bacteriocins. Different nuclease bacteriocins not only affect DNA replication but also participate in the inhibition of RNA and protein synthesis and have a major impact on bacterial energy distribution (Simons *et al*., 2020). For example, gram-negative bacteriocins such as microcin B17, microcin J25, and microcin C act on target strains in this way, binding to various enzymes (DNA gyrase, RNA polymerase, and Asp-tRNA) as direct targets and ultimately inhibiting the growth of their target cells (Simons *et al*., 2020).

2.4 LAB-bacteriocins isolated from fermented mare's milk

Fermented mare's milk is a traditional fermented beverage in Asia and Europe, including parts of China (Inner Mongolia, Xinjiang, and Qinghai), Mongolia, Kazakhstan, Kyrgyzstan, Russia, Turkey, and others. Also known as Airag (Mongolia), Chigee (Inner Mongolia, China), koumiss, or Qymyz (Kazakhstan, Kyrgyzstan, and Russia) (Martuzzi *et al*., 2024).The production of fermented mare's milk dates to around 3500

BC (Outram *et al*., 2009). For centuries, it has not only played a major role in the diet of these regions but has also been regarded as a drink with medicinal properties. Koumiss can improve the functions of the kidneys, digestive tract, nervous systems, and immune systems. It can be used to treat gastric and duodenal ulcers, chronic gastritis, enterocolitis, and other digestive tract diseases. It is also used in the treatment of tuberculosis, cardiovascular diseases, and neurological diseases (Afzaal *et al*., 2021; Dhewa *et al*., 2015; Wang *et al*., 2008; Wu *et al*., 2009). Traditional fermented mare's milk is fermented by the natural microbiota, and its microbial composition is very complex, mainly including lactic acid bacteria and yeast (Voloshyna *et al*., 2021). Various *Lactobacillus* strains, including *L. plantarum*, *L. casei*, *L. paracasei*, *L. coryniformis*, *L. helveticus, and L. kefiranofaciens,* were mainly found in Chigee in Inner Mongolia, and some other strains like *L. lactis*, *Leu. mesenteroides, and Str. thermophilus* have also been identified. *L. helveticus*, *L. plantarum*, *L. kefiri, and Leu. mesenteroides* are frequently found in airbags in Mongolia. *L. helveticus*, *L. kefiranofaciens,* and *L. paracasei* are the main strains found in koumiss in Kyrgyzstan, Uzbekistan, and Kazakhstan. And it is possible to highlight that *L. helveticus* is the mian strain found in fermented mare's milk from different countries and regions. Lactose-fermenting yeast species, *Kluyveromices marxianus*, Kluyveromi*ces fragilis*, and *Candida kefir* are common in koumiss (Martuzzi *et al*., 2024). These microbiotas not only contribute to the unique flavor, texture, and probiotic qualities of fermented mare's milk, but also to be a rich source of autochthonous strains and bacteriocin-producing bacteria. Durancins A5-11A and B produced by *En. durans* A511 werev partially characterized, and this was the first report on the isolation and characterization of bacteriocins produced by LAB isolated from fermented mare's milk (Batdorj *et al*., 2006). It showed a broad inhibitory spectrum, not only having antibacterial acidity but also showing antifungal properties (Belguesmia *et al*., 2013). **Table 2** summarizes the bacteriocin-like inhibitory substance (BLIS) and bacteriocins produced LAB strains isolated from fermented mare's milk in recent years. These bacteriocins are mainly

produced by *L. plantarum*, followed by *L. rhamnosus,* and most of them have wide antibacterial activity, inhibiting both gram-positive and gram-negative strains, particularly against food-borne pathogens such as *L. monocytogenes*, *E. coli*, and *S. typhimurium* et al. Therefore, these bacteriocins could be used as biopreservatives and effective antibiotic alternatives in the food industry.

Table 2 Antibacterial substance produced by LAB isolated from fermented mare milk

Isolated source	Producer strain	Antibacterial substance	Molecular weight	Antimicrobial spectrum	Reference
Airag	*Enterococcus durans* A511	Durancin A5-11A	5206 Da	broad inhibitory spectrum, against several *Lactobacillus spp.* And food-borne pathogens including *Escherichia coli, Staphylococcus aureus* and *Listeria innocua*	(Batdorj *et al*., 2006b; Belguesmia *et al*., 2013)
		Durancin A5-11B	5218 Da		
Airag	*Leu. mesenteroides* 406	BLIS	3.3 Kda	Inhibited the growth of Gram-positive indicator strains, such as *Ec. Faecalis*, *Ped. Acidilactici*, *Lis. monocytogenes* and *C. botulinum*	(Wulijideligen *et al*., 2012)
Airag	*Leu. mesenteroides* 213M0	BLIS	2.6-3.0 kDa	Inhibited the growth of *Listeria sp.* And *Ent. Faecalis*, *Leu. mesenteroides*, *Pediococcus pentosaceus* and *Streptococcus thermophilus*	(Arakawa *et al*., 2016)
Koumiss	*Lactobacillus crustorum* MN047	Bacteriocin MN047 A	1,770.89 Da	broad inhibitory spectrum, against both gram-positive（*Staphylococcus aureus*，*Enterococcus faecalis, Listeria monocytogenes*） and gram-negative bacteria（*Escherichia coli, Salmonella , Sakazakii*）	(Yi *et al*., 2016)
		Bacteriocin BM173	–	broad-spectrum antibacterial activity, against some *Escherichia coli, Cronobacter sakazakii*, *Salmonella*, *Enterococcus faecalis*, *Staphylococcus aureus*	(Qiao *et al*., 2021)

Koumiss	*Lactobacillus plantarum* LB-B1	Pediocin LB-B1	2.5 – 6.2 kDa	Inhibit the growth of *Listeria, Lactobacillus*, *Streptococcus*, *Enterococcus*, *Pediococcus* and *Escherichia*	(Xie *et al*., 2011)
Fermented Sumbawa mare's milk	*Bacillus amyloliquefaciens* BC9	BLIS	48 kDa	broad-spectrum antibacterial activity, inhibit *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Orchrobactrum oryzae*, *Escherichia coli*, *Bacillus cereus*, *Salmonella enterica* sv. *Typhimurium*, *Pseudomonas aeruginosa*, *Bacillus anthrophaeus*	(Mulyawati *et al*., 2019a; Mulyawati et al., 2019b; Rakhmanova *et al*., 2021)
Fermented Sumbawa mare's milk	*Lactobacillus plantarum* SB7	BLIS	17- 48 kDa	broad-spectrum antibacterial activity, inhibit *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Orchrobactrum oryzae*, *Escherichia coli*, *Salmonella enterica* sv. *Typhimurium*, *Pseudomonas aeruginosa*, *Bacillus anthrophaeus*	(Mulyawati *et al*., 2019a; Mulyawati et al., 2019b; Rakhmanova *et al*., 2021)
Fermented Sumbawa mare's milk	*Lactobacillus rhamnosus* DC12	BLIS	–	broad-spectrum antibacterial activity, inhibit *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Orchrobactrum oryzae*, *Escherichia coli*, *Salmonella enterica* sv. *Typhimurium*, *Pseudomonas aeruginosa*, *Bacillus anthrophaeus*	(Mulyawati et al., 2019b)
Koumiss	*Lactobacillus plantarum* MXG-68	Plantaricin MXG-68	6.5 kDa	broad antibacterial spectrum, against *Staphylococcus aureus*, *Clostridium perfringens, Listeria monocytogenes*, *Bacillus subtilis*, *Micrococcus luteus*,	(Man and Xiang, 2019)

				Lactobacillus acidophilus，*Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Salmonella Typhimurium*, *Salmonella enterica Typhimurium*	
Koumiss	*Lactobacillus plantarum* NMD17	Plantaricin MX	–	broad antibacterial spectrum, could inhibit gram-negative and gram-positive bacteria such as *Salmonella Typhimurium*, *Escherichia coli*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Bacillus subtilis*, *Listeria monocytogenes*	(Man and Xiang, 2021)
Koumiss	*Lactobacillus rhamnosus* 1.0320	Bacteriocin 1.0320	1-3.3 kDa	broad antibacterial spectrum, could inhibit gram-negative and gram-positive bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella enterica*, *Enterobacter sakazakii*, *Staphylococcus epidermidis*, *Bacillus cereus*	(Xu *et al*., 2021)

2.5 Bacteriocins produced by *Leuconostoc* . spp

Since Orberg and Sandine's (1984) finding of bacteriocins produced by *Leuconostoc.* spp, a variety of bacteriocins produced by various *Leuconostoc* species have been found during the last nearly 40 years (Orberg and Sandine, 1984). Several bacteriocins produced by different *Leuconostoc* species, such as *Leu*. *rnesenteroides*, *Leu. gelidum*, *Leu. lactis, and Leu*. *pseudomesenteroides,* are summarized in **Table 3**. Most of them are class IIa pediocins-like bateriocins, mesentericin Y105, and leucocin A-

UAL, especially against *Lis. monocytogenes* (Hastings *et al*., 1991; Hechard *et al*., 1992). Two-peptide bacteriocins, such as leucocin H produced by *Leuconostoc* MF215B, which is composed of leucocin and hand leucocin H, act together to inhibit *Lis. monocytogenes*, *Bacillus cereus*, and *Clostridium perfringens* (Blom *et al*., 1999). The first cyclic bacteriocin of the genus *Leuconostoc* was found by Masuda et al., leucocyclicin Q, purified from the culture supernatant of *Leu. mesenteroides* TK41401, isolated from Japanese pickles (Masuda *et al*., 2011). Furthermore, many *Leuconostoc* strains can produce more than one bacteriocin. For example, *Leu. mesenteroides* TA33a, isolated from spoiled, vacuum-packaged processed meats, produces three bacteriocins: leucocins A, B, and C (*A. Papathanasopoulos *, § , Fr et al*., 1997), *Leu. pseudomesenteroides* QU 15 produces leucocin A, Q and N (Sawa *et al*., 2010). *Leu. mesenteroides* FR52 isolated from raw milk produces two distinct bacteriocins, mesenterocin 52A and mesenterocin 52B (Revol-Junelles *et al*., 1996). *Leuconostoc* is commonly found in many naturally fermented foods and using *Leuconostoc* as a starting bacterium makes the bacteriocins produced by them potential food preservatives.

Table 3 Bacteriocins produced by *Leuconostoc* in different studies

Isolated source	Producer strain	Antibacterial substance	Molecular weight	Antimicrobial spectrum	Reference
Goat's milk	*Leuconostoc rnesenteroides* Y105	Mesentericin Y105	3868 Da	Inhibited *Leuconostoc* strains and several strains of *Enterococcus* and *Listeria* spp.	(Hechard *et al*., 1992; Héchard *et al*., 1999)
		Mesentericin B105	3446 Da	Only active against *Leuconostoc* spp. and *Weisella*	
Raw milk	*Leuconostoc rnesenteroides* FR52	Mesenterocin 52A	3868 Da	Inhibited *Leuconostoc* strains and several strains of *Enterococcus* and *Listeria* spp.	(Revol-Junelles *et al*., 1996)
		Mesenterocin 52B	3446 Da	Only active against *Leuconostoc* spp. and *Weisella*	
Cheddar cheese	*Leuconostoc mesenteroides* UL5	Mesentericin 5	4.5 kDa	Inhibit all the *Listeria* strains tested in this study and *Micrococcus flavus*, *Pediococcus pentosaceus*, *Streptococcus faecalis*	(Daba *et al*., 1991)
Brazilian Water Buffalo Mozzarella Cheese	*Leuconostoc mesenteroides* SJRP55	Mesentericin W-SJRP55	3,868 Da	Inhibit *Lis. monocytogenes*	(De Paula *et al*., 2014)
		Mesentericin Z-SJRP55	3,444 Da	Inhibited only *Leu. mesenteroides* subsp. *mesenteroides*	
Algerian dromedary milk	*Leuconostoc mesenteroides* CHBY46	Bacteriocin Bac-CHBY46	3.5 kDa	Inhibit to the *Listeria innocua*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Staphylococcus aureus*, *Bacillus subtilis, Pseudomonas aeruginosa*	(Bellil e*t al.*, 2019)

Cheese	*Leuconostocs mesenteroides* NEF42	Bacteriocin NEF42	1724.6 Da	-	(AL-Jumaily and Al-Bayati, 2016)
Vacuum-packaged meat.	*Leuconostoc gelidum* UAL 187	Leucocin A-UAL 187	3,930.3 Da	-	(Hastings *et al*., 1991)
Dry fermented sausage	*Leuconostoc mesenteroides* L124	Bacteriocin	-	Inhibit the growth of *Carnobacterium, Lactobacillus*, *Leuconostoc*, *Weisella*, *Enterococcus* and *Listeria*	(Mataragas *et al*., 2002.)
Processed meat	*Leuconostoc mesenteroides* TA33a	Leucocin A-TA33a	4598 Da	Active against 14 of the 16 indicators, including *Listeria*, *Enterococcus*, *Pediococcus, Weissella paramesenteroides Carnobacterium*, and *Leuconostoc* strains	(A. Papathanasopoulos *, §, Fr *et al*., 1997)
		Leucocin B-TA33a	3466 Da	only inhibited four *Leuconostoc*, *Weissella* indicator strains	
		Leucocin C-TA33a	4598 Da	Active against 9 of the 16 indicators, including *Listeria*, *Enterococcus*, *Carnobacte rium*, and *Leuconostoc* strains	
Meat	*Leuconostoc lactis* SM2	Leucocin	-	Antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis*, *Escherichia coli*, *Pseudomonas putida*, Klebsiella and *Serratia* sp.	(Lahiri *et al*., 2020)
Wine	*Leuconostoc mesenteroides* subsp. *cremoris* W3	Bacteriocin identical to mesentericin Y105	3869 Da	Inhibited species of the genus of *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Listeria* and *Enterococcus*	(Dündar *et al.*, 2016)

Boza	*Leuconostoc pseudomesenteroides KM432Bz*	Leucocin B-KM432Bz	3931.34 Da	Inhibited the growth of *Lactobacillus* , *Weissella*, several strains of *Leuconostoc* and active against a few enteric pathogens (*Enterococcus faecalis*, *Streptococcus pneumoniae*, *Listeria*)	(Makhloufi *et al*., 2013)
Boza	*Leuconostoc mesenteroides subsp. dextranicum ST99*	Mesentericin ST99	-	Inhibited the growth of *Bacillus subtilis*, *Enterococcus faecalis*, several *Lactobacillus* spp., *Lactococcus lactis* subsp. *cremoris*, *Listeria innocua*, Listeria monocytogenes, *Pediococcus pentosaceus*, *Staphylococcus aureus* and *Streptococcus thermophilus*. *Clostridium* spp., *Carnobacterium* spp., *Leu. mesenteroides*	(Todorov and Dicks, 2004)
Fresh fruits and vegetables	*Leuconostoc mesenteroides* CM135	Bacteriocin identical to mesentericin Y105	-	-	(Trias *et al*., 2008)
	Leuconostoc mesenteroides CM160	Bacteriocin identical to mesentericin Y105	-	-	
Nukadoko (rice bran bed)	*Leuconostoc pseudomesenteroides* QU 15	Leucocin A (ΔC7)	3196.8 Da	Inhibit some species of the genus of *Bacillus*, *Listeria*, *Enterococcus*, *Lactococcus* and *Pediococcus*	(Sawa et al., 2010)
		Leucocin A-QU 15	3929.15 Da	Inhibit some species of the genus of *Bacillus*, *Listeria*, *Enterococcus*, *Lactococcus Lactobacillus*, *Leuconostoc* and *Pediococcus*	
		Leucocin Q	3657.8 Da	Inhibit some species of the genus of *Bacillus*, *Listeria*, *Enterococcus*,	

				Lactococcus Lactobacillus and *Leuconostoc*	
		Leucocin N	3680.8 Da	Inhibit some species of the genus of *Bacillus*, *Listeria*, *Enterococcus*, *Lactococcus Lactobacillus* and *Leuconostoc*	
Japanese pickle	*Leuconostoc mesenteroides* TK41401	Leucocyclicin Q	6,115.59 Da	Activities against *Lactobacillus sakei* , *Bacillus coagulans* , *Lactococcus*, *Weissella paramesenteroides*, *Pediococcus dextrinicus*, *Enterococcus*, *Streptococcus*, and *Leuconostoc*	(Masuda *et al.*, 2011)
Pickle	*Leuconostoc mesenteroides* K7	Leucocin K7	-	*Lis. monocytogenes, Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus thermophilus*, *Lactobacillus brevis*, *Lb. plantarum*, *Lb. mali*, *Lb. curvatus*, *Enterococcus faecium*, *Salmonella*, *Shigella dysenterioe*, *Enterobacter sakazakii* and *Escherichia coli*	(Shi *et al*., 2016)

-: Not mentioned

2.6 Extraction, purification, and identification of bacteriocins

Several steps are involved in the purification and identification of bacteriocins, such as the identification of antimicrobial activity, the concentration, extraction, and purification of the bacteriocins, as well as their identification and characterization. Extraction, purification, and identification are critical steps for further evaluation of the physical and chemical properties, structural characteristics, and mechanisms of bacteriocins. Several processes and a variety of techniques are used in the purification of bacteriocins, depending on the properties and characteristics of the bacteriocin (Kaškonienė *et al*., 2017). The process of concentrating and extracting the antibacterial proteins from the culture of bacteriocin-producing strains is usually the first step in the purification of bacteriocins. Ammonium sulfate precipitation (Meena *et al*., 2016), adsorption-desorption extraction (Tulini and De Martinis, 2010), organic solvent extraction (Gao *et al*., 2016) etc. are commonly used methods in this step. Ion exchange chromatography (IEC) (Rumjuankiat *et al*., 2015), hydrophobic interaction chromatography (HIC) (Song *et al*., 2014), and gel filtration chromatography (Zhu *et al*., 2014) are popular intermediate processes used to purify bacteriocins, either alone or in combination. Reversed-phase HPLC is utilized in numerous studies and is usually the final step in the purification of bacteriocins (Pei *et al*., 2018). Sulfate polyacrylamide gel electrophoresis (SDS-PAGE), ESI-MS, and MALDI-TOF-MS are commonly used to determine the approximate or exact molecular weight and amino acid composition of purified bacteriocins (López *et al*., 2007; Rumjuankiat *et al*., 2015). Due to the complex properties and structures of bacteriocins, there is no extraction or purification technology suitable for all bacteriocins. The efficient purification of bacteriocins has always attracted the attention of researchers and has been continuously optimized.

2.6.1 Extraction of bacteriocin

Depending on the solubility or adsorption-desorption capacity of the sample, ammonium sulfate precipitation, organic solvent extraction, or adsorption-desorption methods can be used to extract bacteriocins. Ammonium sulfate is widely used as a precipitation salting agent due to its advantages of high solubility and low cost. The solubility of proteins varies according to the ionic strength of the solution; adding ammonium sulfate increases the ionic strength of the solution and decreases the solubility of the protein, causing the protein to precipitate out of the solution (Duong-Ly and Gabelli, 2014). Many research use ammonium sulfate as the first step in bacteriocin purification (Bauer *et al*., 2005; Rumjuankiat *et al*., 2015). Although its widespread usage, ammonium sulfate precipitation still has several limitations, including incomplete particle solids in the pellet, uncertain ammonium sulfate saturation concentrations, and particles that float easily in ammonium sulfate concentrations (Yap *et al*., 2022). Another method of purifying bacteriocins is known as pH-mediated cell adsorption/desorption, which includes first altering the pH of broth cultures and using the bacterial cells as an adsorbent material. Initially, this method was used to obtain leucoccin, sakecin A, and pediocin AcH from cell-free cultures (Yang *et al*., 1992). Large volumes of chemical reagents and materials are not needed for this specific method. It is conducive to the subsequent purification of proteins but not suitable for all LAB-bacteriocins extraction (Tulini and De Martinis, 2010; Yang *et al*., 1992). Several organic solvents, including butanol, methanol, ethanol, propanol, and chloroform, can also be used for bacteriocin extraction (Kaškonienė *et al*., 2017). After mixing three liters of cold acetone with the culture supernatant of *P. acidilactici* WRL-1 and freezing it for 2 h, (Chung *et al*., 2011) extracted the bacteriocin-like substances. Broad-spectrum bacteriocins produced by *L. plantarum* zrx03 were extracted by ammonium sulfate precipitation and organic solvents such as n-butanol, n-hexane, dichloromethane, and trichloromethane, in which ethyl acetate was selected as the optimal solution (Lei *et al*., 2020). Organic solvents are usually volatile and easily

removed from the target protein. Acetone, a commonly used organic solvent, can reduce degradation of proteins, and prevent contamination from salt and polyphenols. However, this approach demands the consumption of a huge amount of organic solvents, the majority of which are harmful and flammable (M. Du *et al*., 2022). In general, ammonium sulfate, pH-dependent adsorption, and organic solvent extraction are primarily used for crude extraction, and it is not possible to achieve the desired bacteriocin purity using only one step; therefore, multiple steps are required, such as chromatography separation techniques, to obtain purity bacteriocins.

2.6.2 purification method of bacteriocin

Purification can be performed by a series of steps, including ion exchange, hydrophobic interaction, gel filtration, and reversed-phase high-pressure liquid chromatography. IEC is an important analytical technique for separating and determining ionic compounds. Since most bacteriocins are positive, cation exchange chromatography is usually used to separate bacteriocins (Meena *et al*., 2016). IIEC is a flexible method that can be optimized by using different ion exchangers and controlling the pH and ionic strength of the buffer to achieve better bacteriocin recovery. HIC is also especially effective in isolating hydrophobic bacteriocins because of their amphiphilic character and can significantly increase specific activity. Highly active and low-salt nisin were obtained from whey using HIC without ammonium sulfate precipitation, which reduced processes and costs (Jozala *et al*., 2015). Gel filtration is another widely used method for purifying bacteriocin. due to the differences in molecular weight of proteins, different gel columns can be used, such as Superdex, Sephadex, and Sepharose, while Sepharose being the most widely used column for bacteriocins (Bauer *et al*., 2005; Beaulieu *et al*., 2006; Sawa *et al*., 2013). Reverse phase chromatography is the most popular analytical technique for separating complex mixtures in many fields and is often used as the final step in the purification of bacteriocins (Pei *et al*., 2018). The antibacterial chemicals produced by the metabolism of LAB are

complex and contain various unknown components. As a result, one or more purification methods are required for the subsequent stage in bacteriocin purification. Popular purification methods are the combination of ammonium sulfate precipitation, ion exchange chromatography, hydrophobic interaction chromatography, and RP-HPLC in different forms. For example, the novel plantacin ZJ5 was obtained by a multi-step purification procedure that included ammonium sulfate precipitation, cation exchange chromatography, hydrophobic interaction, and RP-HPLC (Song *et al*., 2014). Three bacteriocins, plantaricin KL-1X, -1Y, and -1Z, were successfully separated from the cultures of *L. plantarum* KL-1 utilizing three stages (Rumjuankiat *et al*., 2015). Plantaricin ZJ008 was purified by macroporous resin column, cation exchange chromatography, gel chromatography, and RP-HPLC without ammonium sulfate precipitation (Zhu *et al*., 2014). Purification methods for bacteriocins are relatively complex and time-consuming, and there is no purification technique that is applicable to all types of bacteriocins. Some researchers have also developed relatively simple methods to purify bacteriocins. Suárez et al. proposed a one-step purification method for nisin based on immunoaffinity chromatography that was rapid and reproducible and achieved a higher final yield of production (Suárez *et al*. 1997).

2.6.3 Identification of bacteriocin

The main methods for identifying bacteriocins are SDS-PAGE and tricine/SDS-PAGE for approximate molecular weight identification, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), electrospray ionization mass spectrometry (ESI-MS), and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) for accurate molecular weight determination. The amino acid composition and structure of bacteriocins were identified using MALDI-TOF-MS/MS, quadrupole time-of-light mass spectrometry (Q-TOF-MS/MS), nano-HPLC electrospray ionization multistage tandem mass spectrometry (nLC-ESI-MS/MS), and liquid chromatograph mass spectrometry (LC-MS/MS) (Cui *et al*.,

2021; Kaškonienė *et al*., 2017). Gel electrophoresis is suitable for the analysis of small molecular-weight protein samples because of its relatively simple operation, rapidity, ease of observation, and safety. After a novel bacteriocin produced by *En. faecalis* L11 was extracted, its molecular weight was estimated to be 6.8 kDa by tris/tricine-SDS-PAGE (Gao *et al*., 2016). Initially, tricine-SDS-PAGE examination of plantaricin ZJ5 revealed that it was a single protein of about 3 kDa, and MALDI-TOF/MS analysis confirmed that the purified peptide's molecular weight was 2572.9 Da (Song *et al*., 2014). MALDI-TOF-MS/MS is a method for protein analysis and identification technology developed in recent years. It can determine the composition and structure of proteins and has become the main technology of proteomics research (Hindré *et al*., 2003). Many researchers have used this method to determine the molecular weight and amino acid sequence of bacteriocins. After purifying enterotoxin LD3, it is revealed to be an N-terminal amino acid sequence of H_2NQGGQANQ-COOH with a m/z of 4114.6 Da by MALDI-TOF-MS (Gupta et al., 2016). Plantaricin ZJ008, a novel bacteriocin found by Zhu et al., with a molecular weight of 1334.77 Da according to MALDI-TOF-MS analysis (Zhu *et al*., 2014). Furthermore, ESI-MS is a widely utilized mass spectrometry approach in laboratories for identifying thermally labile large supramolecules. The ESI-MS mass spectrum only consists of molecular ion peaks, therefore the molecular weight of the sample could be determined quickly and accurately (Banerjee and Mazumdar, 2012). ESI-TOF-MS is a commonly used method for identifying bacteriocins and is used in many studies to determine the structures of novel bacteriocins (Rumjuankiat *et al*., 2015; Sawa *et al.*, 2013). SDS-PAGE analysis of a novel bacteriocin from *En. faecalis* 478 showed that the 45 KDa protein was the major determinant of its antibacterial activity, and nLC-ESI-MS/MS analysis of the corresponding band determined the predicted molecular weight of the protein to be 47,809 Da (Phumisantiphong *et al*., 2017). Q-TOF-MS is an analytical technique that combines the advantages of two different mass analyzers. It uses the high compound fragmentation efficiency of quadrupole technology and the rapid

analysis speed and high mass resolution capabilities of time-of-flight to introduce a unique "hybrid" analyzer (Allen and McWhinney, 2019). Using Q-TOF-MS/MS analysis, estimated the molecular weight of the novel bacteriocin-M4L1 to be 3748.9643 Da (Li *et al*., 2022).

3. Motivation and objectives

Numerous LAB-bacteriocins with varying structures and characteristics have been continuously purified, identified, and used in food industry. Nisin A and pediocin PA-1 produced by some strains *Lactococcus lactis* subsp. *lactis* and *Pediococcus acidi-lactici*, respectively, are commercially used broad-spectrum bacteriocins worldwide, however they are easy to disruption of native, normal bacteria (Umu *et al*., 2017). Narrow-spectrum bacteriocins have been recently reevaluated for their use because they can inhibit certain pathogens and spoilage bacteria without significantly affecting the overall bacterial flora. Some strains of *Leuconostoc* spp. produce Narrow-spectrum bacteriocins especially inhibit the growth of *Listeria* spp. *Leuconostoc mesenteroides* subsp. *mesenteroides* 406 and 213M0 were isolated from different samples of Mongolian traditional fermented milk (Airag). Thier cell-free culture supernatant (CFS) had antibacterial activity especially against *Lis. Monocytogenes* suggesting that both strains probably produce class IIa antilisterial bacteriocin-like inhibitory substance (BLIS) (Wulijideligen *et al.,*2012 ; Arakawa *et al.*, 2016). In addition, draft genome sequences revealed that the two strains had the gene cluster (*mes*) responsible for the production of and immunity to mesentericins Y105 and B105 (*Morita et al*., 2016a, 2016b).This correspondence of the gene clusters was strange to us, because the size of the bacteriocin-like inhibitory substance (BLIS) produced by strain 213M0 was thought to be slightly larger than that from that of strain 406 based on the result of an in situ antilisterial activity assay (Arakawa *et al*., 2016). Therefore, more comparative analysis would be needed.

The major content of study:

To provide more scientific support for the use of strains 406 and 213M0 and their bacteriocins for safe and effective food biopreservation, this study aimed to clarify the differences between them by recomparing the antibacterial properties and bacteriocin-related gene clusters, and by purifying and identifying their bacteriocins.

II Comparison of antibacterial properties between *Leu. mesenteroides* 406 and 213M0

Abstract

Cell growth (pH, tribidity, and viable cell count) of *Leu. mesenteroides* 406 and 213M0 were compared at constant intervals and revealed that their cell growth was almost same. Their antilisterial activity was compared using *Lis. monocytogenes* VTU 206 as an indicator strain, it was shown that *Leu. mesenteroides* 213M0 had lower activity than strain 406. Then, antibacterial spectra of *Leu. mesenteroides* 406 and 213M0 was compared using 9 strains of *Listeria* and 23 strains of LAB as indicators. The results showed that although their antimicrobial spectra were similar, their antimicrobial abilities were different. Both strains inhibited the growth of all tested *Listeria* strains and *En. faecalis* JCM 5803^T, *Leu. mesenteroides subsp. cremoris* NBRC 107766^T, *Leu. mesenteroides subsp. dextranicum* NBRC 100495^T, *Leu. lactis* JCM 6123^T, *W. cibaria* JCM 12495^T, *W.a paramesenteroides* JCM 9890^T. However, for most of the inhibited strains, *Leu. mesenteroides* 406 showed higher antibacterial activity than strain 213M0, except for *Lis.monocytogenes* JCM 7680 and *Leu. lactis* JCM 6123^T.

1. Introduction

Heterofermentative facultative anaerobic *Leuconostoc* is one of the commonly used starter cultures in dairy fermentation processing. *Leuconostoc* metabolizes glucose or galactose via the heterofermentative pathway, producing lactate, ethanol, and carbon dioxide as end products, and even acetate under certain conditions (in the presence of acetaldehyde and pyruvate). Some species of *Leuconostoc*, such as *Leu. mesenteroides* can metabolize citric acid, and the diacetyl produced in this process is the primary flavor compound that imparts flavor to dairy butter (Cogan and Jordan, 1994).

Metabolites generated during fermentation, such as lactic acid, carbon dioxide, and other compounds, including bacteriocins, can inhibit undesirable strains, lengthen the shelf life of fermented foods, and ensure food safety (Stiles, 1994). Bacteriocins produced by *Leuconostoc* may not have broad antibacterial activity, but they are effective against the important food pathogen, *Lis. monocytogenes*. *Leu. mesenteroides* Y105, isolated from goat milk, produce two bacteriocins: mesentericin Y105 and mesentericin B105 (Biet *et al*., 1998). *Leu. mesenteroides* UL5 isolated from cheddar cheese produces thermostable mesentericin 5, a bacteriocin that has antilisterial activity (Daba *et al*., 1991). These bacteriocin-producing strains were isolated from various fermentation products, however only a few studies have been reported on the isolation of bacteriocin-producing *Leuconostoc* from fermented mare's milk. *Leu. mesenteroides* 406 and 213M0 were isolated from airag. They can inhibit the growth of *Lis. monocytogenes* and possess Class IIa bacteriocin properties, such as small heat stable proteins. However, in previous study, their antibacterial properties were studied separately under different conditions. Their cell growth and antibacterial properties were detected at different testing times and using different strains as indicators (Wulijideligen *et al.,*2012 ; Arakawa *et al.*, 2016). Therefore, the aim of this study is to determine the similarities and differences between *Leu. mesenteroides* 406 and 213M0 by re-comparing their antibacterial properties under same experimental conditions.

2. Materials and methods

A. Bacterial Strains and Culture Conditions

Leu. mesenteroides subsp. *mesenteroides* 406 and 213M0 were cultivated with 2% (*v*/*v*) inoculum in MRS broth (Oxoid Limited; Hampshire, UK) at 25 ˚C. Other strains used in this study are listed in **Table 4** along with their culture conditions. Tryptone, yeast extract, lactose, and glucose (TYLG) broth were used to propagate *Listeria* spp. strains. The ingredients of TYLG broth were purchased from Becton, Dickinson and

Company (Franklin Lakes, NJ, USA) and FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). TYLG was prepared according to the (MOK et al., 1998) by dissolving 1% tryptone (w/v), 0.5% yeast extract (w/v), 0.5% glucose (w/v), 0.5% lactose (w/v), 0.10% Tween 80 (w/v), and 0.01% L-cysteine (w/v) hydrochloride in water and sterilized by autoclaving. Before use, all bacterial strains were propagated three times. MRS agar (Oxoid, Hampshire, UK) and Standard Method Agar (Nissui, Japan) were used on the antibacterial assay.

B. Sequential Measurement of Bacterial Growth and Bacteriocin Productivity

The pH, turbidity (at OD_{620}),viable cell count (log CFU /mL) and, antilisterial activity (AU/ ml) were determined at constant intervals (0, 2, 4, 8, 12, 18, 24, 48 and 72 h). The viable cell counting was performed by plating the serially diluted culture solution in the MRS agar plate followed by 48 h incubation at 30°C, pH measurement was done by using pH meter (F-52,HOROBA, Japan).

C. Antibacterial Activity Assay

Antimicrobial activity was tested by agar well diffusion method (Tagg and McGiven, 1976). To prepare CFS as a sample for the assay, the 24 h incubation culture of both strains was neutralized (pH 7.0) with 1 M NaOH, followed by centrifugation at 1600× *g* for 20 min at room temperature, and sterilized through a 0.22 μm filter (Membrane Solutions, LLC; Auburn, WA, USA). CFS was then serially diluted two-fold using sterile saline. Each indicator strain listed in **Table.6** was inoculated at 2.5% (*v*/*v*) into an appropriate agar medium and mixed well. After solidification, wells (6 mm diameter) were punched on the agar plate. CFS or the dilution (50 μL) was aliquoted into the well, and the plate was incubated at 30 or 37 ˚C for 24 h.

A clear zone without cell growth of each indicator around the well indicated the presence of BLIS or bacteriocins. The unit of BLIS/bacteriocin activity (arbitrary units,

AUs) was defined as the reciprocal of the highest dilution inhibiting the growth of each indicator strain. Results presented are the mean ± S.D. of at least three independent experiments.

D. Antibacterial spectra Assay

Antibacterial spectra of strains 406 and 213M0 were evaluated by the antibacterial activity assay of CFS against 32 bacterial strains listed in **Table.5** as indicators. Standard Method Agar (Nissui, Japan) and MRS agar (Oxoid, Hampshire, UK) was used for test *Listeria* and LAB respectively. Antibacterial titers were determined and expressed in activity units per ml (AU/ ml). The sample was serially diluted twofold using sterile distilled water and the bacteriocin activity value was defined as the reciprocal of the highest observed dilution of the growth inhibition zone, and its unit was expressed in Arbitrary Unit (AU). The bacteriocin activity values presented are the results of at least three independent measurements.

Table 4 Indicator strains used in antibacterial spectra

Tested bacteria		Culture conditons[2]
Species	Strain[1]	
Listeria monocytogenes	VTU 206	30°C, TYLG
Listeria monocytogenes	JCM 7671	37°C, TYLG
Listeria monocytogenes	JCM 7672	37°C, TYLG
Listeria monocytogenes	JCM 7673	37°C, TYLG
Listeria monocytogenes	JCM 7674	37°C, TYLG
Listeria monocytogenes	JCM 7675	37°C, TYLG
Listeria monocytogenes	JCM 7679	37°C, TYLG
Listeria monocytogenes	JCM 7680	37°C, TYLG
Listeria ivanovii subsp. ivanovii	JCM 7681^T	37°C, TYLG
Enterococcus faecalis	JCM 5803^T	37°C, MRS
Enterococcus faecium	JCM 5804^T	37°C, MRS
Lactococcus lactis subsp. *lactis*	IFO 12007	30°C, MRS
Lactococcus lactis subsp. *lactis*	NIAI 527	30°C, MRS
Lactococcus lactis subsp. *lactis*	NIAI N-7	30°C, MRS
Lactococcus lactis subsp. *lactis*	NBRC 100933^T	30°C, MRS

Lactococcus lactis subsp. *lactis*	JCM 7638	30°C, MRS
Lactococcus lactis subsp. *cremoris*	NBRC 100676^T	30°C, MRS
Lactococcus lactis subsp. *hordniae*	NBRC 100931^T	30°C, MRS
Leuconostoc mesenteroides subsp. *cremoris*	NBRC 107766^T	30°C, MRS
Leuconostoc mesenteroides subsp. *dextranicum*	NBRC 100495^T	30°C, MRS
Leuconostoc mesenteroides subsp. *mesenteroides*	NBRC 100496^T	30°C, MRS
Leuconostoc lactis	JCM 6123^T	30°C, MRS
Pediococcus acidilactici	JCM 8797^T	30°C, MRS
Pediococcus parvalus	JCM 5889	30°C, MRS
Pediococcus pentosaceus	JCM 5885	37°C, MRS
Pediococcus pentosaceus	JCM 5890^T	37°C, MRS
Streptococcus thermophilus	JCM 17834^T	37°C, MRS
Streptococcus thermophilus	JCM 20026	37°C, MRS
Weissella cibaria	JCM 12495^T	30°C, MRS
Weissella confusa	JCM 1093^T	30°C, MRS
Weissella paramesenteroides	JCM 9890^T	30°C, MRS
Weissella viridescens	JCM 1174^T	37°C, MRS

1 VTU, Department of Veterinary Public Health, University of Tokyo; JCM, Japan Collection of Microorganisms; NBRC, NITE Biological Resource Center, Japan; IFO, Institute for Fermentation, Osaka; and NIAI, National Institute of Animal Industry, Japan.

2 TYLG, Tryptone, yeast extract, lactose, and glucose broth ; and MRS, de Man, Rogosa, and Sharpe broth.

3. Results

A. Comparison of Bacterial Growth and Bacteriocin Productivity

The culture pH, turbidity, and cell viability in the MRS broth were measured sequentially for 72 h to compare the growth of *Leu. mesenteroides* 406 and 213M0. Strain 406 showed better growth than 213M0 except for the decline phase (**Fig. 1a-c**). At 16h incubation, the cell viability of strain 406 was more than twice that of strain 213M0. During the 72 h growth duration, the pH of two strain cultures decreased from approximately 6.0 to 4.1. Overall, CFS- 406 dropped faster than CFS-213M0. The antilisterial activity of their CFSs against strain VTU 206 was also measured

sequentially for 72 h to compare the bacteriocin production of strains 406 and 213M0. The activity of both strains increased in parallel with the cell growth during the exponential phase up to 16 h of incubation (**Fig.1d**). After 16 h of incubation, the activity of strain 406 was twice as high as that of 213M0. In addition, from the end of the stationary phase to the decline phase during 48–72 h of incubation, the activity of both strains was reduced by half compared with the maximum activity at 24 h of incubation. These results suggested that the differences in antibacterial properties of both strains might be due to differences in bacteriocin production affected by cell growth.

B. Comparison of Antibacterial Spectra

The antibacterial spectra of strains 406 and 213M0 were compared against 32 bacterial strains including *Listeria* spp. and LAB. Both CFSs showed similar antibacterial spectra (**Table 5**), in that the CFS inhibited the growth of all tested strains of *Listeria* spp. ,with activity higher than 1700 AU/ml. Despite having weaker inhibitory activity against LAB than spp., they still inhibited some indicator LAB strains, including *En. faecalis* JCM 5803^T, *Leu. mesenteroides* subsp. *cremoris* NBRC 107766^T, *Leu. mesenteroides* subsp. *dextranicum* NBRC 100495^T, *Leu. lactis* JCM 6123^T, *W. cibaria* JCM 12495^T, and *W. paramesenteroides* JCM 9890^T. In addition, the activity of strain 406 was generally higher than that of strain 213M0. However, only against two strains, *Lis. monocytogenes* JCM 7680 and *Leu. lactis* JCM 6123^T, the activity of both strains was reversed; namely, the activity of strain 213M0 was higher than that of strain 406. These results suggested that there were differences not only in bacteriocin productivity as noted above, but also in the antibacterial substances themselves.

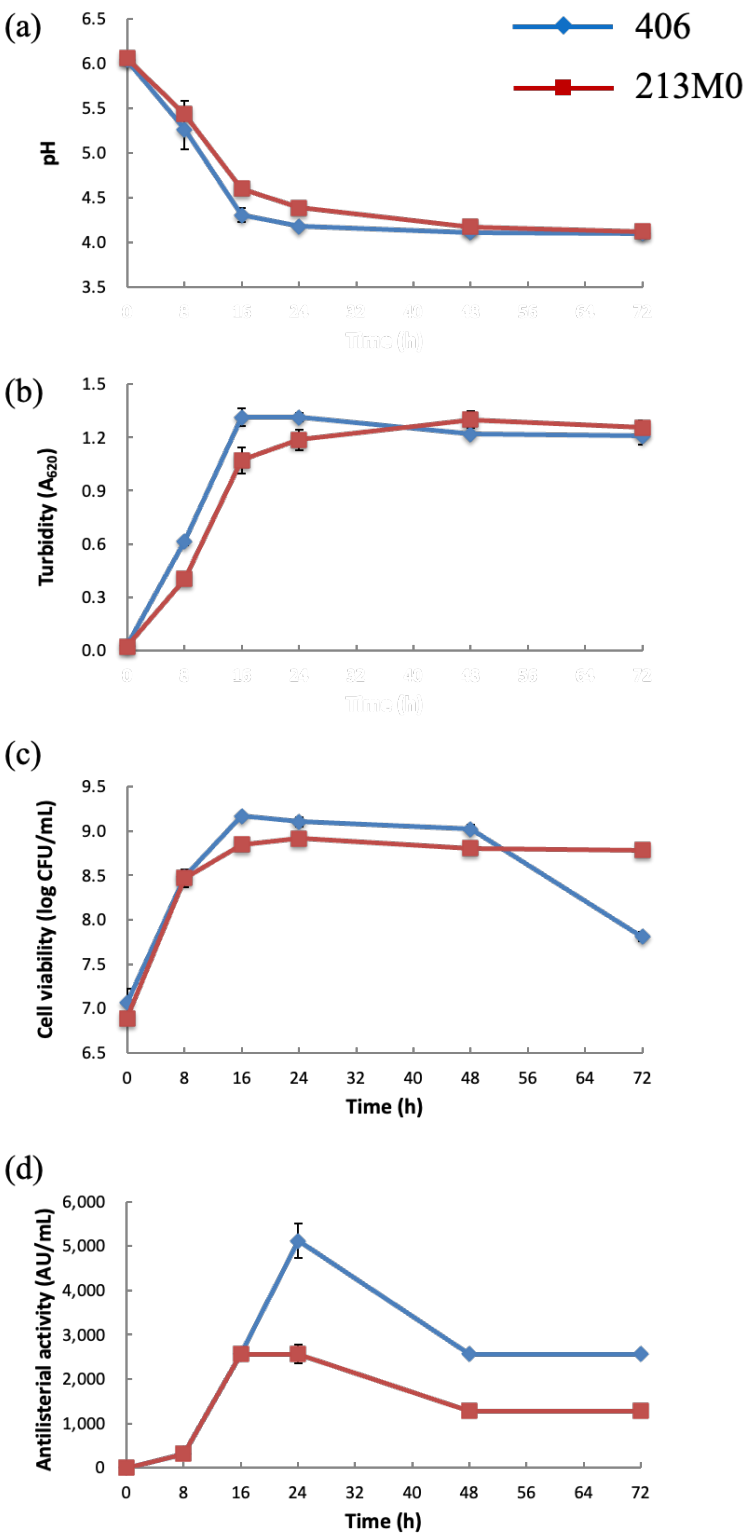

Fig. 1 Changes in culture pH (a), turbidity (b), cell viability (c), and antilisterial activity against *Lis. monocytogenes* VTU 206 (d) of *Leu. mesenteroides* 406 and 213M0.

Table 5 Antibacterial spectra of *Leu. mesenteroides* 406 and 213M0

Tested bacteria		Activity AU/ml[2]	
Species	Strain[1]	*Leu. mesenteroides* 406	*Leu. mesenteroides* 213M0
Listeria monocytogenes	VTU 206	4,969±99.1	2,695±63.1
Listeria monocytogenes	JCM 7671	3,093±111.6	2,560±105.2
Listeria monocytogenes	JCM 7672	3,328±85.9	3,072±97.1
Listeria monocytogenes	JCM 7673	3,584±70.1	2,944±105.2
Listeria monocytogenes	JCM 7674	5,546±188.4	3,584±105.2
Listeria monocytogenes	JCM 7675	2,560±0.0	2,560±0.0
Listeria monocytogenes	JCM 7679	5,120±0.0	5,120±0.0
Listeria monocytogenes	JCM 7680	1,706±37.0	2,560±0.0
Listeria ivanovii subsp. ivanovii	JCM 7681^T	15,360±280.4	9,386±104.5
Enterococcus faecalis	JCM 5803^T	724±13.6	600±6.8
Enterococcus faecium	JCM 5804^T	-	-
Lactococcus lactis subsp. lactis	IFO 12007	-	-
Lactococcus lactis subsp. lactis	NIAI 527	-	-
Lactococcus lactis subsp. lactis	NIAI N-7	-	-
Lactococcus lactis subsp. lactis	NBRC 100933^T	-	-
Lactococcus lactis subsp. lactis	JCM 7638	-	-
Lactococcus lactis subsp. cremoris	NBRC 100676^T	-	-
Lactococcus lactis subsp. hordniae	NBRC 100931^T	-	-
Leuconostoc mesenteroides subsp. cremoris	NBRC 107766^T	72±2.5	32±1.3
Leuconostoc mesenteroides subsp. dextranicum	NBRC 100495^T	471±11.2	235±5.5
Leuconostoc mesenteroides subsp. mesenteroides	NBRC 100496^T	-	-
Leuconostoc lactis	JCM 6123^T	20±0.0	40±1.4
Pediococcus acidilactici	JCM 8797^T	-	-
Pediococcus parvalus	JCM 5889	-	-
Pediococcus pentosaceus	JCM 5885	-	-
Pediococcus pentosaceus	JCM 5890^T	-	-
Streptococcus thermophilus	JCM 17834^T	-	-
Streptococcus thermophilus	JCM 20026	-	-
Weissella cibaria	JCM 12495^T	297±8.6	114±6.1
Weissella confusa	JCM 1093^T	-	-
Weissella paramesenteroides	JCM 9890^T	1,768 ±30.3	522±8.4
Weissella viridescens	JCM 1174^T	-	-

1 VTU, Department of Veterinary Public Health, University of Tokyo; JCM, Japan Collection of Microorganisms; NBRC, NITE Biological Resource Center, Japan; IFO, Institute for Fermentation, Osaka; and NIAI, National Institute of Animal Industry, Japan.

2 AU, arbitrary units (mean ± S.D.); and -, no inhibition.

4. Discussion

Leu. mesenteroides 406 and 213M0 of growth and antimicrobial properties were examined independently in previous studies. After 24 h of incubation, strain 213M0's growth peaked, and its antibacterial activity increased as well, staying for an additional 48 h. When the culture was in the stationary phase, strain 406's antibacterial activity peaked and then declined over a period of 36 h (Arakawa *et al*., 2016; Wulijideligen *et al*., 2012). The findings of this study are mainly consistent with those of previous studies, but strain 406 showed better growth and stronger antilistral activity than strain 213M0. Furthermore, we used the same indicator strain to re-compare the antibacterial spectra of *Leu. mesenteroides* 406 and 213M0, resulting in an interesting finding, although they have similar antibacterial spectra to inhibit the same indicator strains, including *Listeria*, *Enterococcus, Leuconostoc,* and *Weissella*, their inhibitory activity against some indicator strains is opposite. For example, strain 213M0 had higher inhibitory activity against *Lis. monocytogenes* JCM 7680 and *Leu. lactis* JCM 6123^{T}, while strain 406 showed higher inhibitory ability against most inhibited bacteria than strain 213M0 and weaker inhibitory ability against the above two strains. The above results suggest that differences in the antibacterial properties of the two strains might be due not only to differences in bacteriocin production, which is affected by cell growth, but also to differences in the antibacterial substances themselves. Without the purification of bacteriocin, it is impossible to explain the reasons for the different antibacterial activities and properties. Therefore, it is necessary to purify and identify the bacteriocins of *Leu. mesenteroides* 406 and 213M0.

5. Conclusion

Leu. mesenteroides 406 showed better growth and generally higher antibacterial activity than strain 213M0. However, the activity of strain 213M0 was higher against only a few strains.

III. DNA sequencing for the gap-filling, mapping of plasmids, and plasmid curing

Abstract

DNA sequencing revealed that *Leu. mesenteroides* 406 and 213M0, respectively, had two and three plasmids. In addition, one of their plasmids (pLM406A and pLM213M0A) contained a similar gene cluster (*mes*) involved in the mesenterins Y105 and B105. Another plasmid of *Leu. mesenteroides* 406 (pLM406B) contains a series of genes involved in citrate metabolism. The mesenterin secretion genes *mesDE* and bacteriocin immunity gene can be identified on the other plasmids (pLM213M0B) of strain 213M0, whereas pLM213M0C is a cryptic plasmid. Their *mes* genes have high similarity with other *mes*-containing plasmids, pHY30 and pFR38, in *Leu. mesenteroides* Y105 and *Leu. mesenteroides* FR52, respectively. In addition, the *mes* genes of pLM406A and pLM213M0A are 100% identical except for *mesG*; the similarity between *mesDE* of pLM213M0B and pHY30 is higher than 88%. Then, removal of these plasmids led to a loss of activity, indicating that the bacteriocins of both strains were biosynthesized from the plasmids.

1. Introduction

Bacteriocin-related genes are encoded on a plasmid or chromosome in a producer cell. For example, mesentericin production of *Leu. mesenteroides* Y105 and FR52 were associated with the presence of plasmids pHY30 and pFR38, respectively (Héchard *et al.*, 1999 ; Revol-Junelles *et al.*, 1996). The biosynthetic gene clusters of mesentericin OZ were located on the chromosome of *Leu. mesenteroides* OZ (Osmanagaoglu & Kiran, 2011). Previous study suggested that strain 406 and 213M0 contain 2 and 3 plasmid-like sequences, respectively. However, it is unclear that the antibacterial

activity of both strains is due to plasmid-encoded genes. The location of bacteriocin-related genes can be determined by antibacterial activity assays of derivative strains generated by plasmid curing. After plasmid curing, when bacteriocin genes are located on chromosomes, they will have antibacterial activity. If bacteriocin genes are on a plasmid and have been removed, the derivative strains will lose their activity. In this experiment, the location of bacteriocin-related genes was confirmed by gap-filling PCR and DNA sequencing, by gene annotation, and by antibacterial activity assays of derivative strains obtained using plasmid curing.

2. Materials and methods

A. PCR and DNA sequencing to close plasmid gaps

In previously reported draft genome information of strains 406 (BCMP01000000) and 213M0 (BCMO01000000) (Morita *et al*., 2016a, 2016b), two and three plasmid-like sequences are shown as contigs- 33 and -40 in 406, and contigs-26, -30, and -48 in 213M0. The gaps in these contigs were filled by PCR and DNA sequencing to obtain the full-length circular plasmid sequences. Total and plasmid DNA was extracted from *Leu. mesenteroides* 406 and 213M0 using the previous method with some modifications (Klaenhammer, 1993).

PCR was performed with primers (**Table 6**) designed based on the contig sequences and KAPA Taq EXtra HotStart ReadyMix (Kapa Biosystems, Inc.; Wilmington, MA) using SimpliAmp Thermal Cycler (Applied Biosystems; Waltham, MA). The PCR conditions for contigs-33 and -40 in strain 406 and contigs-30 and -48 in strain 213M0 were as follows: Initial heating at 95°C for 2 min; 40 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec; and final extension at 72°C for 7 min. For contig-26 in 213M0, primers 213-26-F5 and -R5 were used first under the following conditions: Initial heating at 95°C for 2 min; 40 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 30 sec; and final extension at 72°C for 7 min. Next, the

PCR amplicon was extracted using Mag Extractor DNA fragment purification kit (Toyobo Co., Ltd.; Osaka, Japan) after agarose gel electrophoresis, and then used as a template for the second PCR with primers 213-26-F4 and -R4 under the following conditions: Initial heating at 95°C for 1 min; 40 cycles of 95°C for 30 sec, 60°C for 1 min, and 72°C for 30 sec; and final extension at 72°C for 7 min.

The resulting PCR amplicons were extracted after electrophoresis and finally submitted to DNA sequencing service at Eurofins Genomics K.K. (Tokyo, Japan). DNA sequences obtained were submitted to the DNA Data Bank Japan (DDBJ) to obtain accession numbers (LC832857-LC832861) after annotation using the DDBJ Fast Annotation and Submission Tool (DFAST). In addition, the putative genes related to bacteriocin biosynthesis and immunity were analyzed using the Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI).

Table 6 Primer used in this study

Primer names	Sequence（5'-3'）	Target
mesY-F	ACCAAAATCCATTTCCACCA	Structural gene(*mesY*) of mesentericin Y105
mesY-R	TCTGTGGAAGCATATCAGCAA	
406-33-F1	CCCAATACACCTTTACCACCAC	Contig-33 (pLM406A) in *Leu. mesenteroides* 406
406-33-R1	CTTGGATTGTGGGAACAAGA	
406-40-F1	AGAAACTGCCCGTGATGGAAAC	Contig- 40 (pLM406B) in *Leu. mesenteroides* 406
406-40-R1	GCTGGTGTTGGATTGTCTTTGCT	
213-26-F4	AGCGGTTGCTATAACGGCTA	Contig-26 (pLM213M0A)in *Leu. Mesenteroides* 213M0
213-26-R4	GCTTCAAATGACGACTGCAA	
213-26-F5	CGAGCTTTAAAGGGTGCTGAAAAAT	Contig-26 (pLM213M0A)in *Leu. Mesenteroides* 213M0
213-26-R5	CGCTACTGAATTTCTTGTCAAGGTTGT	

213-30-F1	TTAGTCCGTGAGCGGTTTATGAGAG	Contig-30 (pLM213M0B) in *Leu. mesenteroides* 213M0
213-30-R1	AATCAAGAAAGGAGCTGTGATGACG	
213-48-F2	TTGCGCTAATCGGTCAATGG	Contig-48 (pLM213M0C) in *Leu. mesenteroides* 213M0
213-48-R2	GTGACCGACCGTAGGGAGACTTTAT	

C. Plasmid curing

Leu. mesenteroides 406 and 213M0 were cultivated stepwise in the modified MRS broth supplemented with novobiocin (1, 2, 5, 10, 20, 50, 100, to 200 μg/mL) to obtain derivative strains.The loss of plasmids pLM406A (contig-33 of strain 406) and pLM213M0A (contig-26 of strain 213M0) in the derivative strains was confirmed by PCR using primers of mesY-F and -R **(Table 6)** to detect the structural gene (*mesY*) of mesentericin Y105. Primer pairs of 406-40-F1 and R1, 213-30-F1 and -R1, and 213-48-F2 and -R2 **(Table 6)** were also used to detect plasmids pLM406B (contig-40 of 406), pLM213M0B (contig-30 of 213M0), and pLM213M0C (contig-48 of 213M0), respectively. PCR was performed using KAPA2G Fast HotStart ReadyMix (Kapa Biosystems). The PCR conditions were as follows: Initial heating at 95°C for 10 min; 35 cycles of 95°C for 15 sec, 53°C for 15 sec, and 72°C for 5 sec; and final extension at 72°C for 1 min. Only for pLM406B, KOD One PCR Master Mix (Toyobo) was used, and the PCR conditions were as follows: Initial heating at 98°C for 10 min; 40 cycles of 98°C for 10 sec, 53°C for 5 sec, and 68°C for 5 sec; and final extension at 72°C for 30 sec. After PCR, agarose gel electrophoresis was performed with FastGene 50-bp DNA Ladder (Nippon Genetics Co., Tokyo, Japan) to visualize the loss of each plasmid.

The antibacterial activity of the derivative strains with loss of plasmids was tested against seven indicator strains: *Lis. monocytogenes* VTU 206, *W. paramesenteroides* JCM 9890^{T}, *Leu. lactis* JCM 6123^{T}, *E. faecalis* JCM 5803^{T}, *Leu. mesenteroides*

subsp. *dextranicum* NBRC 100495^{T}, *W. cibaria* JCM 12495^{T}, and *Leu. mesenteroides* subsp. *cremoris* NBRC 107766^{T}.

3. Result

A. DNA Sequencing and plasmid mapping

Plasmid-like contigs (contigs-33 and -40 in strain 406 and contigs-26, -30, and -48 in strain 213M0) in the previously published draft genome sequences (Morita *et al*., 2016a, 2016b) were gap- filled by PCR and DNA sequencing. Among them, contig-33 in 406 and contig-48 in 213M0 were cyclized without any sequence insertion. Contig-40 in 406 and contig-30 in 213M0 were directly cyclized with 248- and 3-nt sequences, respectively. The other one, contig-26, included a 171-nt wrong sequence at the 5′-terminal side. In addition, another 134-nt sequence (at the 5′-side of contig-54) was added to its 3′-end to be cyclized as a plasmid. As a result, two (named pLM406A and pLM406B) and three (named pLM213M0A, pLM213M0B, and pLM213M0C) plasmids were confirmed in strains 406 and 213M0. The sequences of the five plasmids were annotated using DFAST. **Table 7**, **Fig. 2 and Fig. 3** show an overview and maps of the sequenced and annotated plasmids. Both pLM406A and pLM213M0A contained the mesentericin Y105-B105 gene cluster (**Fig. 2a** and **Fig. 3a**), and pLM213M0B encoded two genes (*mesDE*) related to a transport system of mesentericins (**Fig. 3b**). In addition, pLM406B contained genes related to a citrate metabolism system (**Fig. 2b**), and pLM213M0C was a cryptic plasmid (**Fig. 3c**). Incidentally, no gene sequences thought to be related to bacteriocins were found in the chromosome contigs.

Table 7 Overview of confirmed plasmids in *Leu. mesenteroides* 406 and 213M0

Plasmid	Length (bp)	GC (%)	ORFs	Accession No.
pLM406A	32,775	34.2	42	LC832860
pLM406B	23,705	39.4	24	LC832861
pLM213M0A	41,975	33.5	51	LC832857
pLM213M0B	11,669	33.8	14	LC832858
pLM213M0C	6,449	30.2	8	LC832859

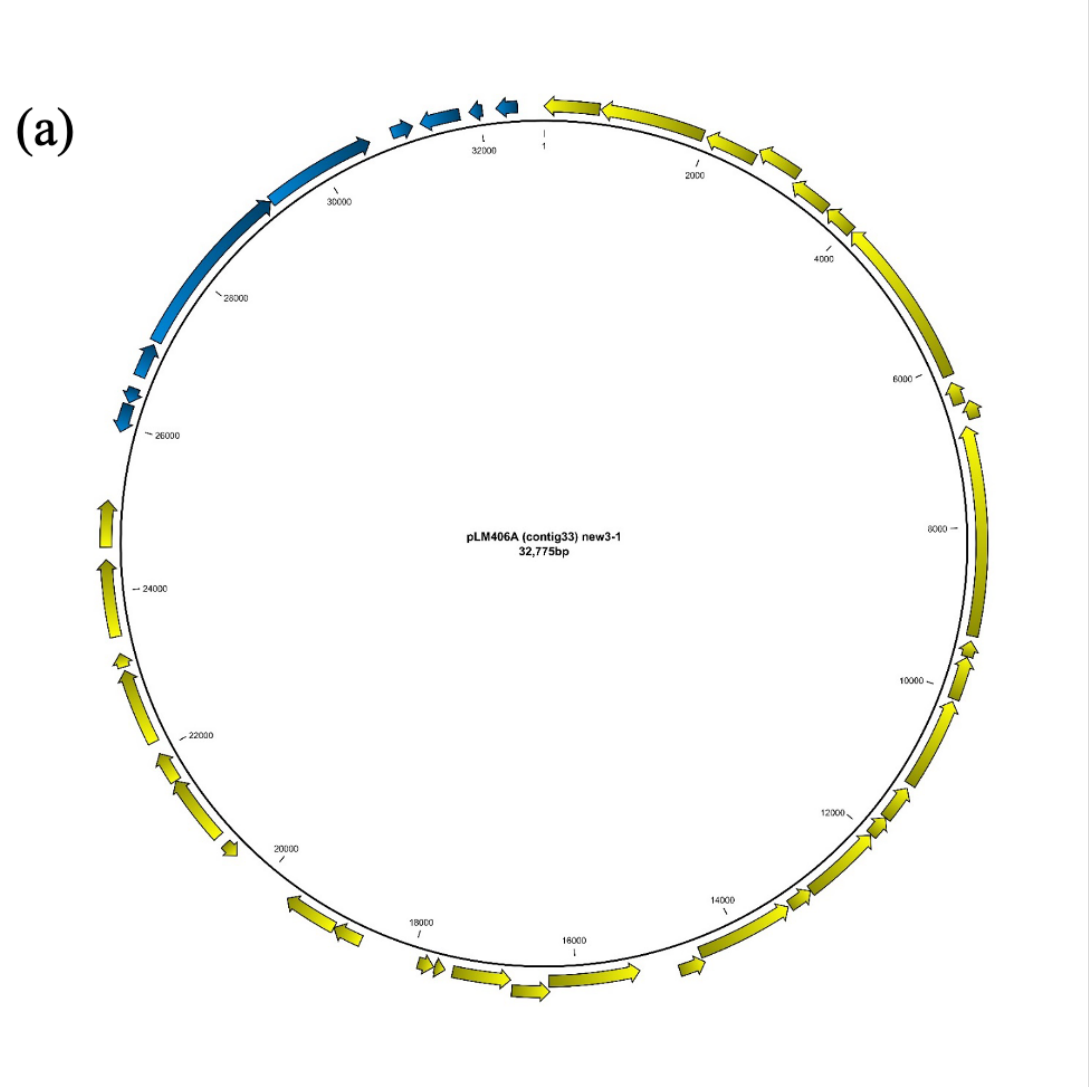

Fig. 2 Maps of plasmids pLM406A (a) and pLM406B (b) in *Leu. mesenteroides* 406

Blue arrows indicate mesentericin Y105-B105-related genes. Yellow allows are others.

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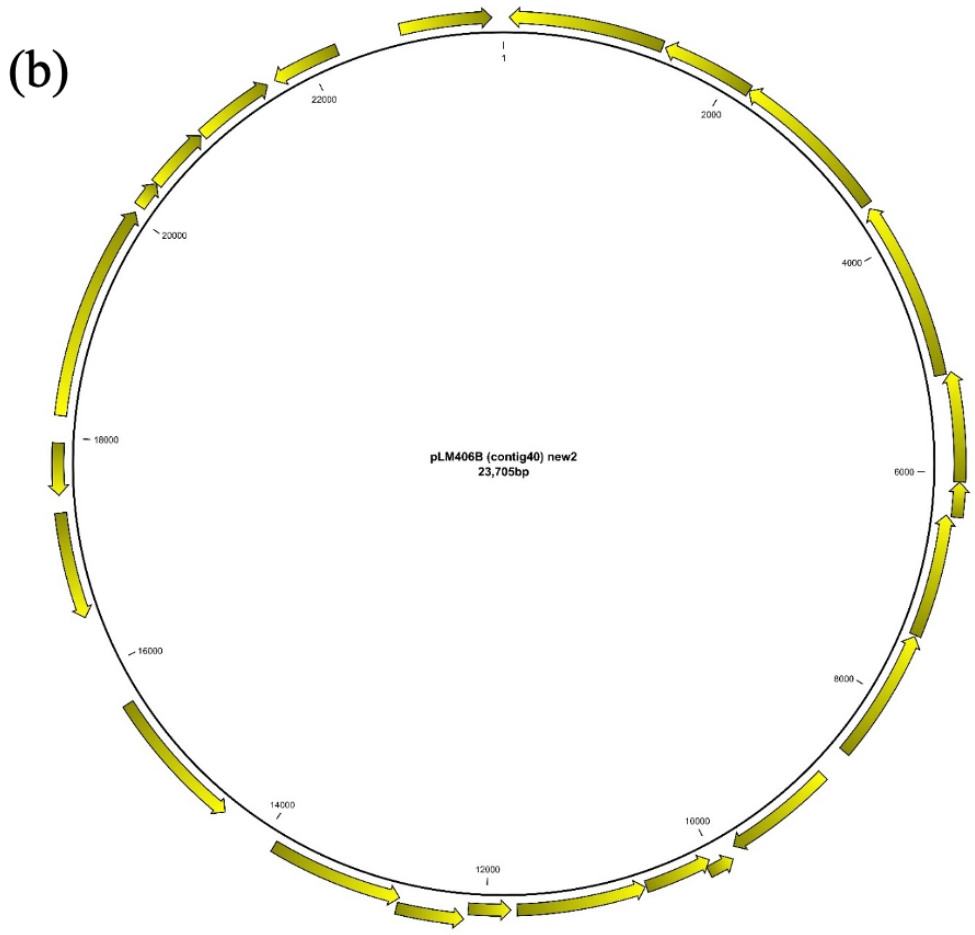

Fig. 2 Maps of plasmids pLM406A (a) and pLM406B (b) in *Leu. mesenteroides* 406

Blue arrows indicate mesentericin Y105-B105-related genes. Yellow allows are others.

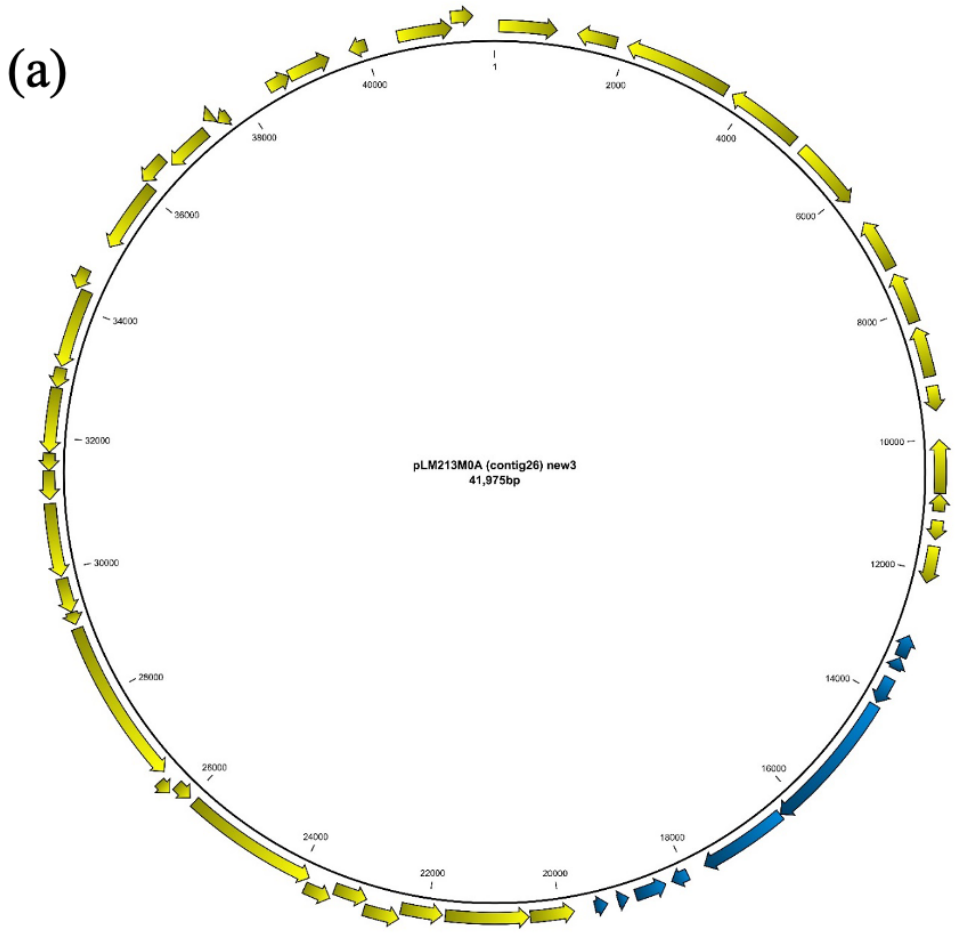

Fig. 3 Maps of plasmids pLM213M0A (a), pLM213M0B (b) and pLM213M0C (c) in *Leu. mesenteroides* 213M0.

Blue arrows indicate mesentericin Y105-B105-related genes. Green arrows indicate mesentericin M-related genes newly estimated in this study. Yellow allows are others.

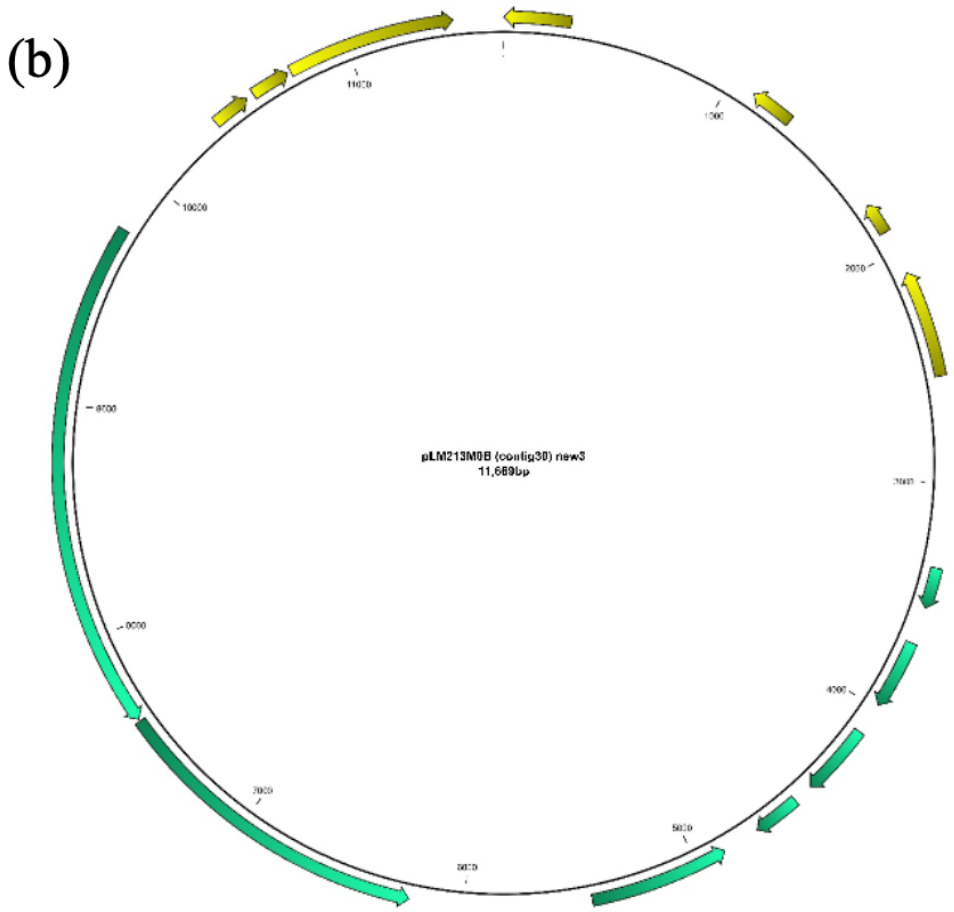

Fig. 3 Maps of plasmids pLM213M0A (a), pLM213M0B (b) and pLM213M0C (c) in *Leu. mesenteroides* 213M0.

Blue arrows indicate mesentericin Y105-B105-related genes. Green arrows indicate mesentericin M-related genes newly estimated in this study. Yellow allows are others.

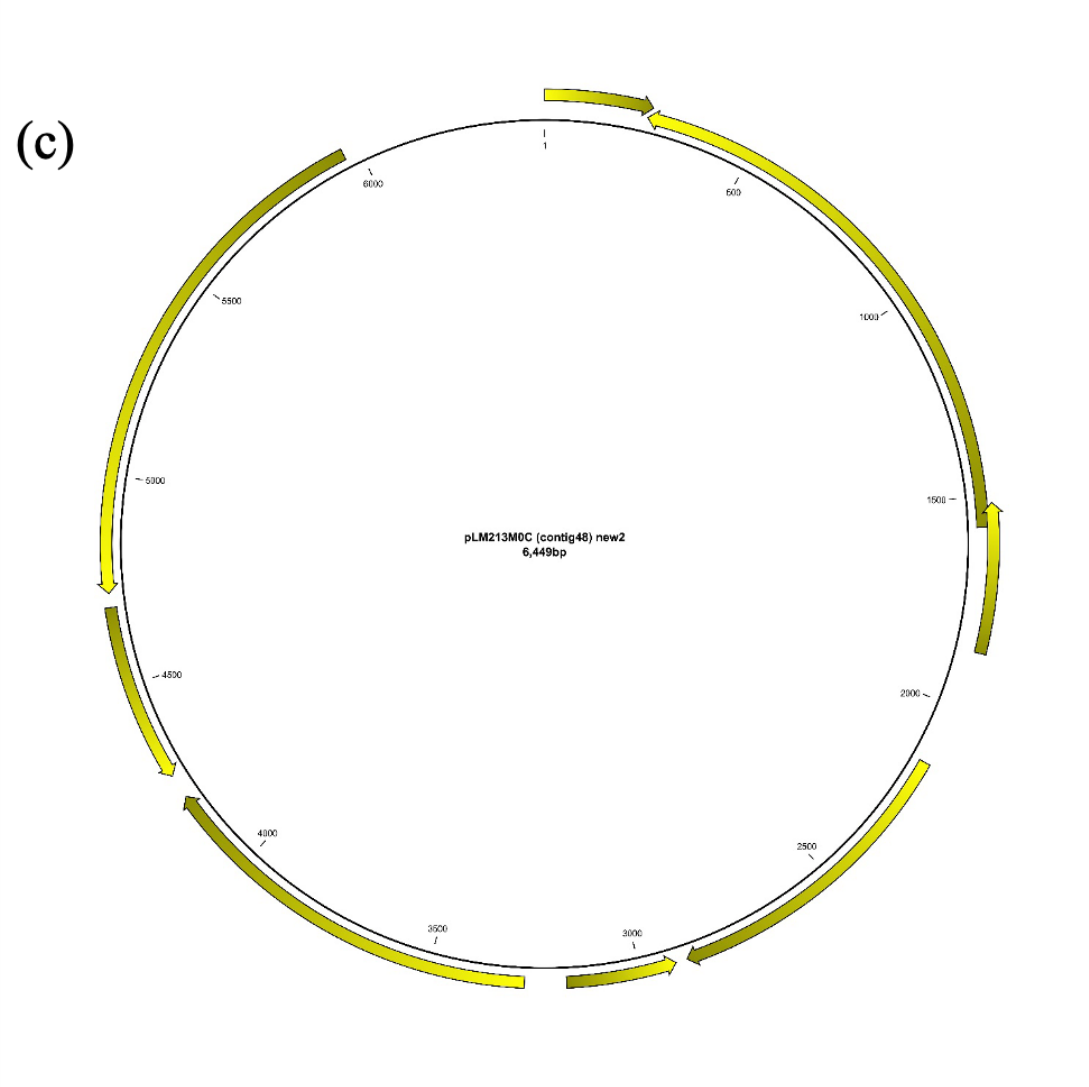

Fig. 3 Maps of plasmids pLM213M0A (a), pLM213M0B (b) and pLM213M0C (c) in *Leu. mesenteroides* 213M0.

Blue arrows indicate mesentericin Y105-B105-related genes. Green arrows indicate mesentericin M-related genes newly estimated in this study. Yellow allows are others.

B. Homology Analysis of Mesentericins Y105-B105-Related Genes

In the confirmed plasmids, the putative nine genes (*mesIYCDEFHBG*) related to biosynthesis and immunity for mesentericins Y105 and B105 were searched for homology using the BLAST program. The gene cluster encoding mesentericins Y105/B105 and mesentriocins FR52A/52B were found to show a similar organization to those found in *Leu. mesenteroides* 406 and 213M0 (**Fig. 4**). The sequences of the nine genes on pLM406A and pLM213M0A were 100% identical except for functionally unknown *mesG*. The homology of *mesG* between them was 77%. This was because the middle region (60 bp) of *mesG* was missing on pLM213M0A, although the other region was 100% identical to that on pLM406A.The nine genes of both strains also had much high similarity to those on plasmid pFR38 (accession no. AY286003) in *Leu. mesenteroides* FR52 (a producer of mesentericins 52A and 52B = mesentericins Y105 and B105) (Revol-Junelles *et al*., 1996) compared to pHY30 (accession no. X81803 and AF143443) on strain Y105 (a producer of mesentericins Y105 and B105) (Hechard *et al*., 1992; Biet *et al*., 1998; Fremaux *et al*., 1995). In addition, the two transporter genes (*mesDE*) on pLM213M0B had less high homology to those on pFR38, pLM406A and pLM213M0A (82 and 78%), and those on pHY30 (92 and 88%). These results suggested that differences in the productivity and antibacterial spectra of bacteriocins between strains 406 and 213M0 might be due to shortened *mesG* and/or duplicated *mesGE* genes.

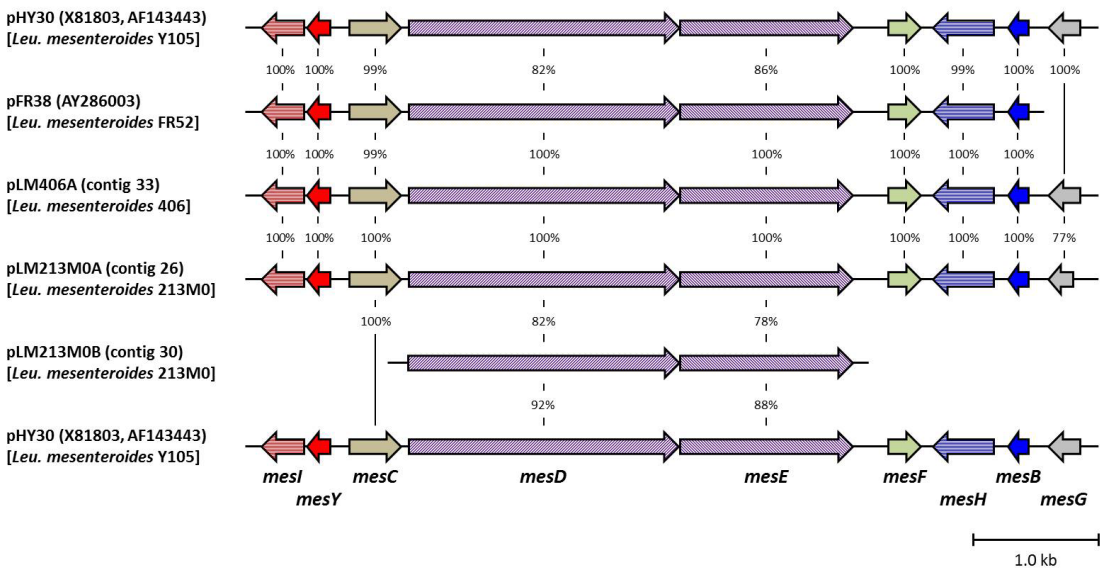

Fig. 4 Sequence alignment of mesentericin Y105-B105-reated genes.

Sequence alignment of mesentericin Y105-B105-reated genes. Gene functions: *mesI*, immunity to mesentericin Y105; *mesY*, structural gene of mesentericin Y105; *mesC*, unknown; *mesDE*, secretion of mesentericins Y105 and B105; *mesF*, unknown; *mesH*, immunity to mesentericin B105; *mesB*, structural gene of mesentericin B105; and *mesG*, unknown.

C. Plasmid Curing

Leu. mesenteroides 406 and 213M0 were stage-cultured in a medium containing novobiocin, a curing agent, to obtain the plasmid-cured strains that lacking antilisterial activity. As a result, strain A derived from strain 406 and strain B derived from strain 213M0 (**Fig. 5**) were isolated and their plasmid profiles were observed by agarose gel electrophoresis after PCR with the specific primers (**Table 7**) for each plasmid (**Fig. 6**).Two bands with approximate size of 169 bp and greater than 500 bp corresponding to pLM406A (*mesY*) and pLM406B (**Fig. 6**) were amplified from *Leu. mesenteroides* 406, while for strain A no corresponding band for *mesY* was amplified suggesting that train A had lost the pLM406A, carrying mesentericin Y105/B105 gene cluster. On the contrary, pLM406B was still present in strain A, despite novobiocin curing treatment. For *Leu. mesenteroides* 213M0, approximate size of 169 bp , approximately 335bp and 491bp (**Fig. 6**) band corresponding to pLM213M0A (*mesY*), pLM213M0B (*mesDE*) and pLM213M0C were obtained by PCR, the opposite, there were no band in the strain B, indicated that all plasmids were eliminated by the novobiocin treatment in plasmid cured strain. Furthermore, *Enterococcus faecalis* JCM 5803^T, *Leu. mesenteroides subsp. cremoris* NBRC 107766^T, *Leu. mesenteroides subsp. dextranicum* NBRC 100495^T, *Leu. lactis* JCM 6123^T, *W. cibaria* JCM 12495^T, *W. paramesenteroides* JCM 9890^T were used as indicator strains to test the antibacterial activity of CFS of plasmid cured strains. The results were the same as *Lis. monocytogenes* VTU206 used as indicator, and no inhibition zone was observed in this test (**Fig. 5).**

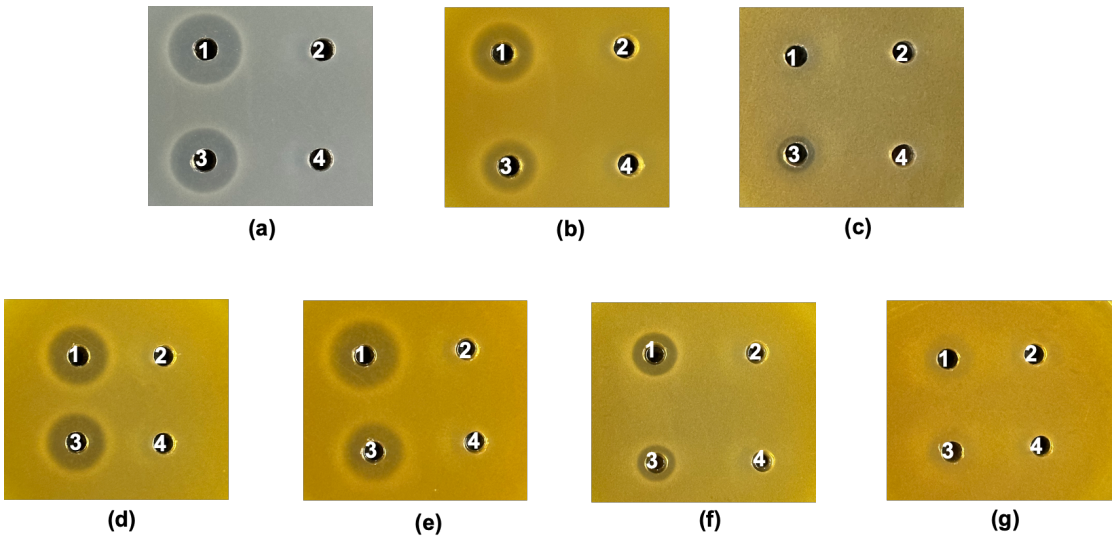

Fig. 5 Antibacterial activity of *Leu. mesenteroides* 406 (1) and 213M0 (3), and their derivative strains (2) and (4) from 406 and 213M0, respectively) obtained by plasmid curing using novobiocin.

Indicator strains: *Lis. monocytogenes* VTU 206 (a), *W. paramesenteroides* JCM 9890T (b), *Leu. lactis* JCM 6123T (c), *En. faecalis* JCM 5803T (d), *Leu. mesenteroides* subsp. *dextranicum* NBRC 100495^{T} (e), *W. cibaria* JCM 12495^{T} (f), and *Leu. mesenteroides* subsp. *cremoris* NBRC 107766^{T} (g).

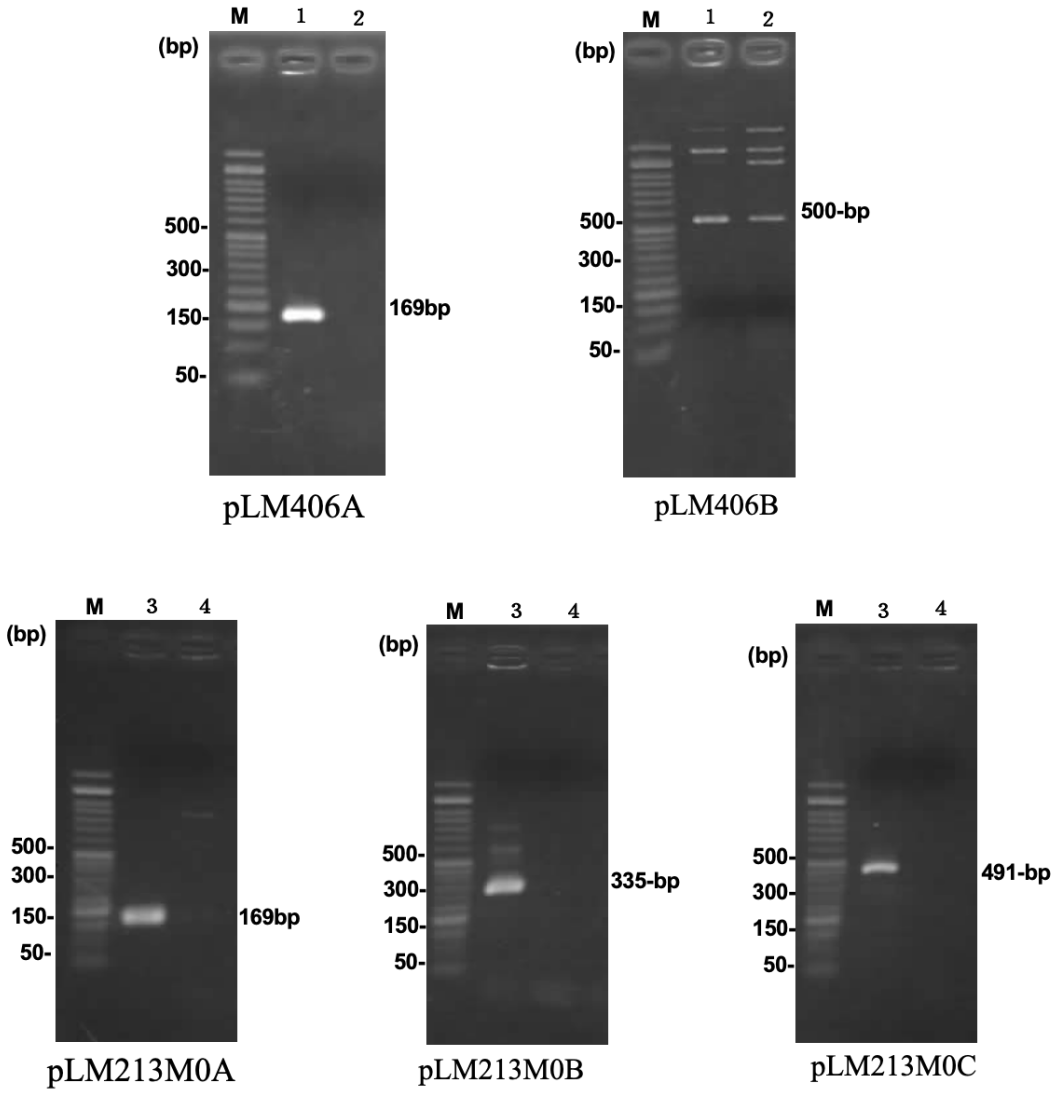

Fig. 6 Profiles of plasmids pLM406A and pLM406B in *Leu. mesenteroides* 406 (lane 1) and the derivative strain (lane 2), and pLM213M0A , pLM213M0B and pLM213M0C in *Leu. mesenteroides 213M0* (lane 3) and the derivative strain (lane 4) using each plasmid-specific primer.

Lane M: FastGene 50-bp DNA Ladder (Nippon Genetics Co.).

4. Discussion

LAB play an important role in the food industry because due to their excellent properties such as lactose fermentation, bacteriocin, and exopolysaccharide production. Among LAB, *Leuconostoc* present in many environments had been isolated a variety of different strains from dairy, plants, and meat (Ahn *et al*., 2023; Lahiri *et al*., 2020; Wang *et al*., 2018). *Leuconostoc* has been considered to be generally recognized as safe (GRAS) because of its long history of safe consumption in traditional fermented foods (Hemme and Foucaud-Scheunemann, 2004), despite the fact that some species of this genus cause food deterioration and have been linked to disease in immunocompromised patients (Menegueti *et al*., 2018; Raimondi *et al*., 2019; Samelis *et al*., 2006). *Leuconostoc* has some beneficial characteristics, their capacity to produce organic acids, carbon dioxide, dextrans and, especially, aromatic compounds, such as diacetyl, acetaldehyde and acetoin improve the flavor of fermented food and contribute to the organoleptic properties of the final product (Cogan and Jordan, 1994). In addition, several numbers of *Leuconostoc* have been reported to produce antimicrobial compounds including bacteriocins and its potential as a bioactive preservative remains extensively studied (Hwang *et al*., 2018; Pujato *et al*., 2014; Veskovi *et al*., 2013). Many of these essential properties for industry are not only encoded by chromosomal genes, but are also present on plasmids (Cotter *et al*., 2003). Plasmids are widely present in *lactobacilli,* carrying various functional genes that confer them advantageous traits and allow them to survive in a competitive environment (Davray *et al*., 2021). The ability to metabolize different carbon and nitrogen sources, as well as tolerance to heavy metals, disinfectants, antibiotics, and other environmental toxins, are additional advantages of plasmid-encoded genes. However plasmid metabolism and replication need energy consumption (Flórez *et al*., 2021). Plasmids are extrachromosomal DNA molecules that can be lost and gained and exchanged within a population. During the evolutionary process over the years, LAB have developed plasmids to adapt to the environment and have also lost some plasmids to adapt to the

new environment. For example, *L. plantarum* P-8 was isolated from fermented milk, and when the strain was transplanted to other environments (rats and humans), it lost important plasmids that allowed it to adapt to dairy products (Davray *et al*., 2021). Closing the sequence gaps between contigs by PCR amplification and subsequent sequencing of the PCR products confirmed that two contigs of *Leu. mesenteroides* 406 and three contigs of 213M0 were indeed circular and were plasmids. It is not uncommon for bacteria to have multiple plasmids. *En. mundtii* QU 25 has five plasmids including pQY182, pQY082, pQY039, pQY024 and pQY003, and pQY024 contains a gene for producing a bacteriocin, mundticin (Shiwa *et al*., 2014). *L. plantarum* WCFS1 contains three plasmids, among which pWCFS103 was demonstrated to be a conjugative plasmid and contains several genes for heavy-metal resistance, NADH oxidase activity (Van Kranenburg *et al*., 2005). *L. lactis* FM03-V1 contains 11 plasmids carrying genes encoding various functions such as lactose and citrate utilization, oligopeptide uptake, ion transport, phage resistance, heavy metal transport, stress resistance, and polysaccharide production (Van Mastrigt *et al*., 2018). Bacteriocin-associated *mes* genes are carried by strains 406 (pLM406A) and 213M0 (pLM213M0A and pLM213M0B). The association between plasmids and bacteriocin production is common in LAB. For example, pediocin PA-1 are coded by plasmid pSRQ11 of *P. acidilactici* PAC 1.0 and a bacteriocin-encoding gene located on a large 10-kb plasmid of *L. curvatus* CWB1-B28 (Malik *et al*., 2016). Similar results, *mes* genes are often encoded by plasmids. Mesentericin Y105 was originally purified from the CFS of *Leu. mesentemides* Y105 and determined to be encoded by pHY30 (Fremaux *et al*., 1995; Hechard *et al*., 1992). Subsequently, it was found that the mesenterocin 52A which has an amino acid sequence identity to mesenterocin Y105 and another bacteriocin, mesenterocin 52B, encoded within a plasmid from *Leu. mesenteroides* FR52 (Revol-Junelles *et al*., 1996). Later, *Leu. mesentemides* Y105 was confirmed to have a gene encoding the structural of mesenterocin B105 present in pH30, and this bacteriocin has the same amino acid sequence as mesenterocin 52B (Biet *et al*., 1998).

Therefore, we attempted to compare the mesenterocin-encoding genes of pHY30 and pFR38 with the similar gene sequences of pLM406A, pLM213M0A, and pLM213M0B , and the results showed that they were similar to each other. It is indicating that the antibacterial ability of *Leu. mesenteroides* 406 and 213M0 is generated by the synthesis of mesenterocin Y105 and B105. Although some cases were given different names, mesentericins Y105 and B105 have been reported to be produced by many strains of *Leuconostoc* spp. such as *Leu. mesenteroides* SJRP55 isolated from Brazilian water buffalo mozzarella (De Paula *et al*., 2014), *Leu. mesenteroides* subsp. *cremoris* W3 isolated from wine (Dündar *et al*., 2016) and *Leu. mesenteroides* E131 isolated from Greek traditional fermented sausage (Xiraphi *et al*., 2008). This means that it is quite common for *Leuconostoc* spp. to possess the genes for production of mesentericins Y105 and B105, and that the genes are well conserved and spread in various environments over a long evolutionary process (Inglis *et al*., 2013). Additionally, mesentericin Y105 as a Class IIa bacteriocin are commonly found to be encoded by genes on plasmids, but also been found on chromosomes like *Leu. mesenteroides* subsp. *mesenteroides* OZ (Osmanagaoglu and Kiran, 2011). *Leu. mesenteroides* 406 and 213M0 were both isolated from Mongolian traditional fermented milk, their *mes*-encoding gene is located on plasmid, further confirmed by the plasmid curing test. *Leu. mesenteroides* is one of the resident strains in Mongolian traditional dairy products (Yu *et al*., 2011), could be simply explained as plasmid can transfer mesentericins genetic elements between *Leu. mesenteroides* in Mongolian region to preserve these genes and able to inhibit other competing species. The permease involved in citrate uptake is also plasmid encoded in strain 406 which gives the strain the ability to utilize citrate. This is one of the important characteristics of the strain to grow and survive in the milk environment. This gene is highly related to the formation of flavor during fermentation (Van Mastrigt *et al*., 2018). Strain 213M0 contains other two plasmids (pLM213M0B and pLM213M0C), pLM213M0B harbored the mesentericin secretion genes, *mesDE* and pLM213M0C with an unknown function. It's possible

that multiple plasmids within the same strain work in cooperation, but it's still unclear whether this influencing the strain's overall antibacterial activity or not.

5. Conclusion

Leu. mesenteroides 406 and 213M0 had two and three plasmids, respectively. Each one (pLM406A and pLM213M0A) of the plasmids in strains 406 and 213M0 harbored the similar mes gene cluster for mesentericin Y105-B105 production and immunity. Plasmid pLM213M0B in strain 213M0 harbored the mesentericin secretion genes, *mesDE*. Differences in the bacteriocin genes between the two strains were shortened *mesG* and duplicated *mesDE* in strain 213M0. Plasmid curing revealed that the antibacterial activity of strains 406 and 213M0 was due to plasmid-encoded bacteriocins.

IV Purification and analysis of bacteriocins produced by *Leu. mesenteroides* 406 and 213M0

Abstract

Bacteriocins produced by *Leu. mesenteroides* 406 and 213M0 were separated using ammonium sulfate precipitation, C18 solid-phase extraction, and reversed-phase HPLC. The N-terminal amino acid sequence and molecular weight of bacteriocins were determined using a protein sequencer and MALDI-TOF/TOF-MS/MS. Mesentericins Y105 and B105 were purified from both strains, while mesenterin M (MesM) was unique to 213M0-CFS. Gene cluster of mesenterin M, *mesKJLMNE$_2$D$_2$*, was coded on the plasmid of strain 213M0 (pLM213M0B), and its amino acid sequence was HWIGDVLGAIGHVYHPADPQKVLDQLNGKTQPKPGHQYSPY, which is different from any known bacteriocins. Interestingly, the three amino acid residues at the C-terminus were cleaved. This is the first report of a bacteriocin with cleaved C-terminal amino acids. In conclusion, the novel bacteriocin should be responsible for the differences in antibacterial properties between the two strains.

1. Introduction

From the results of the above two experiments, we thought that the different antibacterial properties of the *Leu*. *mesenteroides* 406 and 213M0 were owing to their antibacterial substances themselves. However, they contain similar mesentericin Y105-B105 gene cluster. Meanwhile, we thought that the differences in the difference in the antibacterial activity and production of bacteriocins between strains 406 and 213M0 might be due to shortened *mesG* and/or dublicated *mesDE* genes. In order to clarify these speculations, the bacteriocins produced by *Leu. mesenteroides* 406 and 213M0 were needed to purify and identify. Bacteriocins purification and identification are

complex and time-consuming, particularly in the presence of unknown antagonistic peptides. The typical procedure begins with a stage in which the bacteriocin is concentrated from the culture supernatant using methods like ammonium sulfate precipitation (Bauer *et al*., 2005), organic extraction (Gao *et al*., 2016), pH-dependent adsorption of the bacteriocin onto the producer cells (Yang *et al*., 1992) and diatomaceous earth calcium silicate (Micro-Cel) *(Dündar et al*., 2014). However, bacteriocin extraction in one step is insufficient to eliminate contaminants, such as protein and salt. Purification of the crude extraction is required for later studies. According to recent purification studies, cation exchange chromatography, gel filtration chromatography, and reversed-phase high-performance liquid chromatography are the primary methods that can be used alone or in combination to improve bacteriocin purity and ensure structural and functional analysis (Gupta *et al*., 2016; López *et a*l., 2007; Meena *et al*., 2016). The BLIS produced by *Leu. mesenteroides* 406 and 213M0 were initially thought to be Class II bacteriocins and were anticipated to be manufactured by the gene cluster *mes IYCDEFHBG* encoded by their plasmids pLM406A and pLM213M0A, respectively. *Leu. mesenteroides* Y105 produces 37-amino-acid and 32-amino-acid peptides, respectively, known as mesentericins Y105 and B105 (Biet *et al*., 1998). In 1992, He´chard et al. purified mesentericin Y105 by blue agarose affinity chromatography, ultrafiltration, and RP-HPLC (Hechard *et al*., 1992). Later, the CFS of *Leu. mesenteroides* Y105 was separated using an effective approach that involved ammonium sulfate precipitation, solid phase extraction, and RP-HPLC (Biet *et al*., 1998). A three-step process was subsequently developed, beginning with cation exchange chromatography to remove the majority of the colored contaminants, followed by applying the active fraction to a C18 column and finally purifying by RP-HPLC (Guyonnet *et al*., 2000).

2. Materials and methods

A. SDS-PAGE and in situ activity assay

The preparation of *Leu. mesenteroides* 406 and 213M0 CFS was the same as that for the antimicrobial assays described in Part II. The CFS was precipitated with 80% ammonium sulfate, and the crude extract produced by centrifugation was diluted in water to get the samples, which were then analyzed using SDS-PAGE on 4.0% (w/v) spacer gel and 16.5% (w/v) separation gel, as previously described (Arakawa *et al*., 2016). After electrophoresis (100V/67min), half of the gel containing the sample and molecular weight markers (IO-RAD SDS-PAGE Polypeptide 161-0326) was stained with Coomassie brilliant blue R-250 (EzStain Aqua; Atto Corporation, Tokyo, Japan). The other half of the gel was used for the in-situ activity assay, fixed by the solution containing 20% (v/v) methanol and 10% (v/v) acetic acid, and washed with water. 40 ml of MRS agar inoculated with 100 μl of a culture solution of Lis. monocytogenes VTU 206 was overlaid on the gel in a Petri dish, incubated at 30 °C for 24 h, and examined for a zone of inhibition.

B. Bacteriocin purification

Bacteriocin was purified using the method of Biet F et al. (1998), with some modifications (Biet *et al*., 1998). First, each CFS (100 mL) prepared by centrifugation (23,800× *g*, 20 min, 4 °C) of culture solutions of strains 406 and 213M0 was heated at 70 °C for 30 min. Next, ammonium sulfate (35%, *w*/*v*) was added to the heated CFS, and the mixture was stirred at 4 °C for 18 h to precipitate the bacteriocins. The precipitate was collected by centrifugation (5900× *g*, 1 h, 4 °C) and redissolved in 10 mL of distilled water. The solution was then applied to a reverse-phase Sep-Pak C18 cartridge (Waters Corporation; Milford, MA, USA). Elution was performed with 5 mL of 0, 15, 30, and 40% (*v*/*v*) acetonitrile in 20 mM ammonium acetate. All eluates were concentrated to 200 μL using a centrifugal evaporator (model EC-57C, Sakuma;

Tokyo, Japan). The concentrated eluate with antibacterial activity was further subjected to reverse-phase HPLC (Shimadzu Corporation, Kyoto, Japan) with a Wakosil-II 5C8 RS HPLC column (4.6 mm × 250 mm; FUJIFILM Wako Pure Chemical Corporation) equilibrated with solvent A (10% acetonitrile and 0.1% trifluoroacetic acid, *v*/*v*) at 50 °C. Elution was performed at a flow rate of 1 mL/min with a linear gradient from 0 to 100% of solvent B (90% acetonitrile and 0.1% trifluoroacetic acid; *v*/*v*) for 45 min. The eluate was monitored at a wavelength of 220 nm, and the eluate peaks shown were collected. After that, the collected fractions were concentrated and assayed for antibacterial activity against three indicator strains: *Lis. monocytogenes* VTU 206, *W. paramesenteroides* JCM 9890^{T}, *Leu. lactis* JCM 6123^{T}. Peptide content of the collected samples at each purification step was determined using Pierce Micro BCA Protein Assay Kit (Thermo Fisher Scientific Inc.; Waltham, MA, USA).

C. N-terminal Amino Acid Sequencing

The bacteriocins purified by HPLC were denatured at 130°C for 30 min and dissolved in 40% (v/v) acetonitrile. The solution was loaded several times onto a PVDF membrane hydrated with methanol and distilled water, and then completely dried at 55°C. After that, the membrane was applied to N-terminal amino acid sequencing using a PPSQ-31A pepetide sequencer (Shimadzu Corporation) at Department of Instrumental Analysis in Okayama University, Japan.

D. Mass Analysis

Dried samples after purification by HPLC were dissolved in 10 μL of 70% acetonitrile (v/v) containing 0.1% (v/v) formic acid and mixed with saturated solution of α-Cyano-4-hydroxycinnamic acid used as a matrix at a volume ratio of 1:5. The mixture was applied to MALDI-TOF MS or MS/MS analysis using UltrafleXtreme (Bruker, Billerica, MA) at Department of Genomics and Proteomics in Okayama University,

Japan. The data obtained from MS/MS were further analyzed to determine the peptide sequence using the Mascot server.

3. Results

A. comparison of the molecular weight of BLIS

An in situ antibacterial activity assay was used following SDS-PAGE to determine the molecular weight of BLIS of strains 406 and 213M0. Based on the position of the inhibitory zone, the crude bacteriocin molecular weights of strains 406 and 213M0 are substantially identical; they were calculated to be approximately 3.5 kDa, as shown in (**Fig. 7**).

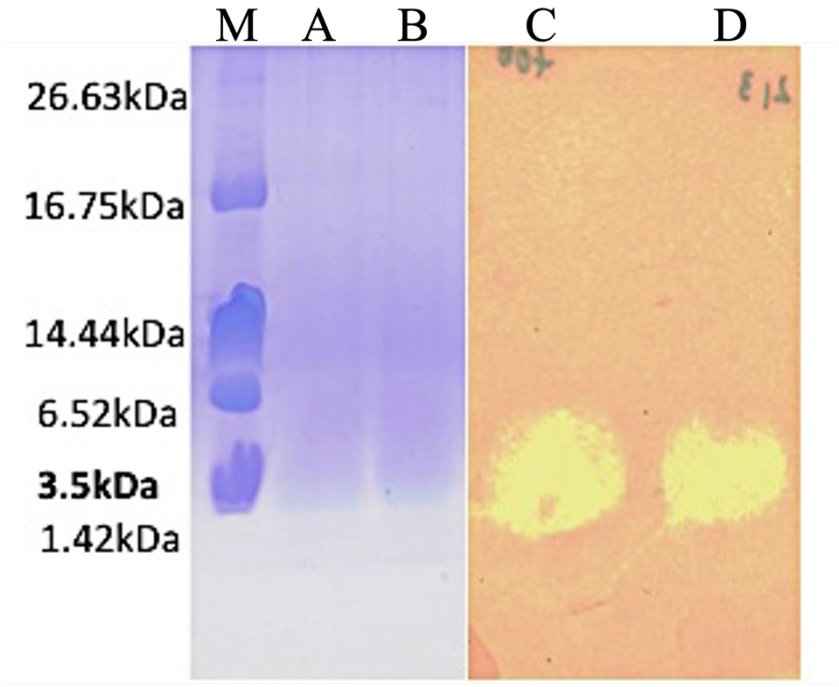

Fig. 7 SDS-PAGE of crude bacteriocins produced by *Leu. mesenteroides* 406 and 213M0

M, molecular weight markers; A, crude bacteriocins produced by strain 406; B, crude bacteriocins produced by strain 213M0; C, inhibition zone of crude bacteriocins produced was strain 406 after in-situ activity assay; D, inhibition zone of crude bacteriocins produced was strain 213M0 after in-situ activity assay

B. Purification of Bacteriocins

To identify the appropriate elution gradient in the solid phase extraction step, the samples were eluted with varying eluent concentrations (0, 10, 20, 30, 40, 60, 80, and finally 100% (v/v) acetonitrile solutions), and their antibacterial activity was evaluated using the agar well diffusion method. *Lis. monocytogenes VTU* 206 was used as an indicator strain, with acetonitrile solutions serving as a control. The active fractions of both strains eluted with 20% to 80% acetonitrile showed antibacterial activity, and the acetonitrile solution did not inhibit the growth of indicator strains (**Fig. 8**). The 40% acetonitrile elations exhibited the strongest antibacterial activity, as shown by the diameter of the inhibition zone. The antibacterial ability of the active fractions eluted with 60% and 30% acetonitrile was also readily apparent. Thus, RP-HPLC was used to further purify the acetonitrile 30%, 40%, and 60% acetonitrile elations. The purification results show the samples eluted with 40% acetonitrile are the best, while the components of the fraction eluted with 30% acetonitrile are complicated and cannot be entirely separated by RP-HPLC shown in **Fig. 9**. Based on the above results, it was determined that 0, 15, 30, and 40% acetonitrile solutions were used to elute the sample during the solid phase extraction step, and the active fraction eluted with 40% acetonitrile was purified by RP-HPLC. The purification status at each step is shown in **Table.8.**

In the final step using HPLC, three and four active peak fractions were collected from sample solutions of strains 406 and 213M0, respectively, while no active peaks were detected in samples of their derivative strains and MRS broth (control) (**Fig.10a**). For strain 406, the active fraction around peak-II was not collected because the peak was

only a trace. Fig.10B and Table8 show the antibacterial activity of the collected fractions against three indicator strains.

The peak-I fractions of both strains had higher activity than the other fractions against *Lis. monocytogenes* VTU 206 and *Leu. lactis* JCM 6123^T, but no activity against *W. paramesenteroides* JCM 9890^T. On the other hand, the peak IV fractions had very high activity against *W. paramesenteroides* JCM 9890^T, but no activity against *Leu. lactis* JCM 6123^T. The peak-III fractions also showed a similar antibacterial spectrum to the peak-IV fractions, but the activity of the peak-III was much lower than that of the peak-IV. The activity of the peak-III and -IV fractions of strain 406 tended to be higher than that of the corresponding peak fractions of 213M0. This indicated that the production of the peak-III and -IV bacteriocins by strain 406 was high compared with that of 213M0.

Peak-II was detected only in the 213M0 sample (**Fig. 10a**). This fraction had antibacterial activity against *Lis. monocytogenes* VTU 206 and *Leu. lactis* JCM 6123^T, but no activity against *W. paramesenteroides* JCM 9890^T (**Fig. 10b**). This spectrum was similar to that of the peak-I fraction. The peak-II fractions had less than one-fifth the activity and one-eighth the specific activity of the peak-I fraction against *Lis. monocytogenes* VTU 206, but more than half the activity and the specific activity of the peak-I fraction against *Leu. lactis* JCM 6123^T (**Table 8**). This result suggested that the peak-II bacteriocin would be different from the peak-I as well as the peaks-III and IV bacteriocins, and that only strain 213M0 would produce another bacteriocin that strain 406 did not produce.

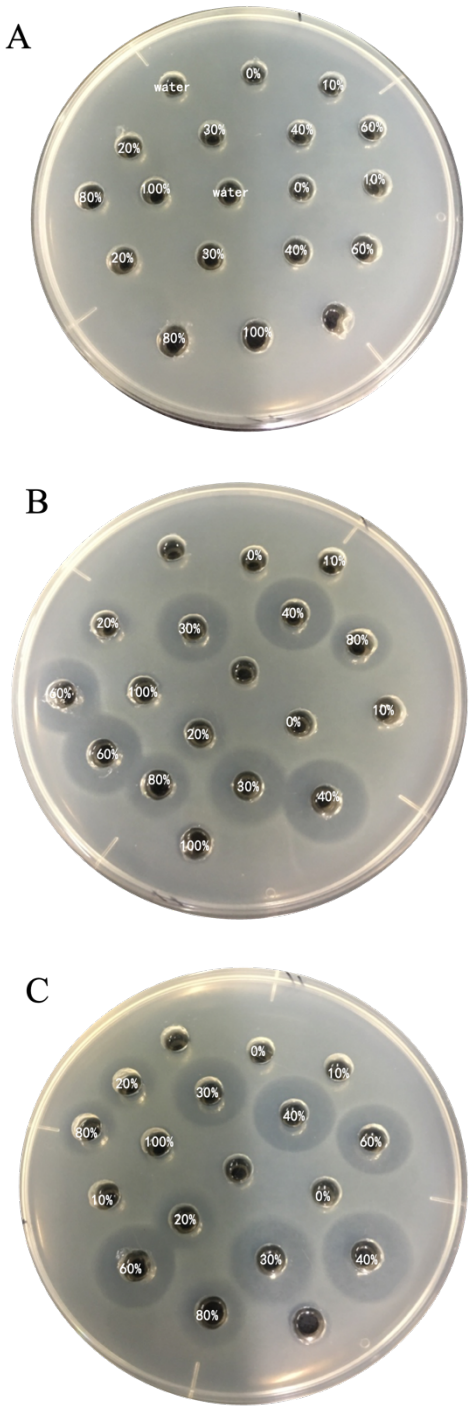

Fig. 8 The antibacterial activity of fractions eluted with varying acetonitrile concentrations.

A. Different concentrations of acetonitrile solutions were employed as controls. B. Strain 406-BLIS fractions C. Strain 213M0-BLIS fractions

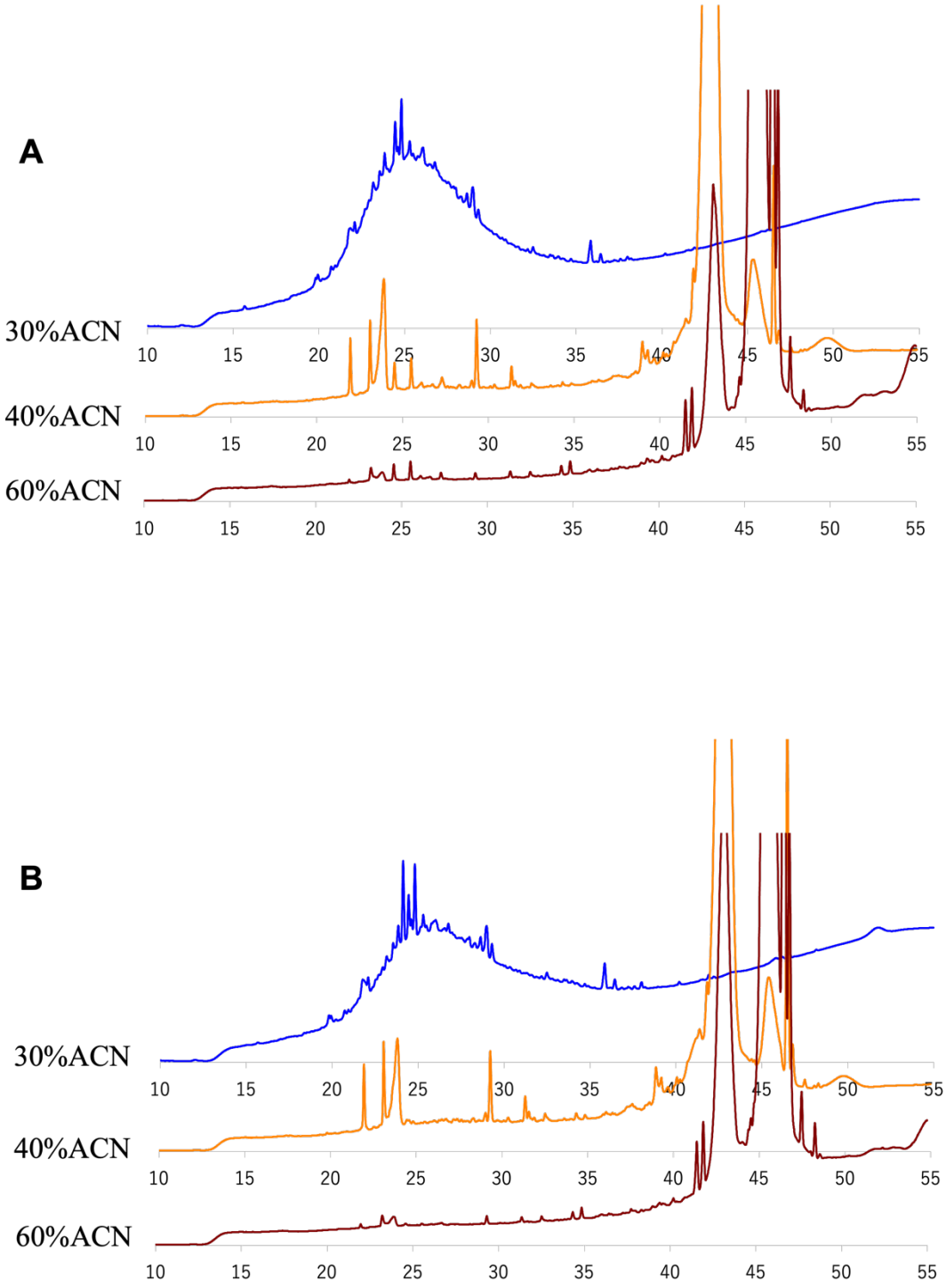

Fig. 9 RP-HPLC purification chromatogram for active fractions eluted with 30%, 40%, and 60% acetonitrile solutions

A. Active fractions of strain 406 B. Active fractions of strain 213M0

Table 8 Purification status of bacteriocins produced by *Leu. mesenteroides* 406 and 213M0

Activity against *Lis.monocytogenes* VTU206

Sample	Purification steps	Volume (mL)	Total protein (mg)	Activity (AU/mL)	Total activity (AU)	Activity recovered (%)	Specific activity (AU/mg)	Purification (-fold)
406	supernatant	100	1,116.58	2,880.00	288,000.00	100.00	257.93	1.00
	precipitation in $(NH_4)_2SO_4$	10	19.72	17,066.67	170,666.67	59.26	8,653.08	33.55
	sep-pak C18 (40%ACN)	5	0.1942	13,084.44	65,422.22	22.72	336,856.74	1,306.00
	C8 RP-HPLC (peak I)	1	0.0026	1,813.33	1,813.33	0.63	685,479.12	2,657.61
	C8 RP-HPLC (peak III)	1	0.0028	113.33	113.33	0.04	40,910.16	158.61
	C8 RP-HPLC (peak IV)	1	0.0045	388.57	388.57	0.13	86,988.84	337.26
213M0	supernatant	100	1,224.10	2,400.00	240,000.00	100.00	196.06	1.00
	precipitation in $(NH_4)_2SO_4$	10	21.28	16,497.78	164,977.78	68.74	7,752.58	39.54
	sep-pak C18 (40%ACN)	5	0.1955	11,946.67	59,733.33	24.89	305,536.11	1,558.36
	C8 RP-HPLC (peak I)	1	0.0033	1,554.29	1,554.29	0.65	474,785.85	2,421.61
	C8 RP-HPLC (peak II)	1	0.0050	270.00	270.00	0.11	54,455.45	277.75
	C8 RP-HPLC (peak III)	1	0.0021	120.00	120.00	0.05	57,191.08	291.70
	C8 RP-HPLC (peak IV)	1	0.0021	220.00	220.00	0.09	103,704.20	528.94

Activity against *W.paramesenteroides* JCM9890^T

Sample	Purification steps	Volume (mL)	Total protein (mg)	Activity (AU/mL)	Total activity (AU)	Activity recovered (%)	Specific activity (AU/mg)	Purification (-fold)
406	supernatant	100	1,116.58	1,780.00	178,000.00	100.00	159.42	1.00
	precipitation in $(NH_4)_2SO_4$	10	19.72	13,653.33	136,533.33	76.70	6,922.47	43.42
	sep-pak C18 (40%ACN)	5	0.1942	2,880.00	14,400.00	8.09	74,145.10	465.11
	C8 RP-HPLC (peak I)	1	0.0026	-	-	-	-	-
	C8 RP-HPLC (peak II)	1	0.0028	262.22	262.22	0.15	94,654.87	593.76
	C8 RP-HPLC (peak IV)	1	0.0045	710.00	710.00	0.40	158,946.53	997.06
213M0	supernatant	100	1,224.10	530.00	53,000.00	100.00	43.30	1.00
	precipitation in $(NH_4)_2SO_4$	10	21.28	4,551.11	45,511.11	85.87	2,138.64	49.39
	sep-pak C18 (40%ACN)	5	0.1955	920.00	4,600.00	8.68	23,529.01	543.43
	C8 RP-HPLC (peak I)	1	0.0033	-	-	-	-	-
	C8 RP-HPLC (peak II)	1	0.0050	-	-	-	-	-
	C8 RP-HPLC (peakIII)	1	0.0021	115.56	115.56	0.22	55,072.89	1,271.98
	C8 RP-HPLC (peak IV)	1	0.0021	188.89	188.89	0.36	89,038.96	2,056.47

Activity against *Leu. lactis* 6123^T

Sample	Purification steps	Volume (mL)	Total protein (mg)	Activity (AU/mL)	Total activity (AU)	Activity recovered (%)	Specific activity (AU/mg)	Purification (-fold)
406	supernatant	100	1,116.58	12.50	1,250.00	100.00	1.12	1.00
	precipitation in $(NH_4)_2SO_4$	10	19.72	120.00	1,200.00	96.00	60.84	54.35
	sep-pak C18 (40%ACN)	5	0.1942	186.67	933.33	74.67	4,805.70	4,292.76
	C8 RP-HPLC (peak I)	1	0.0026	33.33	33.33	2.67	12,600.72	11,255.77
	C8 RP-HPLC (peak III)	1	0.0028	-	-	-	-	-
	C8 RP-HPLC (peakIV)	1	0.0045	-	-	-	-	-
213M0	supernatant	100	1,224.10	68.57	6,857.14	100.00	5.60	1.00
	precipitation in $(NH_4)_2SO_4$	10	21.28	400.00	4,000.00	58.33	187.97	33.55
	sep-pak C18 (40%ACN)	5	0.1955	266.67	1,333.33	19.44	6,820.00	1,217.47
	C8 RP-HPLC (peak I)	1	0.0033	53.33	53.33	0.78	16,291.67	2,908.31
	C8 RP-HPLC (peak II)	1	0.0050	45.00	45.00	0.66	9,075.91	1,620.19
	C8 RP-HPLC (peak III)	1	0.0021	33.33	33.33	0.49	15,886.41	2,835.96
	C8 RP-HPLC (peak IV)	1	0.0021	-	-	-	-	-

"- " : No detection

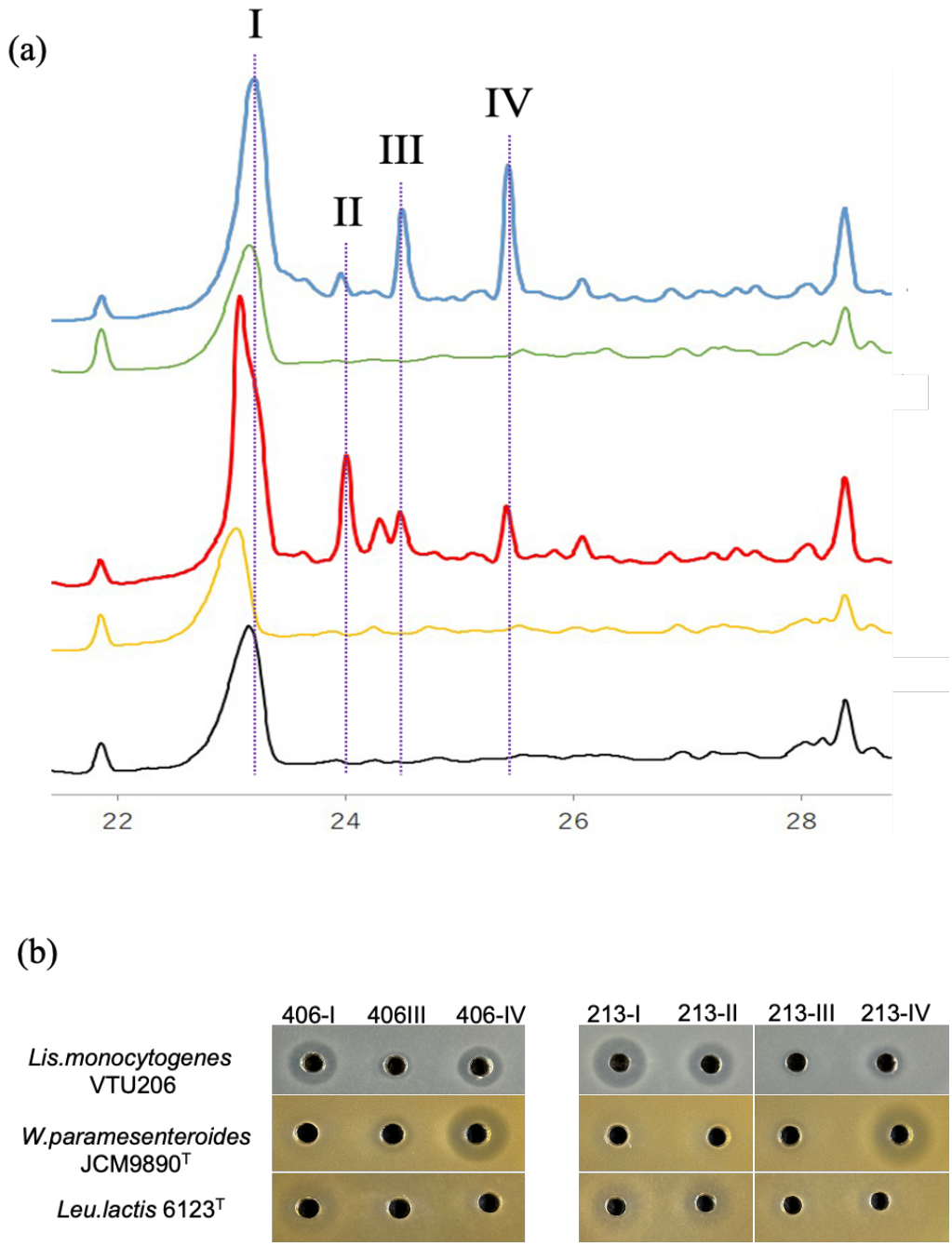

Fig. 10 Elution profile and antibacterial activity of active peptides produced by *Leu. mesenteroides* 406 and 213M0

(a) HPLC chromatogram to purify bacteriocins produced by *Leuconostoc mesenteroides* 406 (blue) and 213M0 (red). The 406- and 213M0-derivative strains (green and yellow, respectively) without antibacterial activity, and MRS broth (black) were used as controls. The purified fractions around peaks-I, -III and -IV in strain 406, and

peaks-I, -II, -III and -IV in strain 213M0 were collected. (b) Antibacterial activity of the three and four peak fractions (406-I, -III and IV, and 213M0-I, -II, -III, -IV) purified by HPLC, against *Listeria monocytogenes* VTU 206, *Weissella paramesenteroides* JCM 9890^T, and *Leuconostoc lactis* JCM 6123^T.

C. Identification of Purified Bacteriocins

The purified bacteriocins in the three and four fractions from 406 and 213M0, respectively, were identified by N-terminal amino acid sequencing and MALDI-TOF MS analysis (**Table 9** and **Fig.14**). From the resulting N-terminal sequences and masses, the 406-I and 213M0-I bacteriocins were identified as mesentericin Y105 (Hechard *et al*., 1992). Although the measured masses ($[M+H]^+$ = 3869) were two less than the expected mass ($[M+H]^+$ = 3871) from the amino acid sequences, the value corresponded to that of mesentericin Y105 with a disulfide bridge as reported previously (Campanero *et al*., 2020). The 406-IV and 213M0-IV bacteriocins were a perfect match to mesentericin B105 (Biet *et al*., 1998) in both the resulting N-terminal sequences and masses. The N-terminal sequence of the 406-III bacteriocin were the same as that of mesentericin B105, but its molecular mass ($[M+H]^+$=3463) corresponded to that of mesentericin B105 ($[M + H]^+$ = 3447) plus 16. This indicated that the 406-III bacteriocin was the oxidized form of mesentericin B105 at one methionine residue (Revol-Junelles *et al*., 1996), as has been well studied, especially with pediocin PA-1produced by *Pediocccus acidilactici* strains (Fimland *et al*., 1996 ; Johnsen L *et al*., 2000 ; Kuniyoshi *et al*., 2022). The N-terminal amino acids of the 213M0-III bacteriocin could not be unambiguously sequenced because of contamination with (an) other peptide/protein(s). However, since its mass was measured to be the same as that of the 406-III bacteriocin, it was also considered to be oxidized mesentericin B105.

The N-terminal sequence of the 213M0-II bacteriocin was HWIGDVLGAIGHV-. This was identical to a part of the translated sequence (66-aa, accession no. WP_061399677 and BFP62698) of a gene on pLM213M0B. In the translated sequence, upstream of the resulting sequence was a putative leader sequence (22-aa) with a double glycine motif at the C-terminus.

The mass of the bacteriocin was predicted to be $[M+H]^+ = 4947.5$ based on the translated sequence excluding the leader peptide but was actually $[M+H]^+ = 4964$ in the MALDI-TOF MS analysis. In order to investigate the cause of this discrepancy, MS/MS analysis followed by Mascot search was next performed for the full-length sequence of the bacteriocin (**Fig. 15 and Table 10**). As a result, a 41-aa peptide sequence was obtained (**Fig. 16**). Surprisingly, the resulting sequence had three C-terminal residues truncated from the translated sequence. To our knowledge, this is the first report of a C-terminal truncated bacteriocin. In addition, the full length of the sequence did not have high homology to any other bacteriocin sequence and was only partially close to some putative bacteriocins represented by infantaricin H (**Fig. 17**) (Campanero et.al., 2020) . These results indicate that mesentericin M is a novel type of bacteriocin with no relatives.

Table 9 N-terminal sequences and molecular masses of active peptides purified stepwise from the cultures of *Leu. mesenteroides* 406 and 213M0

Producer	Fraction	N-terminal sequence	MS $[M+H]^+$	Identification
Leuconostoc Mesenteroides 406	406-I	KYYGNGVH(X)TKSG-	3869	Mesentericin Y105
	406-III	KGVLGWLSMASSA-	3463	Oxicized mesentericin B105
	406-IV	KGVLGWL-	3447	Mesentericin B105
Leuconostoc Mesenteroides 213M0	213M0-I	KYYGNGVH(X)TKSG-	3869	Mesentericin Y105
	213M0-II	HWIGDVLGAIGHV-	4564	Mesentericin M[1]
	213M0-III	-	3463	Oxicized mesentericin B105
	213M0-IV	KGVLGWL-	3447	Mesentericin B105

[1] Novel bacteriocin identified in this study.

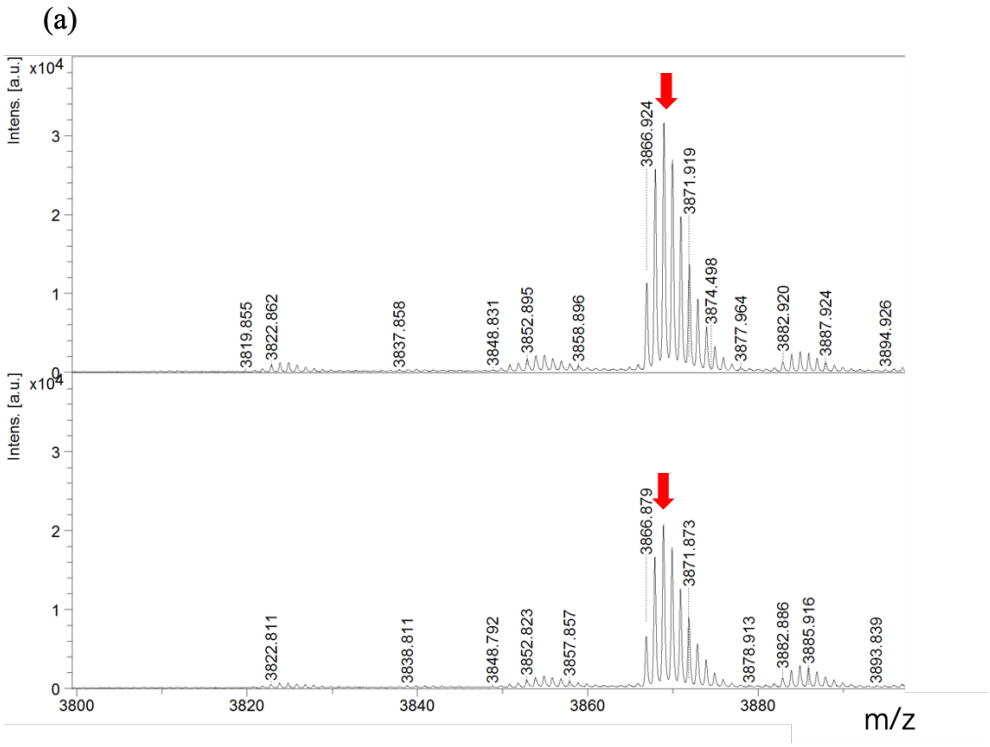

Fig. 11 MALDI-TOF-MS spectra of purified bacteriocins produced by *Leu. mesenteroides* 406 and 213M0

Samples: peak-I of 406 (a), peak-I of 213M0 (b), peak-III of 406 (c1, c2), peak-III of 213M0 (d), peak-IV of 406 (e), peak-IV of 213M0 (f), peak-II of 213M0 (g), and blank (h). Red arrows indicate the highest peak in each analysis.

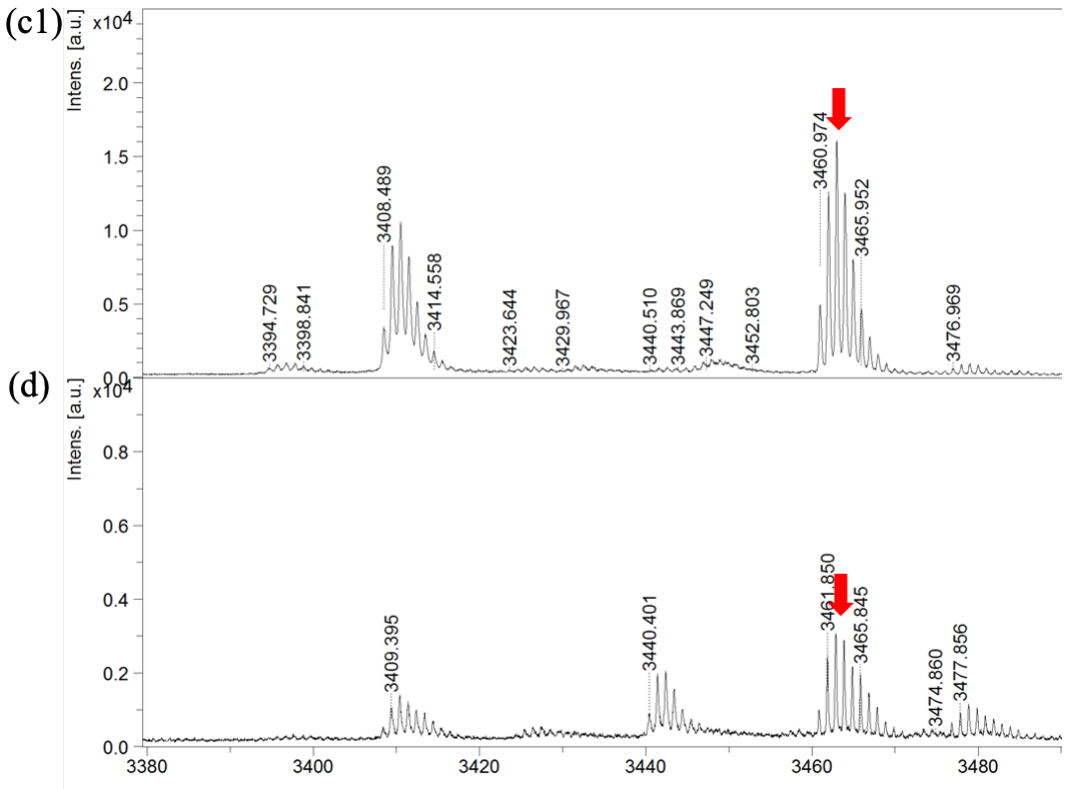

Fig. 11 MALDI-TOF-MS spectra of purified bacteriocins produced by *Leu. mesenteroides* 406 and 213M0

Samples: peak-I of 406 (a), peak-I of 213M0 (b), peak-III of 406 (c1, c2), peak-III of 213M0 (d), peak-IV of 406 (e), peak-IV of 213M0 (f), peak-II of 213M0 (g), and blank (h). Red arrows indicate the highest peak in each analysis.

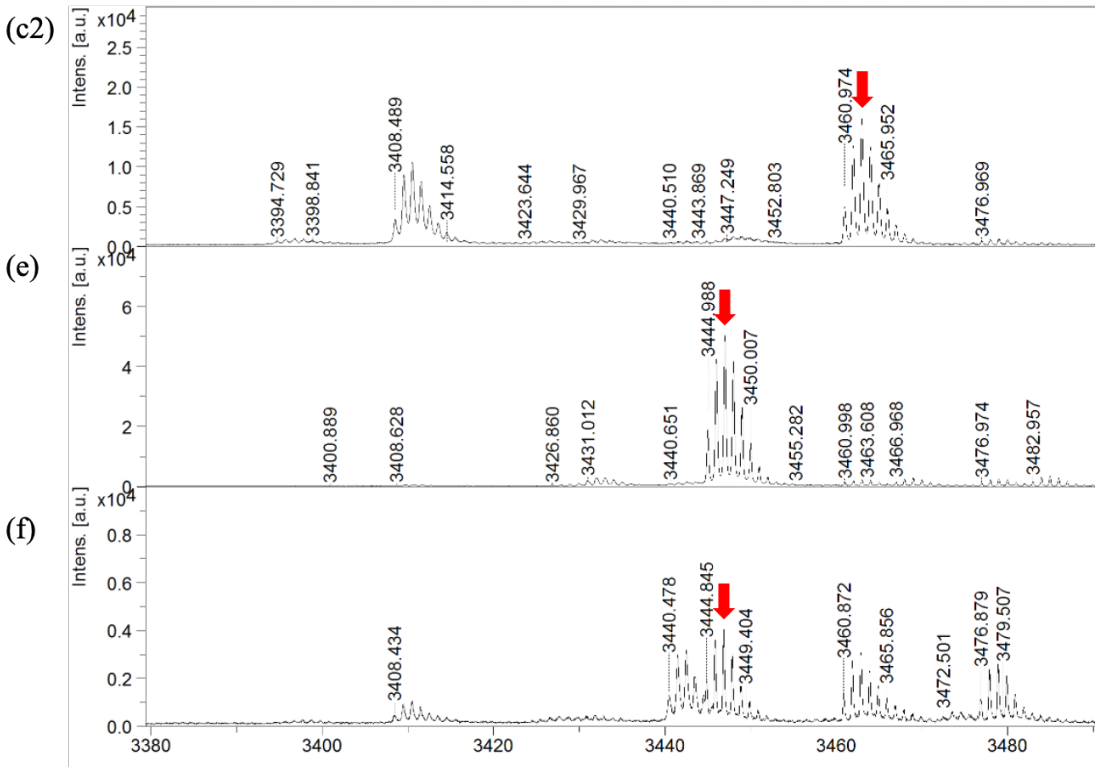

Fig. 11 MALDI-TOF-MS spectra of purified bacteriocins produced by *Leu. mesenteroides* 406 and 213M0

Samples: peak-I of 406 (a), peak-I of 213M0 (b), peak-III of 406 (c1, c2), peak-III of 213M0 (d), peak-IV of 406 (e), peak-IV of 213M0 (f), peak-II of 213M0 (g), and blank (h). Red arrows indicate the highest peak in each analysis.

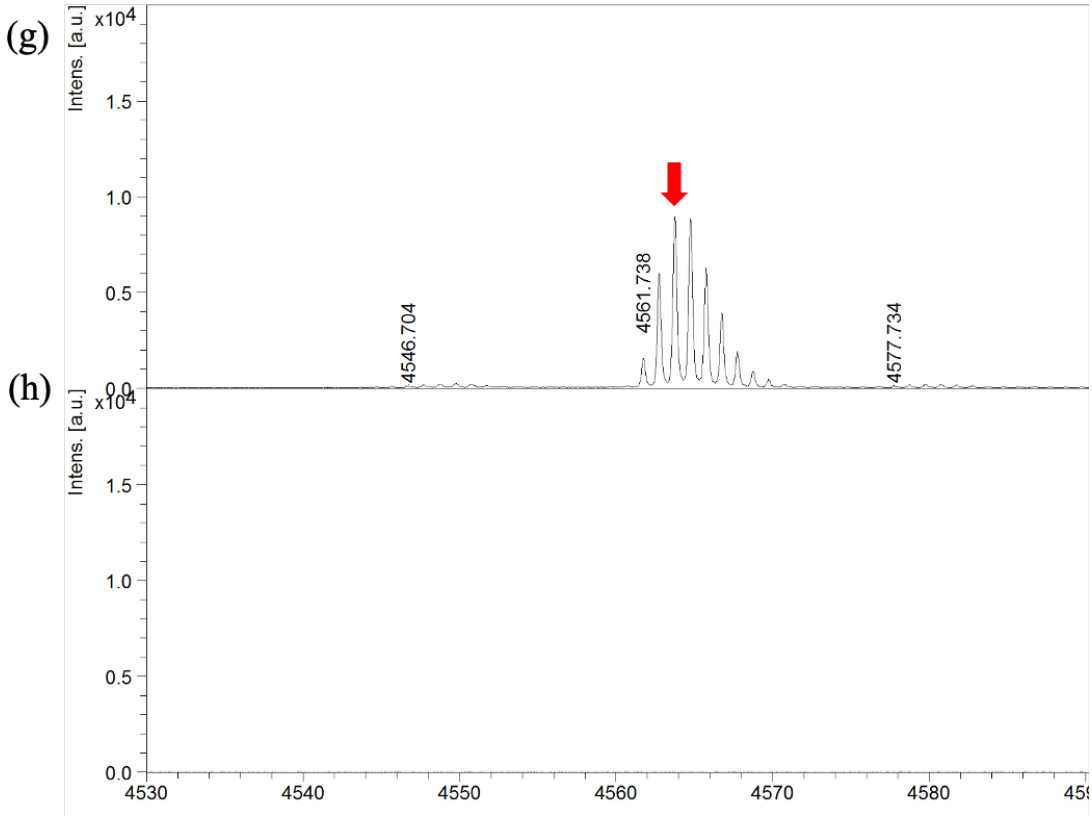

Fig. 11 MALDI-TOF-MS spectra of purified bacteriocins produced by *Leu. mesenteroides* 406 and 213M0

Samples: peak-I of 406 (a), peak-I of 213M0 (b), peak-III of 406 (c1, c2), peak-III of 213M0 (d), peak-IV of 406 (e), peak-IV of 213M0 (f), peak-II of 213M0 (g), and blank (h). Red arrows indicate the highest peak in each analysis.

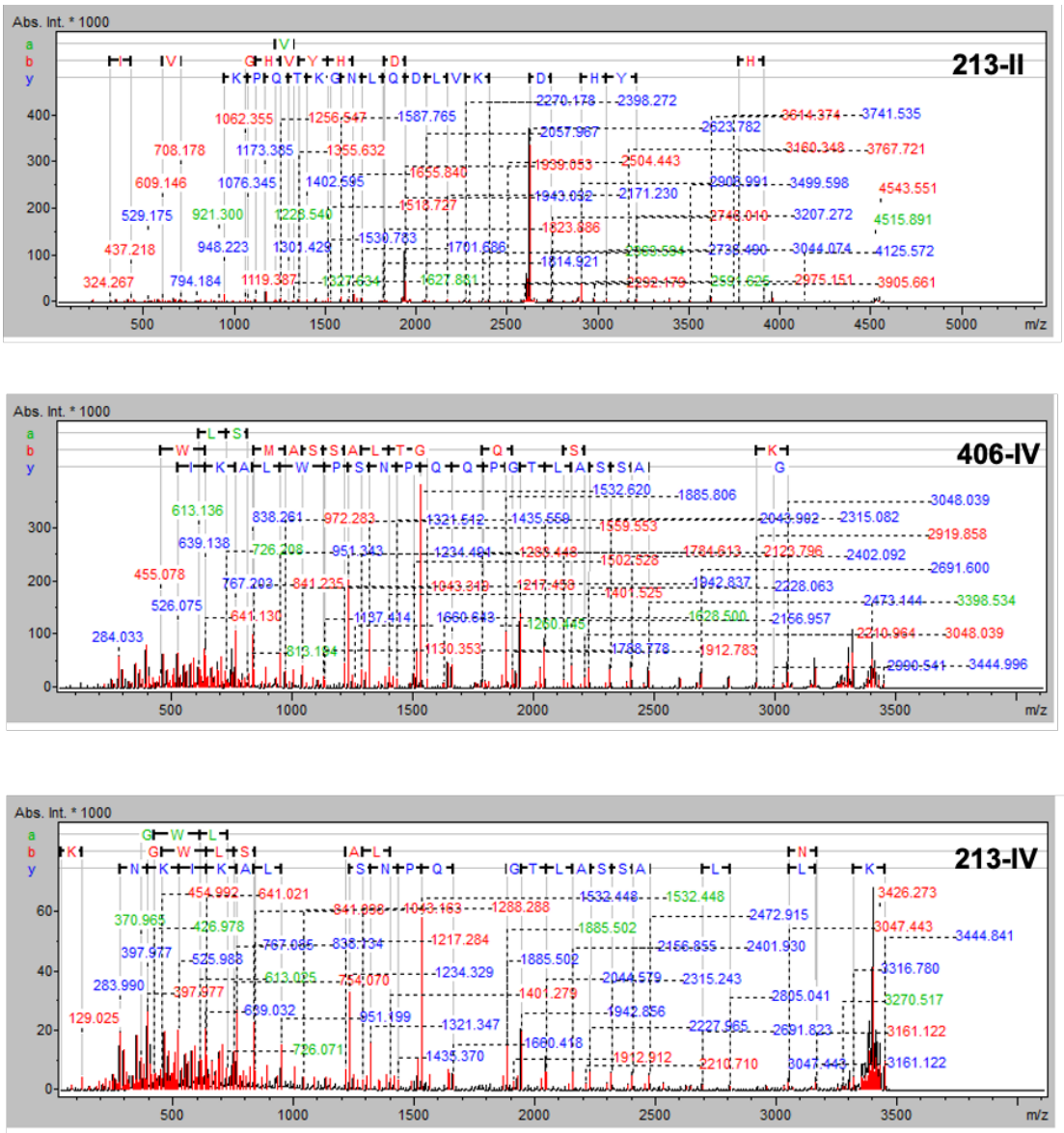

Fig. 12 MALDI-TOF-MS/MS analysis of peaks 213-II, 406-IV, and 213-IV

MKSYLSNYKELSNSECNKISGG HWIGDVLGAIGHVYHPADPQKVLDQLNGKTQPKPGHQYSPY GYY

Fig. 13 Sequence of mesentericin M (solid-underlined) and the prepeptide

Arrows refer to cleavage sites. Dotted- and dashed-underlined sequences indicate a signal peptide and a posttranslationally released tripeptide, respectively.

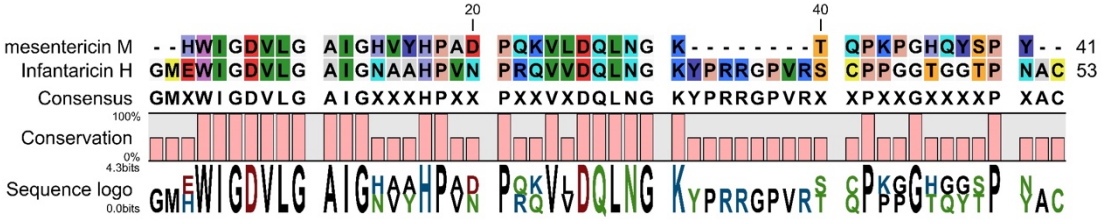

Fig. 14 Sequence alignment of mesentericin M and infantaricin H

Table 10 Monoisotopic mass (m/z) of fragment a-, b-, and y-ions in MS/MS spectra of mesentericin M. (precursor [M+H]+ = 4561.7384)

#	a	a*	b	b*	Seq.	y	y*	#
1	110.0713		138.0662		H			41
2	296.1506		***324.1455***		W	4424.2575	4407.2309	40
3	409.2347		***437.2296***		I	4238.1782	4221.1516	39
4	466.2561		494.2510		G	***4125.0941***	4108.0675	38
5	581.2831		609.2780		D	4068.0726	4051.0461	37
6	680.3515		708.3464		V	3953.0457	3936.0191	36
7	793.4355		821.4305		L	3853.9773	3836.9507	35
8	850.4570		878.4519		G	***3740.8932***	3723.8667	34
9	921.4941		949.4890		A	3683.8717	3666.8452	33
10	1034.5782		***1062.5731***		I	3612.8346	3595.8081	32
11	1091.5996		***1119.5946***		G	***3499.7506***	3482.7240	31
12	1228.6586		***1256.6535***		H	3442.7291	3425.7026	30
13	1327.7270		***1355.7219***		V	3305.6702	3288.6436	29
14	1490.7903		***1518.7852***		Y	***3206.6018***	3189.5752	28
15	1627.8492		***1655.8441***		H	***3043.5384***	3026.5119	27
16	1724.9020		1752.8969		P	***2906.4795***	2889.4530	26
17	1795.9391		1823.9340		A	2809.4268	2792.4002	25
18	1910.9660		***1938.9609***		D	***2738.3897***	2721.3631	24
19	2008.0188		2036.0137		P	***2623.3627***	2606.3362	23
20	2136.0774	2119.0508	2164.0723	2147.0457	Q	2526.3100	2509.2834	22
21	2264.1723	2247.1458	***2292.1672***	2275.1407	K	***2398.2514***	2381.2248	21
22	2363.2407	2346.2142	2391.2357	2374.2091	V	***2270.1564***	2253.1299	20
23	2476.3248	2459.2983	2504.3197	2487.2932	L	***2171.0880***	2154.0614	19
24	2591.3518	2574.3252	2619.3467	2602.3201	D	***2058.0039***	2040.9774	18
25	2719.4103	2702.3838	2747.4052	2730.3787	Q	***1942.9770***	1925.9504	17
26	2832.4944	2815.4678	2860.4893	2843.4628	L	***1814.9184***	1797.8919	16
27	2946.5373	2929.5108	***2974.5322***	2957.5057	N	***1701.8343***	1684.8078	15
28	3003.5588	2986.5322	3031.5537	3014.5272	G	***1587.7914***	1570.7649	14
29	3131.6538	3114.6272	***3159.6487***	3142.6221	K	***1530.7700***	1513.7434	13
30	3232.7014	3215.6749	3260.6963	3243.6698	T	***1402.6750***	1385.6484	12
31	3360.7600	3343.7335	3388.7549	3371.7284	Q	1301.6273	1284.6008	11
32	3457.8128	3440.7862	3485.8077	3468.7811	P	***1173.5687***	1156.5422	10
33	3585.9077	3568.8812	***3613.9027***	3596.8761	K	1076.5160	1059.4894	9
34	3682.9605	3665.9339	3710.9554	3693.9289	P	***948.4210***	931.3945	8
35	3739.9820	3722.9554	***3767.9769***	3750.9503	G	851.3682	834.3417	7
36	3877.0409	3860.0143	***3905.0358***	3888.0092	H	794.3468	777.3202	6
37	4005.0995	3988.0729	4033.0944	4016.0678	Q	657.2879	640.2613	5
38	4168.1628	4151.1362	4196.1577	4179.1311	Y	529.2293		4
39	4255.1948	4238.1683	4283.1897	4266.1632	S	366.1660		3
40	4352.2476	4335.2210	4380.2425	4363.2159	P	279.1339		2
41					Y	182.0812		1

4. Discussion

The crude bacteriocin, prepared using ammonium sulfate precipitation, was separated by SDS-PAGE to determine their molecular weight of about 3.5 K Da, which differs from previous results (Arakawa *et al*., 2016; Wulijideligen *et al*., 2012). However, because a protein with a molecular weight that is comparable to bacteriocins might precipitate alongside bacteriocins in the ammonium sulfate precipitation step, an accurate molecular weight cannot be determined by crude extraction. Therefore, more research is required to precisely purify and describe these bacteriocins. After three steps of purification, it was found that *Leu. mesenteroides* 213M0 had four fractions with antibacterial activity, one more than strain 406. Partial amino acid sequence analysis and MALDI-TOF/TOF mass spectrometry showed peaks I from both strains were identical to mesentericin Y105. As for 406-IV and 213-IV, it was determined from the MS/MS results that they were 100% identical to mesentericin B105. This is consistent with the genome information (*Morita et al*., 2016a, 2016b), suggesting that they efficiently synthesized and secreted mesentericin Y105 and mesentericin B105. Peak 213M0-II was identified as a novel bacteriocin, mesentericin M, with not highly homologous bacteriocins, although partially homologous putative bacteriocins such as infantaricin H produced by *Streptococcus infantarius* LP90 have been reported (Campanero *et al*., 2020). Mesentericin M produced only by strain 213M0, that was revealed for the first time in this study. Furthermore, mesentericin M was, to our knowledge, the first bacteriocin in which the C-terminal three amino acid residues (GYY) were posttranslationally cleaved. One of the identified bacteriocins that underwent a similar post-translational modification at the C-terminus was microcin E492 produced by *Klebsiella pneumoniae* RYC492, but it was not cleaved (Duan *et al*., 2022). After translation, the prepeptide of microcin E492 was modified at the C-terminal serine by the addition of a C-glycosylated trimer of N-(2,3-dihydroxybenzoyl)-L-serine to form the mature bacteriocin with stronger antibacterial activity. This process requires a glycosyltransferase (*mceC*) and an enterobactin esterase (*mceD*)

encoded on the microcin E492 gene cluster (*mceABCDEHGHIJ*). Based on the sequence information of mesentericin M revealed in this study, we here inferred the genes responsible for its biosynthesis and immunity around its structural gene (*mesM*) on pLM213M0B (**Fig. 3b** and **Fig. 18**). There are three genes (named *mesKJL*) and one gene (*mesN*) upstream and downstream near *mesM*, respectively. Among them, *mesJ* was annotated as a bacteriocin immunity gene, and its upstream neighbor is *mesK*. Although the function of *mesK* is unknown, a sequence with 98% homology to *mesK* was found in the vicinity of the ABC transporter genes (*lcaCD*) of leucocin A produced by *Leuconostoc gelidum* UAL 187 (Hastings *et al*., 1991). There is a putative promoter just upstream of *mesK*, and there is a replication initiation gene which is not directly related to bacteriocin production and immunity in further approximately 800-bp upstream. Therefore, *mesK* is thought to be the first gene of the mesentericin M-related gene cluster. Downstream of *mesM* is *mesN*, whose function is unknown, and approximately 770 bp further downstream is $mesE_2D_2$, described as pLM213M0B-*mesDE* in **Fig. 3b**. These two genes had been thought to be responsible for the secretion of mesentericins Y105 and B105 as mentioned above. However, since there are no other mesentericin M-secreting genes in the vicinity, it was speculated that they would play this role. On these grounds, these seven genes were deduced to be the mesentericin M-related genes. As noted above, mesentericin M requires not only cleavage of the N-terminal leader peptide but also cleavage of the C-terminal tripeptide for its maturation. For cleavage at the C-terminus, protease such as a carboxypeptidase would be needed, as well as a signal peptidase at the N-terminus. The three genes (*mesK*, *mesL* and *mesN*) of unknown function on pLM213M0B may possibly play a role in this protease activity. However, this seems unlikely, since the translated sequences of *mesK*, *mesL,* and *mesN* do not have the well-known conserved regions of proteases. we rather focus on the homology between the cleaved tripeptide (GYY) and the three residues (KYY) on the N-terminal side (just after the double glycine motif in the leader sequence) of mesentericin Y105. Namely, we think that the

leader peptidase of mesentericin Y105 may misrecognize and cleave the tripeptide. Further experiments are needed to elucidate the cleavage mechanism of the C-terminal tripeptide and its effects.As for the 406-III bacteriocin, the N-terminal sequence was identical to that of mesentericin B105 (**Table 9**). However, its mass was 16 higher than that of mesentericin B105, identified in the peak-IV fraction (**Fig. 14**) This was consistent with the value obtained via oxidation at the at the ninth amino acid residue, methionine, to sulfoxide of mesentericin B105. Similar methionine oxidation has been well studied with pediocin PA-1 (Fimland *et al*., 1996 ; Johnsen L *et al*., 2000; Kuniyoshi *et al*., 2022). It has been reported that the methionine residue at position 31 of pediocin PA-1 was gradually oxidized to sulfoxide during storage and that the antibacterial activity was greatly reduced by the oxidation. The specific activities of the peak-III bacteriocins were also significantly lower than those of the corresponding peak-IV bacteriocins identified as mesentericin B105 (**Fig. 10b** and **Table 8**). These results indicated that the peak-III bacteriocins were oxidized mesentericin B105, whereas any active peaks of the oxidized forms of mesentericins Y105 and M were not detected (**Fig. 10a**), which must be due to the absence of a methionine residue in them. Incidentally, almost the same results were also observed for mesentericin B105 and 52B (Biet *et. al*., 1998; Revol-Junelles *et. al*., 1996). As shown in **Fig. 10** and **Table 8**, mesentericins Y105 and M had no antibacterial activity, but mesentericin B105 and the oxidized form had activity against *W. paramesenteroides* JCM 9890 ^{T}. The activity of strain 406 against strain JCM 989^{T} was significantly higher than that of strain 213M0, meaning that strain 406 produced more mesentericin B105 than 213M0. In fact, the amount of mesentericin B105 purified from the 406 culture was approximately twice that from the 213M0 culture (**Table 8**). On the other hand, there was not much difference in the amount of mesentericin Y105 produced between strains 406 and 213M0 (**Table 8**). The difference in the production of mesentericin B105, despite roughly equal production of mesentericin Y105, is one of the factors contributing to the generally higher antibacterial activity of strain 406 compared with

strain 213M0. The comparison of production levels also suggests that, unlike mesentericin Y105, the production of mesentericin B105 by strain 213M0 would not reach its maximum. The nucleotide sequence comparison (**Fig. 4**) in this study showed that the genetic differences between strains 406 and 213M0 to produce mesentericins Y105 and B105 were the shortening of *mesG* and the duplication of the transporter genes (*mesDE* and *mesD2E2*). Although *mesG* is rumored to encode an additional putative accessory protein (Wan *et. al*., 2015) , it has no significant similarity to other already-known functional protein-coding genes (Héchard *et. al*., 1999). In Morisset and Frère (2002), the mutant strains expressing the eight *mes* genes (*mesIYCDEFHB*), except *mesG,* produced mesentericin B105 at least as much as the wild strain Y105 (Morisset and Frère, 2002). In addition, no effect of *mesG* on the primary structure of mesentericin B105 was detected by mass analysis in this study (**Table 9** and **Fig. 14**). Therefore, there may be no special function for *mesG*. In other words, the shortening of *mesG* did not seem to affect the low production or the primary structure of mesentericin B105 in strain 213M0. Another possible influencing factor, the duplication of similar secretory genes (*mesDE* and *mesD2E2*), might lead to decreased production of mesentericin B105. The leader sequence of mesentericin M is more like that of mesentericin B105 (50.0%) than that of mesentericin Y105 (33.3%). Such leader sequence similarity might lead to transporter competition, resulting in a decrease in mesentericin B105 secretion instead of an increase in mesentericin M secretion. The effects of the *mesG* incompleteness and transporter duplication on mesentericin production are only predictions, and thus require further study.

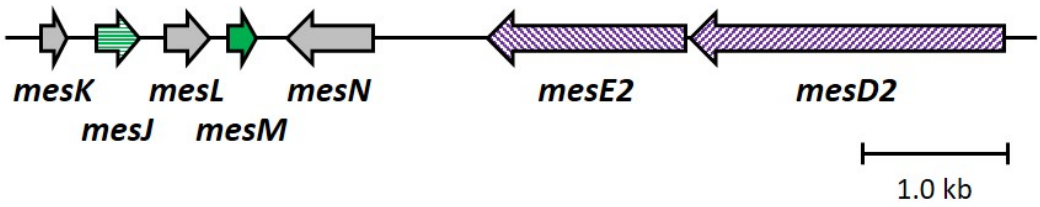

Fig. 15 Sequence of mesentericin M (solid-underlined) and the prepeptide. Arrows refer to cleavage sites. Dotted- and dashed-underlined sequences indicate a signal peptide and a posttranslationally released tripeptide, respectively.

5. Conclusion

Leu. mesenteroides 406 and 213M0 produced mesentericins Y105 and B105 (including the oxidized form).Strain 406 produced more mesentericin B105 and the oxidized form than strain 213M0, although their production of mesentericin Y105 was not significant different. Only *Leu. mesenteroides* 213M0 produced a novel C-terminal truncated bacteriocin, “mesentericin M”.

V. Summery

The most common cause of foodborne illness is the consumption of food contaminated with pathogens or their toxins, which has a significant negative impact on human health and the economy. *Listeria monocytogenes* is recognized as one of the most serious foodborne pathogens due to its survivability under extreme conditions such as a wide pH range, refrigeration temperatures, and high salt concentrations. As *Lis. monocytogenes* can easily proliferate in many foods especially dairy and meat products during storage, various measures including the use of chemical preservatives are taken to protect them from *Listeria* contamination. However, consumers have recently become more aware of the importance of food safety in light of the risk to human health, and have increased their demand for more natural and minimally processed foods. In this context, antibacterial peptides, bacteriocins, produced by lactic acid bacteria (LAB) have attracted attention over the last few decades for their potential as natural alternatives to chemical preservatives, namely as biopreservatives. *Leuconostoc mesenteroides* is a human-familiar LAB species that has long been used for food fermentation, and some strains of the species are known to produce bacteriocins with antilisterial activity. Wulijideligen *et al.* (2012) and Arakawa *et al.* (2016) previously reported that *Leu. mesenteroides* subsp. *mesenteroides* 406 and 213M0 isolated from different samples of Mongolian traditional fermented mare milk, airag, produced bacteriocin-like inhibitory substances (BLIS) with high antilisterial activity and high stability to heating, long storage, and a wide pH range. In addition, Morita *et al.* (2016) released the genome sequence information of the two strains and showed that both strains encode a nearly identical gene cluster responsible for the biosynthesis of and immunity to a set of bacteriocins, mesentericins Y105 and B105. This suggested that strains 406 and 213M0 would produce the same bacteriocins. However, this suggestion didn't agree with the previous results that the BLIS of strains 406 and 213M0 had very similar properties, but their antibacterial spectra and molecular size were slightly

different. Since the previous studies were conducted under different conditions, a comparative study under identical conditions was needed to resolve the discrepancy. In order to provide more scientific support for the use of strains 406 and 213M0 and their bacteriocins for safe and effective food biopreservation, this study aimed to clarify the differences between them by recomparing the antibacterial properties and bacteriocin-related gene clusters, and by purifying and identifying their bacteriocins.

First, cell growth and BLIS productivity were compared between strains 406 and 213M0. The culture pH, turbidity and viable cell count for cell growth, and the antilisterial activity of the cell-free culture supernatant (CFS) for BLIS productivity were measured for 0, 8, 16, 24, 48 and 72 h incubation at 25℃. The antilisterial activity was determined using the agar well diffusion method against *Lis. monocytogenes* VTU 206 as an indicator strain. The cell growth of strain 406 was better than that of strain 213M0 during their exponential phase and the first half of their stationary phase. The antilisterial activity of strain 406 was also higher than that of strain 213M0. Next, the antibacterial spectra of the CFS of both strains were evaluated against 9 *Listeria* spp. and 23 LAB strains used as indicators. Their antibacterial spectra were similar, and they were effective against all *Listeria* spp. strains and 6 of the 23 LAB strains. The activity of strain 406 was generally higher than that of strain 213M0, but conversely lower against only two indicator strains, *Lis. monocytogenes* JCM 7680 and *Leuconostoc lactis* JCM 6123^{T}. These results suggested that the BLIS productivity of strain 406 was generally high as well as its cell growth, compared to strain 213M0, but there is some other difference between their BLIS that would partially reverse their antibacterial spectra.

Secondly, to clarify the antibacterial activity of strains 406 and 213M0 was caused by bacteriocins, cyclization of their plasmids harboring bacteriocin-related genes, comparison of the bacteriocin-related gene clusters, and confirmation of the loss of activity by plasmid-curing were performed. Gaps of the plasmid-like sequences shown in the draft genome information of both strains released by Morita *et*

al. (2016) were filled by PCR and nucleotide sequencing. As a result, strains 406 and 213M0 had two and three plasmids, respectively, and each one (pLM406A and pLM213M0A) of them encoded almost the same *mes* gene cluster (*mesIYCDEFHBG*) responsible for the biosynthesis of and immunity to mesentericins Y105 and B105. The sequences of the nine genes were similar to those in plasmids (pHY30 and pFR38) of two mesentericins Y105 and B105 producers, *Leu. mesenteroides* Y105 and FR52. However, one (*mesG*) of the nine genes in pLM213M0A of strain 213M0 was shorter (77% length) than that in pLM406A of strain 406 and pHY30 of strain Y105. In addition, another plasmid pLM213M0B of strain 213M0 encoded two genes similar to secretion genes, *mesDE*, for mesentericins Y105 and B105. Furthermore, a plasmid pLM406A of strain 406 encoded some genes related to the citrate metabolism system, and a plasmid pLM213M0C of strain 213M0 was a cryptic plasmid. Next, strains 406 and 213M0 were subcultured many times in a medium containing novobiocin for plasmid-curing, namely to obtain derivative strains that lost the plasmids. The elimination of each plasmid in the derivative strains was confirmed by PCR. One derivative strain obtained from 406 lost only pLM406A, and another derivative strain from 213M0 lost all three plasmids. Then, the antibacterial activity of both derivative strains was not detected. These results indicated that the antibacterial activity of strains 406 and 213M0 was caused by plasmid-encoded bacteriocins, probably mesentericins Y105 and B105, and suggested that the difference in the antibacterial properties between the two strains might be due to a partial deletion of *mesG* and duplication of *mesDE* detected only in strain 213M0.

Third, to confirm the qualitative difference in the bacteriocins produced by strains 406 and 213M0, the bacteriocins were purified from their CFS by three steps including ammonium sulfate precipitation, solid-phase extraction using a C18 column, and reversed -phase HPLC using a C8 column, and then analyzed by N-terminal amino acid sequencing and mass spectrometry. The antibacterial activity of each fraction was confirmed against *Lis. monocytogenes* VTU 206, *Weissella*

paramesenteroides JCM 9890^T, and *Leu. lactis* JCM 6123^T. From CFS of strains 406 and 213M0, three and four active fractions were purified, respectively. Three fractions from both CFS were identified as mesentericins Y105 and B105, and oxidized mesentericin B105. Mesentericin Y105 had antibacterial activity against strains VTU 206 and JCM 6123^T, but not against JCM 9890^T. On the other hand, mesentericin B105 and the oxidized form showed the activity against strains VTU 206 and JCM 9890^T, but not against JCM 6123^T. The activity of the oxidized form was half of that of the original mesentericin B105. Another active fraction purified from the CFS of strain 213M0 was identified as a novel bacteriocin, named as mesentericin M, showing activity against strains VTU 206 and JCM 6123^T. Mesentericin M consisted of 41 amino acid residues, and its molecular mass was $[M+H]^+$ = 4,564. The nucleotide sequence of its structural gene, *mesM*, was found on pLM213M0B, and contained a typical 22-amino acids leader sequence with a double glycine motif before the mature peptide sequence. In addition, surprisingly, the amino acid sequence and molecular mass of mesentericin M analyzed on MS/MS spectrometry showed that truncation of the C-terminal three amino acid residues (-GYY) from the propeptide (44 amino acid residues, and its predicted $[M+H]^+$ = 4,947.5) occurred after cleavage of the leader peptide from the translated sequence of the structural gene, *mesM*. There have been no reports of C-terminal truncated bacteriocins, and moreover, mesentericin M is not highly homologous to any other known bacteriocin. These results strongly suggested that mesentericin M produced only by strain 213M0 should be the main cause of the difference in the antibacterial properties between strains 406 and 213M0.

Finally, mesentericin M-related seven genes, *mesKJLMNE$_2$D$_2$*, were presumed around its structural gene, *mesM*, on plasmid pLM213M0B of strain 213M0. An annotation tool suggested that *mesJ* would be a bacteriocin immunity gene and *mesE$_2$D$_2$* would encode ABC trainsporter proteins for bacteriocin secretion. Genes *mesE$_2$D$_2$* were thought to be duplicated secretion genes, *mesDE,* for mesentericins

Y105 and B105 in the previous section. The functions of the other three genes, *mesK*, *mesL*, and *mesN* could not be inferred.

In conclusion, it was found that *Leu. mesenteroides* subsp. *mesenteroides* 406 and 213M0 isolated from different samples of Mongolian traditional fermented milk, airag, produced plasmid-encoding bacteriocins, mesentericins Y105 and B105. The cell growth and total antibacterial activity of strain 406 were generally superior to those of strain 213M0. However, only strain 213M0 additionally produced a novel C-terminal truncated bacteriocin, named as mesentericin M. The slight difference in antibacterial properties between the two strains, especially the difference in antibacterial spectra, should be mainly due to the presence of mesentericin M, although the effect of the *mesG* gene partially deleted only in strain 213M0 has been still unknown. The results of this study, which detailed the antibacterial properties of strains 406 and 213M0, would greatly contribute to the future use of both strains and their bacteriocins as safe and effective food biopreservatives.

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