Study on characterization of antimicrobial resistant Listeria monocytogenes and their biocontrol by using specific bacteriophages and natural antimicrobial substances

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Chapter 1. Introduction

1.1. An overview of *Listeria monocytogenes*

1.1.1. *Listeria monocytogenes* as a pathogen

Listeria monocytogenes was originally described as *Bacterium monocytogenes* by Everitt George Dunne Murray in 1926 and proposed by Pirie in 1940. A few outbreaks were reported in developed countries in 1970 and 1980, and it was verified as the causative agent of foodborne listeriosis in 1983. In the following years, *Listeria* has been the subject of important discoveries among the most researched bacterial infections (Liu, 2008).

The genus *Listeria* spp. is a Gram-positive, rod-shaped, non-spore-forming, facultative intracellular and aero-anaerobic bacteria. It includes twenty currently recognized species, which can be subdivided into two major groups: (i) *Listeria sensu stricto*, constituted by *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii,* and *L. marthii*; and (ii) *Listeria sensu lato*, constituted by *L. grayi*, *L. rocourtiae*, *L. fleischmannii*, *L. weihenstephanensis*, *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia*, *L. grandensis*, *L. booriae*, *L. newyorkensis*, *L. costaricensis*, *L. goaensis* and *L. thailandensis*. Among them, *L. monocytogenes* and *L. ivanovii* are pathogenic species for humans and animals (Allerberger and Wagner, 2010; Quereda et al., 2020; Quereda et al., 2021).

Listeria monocytogenes is a serious foodborne pathogen, widely distributed in the environment and responsible for listeriosis outbreaks. Meloni et al. (2015) reported the outbreaks occurred by consumption of contaminated ready-to-eat meats, seafood, meat and meat products, milk and dairy products, and vegetables. This bacterium can thrive in various conditions including temperatures (0–45 °C), pH ranges (4.3–9.4), high salt concentration (up to 14 %), and water activity (above 0.92). These physiochemical

factors promote the survival and growth of *L. monocytogenes* in varieties of foods (Bouymajane et al., 2021).

The pathogenicity of *L. monocytogenes* is determined by their virulence factors and dangerous serotype distributions. The virulence factors are distributed across the genome or grouped in pathogenicity islands LIPI-1, LIPI-3, and LIPI-4 (Maury et al., 2016). The *hlyA* gene encodes listeriolysin O; *inlA, inlB* genes encode internalin; *clpC* gene encodes *ClpC* ATPase; *plcA* gene encodes phosphatidylinositol-phospholipase C; *iap* gene encodes Iap invasion associated protein; and *prfA* gene for virulence regulator, all these genes responsible for virulence of *L. monocytogenes* (Cao et al., 2018; Soni et al., 2014; Wu et al., 2016). Based on somatic O and flagellar H antigens, *L. monocytogenes* have been classified into 13 serotypes (Maćkiw et al., 2016). Among them, serotypes 1/2a, 1/2b, 1/2c, and 4b can cause 95 % of human listeriosis by contaminated food, animal and human samples. Serotype 4b is the most common in clinical cases, whereas serotypes 1/2a and 1/2b are mainly found in food (Bouymajane et al., 2021; Skowron et al., 2020).

Listeria infections can cause septicemia, encephalitis, meningitis, and abortion in elderly people, fetuses, and pregnant women with a fatality of 20-40 % (Karadal and Yildrim, 2014). Rodríguez-Campos et al. (2019) reported that human listeriosis causes 23,000 cases of invasive infections yearly in global. Listeriosis outbreaks were recorded with mortality rates of 42 % in South Africa in 2017-2018 (Desai et al., 2019) and 17.6 % in the European Union in 2019 (Panera-Martínez et al., 2022). However, only 0.65 listeriosis cases per million inhabitants were reported in Japan in 2010 (Ochiai et al., 2010). Nevertheless, the contamination rates of *L. monocytogenes* in foods in Japan are almost comparable with other countries, including the United States and Europe.

1.1.2. Antimicrobial resistance of *L. monocytogenes*

Antimicrobial resistance (AMR) has been recognized as a serious public health issue worldwide. The AMR pathogens can be distributed between humans, animals,

foods, and environments (water, soil, and air) due to uncleaned facilities, unsecured food processing, and inappropriate infection control (Doyle et al., 2013). Several antibiotics are used in humans and animals for different purposes such as preventing infections, treating diseases, and promoting growth in many countries including Japan (Phillips et al., 2004; Tamura, 2016). The misuse and overuse of antibiotics increased antimicrobial resistance of bacterial pathogens and the proliferation of resistant pathogens through the food chain, leading to increased public health consequences in terms of morbidity, mortality, and treatment costs (Gyles, 2008).

Listeria monocytogenes are susceptible to most antibiotics, however, they are naturally resistant to cephalosporins and fosfomycin (Iwu and Okoh, 2020). Since multidrug-resistant *L. monocytogenes* strains were found in France in 1988 (Poyart-Salmeron et al., 1990), one or multiple antibiotic-resistant strains were continuously isolated from various foods and environmental sources (Aiyedun et al., 2020). Many studies reported that *L. monocytogenes* strains were resistant to gentamycin, streptomycin, kanamycin, erythromycin, rifampin, and sulfonamide, therefore, different antibiotic classes have been alternatively applied (Poros-Gluchowska and Markiewicz, 2003; Prieto et al., 2016; Walsh et al., 2001). The β-lactams group such as ampicillin/ penicillin alone or combined with the aminoglycoside group, especially gentamycin becomes the preferred medication for treating listeriosis (Al-Mashhadany, 2019; Wang et al., 2013). Trimethoprim with sulfamethoxazole, erythromycin, and vancomycin are used as second-choice treatments, especially in pregnant women (Charpentier and Courvalin, 1999; Kemnic and Coleman, 2019).

1.1.3. Biofilm formation of *L. monocytogenes*

Biofilm is a complex microbial community that adheres to surfaces and produces a slimy, protective matrix of extracellular polymeric substances (EPS) (Monds and O'Toolle, 2009). They are ubiquitous and can form on both living and non-living surfaces. Depending on the type of isolates and environmental variables, the biofilm

formation structure varied from interwoven chains and dense, three-dimensional formations resembling mushrooms or honeycombs to cellular monolayers with little or no extracellular matrix, as illustrated in Fig. 1.1 (Borucki et al., 2003; Finn et al., 2023; Milanov et al., 2009).

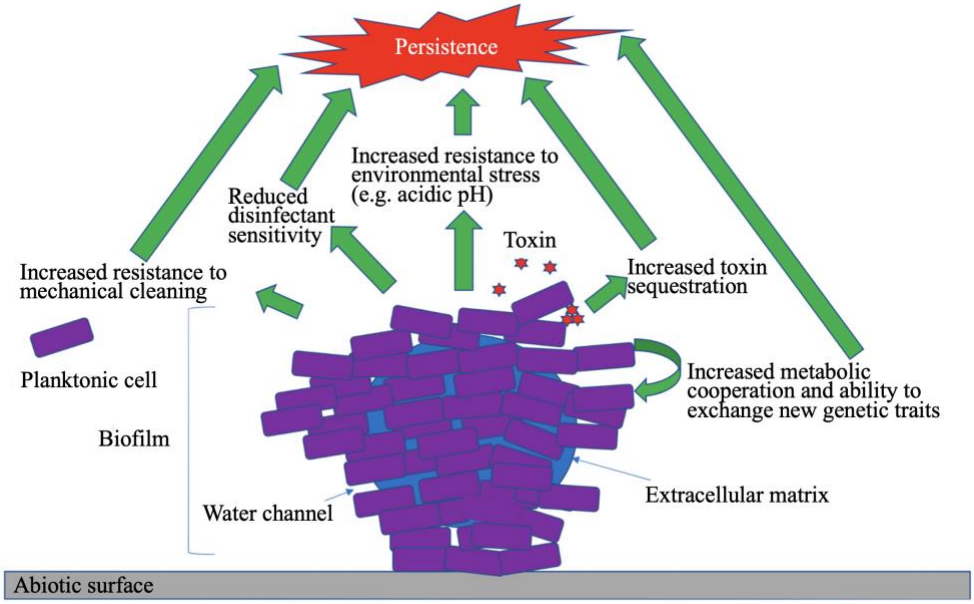


Fig. 1.1. Demonstration of *L. monocytogenes* mature biofilm with a three-dimensional mushroom shape (Finn et al., 2023).

Listeria biofilm production can be influenced by a variety of environmental conditions. Temperature seems to be one of the primary contributors (Colagiorgi et al., 2017). Research typically reports that *L. monocytogenes* adherence and biofilm formation increase as temperature rises to 30-37 °C (Chavant et al., 2002). Topography also influences the growth of listerial biofilm (Møretrø and Langsrud, 2004). Rougher surfaces like corroded or worn offer greater space for biofilm formation because surface flaws can trap water and nutrients to encourage bacterial growth (Chmielewski et al., 2003). In comparison to hydrophobic materials like polystyrene, several investigations have found that biofilm formation occurs more quickly and at higher rates on hydrophilic surfaces like stainless steel (Finn et al., 2023). However, other reports have indicated contradictory results. These may be due to variations in the test strains and/or experimental setups (Djordjevic et al., 2002; Møretrø and Langsrud, 2004; Takahashi

et al., 2010).

The growth of listerial biofilm can also be significantly influenced by NaCl content, pH, and nutrient availability (Valderrama et al., 2013). *L. monocytogenes* cultivated in general-purpose media added with 0 % or 6 % NaCl (w/w) did not affect significantly, while those with 1 % showed a significant reduction in inhibiting listerial biofilm formation (Caly et al., 2001). Additionally, several studies have demonstrated that increasing the acidity of the growing medium by introducing citric or lactic acid, can facilitate the adherence of *L. monocytogenes* onto stainless steel (Briandet et al., 1999a and b). Another study reported that stainless steel was most effectively colonized by *L. monocytogenes* Scott A at pH 7.0 (Smoot and Pierson, 1998a and b).

Other studies demonstrated that nutrient limitation is also important in reducing the production of biofilms (Combrouse et al., 2013; Zhou et al., 2012), but biofilm formation ability of *L. monocytogenes* under various nutritional circumstances may rely on the surface material and strain (Folsom et al., 2006). Moreover, interactions with other bacteria may play an important role in forming listerial biofilms. For instance, *Pseudomonas* spp., which are frequently found in food-related surfaces, have been demonstrated to either encourage or prevent the production of *L. monocytogenes* biofilms, depending on the strain or the circumstances (Carpentier et al., 2004; Finn et al., 2023).

1.2. An overview of bacteriophages

1.2.1. Bacteriophage

Bacteriophages (phages) are viruses that infect bacterial cells and are widely distributed in the biosphere. The term bacteriophage comes from “bacteria” and the Greek word *phagein*, which means “to devour” (Clokie et al., 2011). FW Twort and Félix d'Herelle discovered independently BTwort-d'Herelle phenomena or Bacteriophage phenomena in 1915 and 1917 (Sulakvelidze et al., 2001). The

International Committee on the Taxonomy of Viruses (ICTV) determined the virus taxonomy in 1971 and updated it in March 2021-2022. There were 862 species, 321 genera, 30 subfamilies, 22 families, and one order newly created. The class *Caudoviricetes* was replaced with the order *Caudovirales*, which included all-tailed bacterial and archaeal viruses with icosahedral capsids and a double-stranded DNA (dsDNA) genome (Lefkowitz et al., 2018; Turner et al., 2023).

The genomes of bacteriophages have distinct characteristics such as diversity, mosaicism, and differential gene movement. Phage genomes are extremely diverse, accounting for 15 % of all viruses with known unique sequences. Approximately 750 distinct bacteriophage genomes have been sequenced containing a variety of nucleic acid compositions and virion morphologies. Approximately 90 % of all genetically characterized phages are members of dsDNA phage families. The remaining families of phages are members of single-stranded DNA phage (ssDNA), single-stranded RNA (ssRNA), and double-stranded RNA (dsRNA) (Singh et al., 2024).

Bacteriophages are classified into 13 categories according to the type of encapsulated nucleic acid and the morphology of their virion. There are nine families of dsDNA, including *Myoviridae*, *Siphoviridae*, *Podoviridae*, *Corticoviridae*, *Fuselloviridae*, *Lipothrixviridae*, *Plasmaviridae*, *Rudiviridae*, and *Tectiviridae*; two families of ssDNA, including *Microviridae* and *Inoviridae*; ssRNA, *Leviviridae*; and dsRNA, *Cystoviridae* respectively. Sixty percent of the recognized phages belong to the three major families such as *Siphoviridae*, *Myoviridae*, and *Podoviridae* under the order of *Caudovirales* (Lopes et al., 2018). However, only 3–4 % of the analyzed phages are associated with the ten families under investigation, some of which are incredibly tiny (Ackermann, 2007).

1.2.2. Life cycle of bacteriophages

Bacteriophages have two different life cycles: lytic and lysogenic as shown in Fig. 1.2. Another name for lytic phage is virulent phage. The four stages that distinguish bacteriophage replication cycles are adsorption (attachment), infection, multiplication, and discharge (release) (Guttman et al., 2005). In the attachment phase, phage particles attach to the surface of the bacterial host cell by specific receptors. Then, the phage genetic material enters the host cell through a process akin to injection, which causes the infection phase. After the phage multiplies within the host, the host bacteria lyse and rupture, releasing new phage particles. During the replication process, the chromosome of the host bacterial cell may be crammed into the capsid, resulting in transduction-mediated horizontal gene transfer. The threat posed by pathogenic bacteria resistant to antibiotics can be neutralized with virulent phages (Gamachu and Debalo et al., 2022). In the lysogenic life cycle, temperate phages integrate viral genetic material with the bacterial genome to form prophages, which ensures that the viral genetic material replicates indefinitely without endangering the infected host (Inal, 2003).

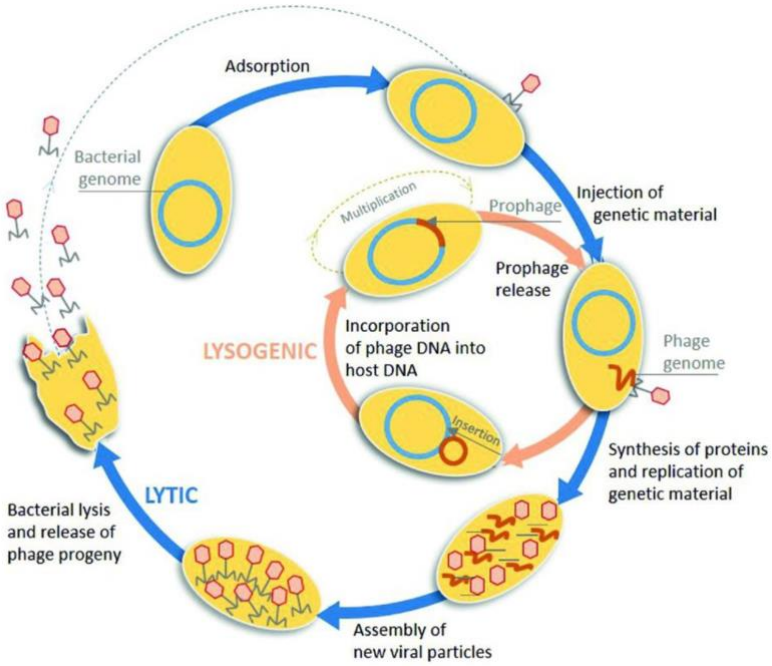


Fig. 1.2. Life cycles of the lytic and lysogenic phages (Costa et al., 2023).

1.2.3. Application of bacteriophage as therapeutic agents

Bacteriophages can infect and kill bacteria without impacting humans or animals, which explores clinical therapy against bacterial pathogens. Previous studies have demonstrated that phage can successfully treat illnesses in humans, animals, and plants (Sulakvelidze et al., 2005a and b). Before the discovery of antibiotics, phages were used to treat many bacterial illnesses such as septicemia, typhoid fever, cholera, dysentery, skin infections, peritonitis, and external otitis (Wittebole et al., 2014).

The rebirth of interest in bacteriophages as antimicrobial agents was largely due to two significant advancements. First, we now have fewer therapeutic alternatives due to AMR bacterial pathogens' emergence and extensive distribution. Furthermore, most newly approved novel antibiotics are modified versions of existing drugs, increasing the likelihood that they may lose their effectiveness quickly (Talbot et al., 2019). Second, antibiotic-mediated disruption of the microbiome is indeed thought to be a factor in several chronic and non-communicable diseases (Dietert and Dietert, 2015).

According to Hyman (2019), bacteriophages have the potential to be exploited therapeutically because (i) the bacteriophage must be lytic and capable of widely damaging the target bacterium by cytotoxicity, (ii) it must remain entirely lytic and refrain from turning into a lysogenic phage, (iii) the host bacterium should be able to be transduced by the bacteriophage, (iv) the intended host range ought to be present, (v) toxin genes that may have an impact on the patient should be examined. Phage can be applied alone or combined with other antibacterial agents to treat infections caused by antibiotic-resistant bacteria (Domingo-Calap and Delgado-Martínez, 2018). Approximately 800 phages are available to combat pathogens such as *Salmonella*, *Escherichia coli*, *Klebsiella*, *Enterococcus*, *Pseudomonas*, *Staphylococcus*, and *Listeria* (Weber-Dabrowska et al., 2016).

1.2.4. Application of bacteriophage as biocontrol agents in the food industry

Globally, foodborne illnesses originating from microbiological cause a serious threat to food safety. Foodborne bacterial infections have a significant influence on public health as well as economic burden (Scharff, 2012). The “One Health” strategy states that providing a safe food supply and preventing bacterial contamination in food are high-priority areas for controlling foodborne disease outbreaks. Bacteriophages are used for pathogen detection and control in the food-producing process, “farm to fork” as promising candidates because of their natural ability to attack and destroy target bacteria without being harmful to humans and animals. Many bacteriophage-based products have been designated as Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (FDA) (Costa et al., 2023). Commercially available phage products such as ListShield™, Listex™ P100, and Phageguard Listex for *L. monocytogenes*; SalmoFresh and Phageguard S for *Salmonella*; and Ecoshield PX and PhageGuard E. for *E. coli*, respectively are used to control foodborne pathogens in the foods (de Melo et al., 2017; Sulakvelidze, 2011).

Since the 1980s, phages have been utilized for the following purposes: (i) phage therapy, which prevents or lessens colonization and diseases in livestock; (ii) phage sanitation and biocontrol, which decontaminates raw foods and disinfects food contact surfaces; and (iii) bio-preservation, which extends the shelf life of perishable manufactured foods (García et al., 2008; Leverentz et al., 2003; Martínez et al., 2008). Owing to their unique characteristics, phages are employed as promising candidates to treat food directly and clean food contact surfaces in farms or food processing plants. This significantly reduces the growth of bacteria colonies on surfaces and biofilm formation, thereby decreasing the risk of pathogen transmission throughout the food supply chain (Endersen et al., 2014; Loc-Carrillo et al., 2011).

1.3. An overview of essential oils

1.3.1. Essential oils (EOs)

EOs are mixtures of volatile lipid-soluble organic compounds extracted from different parts of plants (Matera et al., 2023). In the 16th century, Quinta essential was the term used by Paracelsus von Hohenheim to define "essential oil" (Pichersky et al., 2006). The most common methods for producing EOs for commercial use are steam distillation and hydro distillation, while they can also be obtained by extraction, effleurage, fermentation, expression, or other procedures (Burt, 2004; Tajkarimi et al., 2010). In addition to their unique aroma which makes them useful in perfumery, EOs have a wide range of biological activity which represents the basis for their long history of use in folk and traditional medicines (Dhifi et al., 2016; Mancianti and Ebani, 2020). The applications of EOs in different industries are illustrated in Fig. 1.3 (Herman et al., 2019).

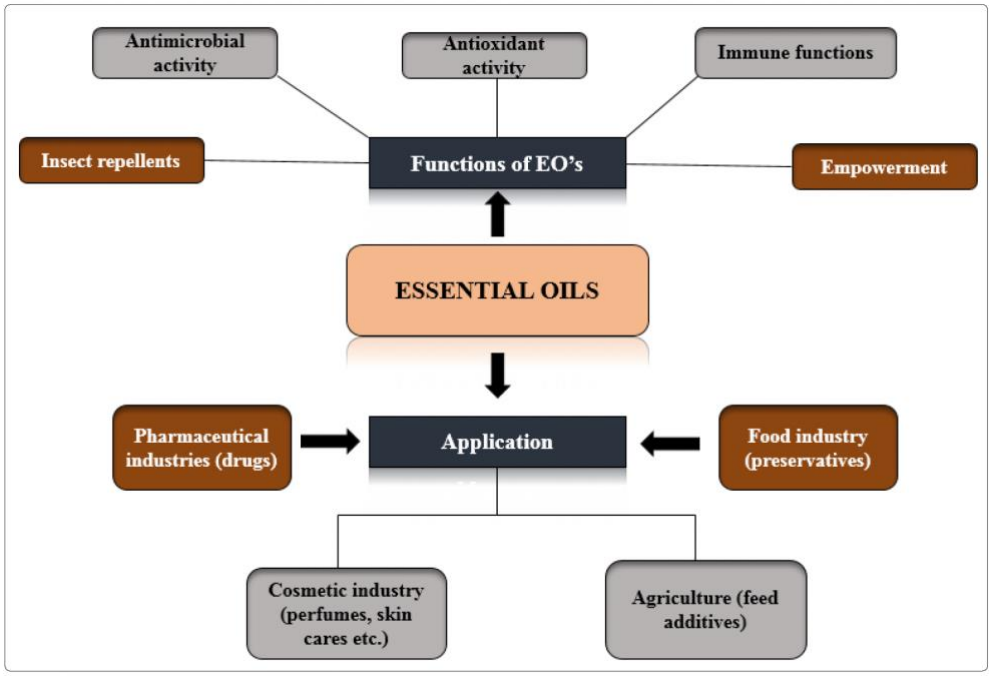


Fig. 1.3. General applications and functions of essential oils in different industries (Herman et al., 2019).

Approximately 3000 EOs have been identified, of which 10 % are employed in or have the potential to be utilized in food products. About 300 EOs were recognized under the US FDA's GRAS lists of food additives (Matera et al., 2023). De Almeida et al. (2010) stated that numerous researchers discovered the biological activities of natural and plentiful herbal plants all over the world, demonstrating that plant-based essential oils and extracts are effective in controlling the growth of a wide range of microorganisms, including bacteria, yeasts, and fungi. Moreover, EOs possess analgesic, anti-inflammatory, antiprotozoal, anticarcinogenic, gastroprotective, and acetylcholinesterase properties. EOs have also shown significant promise in the food industry's efforts to control foodborne microorganisms, therefore, there is currently focusing on using plant extracts as conventional antimicrobials (Herman et al., 2019).

1.3.2. Antimicrobial activity of EOs

Antibacterial activity: Essential oils exhibit potent strong bacteriostatic and bactericidal effects against various bacterial species (Callaway et al., 2011). Cinnamon, clove bud, oregano, thyme, bay leaf, ginger, lemongrass, and allspice oils were shown as the most commonly effective in controlling various foodborne pathogens including *L. monocytogenes*, *S. aureus*, *C. jejuni*, *E. coli,* and *Salmonella* (Friedman et al., 2002). Sheng and Zhu (2014) also investigated the antibacterial activity of cinnamon oils against the "top six" Shiga-toxin-producing *E. coli* O26, O45, O103, O111, O121, and O145. The distinct antibacterial qualities of each dynamic component and their combined effects account for the varied efficacies of the different EOs. Moreover, interactions between different major and minor elements of EOs may also play a significant role in the antibacterial activity (Chouhan et al., 2017; Marchese et al., 2017).

The antibacterial mechanisms of EOs involve damaging the cell membranes of microorganisms, interfering with enzymatic processes, ion transport, and ATP synthesis, as well as reducing the pH of the surrounding environment (Burt, 2004). Furthermore, the concentration and duration of an oil's exposure to microorganisms

determine its effectiveness. The bactericidal effect increases with concentration and duration of contact (Żukowska and Durczyńska, 2024). Kim et al. (2011) reported that most EOs had better antimicrobial activities against Gram-positive than Gram-negative bacteria. It could be due to a direct interaction between the cell membrane and the hydrophobic components of the EOs. On the other hand, Gram-negative bacteria are more resistant to EOs due to their hydrophilic cell wall. As a result, EOs hold great potential as antibiotic substitutes, particularly in the treatment of diseases caused by multidrug-resistant strains.

Antiviral activity: Several EOs such as clove, lemon, oregano, and thyme oils have been shown to have inhibitory effects on many viruses, including influenza, herpes, polioviruses, and SARS-CoV-2 (Elsebai et al., 2022; Sharifi-Rad et al., 2017). According to Bayala et al. (2014), EOs can reduce viral replication more effectively than some typical antiviral drugs when used in quantities that do not harm human cells.

The antiviral mechanisms of EOs involve direct viral inactivation, inhibition of adsorption and penetration into cells, disruption of nucleic acid replication, and viral protein synthesis (Nazzaro et al., 2013). Although *in vitro* trials are promising, further human clinical research is required to demonstrate the safety and efficacy of EOs in treating viral infections. There is inadequate data to propose oils as conventional therapy (Żukowska and Durczyńska, 2024).

Antifungal activity: EOs are also used as promising natural products for fungal inhibition (Hu et al., 2017; Kalemba and Kunicka, 2003). Certain EOs such as cinnamon, lemon, thyme, rosemary eucalyptus, grapefruit, mint, and cedar wood have shown unique properties in addition to preventing the proliferation of mycelia (Hossain et al., 2016). Basil, rosemary, sage, savory, thyme, fennel, lavender, marjoram, oregano, peppermint, and wild mint exhibited potentially significant antifungal activity against *Botrytis cinerea* and *Penicillium expansum* on apple (Fatemi et al., 2013). Additionally, oregano oil has significantly reduced the spores of *Aspergillus terreus* and *Penicillium*

expansum, whereas lavender oils have similarly reduced the spores of *Fusarium oxysporum* and *Penicillium expansum* (Abdi-Moghadam et al., 2023).

The antifungal mechanisms of EOs are preferentially absorbed onto the lipophilic surface of mycelia, with the degree of inhibition rising with the mycelial area. It is thought that EOs establish irreversible cross-links with fungal cell membrane components, resulting in intracellular leakage (Kiran et al., 2016). The differences in water solubility and lipophilic properties of EOs show variable action. Cinnamon, which contains linalool, has a stronger antifungal impact than basil and peppermint, which primarily contain methyl chavicol and menthol. Specific functional groups can disrupt membrane-associated enzyme proteins, influencing the inhibitory processes of EOs (Nazzaro et al. 2017).

1.3.3. Application of essential oils in the food industry

The use of EOs as antimicrobial food additives has been categorized as GRAS by the US FDA. The food industry is interested in these compounds because they are abundant sources of biologically active substances with proven antimicrobial and antioxidant properties (Herman et al., 2019). The fundamental strategy for ensuring food safety is to lower the initial microbiological load and/or restrict the proliferation of residual bacteria during post-process applications, such as manufacturing and storage, by employing active packaging (Yildirim et al., 2018). Cinnamon and clove EOs are included in the most popular natural preservatives due to their active major components. The primary element of cinnamon essential oil is cinnamaldehyde, which effectively inhibits a variety of food spoilage microorganisms. The clove oil contained eugenol, which possesses insecticidal and antibacterial properties. These oils meet the current standards for "natural, safe, and healthy" preservatives as they are naturally antibacterial agents (Ju et al., 2018). According to Haddi et al. (2017), cinnamon EOs have been described as the most significant oil used in the food and cosmetic sectors, particularly as an antibacterial agent because of its several uses including as flavoring

and aromatic agents. Cinnamon oil encapsulation in cyclodextrin nano sponges offers a possible application for antibacterial food packaging (Simionato et al., 2019). Moreover, chicken packed with the linear low-density polyethylene (LLDPE) surface chemically modified by chromic acid and coated with clove oil films exhibited strong antimicrobial activity against *Salmonella* Typhimurium and *Listeria monocytogenes* at refrigeration for 21 days (Mulla et al., 2017). Khaleque et al. (2016) described that high concentrations of clove and cinnamon oils have been shown to control the *L. monocytogenes* in ground beef. Therefore, EOs are natural, safe, and GRAS-recognized for application in the food industry with small or large concentrations based on the impacts of bioactive components that have been shown to enhance product quality and safety without compromising nutritional value or sensory experience (Herman et al., 2019).

1.4. Research objectives

The main purpose of this study is to characterize the antimicrobial-resistant *L. monocytogenes* and their biocontrol by using specific bacteriophages and plant-based essential oils. The specific objectives of this research are:

1) To isolate and characterize the *L. monocytogenes* strains in 2022, and compare their prevalence, antimicrobial resistance, and pathogenicity profiles in 2017 and 2012,
2) To isolate and characterize bacteriophages specific to *L. monocytogenes*,
3) To evaluate the anti-listerial activity of EOs and their application in single and combined with phage vB_LmoS-PLM9 against pathogenic *L. monocytogenes* in broth and milk, and
4) To investigate the effects of phage vB_LmoS-PLM9 and/or cinnamon bark and clove oils on the viability of *L. monocytogenes* in solid foods and their biofilm.

Chapter 2. Occurrence and characterization of *Listeria monocytogenes* in recent 5-year in Fukuoka, Japan

2.1. Introduction

A dangerous foodborne pathogen, *Listeria monocytogenes* is abundantly found in environments and causes listeriosis infection in the elderly and fetuses. A significant death rate of up to 30 % is linked to contaminated foods including milk, meats, seafood, and vegetables (Ai et al., 2022; Bouymajane et al., 2021). Among the identified 13 serotypes, serotypes 1/2a, 1/2b, and 4b occurred in more than 95 % of human listeriosis cases; of these, 1/2a and 1/2b have primarily been isolated from food, while 4b from clinical samples (Jamali and Thong, 2014). Since the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) became the modernized technique to classify and identify microorganisms quickly and accurately, many researchers are approaching to characterize the *L. monocytogenes* by this method (Li et al., 2022; Thouvenot et al., 2018).

Antimicrobial-resistant (AMR) *L. monocytogenes* have emerged because of antibiotic misuse in human and veterinary medicine, raising concerns for global public health. Since the first antimicrobial-resistant *L. monocytogenes* was discovered in France in 1988, certain multidrug-resistant strains were commonly isolated from food and environment (Matereke and Okoh, 2020; Maung et al., 2019; Poyart-Salmeron et al., 1990). Antimicrobial resistance determinants can be exchanged between *L. monocytogenes* and other bacterial species through efflux pumps, horizontal gene transfer, and the ability to build biofilms. The biofilm capability of bacterial pathogens can increase their resistance to antibiotics, detergents, and environmental stressors compared to their planktonic counterparts (Henriques and Fraqueza, 2017; Lee et al., 2019). The pathogenicity of *L. monocytogenes* varied depending on biofilm formation intensity and virulence factors of the types of the isolates. The virulence genes, including *hlyA*, *inlA*, *plcA*, and *clpC*, play important roles during the various phases of

Listeria infections (Quereda et al., 2021).

The isolation source, and environmental, temporal, seasonal, and regional variations influence the incidence, AMR, virulence, and biofilm formation capability of foodborne pathogens such as *L. monocytogenes* (Matereke and Okon, 2020). The AMR profiles of *L. monocytogenes* isolated from raw chicken meat in 2012 and 2017 were described in our previous investigation (Maung et al., 2019), however, an assessment of the current circumstances is still necessary. Therefore, this study aimed to (i) determine the occurrence, characterization in serotyping and MALDI-TOF analysis, and AMR of *L. monocytogenes* isolates in 2022; (ii) compare their profiles to those of the previous isolates from 2012 and 2017; and (iii) examine the virulence genes and biofilm development of *L. monocytogenes* strains isolated in 2012, 2017 and 2022.

2.2. Materials and methods

2.2.1. Sample collection

Eighty-five raw chicken meat and internal organs samples were randomly purchased from 11 supermarkets (A-J) in Fukuoka City, Japan from February to June 2022. On the same day as sampling, all samples were delivered to the Laboratory of Food Hygienic Chemistry, Kyushu University for analysis.

2.2.2. Isolation and identification of *L. monocytogenes*

The bacterial strains of *L. monocytogenes* were isolated using the method reported by Maung et al. (2019). Briefly, each of the samples (10 g) was weighed in a stomacher bag, added 90 mL of Fraser broth (CM0895, Oxoid Ltd., UK) containing Half-Fraser supplement (SR0166E, Oxoid Ltd., UK), homogenized and incubated at 30 °C for 24 h. Later, the secondary enrichment culture (SEC) was prepared by mixing 0.1 mL of primary enrichment culture (PEC) and 10 mL of Fraser broth including Fraser supplement (SR0156E, Oxoid Ltd., United Kingdom), and the mixture was incubated at 37 °C for 48 h. Both enrichment cultures were streaked on CHROMagar™ *Listeria*

(CHROMagar, Paris, France) and kept at 37 °C for 24-48 h. Suspected colonies were shown as blue with white halos and continuously identified using CHROMagar™ Identification *Listeria* (CHROMagar, Paris, France). After 24 h incubation at 37 °C, mauve colonies with halos were detected as *L. monocytogenes*. The laboratory strain no.174 (Liu et al., 2012) and ATCC15313™ standard strain were used as positive controls.

2.2.3. MALDI-TOF MS analysis

The isolates in 2022 (n=17), 2017 (n=12), and 2012 (n=12) were analyzed using the MALDI-TOF MS (Bruker Daltonik GmbH., Bremen, Germany) and the profile spectrum is compared to that of a library of well-characterized bacteria. In brief, the isolates were grown on TSA agar at 37 °C for 24 h. Colonies were suspended in sterilized water (300 µL) and vortexed for 30 s before adding 900 µL of 99.5% high-graded ethanol. The prepared samples were promptly stored at –20 °C until analysis at Fukuoka Sangyo University. The manufacturer's (Bruker) recommendations were followed when applying the identification score criteria. As a result, scores of ≥ 2.000 indicate species-level identification, 1.700–1.999 for genus-level identification, and < 1.700 for no adequate identification. Three separate MALDI-TOF MS analyses were performed on the samples of isolates. Strains with scores > 2.0 were used to build dendrograms. Principal component analysis (PCA) dendrograms were constructed to assess the protein similarity of MALDI-TOF MS on the tested isolates using the Biotyper software.

2.2.4. Serotyping

The serotyping of *L. monocytogenes* isolates (n=17) was performed using a *Listeria* antisera kit (Denka Seiken Co., Tokyo, Japan) followed by the manufacturer's instructions and Maung et al. (2019). *L. monocytogenes* possessed serotype 1/2a (strain no.174) was employed as a control.

2.2.5. Antimicrobial susceptibility testing (AST)

As previously reported by Maung et al. (2019), AST was determined by broth microdilution method using a 96-well DP 32 dry plate (Eiken Chemical Co., Tokyo, Japan). Briefly, the colonies on BHI agar were suspended in 0.9 % NaCl and adjusted to attain an OD_{600} of 0.1. Bacterial inoculum was prepared by diluting with Mueller Hinton broth to yield a cell suspension with 5×10^4 CFU/mL and added 100 µL to each well of the DP 32 containing different concentrations of 18 antimicrobial agents: oxacillin, ampicillin, cefazolin, cefmetazole, flomoxef, imipenem, gentamycin, arbekacin, minocycline, cefoxitin, erythromycin, clindamycin, vancomycin, teicoplanin, linezolid, fosfomycin, sulfamethoxazole/trimethoprim and levofloxacin. The suspension was added to a well without any antibiotics as a control. After incubation at 37 °C for 18-24 h, culture absorbance was measured with a microplate reader Infinite F50/Robotic (Tecan Trading AG, Switzerland). The minimum inhibitory concentrations (MIC), MIC_{50}, and MIC_{90} were evaluated. Based on CLSI (2021) and Maung et al. (2019), the interpretation was classified as resistant, intermediate, or susceptible.

2.2.6. Detection of virulence genes

A total of 36 isolates were used from selected 12 isolates in each year of 2012, 2017, and 2022 (Tables 2.1 and 2.4). The polymerase chain reaction (PCR) was employed to detect the virulence genes such as *hlyA*, *plcA*, *clpC,* and *inlA* using the primer sequences shown in Table 2.2. The PCR mixture (25 µL) was prepared by adding 12.5 µL of GoTaq Green Master Mix (Promega, Madison, WI, USA), 8 µL of double-distilled water, 1.25 µL of each 10 µM primer, and 2 µL of template DNA. The PCR conditions were set up with initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 sec, extension at 72 °C for 1 min, and final extension at 72 °C for 2 min. The amplified PCR products

were electrophoresed on a 1 % agarose gel stained with Midori Green Advance DNA stain (Nippon Genetics Europe GmbH, Germany), and visualized on WSE-6100H LuminoGraph I (ATTO, Tokyo, Japan). *L. monocytogenes* strain no.1 (serotype 1/2a) harboring *hlyA*, *plcA*, *clpC*, and *inlA* genes (Honjoh et al., 2008) was used as a positive control.

2.2.7. Determination of biofilm-forming ability

The biofilm development of the 36 isolates was assessed using a microtiter plate assay, as previously described by Miyamoto et al. (2011). In brief, overnight cultures of each isolate were adjusted with BHI broth to achieve OD_{660} of 0.7. The adjusted culture (200 µL) was applied to each 96-well polystyrene microtiter plate, which was then incubated at 30 °C for 24 h. Following biofilm formation, the suspension was carefully pipetted out and planktonic cells were rinsed twice with phosphate-buffered saline (PBS). After drying at room temperature, 200 µL of 0.1 % crystal violet (CV) was added to each well and allowed for 30 min to stain bacterial biomass. The plates were rinsed 4 times with PBS to remove excess stains. Then, 95 % ethanol (200 µL) was applied to solubilize the residual CV and measured with OD_{595} using an Infinite F50/Robotic microplate reader (Tecan Trading AG, Switzerland) to assess biofilm-forming capacity. The cut-off value (ODc: the average OD of the negative control) was ascertained by measuring the OD of sterile BHI broth, which was utilized as the negative control. The results were classified as follows: weak ($ODc < OD_{595} \leq 2 \times ODc$), moderate ($2 \times ODc < OD_{595} \leq 4 \times ODc$), or strong ($4 \times ODc < OD_{595}$) biofilm production (Stepanović et al., 2000).

2.2.8. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, New York). Significant differences ($P < 0.05$) between isolates from

5-year intervals were analyzed by one-way ANOVA and Tukey's multiple comparison test.

2.3. Results

2.3.1. Occurrence of *L. monocytogenes* in food

Nine out of the 85 samples, 10.6 % were positive for *L. monocytogenes*, and 17 strains were isolated from PEC and SEC of various chicken samples. The sample numbers (1–85) and enrichment cultures (PEC and SEC) were used to create the names of the isolated *L. monocytogenes*. The isolates were found in the samples of 3 breast meats, 2 chicken wings, 2 skins, 1 minced meat, and 1 thigh, but it was not observed in internal organs and bone samples (Table 2.3).

2.3.2. Identification of the isolates by MALDI-TOF MS analysis

Tables 2.1 and 2.4 display the best match scores ranging from 2.112 to 2.511 of the MALDI-TOF MS analysis for *L. monocytogenes* isolates from different years. Figure 2.1 shows the PCA dendrogram of 17 isolates in 2022, split into four clusters upon protein similarity in MALDI-TOF MS profiles. There were 6 isolates (35.29 %), 4 (23.53 %), 4 (23.53 %), and 3 (17.65 %) in each of the four clusters, I through IV. The isolates (5PEC and 5SEC) in cluster-I, (25PEC and 25SEC), and (28PEC and 28SEC) in cluster-II had high MALDI-TOF MS similarity and shared the same serotype, AMR pattern, and source. The isolates (45PEC and 45SEC) in cluster-I and (55PEC and 55SEC) in cluster-III originated from the same source. The isolates 83PEC and 54SEC from the same cluster-IV shared the same serotype and AMR characteristics.

Figure 2.2 depicts the PCA dendrogram of 36 isolates from various years, which were divided into six clusters (I–VI). Isolates (16FO, S18HO, 12HO, S41HC, 54PEC) had the same serotype (1/2b) and AMR patterns (MPIPC, CFX, FOM), while isolates (48FA and 6HA) had serotype 1/2b and AMR patterns (MPIPC, CFX). Furthermore, some of the isolates from the same clusters (50FO and 68HA in cluster-V, 59HO and

55HA in cluster-VI) possessed the same serotype, AMR patterns, source, and year. The isolates 2FA and S24FC shared the same serotype (1/2b) and AMR patterns (MPIPC, CFX, and FOM) in MALDI-TOF MS cluster VI.

2.3.3. Serotyping of the isolates

Based on serotype investigation of 17 *L. monocytogenes* isolates, serotypes 1/2b, 3a, 3b, and 1/2a were found to be 41.2, 29.4, 23.5, and 5.9 % respectively (Table 2.4).

2.3.4. Antimicrobial susceptibility of the isolates

Four AMR patterns (a) CFX (b) MPIPC, CFX (c) CFX, FOM, and (d) MPIPC, CFX, FOM were observed in 17 isolates from 2022 (Table 2.4). Antimicrobial susceptibility profiles were calculated depending on 12 different isolates since they were isolated from the same samples that shared the same serotypes and AMR patterns. The antibiogram of the isolates is shown in Table 2.5. The AMR profiles including MIC_{50} and MIC_{90} of the isolates from 2022 are displayed in Table 2.6. Most of the isolates were susceptible to multiple antibiotics except intermediate resistance to cefmetazole (75 %), and resistance to cefoxitin (100 %), oxacillin (41.7 %), and fosfomycin (41.7 %). For multidrug resistance (MDR), only 2 isolates (16.7 %) were resistant to 3 antibiotics (Table 2.5c).

2.3.5 Comparison of occurrence, serotyping, and AMR of *L. monocytogenes* isolates in 2012, 2017, and 2022

As shown in Fig. 2.3, the prevalence of *L. monocytogenes* isolated from raw chicken samples in 2022 (10.6 %, 9/85 samples) was significantly ($P < 0.05$) lower than those in 2017 (24.0 %, 12/50) and 2012 (52.9 %, 45/85). The serotype distributions of the isolates in different years indicated that serotypes 1/2b, 3a, 3b and 1/2a were 41.2, 29.4, 23.5, and 5.9 % in 2022; serotypes 1/2b and 1/2a were 51.7 and 48.3 % in 2017; and serotypes 1/2b, 1/2a, 4b, and 1/2c were 73.9, 21.5, 3.1, and 1.5 % in 2012 respectively. The pathogenic serotypes 1/2a and 1/2b responsible for more than 95 %

of human listeriosis decreased over time, and 4b was not detected in 2022. Non-pathogenic serotypes 3a and 3b were commonly found only in 2022 isolates (Fig. 2.4).

The changes in AMR of the isolates from the recent 5-year were demonstrated in Fig. 2.5. The isolates in different years showed susceptibility to various tested antibiotics. However, 100 % in 2022 and 2017, and 98.7 % in 2012 were highly resistant to cefoxitin, followed by 72 % in 2012, 82.6 % in 2017, and 41.7 % in 2022 to oxacillin, and 57.3 % in 2012, 95.7 % in 2017 and 41.7% in 2022 to fosfomycin respectively. Furthermore, clindamycin (8 %) and flomoxef (2.7 %) resistance were detected in 2012 isolates, as well as clindamycin (17.4 %) and linezolid (4.3 %) resistance in 2017, but these antibiotic resistances were not found in 2022 (Fig. 2.5A). Figure 2.5B indicates that isolates in 2012 were resistant to one (12 %), two (40 %), three (40 %), and four (6.7 %) antibiotics, whereas those in 2017 were resistant to one (3.5 %), two (10.3 %), three (72.4 %), four (3.0 %), and five (3.5 %) medicines. However, the isolates obtained in 2022 demonstrated resistance to one (33.3 %), two (50.0 %), and three (16.7 %) antibiotics. In 2022, the MDR rate for *L. monocytogenes* was 16.7%, significantly lower ($P < 0.05$) than in 2017 (82.6 %) and 2012 (46.7 %) isolates (Fig. 2.5C).

2.3.6. Comparison of virulence genes possession and biofilm-forming ability of *L. monocytogenes* isolates in 2012, 2017, and 2022

All four virulence genes were detected in different years of the isolates except for the *clpC* and *inlA* genes in 2017 (S15FC) and the *clpC* gene in 2022 (83PEC).

Figure 2.6A shows the median value (interquartile range) of biofilm mass indicated 0.17 (0.10, 0.25) in 2022, which were significantly ($P < 0.05$) weaker than 0.26 (0.13, 0.34) in 2017, and 0.23 (0.08, 0.33) in 2012. The biofilm formation abilities of the 2012 isolates were estimated as weak (8.3 %), moderate (50 %), and strong (41.7 %); those of the 2017 isolates were found to be moderate (41.7 %) and strong (58.3 %); and those of the 2022 isolates were found to be weak (8.3 %), moderate (75 %), and strong (16.7 %) (Fig. 2.6B).

2.4. Discussion

The study related to *L. monocytogenes* contamination in foods has been lacking in Japan since 2017. This study is the first report to compare the pathogenicity in virulence gene detection and biofilm-producing capability of the *L. monocytogenes* isolates in 5-year differences. The incidence of *L. monocytogenes* in 2022 was 10.6 % (9/85) of raw chicken samples. This finding was consistent with the contamination range (8.7-15.1 %) of raw chicken that was recently investigated in various nations including 8.7% in China, 11.1 % in Ethiopia, 13 % in Brazil, and 15.1 % in South Africa (Cavalcanti et al., 2022; Diriba et al., 2021; Kaptchouang Tchatchouang et al., 2022; Liu et al., 2020b). However, our result was greater than that of 3.9 % of poultry in Poland (Skowron et al., 2020), 1.9%-5.7 % of chicken meat in Morocco (Bouymajane et al., 2021) but lower than that of 56 % of poultry in Spain (Panera-Martínez et al., 2022). The variances in the prevalence of *L. monocytogenes* between this study and other investigations could be attributed to differences in sampling procedures, seasonal and geographical fluctuations, and differing sanitary conditions during food preparation and storage (Bouymajane et al., 2021). In a 5-year difference surveillance, the contamination rate of *L. monocytogenes* contamination in 2022 was significantly lower ($P < 0.05$) than those in 2012 (52.9 %, 45/85) and 2017 (24 %, 12/50). The yearly trend of declining contamination rates of *L. monocytogenes* in Japan could be linked to improved hygienic conditions in poultry processing facilities as well as temperature control during transport and storage. Other studies also concurred that the *L. monocytogenes* infection rate in ready-to-eat meat and poultry products had decreased globally, including in the United States (Mamber et al., 2020; Sampedro et al., 2022).

MALDI-TOF MS analysis provides a fast and accurate way to identify bacteria (Thouvenot et al., 2018). In the current investigation, MALDI-TOF MS accurately identified all the isolates as *L. monocytogenes* at the species level, with a score of > 2.0. The PCA dendrogram of the isolates, particularly 17 strains from 2022, revealed that some

of the isolates included in the same cluster by MALDI-TOF MS were identical in serotype, source, year of isolation, and antibiotic resistance profile (Figs. 2.1 and 2.2). This result demonstrated that *L. monocytogenes* strains isolated from raw chickens were successfully recognized and clustered into a group of closely related strains using PCA analysis of MALDI-TOF MS data. It was claimed that PCA analysis of MALDI-TOF MS data from bacterial strains could be relevant in epidemiological studies on infectious diseases.

According to our findings, serotypes 1/2b and 1/2a were most isolated from raw chickens in various years, while serotypes 3a and 3b were only discovered in 2022. Serotypes 4b and 1/2c were identified in 2012, but not in 2017 or 2022. Wang et al. (2017) found that 90 % of the isolates belonged to serotypes 1/2a and 1/2b according to their proclivity to multiply or survive in foods. Shimojima et al. (2016) also noted that these serotypes are common in a variety of foods in Japan. Moreover, human listeriosis-related serotypes, 1/2a and 1/2b are most common in food, and 4b is in clinical cases (Bouymajane et al., 2021; Buchanan et al., 2017; Cavalcanti et al., 2022). These results suggested that the epidemiologically relevant serotypes 1/2a and 1/2b were reduced in 2022 compared to 2012 and 2017, indicating public health risk of listeriosis in Japan seems lower than in other countries.

The pathogenicity levels of *L. monocytogenes* were determined in virulence gene detection and the biofilm-forming ability of the isolates. The *hlyA* and *plcA* genes were detected in all isolates in different years using the primer sequences designed in the current investigation. The listeriolysin O encoding gene (*hlyA*) is used to detect the virulence of *L. monocytogenes* (Liu et al., 2012; Mohamed et al., 2016). In our earlier investigation, we employed primer sequences generated by Liu et al. (2012) to detect *hlyA* in 2017 isolates, and only 58.6 % (17/29) of the isolates tested positive due to spontaneous mutations (Maung et al., 2019). However, all isolates could be detected for the *hlyA* gene when I used the primer sequence designed in this study. As a result, the *hlyA* and *plcA* primer sequences used in this experiment were suitable for

amplifying the genes of *L. monocytogenes* from various years, which may be beneficial for detecting *L. monocytogenes* and virulence genes in future research. Furthermore, *inlA* and *clpC* were found in many isolates obtained throughout a range of years. These findings are in line with research from other countries, including Turkey (AI et al., 2022), Morocco (Bouymajane et al., 2021), Brazil (Iwa and Okoh, 2020), and Poland (Wiśniewski et al., 2022).

According to Wang et al. (2017), the development of biofilm varies based on the types of strain, nutrients of the medium, and environment. In this study, the biofilm-forming ability of the isolates in different years was carried out using the same conditions. All the isolates from various years could produce biofilms, however, the 2022 isolates exhibited notably reduced biofilm formation ($P < 0.05$) in comparison to the isolates from 2012 and 2017. Wiśniewski et al. (2022) documented that *L. monocytogenes'* capacity to form biofilms can boost their survival in food and food processing environments, complicating decontamination and increasing the risk of listeriosis. These findings imply that the isolates from 2022 are less pathogenic than the isolates from earlier batches.

The isolates in 5 different years were commonly resistant to cefoxitin, oxacillin, and fosfomycin. These resistant antibiotics suppress the peptidoglycan formation in bacterial cell walls (Maung et al., 2019; Panera-Martínez et al., 2022). Most of the 2022 isolates were highly resistant to cefoxitin, followed by oxacillin and fosfomycin, which were lower than the 2017 and 2012 isolates. Additionally, 2022 isolates had a significantly ($P < 0.05$) decreased MDR rate compared to 2017 and 2012. These findings contrast with recent studies in other nations including Morocco, South Africa, the United States, and Spain which showed high resistance to various antibiotics in *L. monocytogenes* (Kaptchouang Tchatchouang et al., 2022; Kayode and Okoh, 2022; Panera-Martineaz et al., 2022). This can be justified by advances in public health knowledge, understanding of AMR, and antimicrobial use in healthcare, nursing care,

food, livestock production, and aquaculture under the guidelines of the Japanese Government's National Action Plan on Antimicrobial Resistance (2016-2020). Noritake and Hodin (2022) confirmed that these guidelines effectively lowered total antimicrobial usage nationwide. Furthermore, food additives and other alternative antimicrobials may be recommended in Japan's food and processing facilities to help prevent *L. monocytogenes* antimicrobial resistance in foods.

2.5. Summary

This study examined the prevalence, serotype, MALDI-TOF MS characterization, antimicrobial resistance, virulence gene detection, and biofilm formation of *Listeria monocytogenes* isolated from raw chickens in 2022. The profiles of these isolates were compared with those of isolates from 2012 and 2017. In the investigation in 2022, 9 out of 85 chicken samples (10.6 %) were positive, and 17 *L. monocytogenes* were detected. The serotype distributions of 1/2b, 3a, 3b, and 1/2a indicated 41.2 %, 29.4 %, 23.5 %, and 5.9 % respectively. The isolates in various years were successfully identified and clustered using PCA dendrograms of MALD-TOF MS analysis. In the AMR detection, all isolates were susceptible to most of the tested antibiotics except cefoxitin, oxacillin, and fosfomycin. In a 5-year difference surveillance, the *L. monocytogenes* occurrence in 2022 (10.6 %) was significantly lower ($P < 0.05$) than those in 2017 (24 %) and 2012 (52.9 %). The epidemiologically important serotypes 1/2a, 1/2b, and 4b decreased over time. The MDR rate in 2022 (16.7 %) was significantly lower than those in 2017 (82.6 %) and 2012 (46.7 %). Furthermore, *hlyA*, *plcA*, *clpC*, and *inlA* virulence genes were detected among 36 isolates from different years. Most of the isolates in different years harbored 4 virulence genes. The isolates in 2022 isolates exhibited significantly ($P < 0.05$) weaker biofilm formation than in 2012 and 2017. Despite the low levels of contamination in chicken flesh and AMR of the isolates, virulent *L. monocytogenes* contamination in food should be recognized.

Table 2.1. Conventional and MALDI-TOF MS identification, serotype, ribotype, and antimicrobial resistance patterns of *L. monocytogenes* isolates in 2012 and 2017

No.	Isolate	Source	Conventional method	MALDI-TOF MS		Serotype	Ribotype	AMR patterns
				Organism	Score value			
2012 Isolates								
1	2FA	Minced meat	*L. monocytogenes*	*L. monocytogenes*	2.494	1/2b	ECORI 422-249-S-4	MPIPC, CFX, FOM
2	4FA	Thigh	*L. monocytogenes*	*L. monocytogenes*	2.282	1/2b	ECORI 422-62-S-7	MPIPC, CFX, FOM
3	6HA	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.302	1/2b	ECORI 422-245-S-2	MPIPC, CFX
4	8HO	Wing	*L. monocytogenes*	*L. monocytogenes*	2.256	1/2b	ECORI 422-260-S-2	MPIPC, CFX, FOM
5	11HO	Minced meat	*L. monocytogenes*	*L. monocytogenes*	2.396	1/2a	ECORI 422-257-S-2	MPIPC, CFX, FOM
6	12HO	Thigh	*L. monocytogenes*	*L. monocytogenes*	2.301	1/2b	ECORI 422-257-S-2	MPIPC, CFX, FOM
7	21FA	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.4	1/2b	ECORI 422-261-S-3	MPIPC, CFX
8	48FA	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.248	1/2b	ECORI 422-240-S-3	MPIPC, CFX
9	50FO	Minced meat	*L. monocytogenes*	*L. monocytogenes*	2.301	1/2a	ECORI 422-242-S-3	CFX
10	55HA	Minced meat	*L. monocytogenes*	*L. monocytogenes*	2.351	1/2a	ECORI 422-247-S-8	MPIPC, CFX
11	59HO	Minced meat	*L. monocytogenes*	*L. monocytogenes*	2.356	1/2a	ECORI 422-244-S-8	MPIPC, CFX
12	68HA	Minced meat	*L. monocytogenes*	*L. monocytogenes*	2.315	1/2a	ECORI 422-242-S-3	CFX
2017 Isolates								
1	S4FO	Broiler	*L. monocytogenes*	*L. monocytogenes*	2.342	1/2a	ECORI 422-320-S-2	MPIPC, CFX, FOM
2	S8HC	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.39	1/2a	ECORI 422-317-S-5	MPIPC, CFX, CLDM, LZD, FOM
3	S15FC	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.424	1/2a	ECORI 422-63-S-6	CFX, FOM
4	S16FO	Wing	*L. monocytogenes*	*L. monocytogenes*	2.424	1/2b	ECORI 422-62-S-7	MPIPC, CFX, FOM
5	S18HO	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.3	1/2b	ECORI 422-318-S-8	MPIPC, CFX, FOM
6	S24FC	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.112	1/2b	ECORI 422-75-S-3	MPIPC, CFX, FOM
7	S25FC	Minced meat	*L. monocytogenes*	*L. monocytogenes*	2.398	1/2b	ECORI 422-320-S-6	MPIPC, CFX, CLDM, FOM
8	S31HO	Thigh	*L. monocytogenes*	*L. monocytogenes*	2.358	1/2a	ND	MPIPC, CFX, FOM
9	S38HC	Thigh	*L. monocytogenes*	*L. monocytogenes*	2.327	1/2a	ECORI 422-320-S-6	MPIPC, CFX, CLDM, FOM
10	S39HC	Wing	*L. monocytogenes*	*L. monocytogenes*	2.198	1/2b	ECORI 422-62-S-7	CFX
11	S41HC	Skin	*L. monocytogenes*	*L. monocytogenes*	2.466	1/2b	ND	MPIPC, CFX, FOM
12	S42HC	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.511	1/2b	ND	MPIPC, CFX, FOM

ND= not detected

MPIPC: Oxacillin, CFX: Cefoxitin, FOM: Fosfomycin, CLDM: Clindamycin, LZD: Linezolid

Table 2.2. Primer sequences used for the detection of virulence genes

Target gene	Primer sequence (5'-3')	Target size (bp)	References
hlyA	F: GCATTGATTTGCCAGGTATG R: TTTCTGCATTCACTCCAAGC	399	This study*
plcA	F: TGATTATCGGATCCAACCAC R: GTTTGTTTTTCGGGGAAGTC	447	This study*
clpC	F: TCTTGGTATTAGTTTGAATAAAGCTC R: TCAAACGTACTTTAGAACCAGATT	833	Honjoh et al. (2008)
inlA	F: TTTTTCTATAATAACAAGGTAAGTGAC R: CTGTATAGCTATTGGCGCTAT	820	Honjoh et al. (2008)

*Designed based on nucleotide sequences from NCBI database (www.ncbi.nlm.nih.gov).

Table 2.3. Occurrence of *L. monocytogenes* in raw chicken and viscera samples

No.	Parts of samples	No. of collected samples (n)	No. (%) of positive samples	Occurrence (%)
1	Breast meat	17	3 (17.6)	3.5
2	Minced/ chopped meat	13	1 (7.7)	1.2
3	Thigh	12	1 (8.3)	1.2
4	Wing	9	2 (22.2)	2.4
5	Liver and heart	11	0 (0.0)	0.0
6	Skin	8	2 (25)	2.4
7	Fillet	7	0 (0.0)	0.0
8	Gizzard	4	0 (0.0)	0.0
9	Ovary and oviduct	2	0 (0.0)	0.0
10	Bone	2	0 (0.0)	0.0
Total		85	9	10.6

Table 2.4. Conventional and MALDI-TOF MS identification, serotype, and antimicrobial resistance patterns of *L. monocytogenes* isolates in 2022

No.	Isolate **	Source	Conventional method	MALDI-TOF MS		Serotype	AMR patterns
				Organism	Score value		
1	5PEC*	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.344	3a	CFX
2	5SEC	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.204	3a	CFX
3	25PEC*	Skin	*L. monocytogenes*	*L. monocytogenes*	2.355	1/2b	MPIPC, CFX
4	25SEC	Skin	*L. monocytogenes*	*L. monocytogenes*	2.474	1/2b	MPIPC, CFX
5	26PEC*	Thigh	*L. monocytogenes*	*L. monocytogenes*	2.43	3b	CFX
6	26SEC	Thigh	*L. monocytogenes*	*L. monocytogenes*	2.342	3b	CFX
7	28PEC*	Wing	*L. monocytogenes*	*L. monocytogenes*	2.284	3a	MPIPC, CFX
8	28SEC	Wing	*L. monocytogenes*	*L. monocytogenes*	2.289	3a	MPIPC, CFX
9	45PEC*	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.257	3a	MPIPC, CFX
10	45SEC*	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.355	1/2a	CFX
11	54PEC*	Wing	*L. monocytogenes*	*L. monocytogenes*	2.452	1/2b	MPIPC, CFX, FOM
12	54SEC*	Wing	*L. monocytogenes*	*L. monocytogenes*	2.147	1/2b	CFX, FOM
13	55PEC*	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.318	3b	CFX
14	55SEC*	Breast meat	*L. monocytogenes*	*L. monocytogenes*	2.373	1/2b	CFX, FOM
15	81PEC*	Skin	*L. monocytogenes*	*L. monocytogenes*	2.329	3b	MPIPC, CFX, FOM
16	83PEC*	Minced meat	*L. monocytogenes*	*L. monocytogenes*	2.236	1/2b	CFX, FOM
17	83SEC	Minced meat	*L. monocytogenes*	*L. monocytogenes*	2.43	1/2b	CFX, FOM

*12 different isolates in 2022 used for detection of virulence genes and biofilm forming ability

CFX: Cefoxitin, MPIPC: Oxacillin, FOM: Fosfomycin

** The names of the isolated *L. monocytogenes* were designed according to their sample number (1-85) and enrichment cultures (primary enrichment culture, PEC and secondary enrichment culture, SEC).

Table 2.5. Phenotypic antibiogram profile with minimum inhibitory concentration values of *L. monocytogenes* isolated in 2022

No.	Isolate	MIC values (µg/mL)																		
		Oxacillin	Ampicillin	Cefazolin	Cefmetazole	Flomoxef	Imipenem	Gentamycin	Arbekacin	Minocycline	Cefoxitin	Erythromycin	Clindamycin	Vancomycin	Teicoplanin	Linezolid	Fosfomycin	Sulfamethoxazole-	Trimethoprim	Levofloxacin
1	5PEC	2	0.25	1	32	8	0.25	0.25	0.25	2	>16	0.12	1	1	0.5	2	32	9.5/0.5		1
2	5SEC	2	0.25	1	32	8	0.25	0.25	0.25	2	>16	0.12	1	1	0.5	2	32	9.5/0.5		1
3	25PEC	4	0.25	2	32	8	0.25	0.5	0.25	2	>16	0.12	0.25	1	0.5	2	32	9.5/0.5		1
4	25SEC	4	0.25	2	32	8	0.25	0.5	0.5	2	>16	0.12	0.25	1	0.5	2	32	9.5/0.5		1
5	26PEC	2	0.25	2	32	8	0.25	0.25	0.5	2	>16	0.12	0.5	1	0.5	2	32	9.5/0.5		1
6	26SEC	2	0.25	2	32	8	0.25	0.25	0.5	2	>16	0.12	0.5	1	0.5	2	32	9.5/0.5		1
7	28PEC	4	0.25	2	32	8	0.25	0.25	0.25	2	>16	0.12	1	1	0.5	2	32	9.5/0.5		1
8	28SEC	4	0.25	2	32	8	0.25	0.25	0.25	2	>16	0.25	1	1	0.5	2	32	9.5/0.5		1
9	45PEC	4	0.25	2	32	8	0.25	0.25	0.25	2	>16	0.12	1	1	0.5	2	32	9.5/0.5		1
10	45SEC	2	0.12	1	16	8	0.25	0.25	0.25	2	>16	0.25	1	1	0.5	2	32	9.5/0.5		1
11	54PEC	4	0.25	1	16	8	0.25	0.25	0.5	2	>16	0.25	1	1	0.5	2	128	9.5/0.5		1
12	54SEC	2	0.25	1	16	8	0.25	0.25	0.5	2	>16	0.25	2	1	0.5	2	128	9.5/0.5		1
13	55PEC	2	0.25	2	32	8	0.25	0.25	0.25	2	>16	0.12	0.5	1	0.5	2	32	9.5/0.5		1
14	55SEC	2	0.25	2	32	8	0.25	0.5	0.25	2	>16	0.12	0.5	1	0.5	2	128	9.5/0.5		2
15	81PEC	4	0.25	2	32	8	0.25	0.25	0.25	2	>16	0.25	1	1	0.5	2	128	9.5/0.5		0.5
16	83PEC	2	0.5	2	32	8	0.25	0.25	0.25	2	>16	0.25	2	1	0.5	2	128	9.5/0.5		1
17	83SEC	2	0.5	2	32	8	0.25	0.25	0.25	2	>16	0.25	2	1	0.5	2	128	9.5/0.5		1

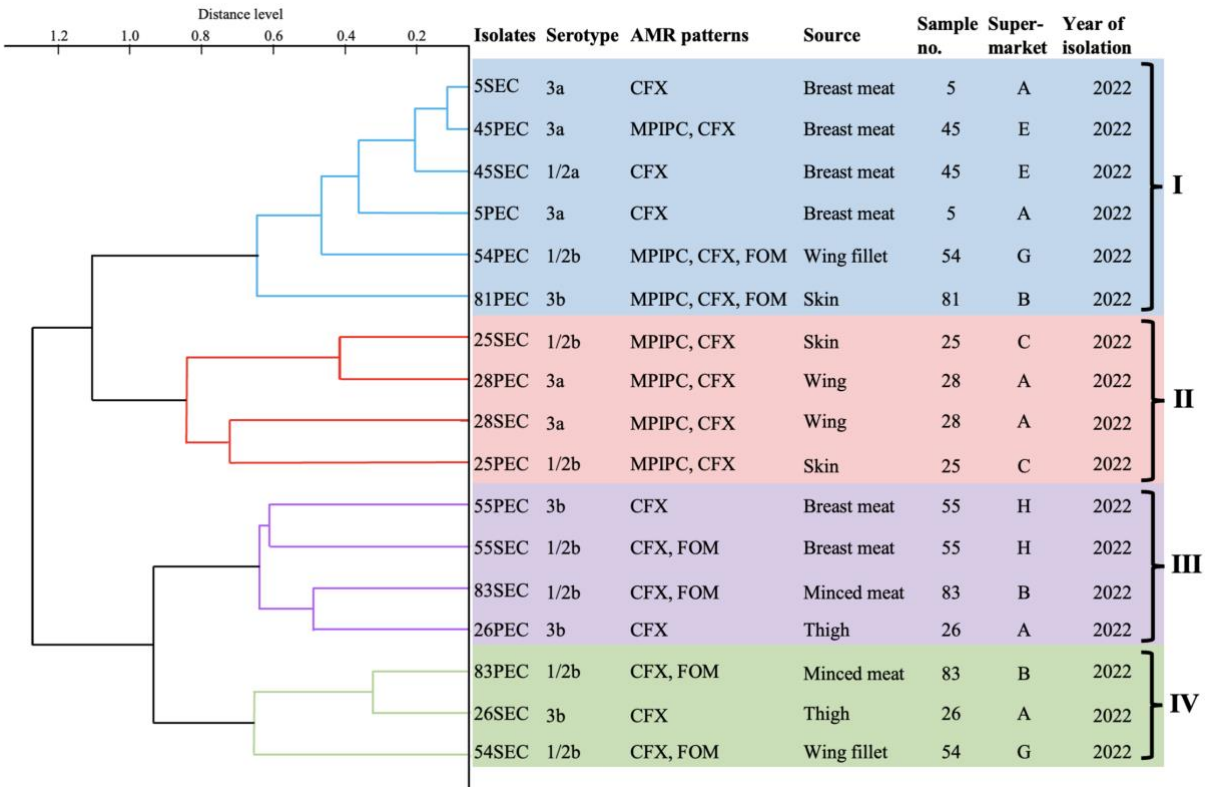
Susceptible
Intermediate
Resistance

Table 2.6. Antimicrobial resistance profiles of *L. monocytogenes* strains isolated in 2022

Antimicrobial agents	Concentrations (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	Number of Isolates (%)		
				S	I	R
Oxacillin	0.12, 0.25, 0.5, 1, 2, 4	2	4	7 (58.3)	-	5 (41.7)
Ampicillin	0.12, 0.25, 0.5, 4, 8, 16	0.25	0.25	12 (100)	-	-
Cefazolin	0.5, 1, 2, 4, 8, 16	2	2	12 (100)	-	-
Cefmetazole	1, 2, 4, 8, 16, 32	32	32	3 (25)	9 (75)	-
Flomoxef	0.5, 1, 2, 4, 8, 16	8	8	12 (100)	-	-
Imipenem	0.25, 0.5, 1, 2, 4, 8	0.25	0.25	12 (100)	-	-
Gentamycin	0.25, 0.5, 1, 2, 4, 8	0.25	0.5	12 (100)	-	-
Arbekacin	0.25, 0.5, 1, 2, 4, 8	0.25	0.5	12 (100)	-	-
Minocycline	2, 4, 8	2	2	12 (100)	-	-
Cefoxitin	4, 8, 16	>16	>16	-	-	12 (100)
Erythromycin	0.12, 0.25, 0.5, 1, 2, 4	0.12	0.25	12 (100)	-	-
Clindamycin	0.06, 0.12, 0.25, 0.5, 1, 2	1	2	12 (100)	-	-
Vancomycin	0.5, 1, 2, 4, 8, 16	1	1	12 (100)	-	-
Teicoplanin	0.5, 1, 2, 4, 8, 16	0.5	0.5	12 (100)	-	-
Linezolid	0.25, 0.5, 1, 2, 4, 8	2	2	12 (100)	-	-
Fosfomycin	32, 64, 128	32	128	7 (58.3)	-	5 (41.7)
Sulfamethoxazole-Trimethoprim	9.5/0.5, 19/1, 38/2	0.5	0.5	12 (100)	-	-
Levofloxacin	0.25, 0.5, 1, 2, 4	1	1	12 (100)	-	-

MIC- Minimum Inhibitory Concentration

S- Susceptible, I- Intermediate, R- Resistance



Isolates	Serotype	AMR patterns	Source	Sample no.	Super-market	Year of isolation	
5SEC	3a	CFX	Breast meat	5	A	2022	I
45PEC	3a	MPIPC, CFX	Breast meat	45	E	2022	
45SEC	1/2a	CFX	Breast meat	45	E	2022	
5PEC	3a	CFX	Breast meat	5	A	2022	
54PEC	1/2b	MPIPC, CFX, FOM	Wing fillet	54	G	2022	
81PEC	3b	MPIPC, CFX, FOM	Skin	81	B	2022	
25SEC	1/2b	MPIPC, CFX	Skin	25	C	2022	II
28PEC	3a	MPIPC, CFX	Wing	28	A	2022	
28SEC	3a	MPIPC, CFX	Wing	28	A	2022	
25PEC	1/2b	MPIPC, CFX	Skin	25	C	2022	
55PEC	3b	CFX	Breast meat	55	H	2022	III
55SEC	1/2b	CFX, FOM	Breast meat	55	H	2022	
83SEC	1/2b	CFX, FOM	Minced meat	83	B	2022	
26PEC	3b	CFX	Thigh	26	A	2022	
83PEC	1/2b	CFX, FOM	Minced meat	83	B	2022	IV
26SEC	3b	CFX	Thigh	26	A	2022	
54SEC	1/2b	CFX, FOM	Wing fillet	54	G	2022	

Figure 2.1. A principal component analysis (PCA) dendrogram of MALDI-TOF MS analysis for 17 *L. monocytogenes* in 2022. Strains are grouped into four clusters (numbered I–IV) by PCA analysis.

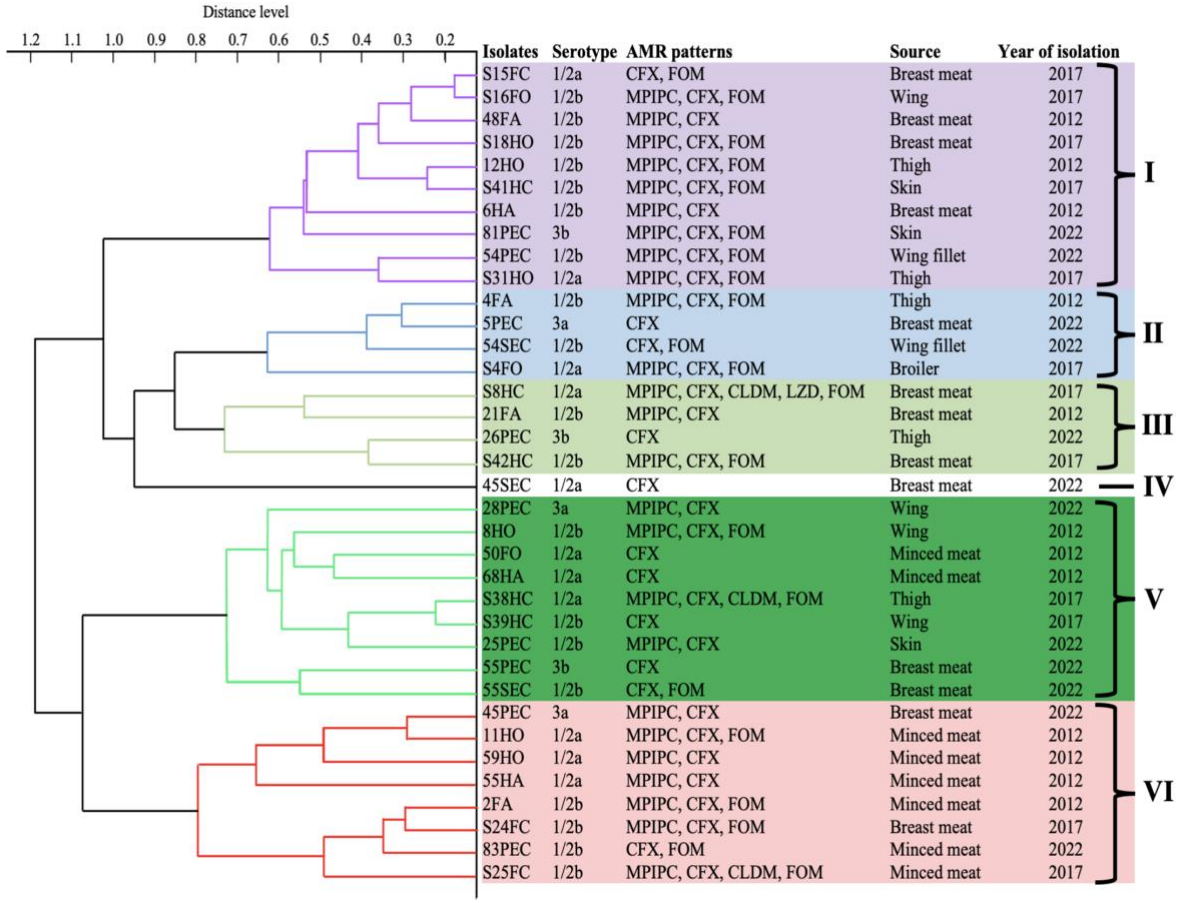


Figure 2.2. A principal component analysis (PCA) dendrogram of MALDI-TOF MS analysis for 36 *L. monocytogenes* strains in 2012, 2017, and 2022. Strains are grouped into six clusters (numbered I–VI) by PCA analysis.

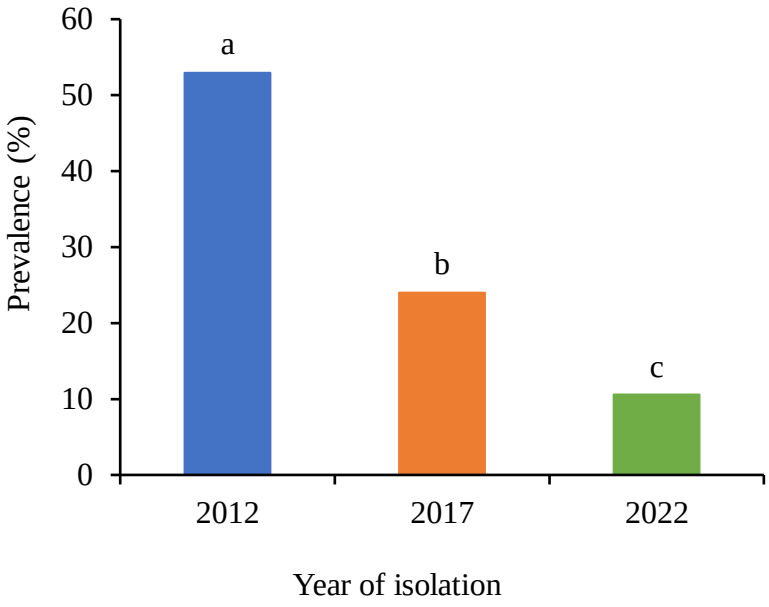


Fig. 2.3. Changes of occurrence of *L. monocytogenes* isolated in 2012, 2017, and 2022. Different letters (a-c) signify significant differences, $P < 0.05$.

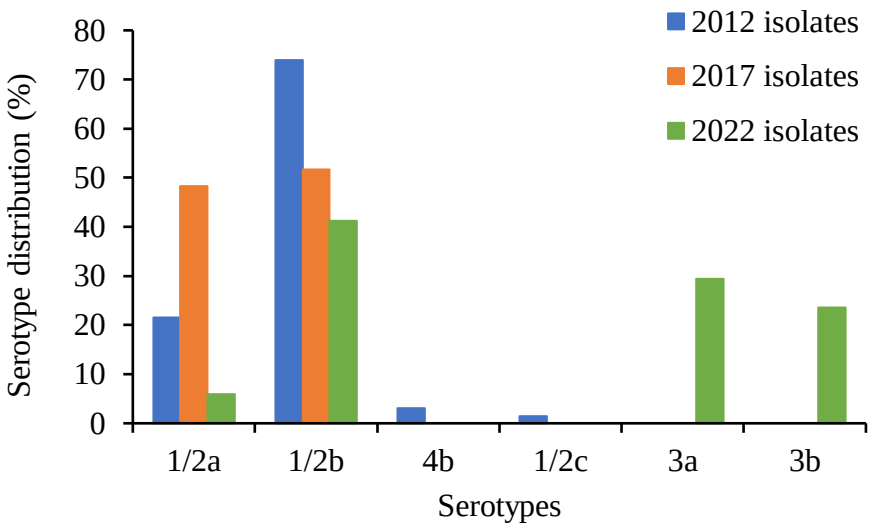


Fig. 2.4. Changes of serotype distribution of *L. monocytogenes* isolated in 2012, 2017, and 2022.

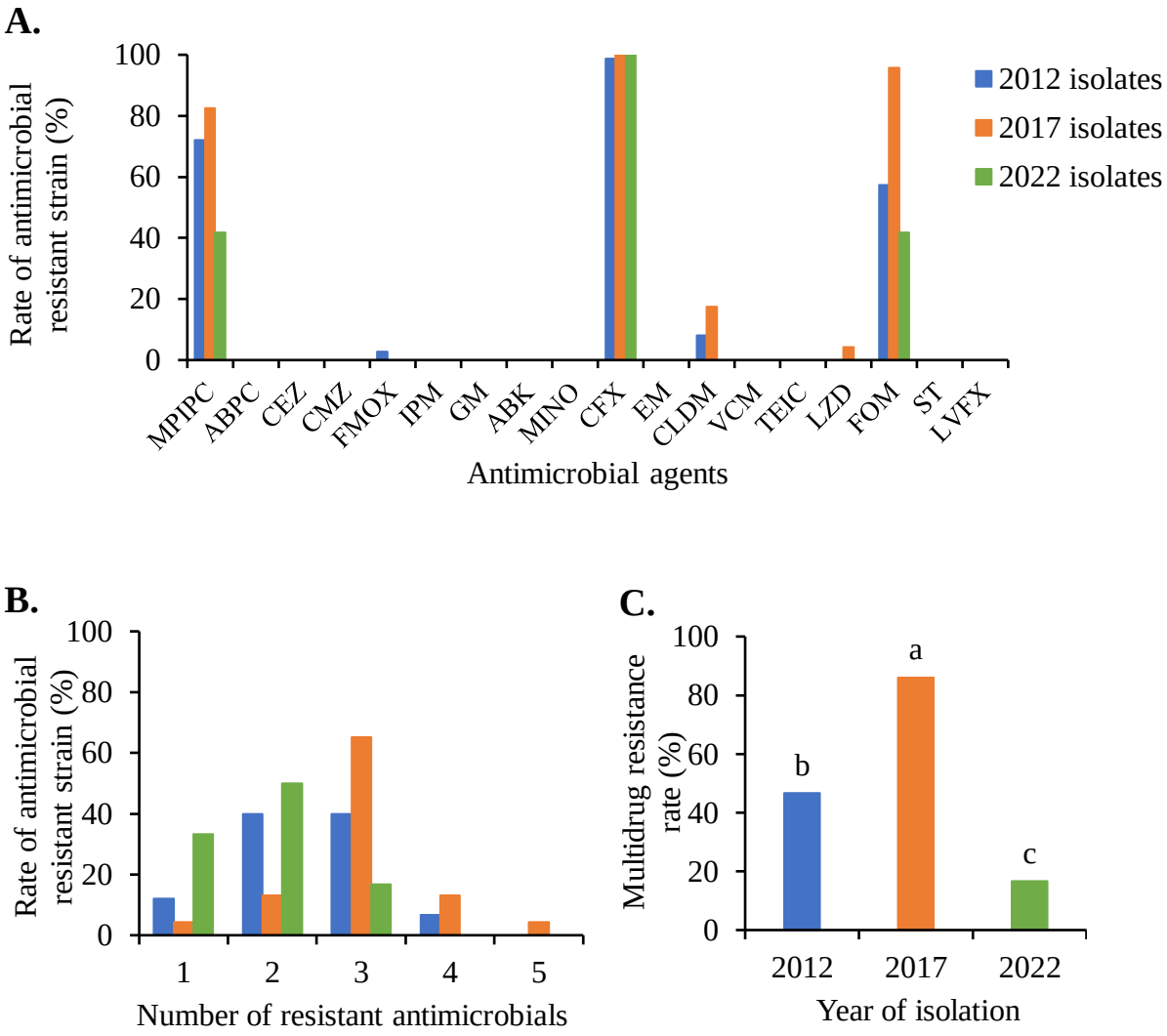


Fig. 2.5. Changes of antimicrobial resistance profiles of *L. monocytogenes* isolated in 2012, 2017, and 2022. (A) Rate of *L. monocytogenes* resistant to antibiotics tested [MPIPC- Oxacillin, ABPC- Ampicillin, CEZ- Cefazolin, CMZ- Cefmetazole, FMOX- Flomoxef, IPM- Imipenem, GM- Gentamycin, ABK- Arbekacin, MINO- Minocyline, CFX- Cefoxitin, EM- Erythromycin, CLDM- Clidamycin, VCM- Vancomycin, TEIC- Teicoplanin, LZD- Linezolid, FOM- Fosfomycin, ST- Sulfomethoxazole-Trimethoprim, LVFX- Levofloxacin] (B) *L. monocytogenes* distribution in number of resistant antimicrobials (C) Multidrug resistance rate of *L. monocytogenes*. The letters (a-c) show a significant difference at $P < 0.05$.

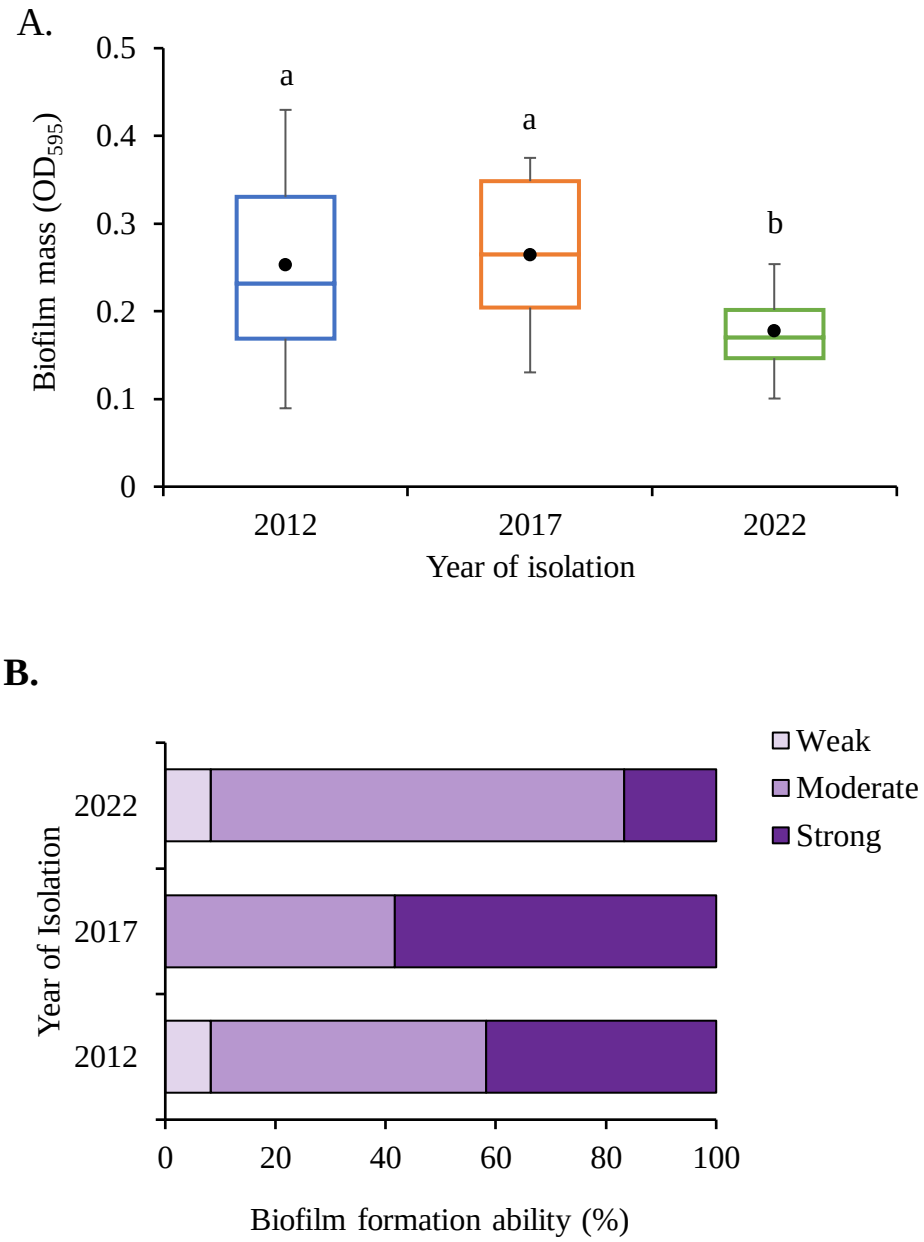


Fig. 2.6. Changes of biofilm formation capability of *L. monocytogenes* isolated in 2012, 2017, and 2022. (A) Biofilm mass (OD_{595}) of the isolates in different years. Whiskers extend to minimum and maximum values, and the horizontal line and the dot in the box represent median and mean values, respectively. The letters (a-c) show a significant difference at $P < 0.05$. (B) Biofilm formation intensity in different years of isolates.

Chapter 3. Isolation and characterization of bacteriophages specific to *Listeria monocytogenes*

3.1. Introduction

Listeria monocytogenes is a Gram-positive foodborne pathogen, widely distributed in soil, water, farms, industrial plants, and various food sources. It can cause listeriosis infection with the highest mortality rate of about 20-30 % among foodborne outbreaks (Santativongchai et al., 2023). *Listeria* infections can lead to several problems, with fatality rates of 50 %, 70 %, and 80 % for sepsis, meningitis, and maternal-fetal infections, respectively (Pushkareva and Ermolaeva, 2010; Shamloo et al., 2019). Due to the misuse and overuse of antibiotics for various purposes, establishing antimicrobial resistance (AMR) in foodborne bacteria has become a global public health issue. If the AMR is not tackled now, the predicted death rate will climb to 10 million and economic losses of over $100 trillion by 2050 (Elsayed et al., 2023). Therefore, efforts to find appropriate alternative antibiotics have increased interest in recent years.

Bacteriophages or phages are bacterial viruses that have been considered promising antibacterial agents. Lytic phages are utilized securely and successfully because they solely interact with the target host bacteria and are not harmful to humans or animals (Garvey, 2020). They are also useful in phage-based biosensors, which are used to rapidly, precisely, and specifically identify hazardous bacteria in environmental samples (Marzban et al., 2022). Furthermore, they are effective as bio-decontamination agents against harmful bacteria in hospitals, livestock, and food supply systems (Gamachu and Debalo, 2022).

The control strategies of *L. monocytogenes* by using the potential bacteriophages in fruits, ready-to-eat foods, milk, and cheese have been studied previously (Guenther and Loessner, 2011; Leverentz et al., 2003; Rodríguez-Rubio et al., 2015). Some

countries, including the United States, Switzerland, and Canada, permit the use of phages in food production. *Listeria* phage-based products such as Listex™ P100 (bacteriophage P100), and ListShield™ (a mixture of six different bacteriophages) are available in commercial (Scattolini et al., 2021). Additionally, there has been an increase in research on the use of phages against pathogenic bacteria in food processing environments and clinical settings to eliminate and prevent the formation of biofilms (Sulakvelidze, 2011).

Many phages are limited in their ability to lyse specific bacterial species, making them less useful as a wide biocontrol for bacterial infections in the food sector (Zhou et al., 2020). Therefore, this chapter aimed to isolate the specific bacteriophages against *L. monocytogenes* and to characterize the isolated bacteriophages phenotypically and genotypically.

3.2. Materials and methods

3.2.1. Bacterial strains and culture conditions

The bacterial strains used in this study are shown in Table 3.1. Bacterial cells were prepared by streaking the frozen stock (−80 °C) onto tryptic soy agar (TSA, Becton, Dickinson and Company, Sparks, MD, USA) and incubated overnight at 30 °C. Then, a single colony was picked and inoculated into 5 mL of Brain Heart Infusion broth (BHI, Becton, Dickinson and Company, Sparks, MD, USA). After incubation at 30 °C for 18-24 h, the cultures were used for subsequent experiments.

3.2.2. Isolation, purification, and propagation of bacteriophages

For the isolation of bacteriophages, 65 raw chicken meats and visceral samples were bought from supermarkets in Fukuoka, and 15 cattle farm samples including raw milk, faeces, compost, soil, and bedding were collected from the cattle farm in Kyushu University. Ten *L. monocytogenes* strains with varied serotypes, ribotypes, and AMR patterns, isolated from different sources, were employed as host strains (Table 3.1).

Briefly, each sample (50 g) was inoculated with an equivalent mixture (100 μL) of 10 bacterial suspensions, homogenized with 100 mL of Luria Bertani broth (Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 10 mmol/L $CaCl_2$ in a sterile stomacher bag, and cultured at 30 °C for 24 h. The culture (10 mL) was centrifuged at 12,000 × *g* for 20 min at 4 °C, and the supernatant was filtered through a membrane filter with 0.22-μm pore size (Merck Millipore, Ireland). As a preliminary step to isolate phages, spot assays were used to screen the filtrates. The overnight culture of each strain (100 μL) was mixed with 4 mL of molten top agar (Luria Bertani broth containing 0.5 % agar) with 1 mmol/L $CaCl_2$, maintained at 55 °C, and poured onto a TSA plate. After that, 10 μL of the filtrates were spotted on the bacterial lawn plate, dried, and incubated at 30 °C for 24 h. The filtrate that had shown clear plaque was used to isolate the individual phage using the plaque assay. The filtrate (100 μL) was mixed with the same volume of host cell suspension and left at 30 °C for 1 h. The mixture (200 μL) was added to top agar (4 mL), poured onto a TSA plate, and kept at 30 °C for 24 h.

For the purification of phages, a single plaque was picked and suspended in 1 mL of saline magnesium (SM) buffer (0.05 M Tris-HCl buffer, 0.008 M $MgSO_4$, 0.1 M NaCl, and 0.01 % gelatin). The phage suspension was 10-fold serially diluted with the same buffer. The diluted phage suspension (100 μL) was mixed with its host culture (100 μL) in 4 mL top agar. Then, the mixture was immediately poured onto a TSA plate, and kept overnight at 30 °C for plaque formation. This step was repeated at least 3 times to ensure the purity of the phages.

For the propagation of phages, the purified phage suspensions (10^4-10^6 PFU/ mL) were mixed with their host strains, added in 4 mL top agar, and poured onto TSA plates, then incubated at 30 °C for 24 h. The SM buffer (5 mL) was added to the dense plaque plates and shaken overnight at room temperature (25 °C). The suspensions were collected by centrifugation at 12,000 × *g* at 4 °C for 20 min. The supernatants were

filtered, and the filtrates were stored at 4 °C as phage stocks (10^8-10^{10} PFU/mL) for further experiment.

3.2.3. Host range determination of isolated phages

The host ranges of the isolated phages against 21 *L. monocytogenes* strains, 8 *Listeria* spp., and other bacterial strains (Tables 3.1, 3.4, and 3.5) were determined by spot assay. Bacterial lawns were prepared by spreading 100 µL of each bacterial culture on TSA plates, and then 10 µL of phage suspensions (10^8 PFU/mL) were spotted onto the plate and maintained at 30 °C for 24 h. The lytic spectra were interpreted as (+) clear lysis, (±) turbid, and (-) no lysis.

3.2.4. Morphological observation of plaques

According to host range determination, 3 different phages: vB_LmoS-PLM9, which was isolated from chicken chopped meat; vB_LmoS-PLM26 from cattle faeces; and vB_LmoS-PLM34 from chicken skin were selected and observed their morphological differences from the plaque assay plates.

3.2.5. Transmission electron microscopy (TEM) observation

The widest host bacteriophage vB_LmoS-PLM34 was observed with TEM. The phage stock (10^{10} PFU/mL) was placed onto carbon-coated grids, stained with 2 % uranyl acetate, and visualized at a voltage of 100 kV using Hitachi TEM H-7650 (Hitachi Co., Ltd., Japan).

3.2.6. Temperature and pH stability tests

The stability tests were performed for 3 different phages. For temperature stability, 1 mL of phage suspension in SM buffer at the final concentration (10^8 PFU/mL) was placed in a 1.5 mL Eppendorf tube and incubated from 4 to 90 °C for 1 h using a mini cooling dry bath incubator, MC-0203 (Major Science Co., Ltd., Taiwan). For pH stability, 100 µL of phage suspension (10^9 PFU/mL) was mixed with 900 µL of pH

values ranging from 2 to 10 of SM buffer and incubated at 30 and 4 °C for 24 h. After that, the viable phage counts were determined using plaque assay.

3.2.7. Genomic DNA sequencing and bioinformatic analysis

The genomic DNA of 3 phages was extracted using a High Pure Viral Nucleic Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The whole genomic sequencing was performed using the Illumina HiSeq system, read sequences were assembled using DFAST version 1.2.18, and the completed sequences were annotated using Prokka 1.14.6 (Seemann, 2014). Open reading frames (ORF) were predicted using the NCBI nucleotide and protein BLAST databases. The REsfinder-3.1 server (Zankari et al., 2012) and VirulenceFinder-2.0 server (Joensen et al., 2014) were used to search for antibiotic resistance and virulence factors genes.

3.2.8. Determination of latent period and burst size

The one-step growth curve of lytic phage vB_LmoS-PLM9 was performed using the protocol by Zhao et al. (2023). The target host strain of phage vB_LmoS-PLM9, *L. monocytogenes* 193 was used in this experiment. Briefly, bacterial culture (900 μL) was mixed with phage suspension (100 μL) to obtain a multiplicity of infection (MOI) of 0.01 and incubated at 30 °C for 15 min to allow phage adsorption. The mixture was centrifuged at 12,000 × *g* for 5 min. Then, the pellet was resuspended in 10 mL of pre-warmed BHI broth and incubated at 30 °C with shaking for 150 min. The sample (200 μL) was withdrawn every 10 min, centrifuged, and then the supernatant was recovered and analyzed using a plaque assay. The latent period was determined as the time interval from infection to the initial release of phage progeny. The burst size was calculated by dividing the number of phages at the end of the rise period by the number of phages at the beginning of the rise period.

3.3. Results

3.3.1. Isolation of *L. monocytogenes* specific phages

Nine phages (named PLM1-PLM9) targeting *L. monocytogenes* 193 (serotype 4b) and 2 phages (PLM31 and PLM34) targeting *L. monocytogenes* 174 (serotype 1/2a) were isolated from raw chicken samples (Table 3.2). From the cattle farm samples, 8 phages (PLM10-PLM17) were isolated using the host strain *L. monocytogenes* 193 (serotype 4b), 5 phages (PLM26-PLM30) against *L. monocytogenes* ATCC15313^{T}, and 2 phages (PLM32 and PLM33) against *L. monocytogenes* 174 (serotype 1/2a) as described in Table 3.3.

3.3.2. Host range of isolated phages

The lytic spectra of the isolated phages are shown in Tables 3.4 and 3.5. The phages (PLM1-PLM9 and PLM10-PLM17) showed similar lytic spectra (41.4 %), phages (PLM26-PLM30) were able to lyse (37.9-51.7 %), and phages (PLM31-34) exhibited lytic activities (89.7-96.6 %). All phages were active against *L. monocytogenes* and other *Listeria* spp., such as *L. innocua, L. seeligeri, L. welshimeri,* and *L. grayi,* but not against *L. ivanovii* and other bacterial genera.

3.3.3. Morphology of plaques

Phage vB_LmoS-PLM9 formed a wide and clear plaque (2.5-3 mm) with a clear boundary. Phages vB_LmoS-PLM26 and vB_LmoS-PLM34 formed medium-size plaques (1-1.3 mm) and pin-size plaques (0.5-0.6 mm).

3.3.4. Morphology of isolated phage

The TEM picture of the phage vB_LmoS-34 is shown in Fig. 3.1. This phage was characterized as a member of the *Siphoviridae* family under the *Caudovirilae* order, presenting an icosahedral head (diameter ~ 60 nm) and a long and noncontractile tail (length ~ 230 nm).

3.3.5. Temperature and pH stability

Phages vB_LmoS-PLM9 and vB_LmoS-PLM 26 were stable at temperatures ranging from 4 to 50 °C but gradually declined as temperatures increased from 60 to 90 °C. Phage vB_LmoS-PLM34 was stable at 4–40 °C, but its activity reduced at 50 °C and was not identified at 60–90 °C (Fig. 3.2A). The titers of all phages were not changed at pH from 3 to 10 at 4 °C for 24 h, while they were stable at pH from 4 to 10 at 25 °C for 24 h (Fig. 3.2B, C, and D).

3.3.6. Genetic characterization of phages

A genome map of phage vB_LmoS-PLM9 is shown in Fig. 3.3. The nucleotide sequence of vB_LmoS-PLM9 was registered in the Gene bank under the accession number OR371505. This phage was a circular double-stranded DNA genome composed of 38,345 bp with 36 % G+C content. A total of 62 ORFs were predicted in the genome, of which 32 (51.6 %) were related to known functions and 30 (48.4 %) were assigned to hypothetical functions. The genome did not contain tRNA, antibiotic resistance, virulence, or toxin genes. Phage vB_LmoS-PLM9 had an N-acetylmuramoyl-L-alanine amidase endolysin gene. The genome annotation results are presented in Table 3.6. These proteins were classified into five groups: (i) phage structure and packing, (ii) DNA replication, (iii) host lysis, (iv) hypothetical proteins, and (v) additional functions (Fig. 3.3). The BLAST search indicated that the genomic DNA of vB_LmoS-PLM9 had high sequence identity with those of *Listeria* phage vB_LmoS_188 (query cover 66 %; identity 96.45 %; accession number NC_028871.1) and *Listeria* phage PSU-VKH-LP019 (query cover 77 %; identity 92.49 %; accession number MH341451.1).

A genome map of phage vB_LmoS-PLM26 is also shown in Fig. 3.4. The nucleotide sequence of vB_LmoS-PLM26 was registered in the Gene bank under the accession number OP926971. It had a circular double-stranded DNA genome

composed of 41,221 bp with 35 % G+C content. A total of 62 ORFs were predicted in the genome, of which 35 (59.3 %) were related to known functions and 24 (40.7 %) were assigned to hypothetical functions. Phage vB_LmoS-PLM26 contained peptidoglycan l-alanyl-d-glutamate endopeptidase endolysin gene. And the genome did not contain tRNA, antibiotic resistance, virulence, or toxin genes. The genome annotation results are presented in Table 3.7. The BLAST search indicated that the genomic DNA of vB_LmoS-PLM26 had high sequence identity with those of *Listeria* phage LP-101 (query cover 84 %; identity 93.49 %; accession number NC_024387.1) and *Listeria* phage LP-P111 (query cover 70 %; identity 91.93 %; accession number OP779214.1).

As illustrated in Fig. 3.5, phage vB_LmoS-PLM34 had a circular double-stranded DNA genome of 32,895 bp with 37 % G+C content. A total of 53 ORFs were predicted in the genome, of which 35 (66 %) were related to known functions and 18 (34 %) were assigned to hypothetical functions. Phage vB_LmoS-PLM34 also contained a gene encoding lysin with N-acetylmuramoyl-L-alanine amidase activity, and the genome did not contain tRNA, antibiotic resistance, virulence, or toxin genes. The genome annotation results are presented in Table 3.8. The nucleotide sequence of vB_LmoS-PLM34 was also registered in the Gene bank under the accession number OP926972. The BLAST search indicated that the genomic DNA of phage vB_LmoS-PLM34 had somewhat similar sequences with those of *Listeria* phage A118 (query cover 5 %; identity 96.62 %; accession number NC_003216.1) and *Listeria* phage A500 (query cover 4 %; identity 82.91 %; accession number NC_00981.1).

3.3.7. Latent period and burst size of a lytic phage vB_LmoS-PLM9

As shown in Fig. 3.6, the one-step growth curve of phage vB_LmoS-PLM9 indicated a latent period of 70 min and a burst size of 51.2 ± 2.1 PFU/cell.

3.4. Discussion

Bacteriophages have recently been identified as potential antibacterial agents in the food sector due to their ability to kill dangerous microorganisms (Zhou et al., 2020). However, phage-based biocontrol approaches have a severely limited antibacterial spectrum because most phages are generally exclusive to a bacterial strain or species, and occasionally to a genus (Hagens and Loessner, 2010). Therefore, a phage capable of infecting various target bacterial strains still needs to be discovered as the best candidate for developing a phage-based biocontrol strategy.

In this study, bacteriophages were isolated from the same set of raw chicken samples used for *L. monocytogenes* isolation in previous Chapter 2. Since chicken meat is the most consumable meat and abundantly sold with varieties of brands in supermarkets, we used raw meat samples to isolate naturally, environmentally friendly, and beneficial phages. Cattle farm samples including raw milk, faeces, compost, pasture soil, and bedding were also used to isolate the phages because phages are naturally abundant in livestock environments. A total of 11 phages from various parts of raw chicken samples and 15 phages from cattle farm samples were isolated by targeting the 3 different *L. monocytogenes*' host strains no. 193 (serotype 4b), no. 174 (serotype 1/2a), and standard strain ATCC15313^{T}. The titrations of all phages in this study were 3.5×10^{8} – 1.7×10^{10} PFU/mL, which were consistent with many studies, phage lysates were between 10^{6} and 10^{11} PFU/mL (Stephenson, 2016; Marzban et al., 2022). Most of the isolated phages in this investigation showed lytic activities against *L. innocua, L. seeligeri, L. welshimeri,* and *L. grayi*, as well as *L. monocytogenes*. A similar study by Zhou et al. (2020) found that the *Listeria* phage vB_LmoM-SH3-3 can lyse *L. monocytogenes*, *L. innocua*, and *L.welshimeri*. Despite other *Listeria* spp. lacking pathogenic characteristics, they might nevertheless be sources of virulence and resistance-associated genes. Gómez et al. (2014) documented pathogenic bacteria may be able to acquire resistance genes from the reservoir of resistance genes formed by *L.*

innocua. In this instance, broad-host-range phages will have a wider range of applications.

According to host range determination and plaque morphology, the broadest host range phage vB_LmoS-PLM34, followed by vB_LmoS-PLM9 and vB_LmoS-PLM26 were selected for further characterization. Temperature and pH are generally regarded to be the key factors influencing phage activity in the food environment. Phages typically become inactive at high temperatures, but some phages have been shown to survive temperatures as high as 60 °C. Previous studies reported that *Listeria* phages were stable at pH ranging from 4 to 10 (Byun et al., 2022; Deniz Ayaz and Cufaoglu, 2016). These findings agree with our results. Phage vB_LmoS-PLM34 was stable at temperatures between 4 and 40 °C, while phages vB_LmoS-PLM9 and vB_LmoS-PLM26 were relatively stable at temperature range from 4 to 50 °C. Furthermore, all three phages remained stable at pH levels ranging from 4 to 10, indicating their efficacy in various foods processed under different conditions.

Genomic sequencing analysis indicated that all 3 phages were circular and double-stranded DNAs. All the phages did not contain tRNA, virulence, and antimicrobial resistance genes. However, phages vB_LmoS-PLM26 and vB_LmoS-PLM34 contained lysogeny genes, while phage vB_LmoS-PLM9 did not. Therefore, phage vB_LmoS_PLM9 seems to be a lytic phage, suggesting it can be safely used in food applications. Nevertheless, 3 different phages possessed the important features that their genomes encoded with lytic proteins such as endolysin and holin. Phage vB_LmoS-PLM9 and vB_LmoS-PLM34 genomes encoded N-acetylmuramoyl-l-alanine amidases, whereas phage vB_LmoS-PLM26 genome encoded peptidoglycan l-alanyl-d-glutamate endopeptidase. These phage-derived lysins will be expressed into recombinant proteins, and the mechanism by which the phage rapidly lyses bacterial cells will be examined in our future study. The genetic engineering of lysogenic phage to be promising virulent phage should also be investigated.

3.5. Summary

This chapter investigated the isolation and characterization of bacteriophages specific to *L. monocytogenes*. Eleven phages from raw chicken samples and 15 phages from cattle farm samples were isolated. All the isolated phages exhibited lytic activities against *L. monocytogenes* as well as *L. innocua, L. seeligeri, L. welshimeri,* and *L. grayi.* The TEM observation of the widest host range phage vB_LmoS-PLM34 showed an icosahedral head (diameter ~ 60 nm) and a long and noncontractile tail (length ~ 230 nm) under the *Siphoviridae* family. Phages vB_LmoS-PLM9 and vB_LmoS-PLM26 indicated high-temperature stability at 4-50 °C, while vB_LmoS-PLM9 was 4-40 °C. All 3 phages were stable at pH ranging from 4 to 10. Genomic sequencing analysis revealed that phage vB_LmoS-Plm9 was a lytic phage however, phages vB_LmoS-PLM26 and vB_LmoS-PLM34 were lysogenic phages. All phages lacked tRNA, virulence, or antimicrobial resistance genes and were composed of endolysin and holin. Phages vB_LmoS-PLM9 and vB_LmoS-PLM34 encoded N-acetylmuramoyl-l-alanine amidases while phage vB_LmoS-PLM26 encoded peptidoglycan l-alanyl-d-glutamate endopeptidase. One-step growth curve analysis of lytic phage vB_LmoS-PLM9 with MOI of 0.01 indicated a latent period of 70 min and a burst size of 51.2 ± 2.1 PFU/cell. Therefore, this study was successfully investigated in isolating and characterizing bacteriophages against *L. monocytogenes*, specifically three different phages.

Table 3.1. Bacterial strains used in this study

Bacterial strains	Serotype	Ribotype	Antimicrobial resistance patterns	Source
L. monocytogenes S8HC*	1/2a	ECORI 422-90-S-1	MPIPC,CFX,CLDM,LZD,FOM	Breast
L. monocytogenes S4HO	1/2a	ECORI 422-75-S-3	MPIPC,CFX,CLDM,FOM	Broiler
L. monocytogenes S25FC*	1/2b	ECORI 422-318-S-8	MPIPC,CFX,CLDM,FOM	Minced meat
L. monocytogenes S38HC*	1/2a	ECORI 422-320-S-6	MPIPC,CFX,CLDM,FOM	Thigh
L. monocytogenes S24FC	1/2b	ECORI 422-75-S-3	MPIPC,CFX,FOM	Breast
L. monocytogenes S31FC	1/2a	ND	MPIPC,CFX,FOM	Thigh
L. monocytogenes 8FO*	1/2b	ECORI 422-260-S-4	MPIPC,FMOX,CFX,FOM	Fillet
L. monocytogenes 76HA	1/2a	ECORI 422-246-S-5	MPIPC,FMOX,CFX,FOM	Wing drummette
L. monocytogenes 11FA	ND	ECORI 422-62-S-1	MPIPC,CFX,CLDM,FOM	Minced
L. monocytogenes 37HA	ND	ECORI 422-258-S-2	MPIPC,CFX,CLDM,FOM	Minced
L. monocytogenes 38FO	ND	ECORI 422-265-S-7	MPIPC,CFX,CLDM,FOM	Thigh fillet
L. monocytogenes 11HO	1/2a	ECORI 422-258-S-2	MPIPC,CFX,FOM	Minced
L. monocytogenes 12HA	1/2b	ECORI 422-258-S-5	MPIPC,CFX,FOM	Thigh
L. monocytogenes 26FA*	4b	ECORI 422-253-S-8	MPIPC,CFX,CLDM	Thigh
L. monocytogenes 56HO	1/2c	ECORI 422-244-S-3	MPIPC,FOM	Thigh
L. monocytogenes 152*	1/2c	ECORI 422-64-S-5	CFX	Beef
L. monocytogenes 174*	1/2a	ECORI 422-72-S-1	CFX	Human cerebrospinal fluid
L. monocytogenes 178*	1/2b	ECORI 422-63-S-6	CFX	Human blood
L. monocytogenes 185	4b	ECORI 422-78-S-3	CFX, FOM	Human cerebrospinal fluid
L. monocytogenes 193*	4b	ECORI 422-78-S-3	CFX, FOM	Minced pork
L. monocytogenes ATCC 15313^{T}*		American Type Culture Collection (USA)		
L. innocua ATCC 33090		American Type Culture Collection (USA)		
L. innocua LIS31		Obihiro University of Agriculture and Veterinary Medicine, Japan		
L. ivanovii ATCC 19119		American Type Culture Collection (USA)		
L. seeligeri ATCC 35967		American Type Culture Collection (USA)		
L. seeligeri LIS34		Obihiro University of Agriculture and Veterinary Medicine, Japan		
L. welshimeri ATCC 35897		American Type Culture Collection (USA)		
L. welshimeri LIS36		Obihiro University of Agriculture and Veterinary Medicine, Japan		
L. grayi ATCC 25401		American Type Culture Collection (USA)		
Bacillus cereus JCM152		Japan Collection of Microorganisms (RIKEN Saitama, Japan)		
Staphylococcus aureus ATCC 25923		American Type Culture Collection (USA)		
Enterococcus fecalis JCM 7783		Japan Collection of Microorganisms (RIKEN Saitama, Japan)		
Clostrium perfrigenes JCM 1290^{T}				
Salmonella Typhimurium NBRC 12529		National Institute of Technology and Evaluation Biological Resource Centre, Chiba, Japan		
Salmonella Enteriditis NBRC 3313				

L. monocytogenes strains were selected depend on different serotype, ribotype, and AMR patterns isolated from different sources

**L. monocytogenes* strains used for screening of bacteriophages isolation

Table 3.2. *L. monocytogenes* phages isolated from raw chicken samples

No.	Phage name	Sample code	Sources	Titer (PFU/mL)	Host strain
1	PLM1	CM-4	Chicken wing	1.7×10^{10}	*L. monocytogenes* 193, serotype 4b
2	PLM2	CM-6	Chicken ovary and oviduct	7.9×10^{9}	
3	PLM3	CM-7	Chicken thigh cartilage	1.7×10^{10}	
4	PLM4	CM-8	Chicken thigh fillet	4.9×10^{9}	
5	PLM5	CM-9	Chicken liver	8.2×10^{9}	
6	PLM6	CM-21	Chicken chopped meat	6.1×10^{9}	
7	PLM7	CM-59	Chicken breast (sasami)	5.8×10^{9}	
8	PLM8	CM-60	Chicken skin	2.6×10^{9}	
9	PLM9	CM-61	Chicken chopped meat	9.5×10^{9}	
10	PLM31	CM-9	Chicken liver	7.0×10^{8}	*L. monocytogenes* 174, serotype 1/2a
11	PLM34	CM-32	Chicken skin	1.2×10^{9}	

Table 3.3. *L. monocytogenes* phages isolated from cattle farm samples

No.	Phage name	Sample code	Sources	Titer (PFU/mL)	Host strain
1	PLM10	F-1	Raw milk	1.2×10^{10}	*L. monocytogenes* 193, serotype 4b
2	PLM11	F-3	Raw milk	5.1×10^{9}	
3	PLM12	F-5	Faeces	9.2×10^{9}	
4	PLM13	F-8	Compost	1.0×10^{10}	
5	PLM14	F-10	Pasture soil	8.4×10^{9}	
6	PLM15	F-12	Pasture soil	6.1×10^{9}	
7	PLM16	F-14	Bedding	1.4×10^{10}	
8	PLM17	F-15	Bedding	1.5×10^{10}	
9	PLM26	F-5	Faeces	5.7×10^{9}	*L. monocytogenes* ATCC 15313^{T}
10	PLM27	F-7	Compost	3.2×10^{9}	
11	PLM28	F-10	Pasture soil	4.2×10^{8}	
12	PLM29	F-14	Compost	7.5×10^{9}	
13	PLM30	F-6	Faeces	3.5×10^{8}	
14	PLM32	F-7	Compost	8.8×10^{8}	*L. monocytogenes* 174, serotype 1/2a
15	PLM33	F-10	Pasture soil	1.9×10^{9}	

Table 3.4. Lytic spectra of isolated phages from raw chicken samples

No	Bacterial strains	PLM1	PLM2	PLM3	PLM4	PLM5	PLM6	PLM7	PLM8	PLM9	PLM31	PLM34
1	*L. monocytogenes* S8HC*	-	-	-	-	-	-	-	-	-	±	±
2	*L. monocytogenes* S4HO	±	±	±	±	±	±	±	±	+	±	+
3	*L. monocytogenes* S25FC*	-	-	-	-	-	-	-	-	-	±	±
4	*L. monocytogenes* S38HC*	-	-	-	-	-	-	-	-	-	+	+
5	*L. monocytogenes* S24FC	-	-	-	-	-	-	-	-	-	±	±
6	*L. monocytogenes* 31FC	-	-	-	-	-	-	-	-	-	±	+
7	*L. monocytogenes* 8FO*	-	-	-	-	-	-	-	-	-	+	+
8	*L. monocytogenes* 76HA	+	+	+	+	+	+	+	+	+	+	+
9	*L. monocytogenes* 11FA	+	+	+	±	±	+	+	±	+	+	+
10	*L. monocytogenes* 37HA	-	-	-	-	-	-	-	-	-	+	+
11	*L. monocytogenes* 38FO	±	+	+	+	+	±	+	+	+	+	+
12	*L. monocytogenes* 11HO	-	-	-	-	-	-	-	-	-	+	+
13	*L. monocytogenes* 12HA	-	-	-	-	-	-	-	-	-	+	+
14	*L. monocytogenes* 26FA*	-	-	-	-	-	-	-	-	-	-	-
15	*L. monocytogenes* 56HO	-	-	-	-	-	-	-	-	-	+	+
16	*L. monocytogenes* 152*	-	-	-	-	-	-	-	-	-	+	+
17	*L. monocytogenes* 174*	-	-	-	-	-	-	-	-	-	+	+
18	*L. monocytogenes* 178*	-	-	-	-	-	-	-	-	-	+	+
19	*L. monocytogenes* 185	+	+	+	+	+	+	+	+	+	±	+
20	*L. monocytogenes* 193*	+	+	+	+	+	+	+	+	+	+	+
21	*L. monocytogenes* ATCC 15313^{T}*	-	-	-	-	-	-	-	-	-	+	+
22	*L. innocua* ATCC 33090	+	+	+	+	+	+	+	+	+	+	+
23	*L. innocua* LIS31	+	+	+	+	+	+	+	+	+	±	±
24	*L. ivanovii* ATCC 19119	-	-	-	-	-	-	-	-	-	-	-
25	*L. seeligeri* ATCC 35967	+	+	+	+	+	+	+	+	+	±	±
26	*L. seeligeri* LIS34	-	-	-	-	-	-	-	-	-	+	+
27	*L. welshimeri* ATCC 35897	+	+	+	+	+	+	+	+	+	±	±
28	*L. welshimeri* LIS36	+	+	+	+	+	+	+	+	+	±	±
29	*L. grayi* ATCC 25401	+	+	+	+	+	+	+	+	+	-	±
(+) clear lysis		10	11	11	10	10	10	11	10	12	16	19
(±) turbid		2	1	1	2	2	2	1	2	0	10	8
Total no. of lysed strains		12	12	12	12	12	12	12	12	12	26	27
(%)		41.4	41.4	41.4	41.4	41.4	41.4	41.4	41.4	41.4	89.7	93.1

*Bacterial strains used for screening of bacteriophages isolation

(+) clear lysis, (±) turbid, (-) No lysis

All of the phages are inactive against *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* JCM152, *Enterococcus fecalis* JCM 7783, *Clostrium perfrigenes* JCM 1290T, *Salmonella* Typhimurium NBRC 12529, *Salmonella* Enteriditis NBRC 3313.

Table 3.5. Lytic spectra of isolated phages from cattle farm samples

No.	Bacterial strains	PLM10	PLM11	PLM12	PLM13	PLM14	PLM15	PLM16	PLM17	PLM26	PLM27	PLM28	PLM29	PLM30	PLM32	PLM33
1	*L. monocytogenes* S8HC*	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
2	*L. monocytogenes* S4HO	±	+	+	±	+	+	±	±	+	+	+	+	+	+	+
3	*L. monocytogenes* S25FC*	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±
4	*L. monocytogenes* S38HC*	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
5	*L. monocytogenes* S24FC	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±
6	*L. monocytogenes* 31FC	-	-	-	-	-	-	-	-	+	-	-	±	-	+	+
7	*L. monocytogenes* 8FO*	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
8	*L. monocytogenes* 76HA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	*L. monocytogenes* 11FA	±	+	+	±	+	+	+	+	+	+	+	+	+	±	+
10	*L. monocytogenes* 37HA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
11	*L. monocytogenes* 38FO	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+
12	*L. monocytogenes* 11HO	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
13	*L. monocytogenes* 12HA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
14	*L. monocytogenes* 26FA*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	*L. monocytogenes* 56HO	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
16	*L. monocytogenes* 152*	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
17	*L. monocytogenes* 174*	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
18	*L. monocytogenes* 178*	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
19	*L. monocytogenes* 185	+	+	+	+	+	+	+	+	+	+	+	+	+	±	+
20	*L. monocytogenes* 193*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	*L. monocytogenes* ATCC 15313^{T}*	-	-	-	-	-	-	-	-	+	±	±	+	-	+	+
22	*L. innocua* ATCC 33090	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
23	*L. innocua* LIS31	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24	*L. ivanovii* ATCC 19119	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-
25	*L. seeligeri* ATCC 35967	+	+	+	+	+	+	+	+	±	-	-	±	-	±	±
26	*L. seeligeri* LIS34	-	-	-	-	-	-	-	-	±	-	-	±	-	+	+
27	*L. welshimeri* ATCC 35897	+	+	+	+	+	+	+	+	+	±	+	+	+	±	±
28	*L. welshimeri* LIS36	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±
29	*L. grayi* ATCC 25401	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±
(+) clear lysis		10	12	12	9	12	12	11	11	13	10	11	12	11	19	21
(±) turbid		2	0	0	3	0	0	0	1	2	2	1	3	0	9	6
Total no. of lysed strains		12	12	12	12	12	12	12	12	15	12	12	15	11	28	27
(%)		41.4	41.4	41.4	41.4	41.4	41.4	41.4	41.4	51.7	41.4	41.4	51.7	37.9	96.6	93.1

*Bacterial strains used for screening of bacteriophages isolation
(+) clear lysis, (±) turbid, (-) No lysis
All of the phages are inactive against *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* JCM152, *Enterococcus fecalis* JCM 7783, *Clostrium perfrigenes* JCM 1290T, *Salmonella* Typhimurium NBRC 12529, *Salmonella* Enteriditis NBRC 3313.

Table 3.6. Annotation of phage vB_LmoS-PLM9 genome

ORF	Position (5'-3')	Length (bp)	No. of amino acids	Size (kDa)	Predicted function
1	23-256	234	77	9.4	hypothetical protein
2	253-450	198	65	8	hypothetical protein
3	589-1947	1359	452	53.2	recombinase family protein
4	2012-2719	708	235	26.2	hypothetical protein
5	2740-3462	723	240	27.6	hypothetical protein
6	3478-3696	219	72	7.8	zinc ribbon domain-containing protein
7	3752-3919	168	55	6.5	hypothetical protein
8	4076-4558	483	160	18.5	helix-turn-helix transcriptional regulator
9	4707-4958	252	83	9.5	helix-turn-helix transcriptional regulator
10	4962-5156	195	64	7.3	hypothetical protein
11	5168-5434	267	88	9.9	hypothetical protein
12	5434-5595	162	53	6.1	hypothetical protein
13	5588-5830	243	80	9.2	hypothetical protein
14	5894-6664	771	256	29.1	phage repressor protein/ antirepressor Ant
15	6787-7320	534	177	19.8	hypothetical protein
16	7317-7532	216	71	8.4	hypothetical protein
17	7640-7828	189	62	7.3	gp45 family putative tail fiber system protein
18	8060-9019	960	319	36.7	phage exonuclease
19	9019-9834	816	271	30.6	recombinase RecT
20	9854-10,786	933	310	36.9	DnaD domain-containing protein
21	10,783-10,950	168	55	6.4	hypothetical protein
22	10,965-11,249	285	94	11.3	hypothetical protein
23	11,246-11,455	210	69	8.1	hypothetical protein
24	11,452-12,060	609	202	23.3	YopX family protein
25	12,057-12,425	369	122	14.2	hypothetical protein
26	12,422-12,598	177	58	6.6	hypothetical protein
27	12,585-13,028	444	147	17	hypothetical protein
28	13,029-13,202	174	57	6.4	hypothetical protein
29	13,199-13,618	420	139	15.7	hypothetical protein
30	13,618-14,100	483	160	17.8	single-stranded DNA-binding protein
31	14,132-14,437	306	101	11.7	response regulator
32	14,540-14,944	405	134	15.5	endodeoxyribonuclease
33	15,089-15,244	156	51	5.7	DUF3954 domain-containing protein
34	15,332-15,904	573	190	22.3	sigma-70 family RNA polymerase sigma factor
35	16,186-16,413	228	75	8.4	hypothetical protein
36	16,453-17,193	741	246	27.7	terminase small subunit
37	17,186-18,505	1320	439	50.5	terminase large subunit
38	18,520-20,076	1557	518	58.8	hypothetical protein

Table 3.6. *Cont.*

ORF	Position (5'-3')	Length (bp)	No. of amino acids	Size (kDa)	Predicted function
39	20,081-21,124	1044	347	40.8	minor capsid protein
40	21,219-21,773	555	184	21	BREX-1 system adenine-specific DNA-methyltransferase
41	21,796-22,668	873	290	32.1	hypothetical protein
42	22,682-22,837	156	51	6	hypothetical protein
43	22,838-23,191	354	117	12.6	hypothetical protein
44	23,191-23,556	366	121	13.6	hypothetical protein
45	23,546-23,863	318	105	12	HK97 gp10 family phage protein
46	23,860-24,231	372	123	14.2	hypothetical protein
47	24,236-24,922	687	228	23.6	Ig-like domain-containing protein
48	24,972-25,403	432	143	16.2	hypothetical protein
49	25,508-25,711	204	67	7.7	Gp15 family bacteriophage protein
50	25,716-30,521	4806	1601	174.3	tail tape measure protein
51	30,525-31,349	825	274	31.2	tail family protein
52	31,362-32,375	1014	337	37.7	phage tail protein
53	32,372-33,400	1029	342	37.9	hypothetical protein
54	33,397-34,470	1074	357	39.2	BppU family phage baseplate upper protein
55	34,467-34,808	342	113	12.5	hypothetical protein
56	34,808-34,954	147	48	5.6	XkdX family protein
57	34,982-35,356	375	124	14.7	gp23 protein
58	35,369-35,647	279	92	9.7	holin
59	35,650-36,519	870	289	33.2	N-acetylmuramoyl-L-alanine amidase
60	36,544-36,960	417	138	15.4	hypothetical protein
61	37,239-37,742	504	167	19.8	DUF4065 domain-containing protein
62	37,739-38,131	393	130	15.2	type II toxin-antitoxin system PemK/ Maz family toxin

Table 3.7. Annotation of phage vB_LmoS-PLM26 genome

ORF	Position (5'-3')	Length (bp)	No. of amino acids	Size (kDa)	Predicted function
1	1-1644	1644	547	63.3	terminase large subunit
2	1656-2786	1131	376	43.5	portal protein
3	2783-3580	798	265	28.6	Clp protease
4	3607-4758	1152	383	42.5	major capsid protein
5	4765-4935	171	56	6.6	hypothetical protein
6	4945-5244	300	99	11.5	gp6-like head-tail connector protein
7	5228-5593	366	121	13.8	head-tail adapter protein
8	5590-5991	402	133	14.8	hypothetical protein
9	5988-6371	384	127	14.6	DUF3168 domain-containing protein
10	6393-6980	588	195	21.1	tail protein
11	7052-7384	333	110	13.2	hypothetical protein
12	7435-7584	150	49	5.9	hypothetical protein
13	7600-12,525	4926	1641	174.8	tail tape measure protein
14	12,518-14,167	1650	549	62	tail protein
15	14,180-16,474	2295	764	87	tail protein
16	16,464-17,555	1092	363	39.5	carbohydrate-binding protein CenC
17	17,603-18,046	444	147	16.5	hypothetical protein
18	18,025-18,441	417	138	15.3	hypothetical protein
19	18,462-18,728	267	88	9.8	holin
20	18,725-19,435	711	236	25.8	Peptidoglycan L-alanyl-D-glutamate endopeptidase
21	19,590-20,162	573	190	22.3	hypothetical protein
22	20,230-20,679	450	149	17.2	anti-CRISPR protein AcrIIA1
23	20,684-21,055	372	123	14.2	anti-CRISPR protein AcrIIA2
24	21,089-21,466	378	125	14.6	anti-CRISPR protein AcrIIA3
25	21,796-22,005	210	69	7.9	hypothetical protein
26	22,447-22,680	234	77	9.3	hypothetical protein
27	22,677-22,868	192	63	7.8	hypothetical protein
28	23,063-24,217	1155	384	44.9	integrase
29	24,350-24,922	573	190	21.3	hypothetical protein
30	24,974-25,426	453	150	17.9	ImmA/IrrE family metallo-endopeptidase
31	25,443-25,766	324	107	12.4	helix-turn-helix transcriptional regulator
32	26,166-26,369	204	67	7.8	YozE family protein
33	26,436-26,627	192	63	7.1	helix-turn-helix transcriptional regulator
34	26,649-26,891	243	80	9.5	hypothetical protein
35	26,894-27,079	186	61	7.5	hypothetical protein
36	27,182-27,304	123	40	4.9	hypothetical protein
37	27,314-27,466	153	50	5.5	hypothetical protein
38	27,829-28,059	231	76	9	hypothetical protein
39	28,345-28,560	216	71	8.1	hypothetical protein

Table 3.7. *Cont.*

ORF	Position (5'-3')	Length (bp)	No. of amino acids	Size (kDa)	Predicted function
40	28,638-29,189	552	183	21.6	DUF1642 domain-containing protein
41	29,248-29,763	516	171	19.7	hypothetical protein
42	29,942-30,412	471	156	18.6	DUF1642 domain-containing protein
43	30,413-30,835	423	140	16.4	hypothetical protein
44	30,832-31,005	174	57	6.6	hypothetical protein
45	31,002-31,385	384	127	14.2	hypothetical protein
46	31,387-31,866	480	159	18.4	Siphovirus Gp157 family protein
47	31,886-32,575	690	229	26.1	AAA family ATPase
48	32,753-33,895	1143	380	43.3	DEAD/DEAH box helicase
49	33,920-34,405	486	161	18.4	DUF669 domain-containing protein
50	34,428-36,701	2274	757	87.2	phage/plasmid primase, P4 family
51	37,036-37,350	315	104	11.7	VRR-NUC domain-containing protein
52	37,418-37,996	579	192	22.2	putative phage-related protein (DUF3310)
53	37,997-38,152	156	51	5.8	hypothetical protein
54	38,214-38,441	228	75	9.2	hypothetical protein
55	38,454-38,879	426	141	16.7	DUF722 domain-containing protein
56	38,977-39,720	744	247	29.2	endonuclease domain-containing protein
57	40,180-40,506	327	108	12.2	hypothetical protein
58	40,506-40,820	315	104	12.8	HNH endonuclease
59	40,926-4	300	99	11.5	terminase small subunit

Table 3.8. Annotation of phage vB_LmoS-PLM34 genome

ORF	Position (5'-3')	Length (bp)	No. of amino acids	Size (kDa)	Predicted function
1	27-389	363	120	14.1	HNH endonuclease
2	386-712	327	108	12.4	hypothetical protein
3	809-940	132	43	5.1	hypothetical protein
4	983-1315	333	110	12.7	hypothetical protein
5	1331-1873	543	180	21.3	integrase
6	1870-2388	519	172	19.9	RNA polymerase subunit sigma
7	2459-2629	171	56	6.8	hypothetical protein
8	2635-2874	240	79	9.4	hypothetical protein
9	2895-3374	480	159	17.5	single-stranded DNA-binding protein
10	3374-3955	582	193	22.4	DUF3310 domain-containing protein
11	3955-4203	249	82	9.7	DUF3850 domain-containing protein
12	4224-4607	384	127	14.6	preprotein translocase subunit YajC
13	4604-5065	462	153	18.1	hypothetical protein
14	5062-5250	189	62	7	SAM-benzoic acid carboxyl methyltransferase
15	5263-5973	711	236	27	hypothetical protein
16	5973-6374	402	133	15.4	YopX family protein
17	6371-6673	303	100	11.1	hypothetical protein
18	6677-7396	720	239	27.7	DUF2786 domain-containing protein
19	7411-7587	177	58	6.8	hypothetical protein
20	7584-8000	417	138	15.6	hypothetical protein
21	7997-8353	357	118	13.6	MerR family transcriptional regulator
22	8355-9269	915	304	35.7	phage replication initiation protein
23	9329-9442	114	37	4.1	hypothetical protein
24	9442-9768	327	108	12.8	DUF771 domain-containing protein
25	9866-10,057	192	63	7.5	hypothetical protein
26	10,167-10,307	141	46	5.3	helix-turn-helix domain-containing protein
27	10,273-10,557	285	94	10.8	hypothetical protein
28	10,600-11,070	471	156	18	ImmA/IrrE family metallo-endopeptidase
29	11,125-11,838	714	237	26.4	hypothetical protein
30	11,860-13,011	1152	383	44.8	integrase
31	13,013-13,123	111	36	4.4	hypothetical protein
32	13,161-13,348	186	61	7.3	hypothetical protein
33	13,328-14,176	849	282	31.2	N-acetylmuramoyl-L-alanine amidase
34	14,173-14,466	294	97	10.4	holin
35	14,470-14,835	366	121	14.5	conserved phage protein
36	14,874-15,935	1062	353	38.2	minor structural protein
37	15,928-18,219	2292	763	84.6	tail protein
38	18,225-20,468	2244	747	84.6	tail protein

Table 3.8. *Cont.*

ORF	Position (5'-3')	Length (bp)	No. of amino acids	Size (kDa)	Predicted function
39	20,461-24,534	4074	1357	146.8	phage tail length tape-measure protein
40	24,678-24,773	96	31	3.6	hypothetical protein
41	24,857-25,204	348	115	13.2	hypothetical protein
42	25,294-25,869	576	191	21	major tail protein
43	25,890-26,288	399	132	15.3	hypothetical protein
44	26,291-26,656	366	121	13.6	HK97 gp10 family phage protein
45	26,650-26,970	321	106	12.5	hypothetical protein
46	26,940-27,278	339	112	12.9	head-tail connector protein
47	27,327-28,496	1170	389	43.4	major capsid protein
48	28,560-29,126	567	188	21	head maturation protease
49	29,123-30,358	1236	411	45.8	portal protein
50	30,361-30,561	201	66	7.1	DUF1056 family protein
51	30,568-32,319	1752	583	67.5	terminase large subunit
52	32,288-32,815	528	175	20.5	terminase small subunit
53	32,791-32,889	99	32	3.6	hypothetical protein

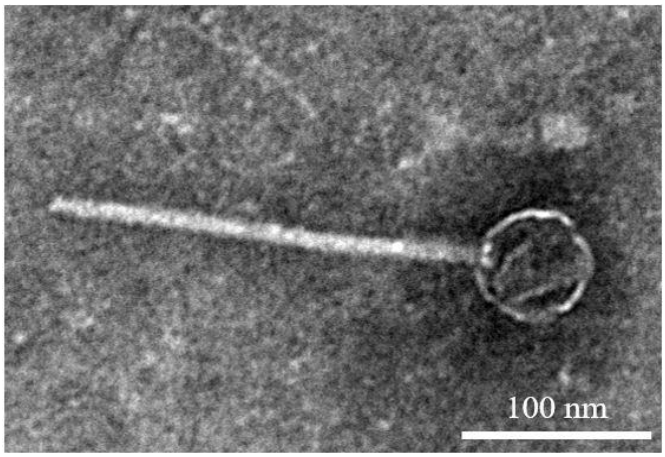


Fig. 3.1. Transmission electron micrograph of phage vB_LmoS-PLM34.

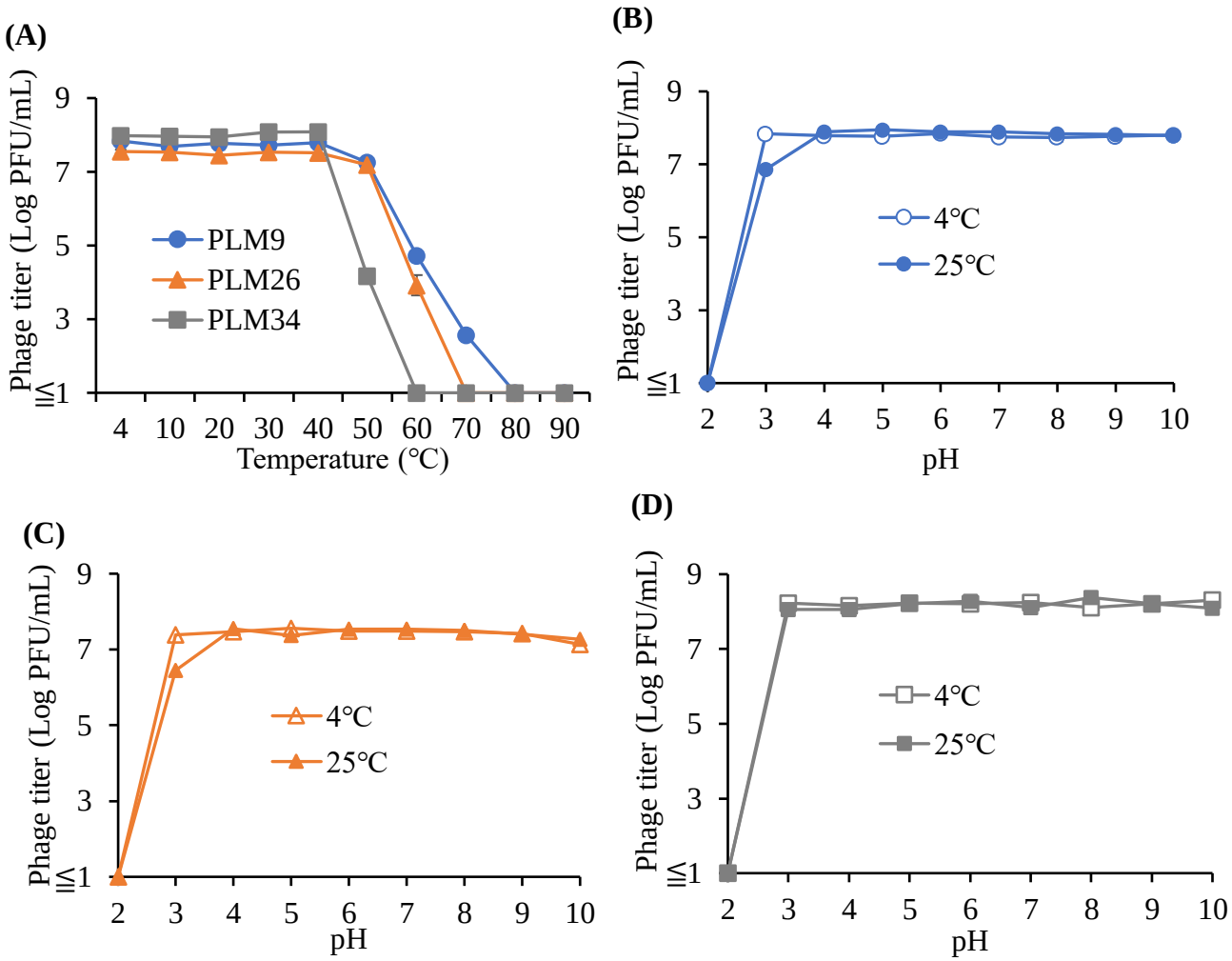


Fig. 3.2. (A) Temperature stability of phages vB_LmoS-PLM9, vB_LmoS-PLM26, and vB_LmoS-PLM34. (B) pH stability of phage vB_LmoS-PLM9. (C) pH stability of phage vB_LmoS-PLM26. (D) pH stability of phage vB_LmoS-PLM34. The error bars show the mean ± S.D. of the three replicates.

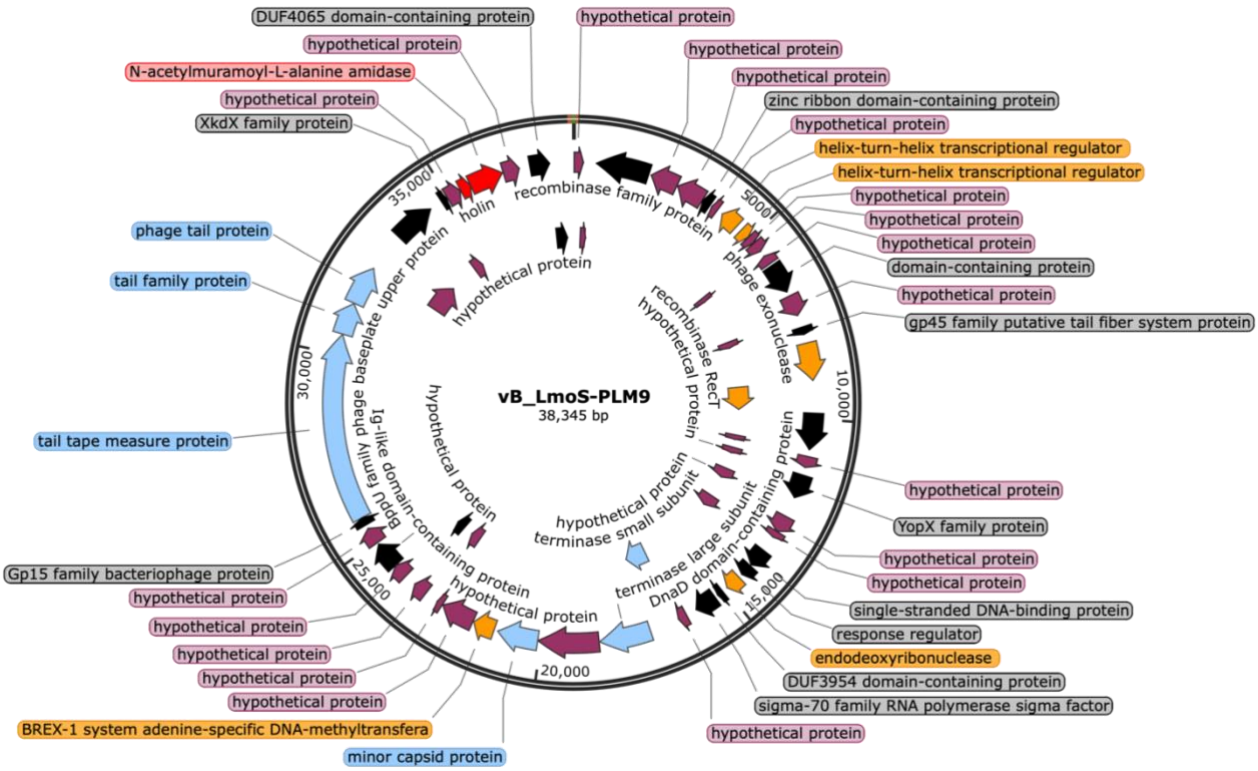


Fig. 3.3. Genome map of *Listeria* phage vB_LmoS-PLM9. GenBank accession number is OR371505. Arrows: Blue- phage structure and packaging, Orange- DNA replication, Red- host lysis, Purple- hypothetical protein, Black- additional functions.

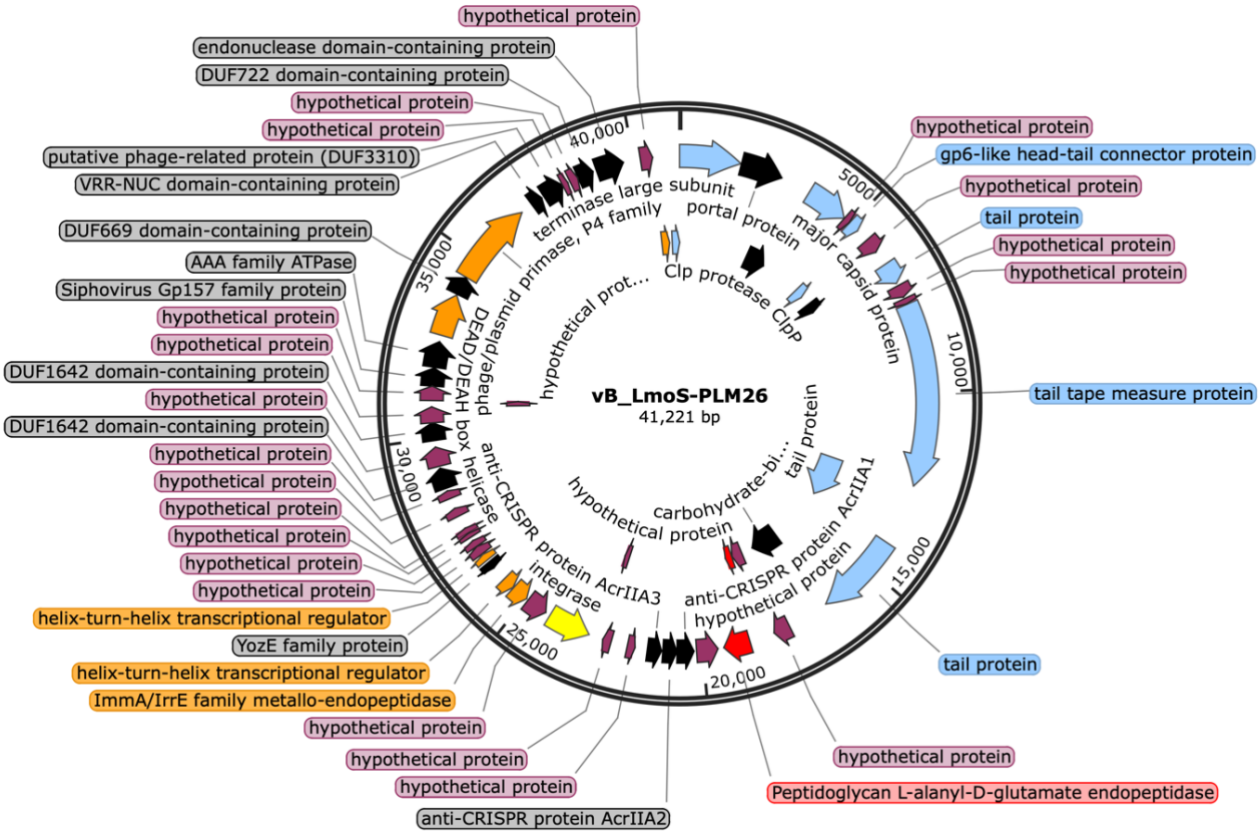


Fig. 3.4. Genome map of *Listeria* phage vB_LmoS-PLM26. GenBank accession number is OP926971. Arrows: Blue- phage structure and packaging, Orange- DNA replication, Red- host lysis, Yellow- Integrase, Purple- hypothetical protein, Black- additional functions.

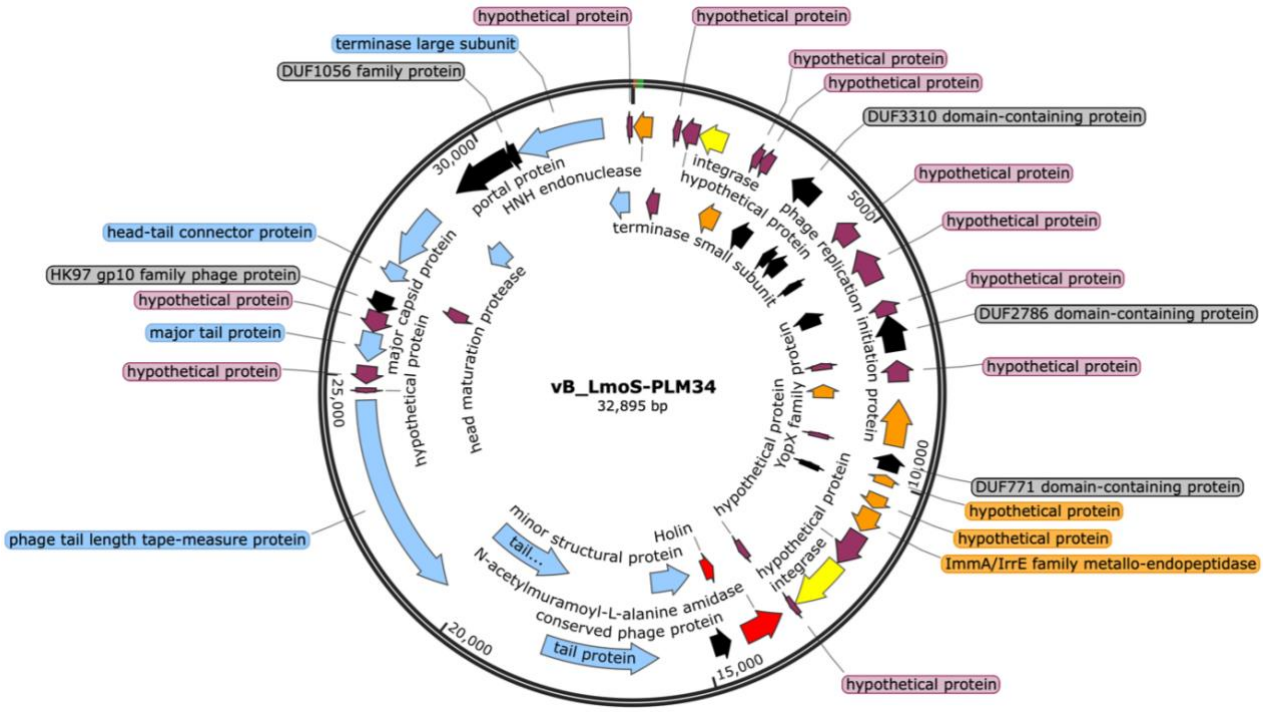


Fig. 3.5. Genome map of *Listeria* phage vB_LmoS-PLM34. GenBank accession number is OP926972. Arrows: Blue- phage structure and packaging, Orange- DNA replication, Red- host lysis, Yellow- Integrase, Purple- hypothetical protein, Black- additional functions.

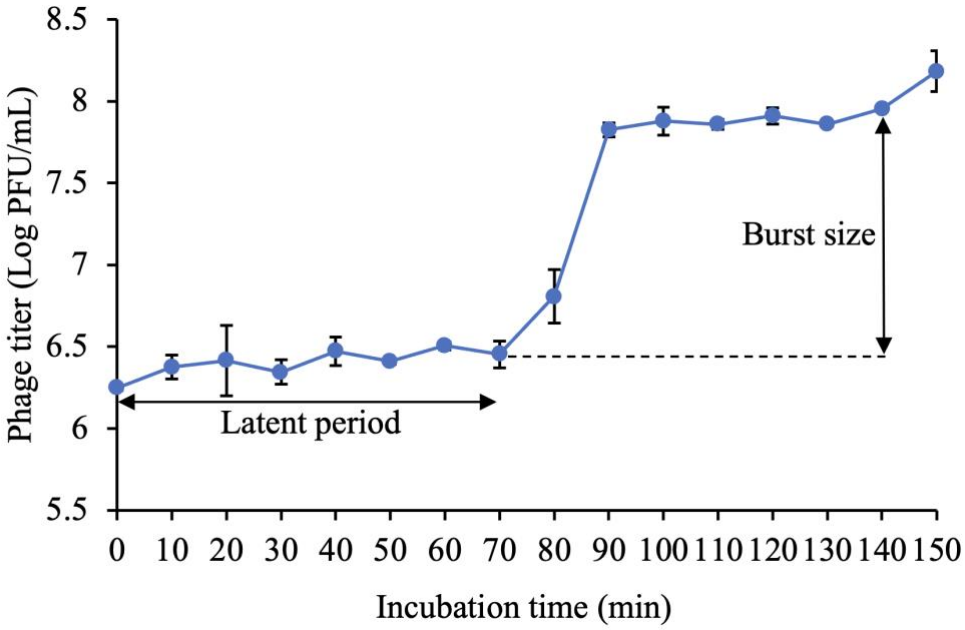


Fig. 3.6. One-step growth curve of phage vB_LmoS-PLM9.

Chapter 4. Evaluation of plant-based essential oils and their application in single or combined with phage vB_LmoS-PLM9 against pathogenic *Listeria monocytogenes* in broth and milk

4.1. Introduction

A foodborne *L. monocytogenes* is a serious bacterium and causes listeriosis in humans and animals. Among the 13 serotypes, serotype 4b accounts for between 30 and 50 percent of sporadic human listeriosis cases globally (Akhtar et al., 2017; Kawacka et al., 2020). The contamination of *L. monocytogenes* in meat, fish, vegetables, and milk and milk products, remains a severe threat to the global food safety problem (Elafify et al., 2022). Hence, applicable techniques are promoted to control *L. monocytogenes* infection in foods.

Antimicrobial resistance (AMR) is becoming an increasingly concerning problem for global public health, prompting a rise in studies exploring antimicrobial substitutes. Bacteriophages (phages) are viruses that target specific bacteria without causing harm to humans, and they are gaining popularity as potential biocontrol agents against foodborne pathogens like *L. monocytogenes,* with numerous studies applying them to various food products (Akhtar et al., 2017; Byun et al., 2022). In certain nations, particularly in the United States, phage-based products like ListShield™ and Listex™ P100 are employed to combat *L. monocytogenes* (Byun et al., 2022; Perera et al., 2015).

Essential oils (EOs) are natural chemicals derived from various parts of plants. They have strong antimicrobial and antioxidant properties and have been recognized as potential agents against microorganisms (Paudel et al., 2019; Van Haute et al., 2017). Most EOs are classified as GRAS and can even be used as decontaminants (Cui et al., 2016; Somrani et al., 2020). Cinnamon bark (*Cinnamomum zeylanicum*) and Cinnamon cassia (*Cinnamomum cassia*) are both referred to as "Cinnamon" and they are part of the same "Lauraceae" family and "*Cinnamomum*" genus, however they are distinct plant species. Cinnamon bark (C.b) has a lighter color, flavor, and scent than cinnamon

cassia (C.c). Cinnamaldehyde, the primary constitute of cinnamon oil, has a broad antibacterial spectrum that protects against spoilage microorganisms and foodborne pathogens (Brnawi et al., 2019; Paudel et al., 2019; Zhang et al., 2016).

The combination of several antibacterial agents presents a new method for expanding the range of antimicrobial effectiveness, addressing AMR, and increasing the effectiveness of antibacterial activity by leveraging synergistic effects (Corrêa et al., 2021; Duc et al., 2018). The potential synergistic effect between phage and EOs may impede the development of phage-resistant bacteria and diminish the potent, distinct aroma of the oil (Corrêa et al., 2021; Ni et al., 2020). This chapter aims to evaluate eight kinds of EOs against various *Listeria* spp. and to investigate the most effective EOs in single and combined with phage vB_LmoS-PLM9 against pathogenic *L. monocytogenes* in broth and milk.

4.2. Materials and methods

4.2.1. Preparation of bacterial culture

The pathogenic *L. monocytogenes* strain no.193 (serotype 4b) isolated from minced pork and well-characterized in our Laboratory (Chapters 2 and 3) was used for this experiment. Bacterial strain from −80 °C frozen stock was streaked onto TSA and cultured in 5 mL of BHI broth overnight at 30 °C. The culture was used for further experiments.

4.2.2. Preparation of bacteriophage vB_LmoS-PLM9

The isolated bacteriophage vB_LmoS-PLM9 in our Laboratory (Chapter 3) was used in this experiment. This phage showed lytic activity against different species of *Listeria* including *L. monocytogenes*. Strain no.193 was used to refresh the phage suspension since it was the target host strain for phage isolation. The fresh phage stock (approximately 10^{10} PFU/mL) was prepared using the double-layer agar method. Briefly, 100 µL phage (10^4 PFU/mL) and 100 µL host cell were added in 4 mL of LB

top agar containing 1 mmol/L $CaCl_2$, then the mixture was poured onto TSA. After keeping at 30 °C for 24 h, the phage was collected by adding 5 ml aliquots of saline magnesium (SM) buffer onto the top of dense-plaque plates. After shaking at 25 °C overnight, the top agar layer was scraped and centrifuged. The supernatant was filtered and maintained as a phage stock at 4°C until use.

4.2.3. Preparation of EOs

Eight 100 % pure EOs; cinnamon bark (C.b), cinnamon cassia (C.c), clove, lemon grass, ginger, turmeric, basil, and lemon were bought from iHerb (NowFoods, Japan). To prepare the 4 and 10 % (v/v) stock oil solutions, 0.5 % (w/v) Tween 80 (FIJIFILM, Wako Pure Chemical Corporation, Japan) was used. These stock solutions were then diluted in BHI broth to create working solutions.

4.2.4. Antibacterial activity of EOs

Disc diffusion test was used to determine the antibacterial activities of 10 % of 8 EOs against *Listeria* spp., including *L. monocytogenes* 193 (Table 4.1). One hundred microliters of bacteria at a density of 10^6 or 10^8 CFU/mL were spread using a sterilized cotton swab onto Muller Hinton agar. Subsequently, 6 mm paper discs (Whatman™, China) were positioned on the plate. Then, 10 µL of 10 % EO was added to the disc and kept at 4 °C for 30 min to ensure better adsorption before being kept at 30 °C for 24 h. Sterilized broth and Gentamycin disc (30 µg/mL, BD BBL™ Sensi-Disc™, USA) were used for controls. The zone diameters (mm) were measured to interpret the degree of inhibition. All experiments were performed in triplicates.

Following the antibacterial test on various EOs, the strong anti-listerial activities of C.b, C.c, and clove were used for further experiments. The minimum inhibitory concentration (MIC) values of EOs (4-0.015 %) against *L. monocytogenes* 193 (10^6 or 10^8 CFU/mL) were investigated using the broth microdilution method (CLSI, 2021). The negative control utilized was sterilized broth. The cells from transparent wells were

10-fold serially diluted with phosphate buffer saline (PBS). The dilution (100 μL) was spread onto TSA plates to determine the MBC values.

4.2.5. Determination of phage activity in the presence of EOs

The survivability of phage was assessed in the presence of various EO concentrations (0.03, 0.0625, 0.125, 0.25, 0.5, and 1 %). The phage suspension (180 μL) was mixed with EOs (20 μL), which was kept at 4 °C for 24 h. Phage titer was determined using a plaque assay.

4.2.6. Determination of viability of *L. monocytogenes* treated with phage and/or EOs in broth

In broth at 30 °C, single and combined phage (MOI 10) and C.b or C.c at 1/3 or 1/2 × MIC value (0.02 or 0.03 %) were applied. Moreover, phage (MOI 1) and/or clove oil at 1/2 or 1 × MIC value (0.125 or 0.25 %) was used. The bacterial suspension (500 μL) was prepared in BHI broth (4 mL) to obtain the cell at 10^6 CFU/mL. An equal volume of phage and/or EOs (500 μL) was added for treatment groups and sterilized broth for control. Samples were taken every 2 h and thinned out with PBS. The viability was checked by the plate count method on TSA.

4.2.7. Determination of viability of *L. monocytogenes* treated with phage and/or EOs in milk

In this trial, commercially available 100 % pasteurized milk including fat content of 3.5 % or more, was purchased from the supermarket. For sample preparation, 500 μL of bacterial culture was contaminated into 4 mL of milk to attain 10^5 CFU/mL. Then, 500 μL of phage and/or EOs alone and in combination was added to the artificially contaminated milk and stored at 30 °C for 24 h and 4 °C for 7 d. Only broth was employed for the control sample. At 30 °C, phage (MOI = 100), C.b or C.c (0.0625 % or 0.125 %), and clove oil (0.5 %) were used, and the viable counts were checked every 2 h. At 4 °C, phage (MOI = 100), C.b or C.c (0.125 %), and clove oil (0.5 %) were

treated, and the viable counts were determined every single day.

4.2.8. Statistical analysis

Analysis of variance (ANOVA) followed by post-hoc least significant difference (LSD) pairwise multiple comparison test was performed to assess significant differences between essential oils groups. Student's t-test was utilized to ascertain statistically significant differences between control and treatment groups. Differences were considered statistically significant when $P < 0.05$.

4.3. Results

4.3.1. Antibacterial activity of EOs against *L. monocytogenes*

Table 4.1 shows that 10 % of C.b, C.c, clove, and lemongrass oils presented anti-listerial efficiencies, but others did not. Among eight EOs, C.b and C.c oils indicated the widest inhibition zone, then clove and lemongrass oils against different species of *Listeria* including *L. monocytogenes* 193.

4.3.2. MIC and MBC values of EOs against *L. monocytogenes*

The MIC values of C.b and C.c were found to be 0.0625 % at 10^6 CFU/mL and 0.125 % at 10^8 CFU/mL. The MBC of C.b and C.c were 0.125 % at 10^6 CFU/mL and 0.25 % at 10^8 CFU/ mL. For the clove oil, the MIC values were 0.25 % at 10^6 CFU/mL and 0.5 % at 10^8 CFU/mL, and the MBC were 0.5 % at 10^6 CFU/mL and 1 % at 10^8 CFU/mL (Table 4.2).

4.3.3. Effects of EOs on phage survivability

Figure 4.1 demonstrates the titers of phage vB_LmoS-PLM9 remained constant when exposed to C.b, C.c, or clove oils in concentrations between 0.03 to 1 % at 4 °C for 24 h.

4.3.4. Effects of phage and/or EOs against *L. monocytogenes* in broth

In applications of phage and/or C.b or C.c at 30 °C, viable cells were reduced by 3.2 log after 10 h and resistant cells regrew after 24 h in phage therapy at MOI of 10 compared to the control. The surviving cells were decreased by 1.7 and 1.1 log at 10 h in a single treatment of 1/2 × MIC (0.02 %) of C.b/ C.c but regrew later. Phage (MOI = 10) plus 0.02 % C.b/ C.c reduced surviving cells by approximately 2 log at 4 h, and continuously supressed the regrowth till 10 h, and then reached the same level at 24 h (Fig. 4.2A). The treatment of 0.03 % of C.b/ C.c retarded the growth of cells up to 24 h. Combined phage (MOI = 10) and 0.03 % C.b/ C.c was highly effective compared to individual ones, reducing the viable cells by 3 log at 24 h incubation (Fig. 4.2B).

Fig.4.3 shows the application of individual and combined phage and clove oil at 30 °C. The surviving cells were decreased by a maximum of 2.7 log in phage treatment at MOI of 1 after 8 h but regrowth was similar to control later time. Clove oil at 0.125 % reduced the cell viability by 1.1 log at 2 h and continued to decline for 24 h. Combining phage (MOI of 1) with clove oil (0.125 %) reduced surviving cells by 3.6 log after 10 h compared to the initial cell counts of the control sample and was undetectable at 24 h. These results showed that combined therapy was more effective than the single one. Moreover, the bacterial cells were not detected in the treatments with 1 × MIC value (0.25 %) clove oil alone or combined with phage after 2 h (Fig. 4.3).

4.3.5. Effects of phage and/or EOs against *L. monocytogenes* in milk

The applications of single and combined phage and C.b or C.c in milk are demonstrated in Fig. 4.4. At 30 °C, phage alone (MOI = 100) decreased the cell survivability by 1.4 log at 2 h but reached the level as the control after 24 h. In C.b or C.c oil (0.0625 %) alone, the surviving cells were reduced by 1.5 log at 10 h but later regrew to the same level as the control. Phage (MOI 100) plus 0.0625 % C.b/ C.c treatments decreased the cell growth by approximately 1.5 log at 2 h and 3.7 log at 10

h respectively (Fig. 4.4A). Both cinnamon oil treatments at 0.125 % controlled the initial cell counts up to 24 h. However, surviving cells were lowered by 2.3 and 1.5 log at 2 h and prohibited the resistant cells in phage (MOI = 100) plus 0.125 % C.b/ C.c (Fig. 4.4B). At 4 °C, phage alone at MOI 100 lowered surviving cells by approximately 1 log at 1 d and was stable until the end of the trial. In single 0.125 % of both cinnamon oils, viable cells were inhibited for 7 d. Phage at MOI 100 united with C.b/ C.c (0.125 %) gradually decreased the viable counts from 1 d and arrived under detection levels at 4 and 5 d, respectively (Fig. 4.4C).

The use of phage and/or clove oil in milk at 30 and 4 °C shown in Fig. 4.5. At 30 °C, clove oil (0.5 %) treatment did not show an obvious effect on the survivability of *L. monocytogenes*. However, viable cells were reduced by 2.7 log at 4 h and 3.4 log at 10 h when compared with the growth of the control sample, but resistant bacteria grew at 24 h in phage (MOI of 100) plus 0.5 % clove oil (Fig. 4.5A). At 4 °C, phage treatment (MOI = 100) decreased the viable counts after 1 d, although 0.5 % clove oil did not show the effect. Combining phage at MOI of 100 and 0.5 % clove oil reduced viable numbers by 1.7 log at 1 d and 3.5 log at 7 d (Fig. 4.5B).

4.4. Discussion

Recently, phage application has been proposed as a promising strategy to control foodborne infections, including *L. monocytogenes*. Phage combined with other antibacterial substances is becoming increasingly popular due to the establishment of phage-resistant mutants in single-phage therapy (Lima et al., 2019; Pinheiro et al., 2019). Many researchers have worked on phage cocktails, phage and antibiotics, phage and nisin, phage and carvacrol, etc. to combat phage-resistant bacteria (Cufaoglu and Ayaz, 2019; Duc et al., 2020; Lopes et al., 2018; Noor Mohammadi et al., 2022b). Corrêa et al. (2021) reported *L. monocytogenes* and *Salmonella* Enteritidis were effectively reduced by binding cinnamon oil and phenolic acid. Additionally, commercial phage (SALMONELEX) combined with thymol or carvacrol exhibited

synergism against *Salmonella* in chicken as reported by Moon et al. (2020).

In this investigation, eight different EOs at 10 % were employed to assess anti-listerial activity. The most potent effects were observed in C.b and C.c oils, following clove and lemongrass oils against different *Listeria* spp., including *L. monocytogenes* (Table. 4.1). Similar findings reported by dos Santos et al. (2022) cinnamon oil indicated the strongest antibacterial efficiency to *L. monocytogenes* when they tested with 10 % of five EOs (dos Santos et al., 2022). Tomičić et al. (2018) also reported that cinnamon oils showed the highest anti-listerial efficiency among tested various essential oils including cinnamon, dill, clove, mentha, red thyme, rosemary, sage, and summer savory. Mahfuzul Hoque et al. (2008) and Paudel et al. (2019) documented that cinnamon and clove EOs are potential antimicrobial substances for use in food. Cinnamon and clove oils compose cinnamaldehyde and eugenol, which have potential antimicrobial properties to Gram +ve and -ve pathogens. Other studies have indicated that cinnamaldehyde hindered ATPase, caused damage to cell membranes, and impeded cytokinesis. On the other hand, eugenol facilitated the permeabilization of cell membranes through its interaction with proteins (Hyldgaard et al., 2012; Rogiers et al. 2017; Somrani et al., 2020).

The MIC values for cinnamon oils (0.0625 %) in the current study were found to be lower compared to earlier studies: 0.1 % (Somrani et al., 2020), 0.5 % (Brnawi et al., 2018), 1-10 % (dos Santos et al., 2022), and 1.25-2.5 % (Mahfuzul Hoque et al., 2008). Prior research reported by Mahfuzul Hoque et al. (2008) that MIC values of clove oil ranged from 2.5 to 5 %, which was higher than the values (0.25 %) found in this investigation. These discrepancies are most likely attributable to the various strains and methodologies used in numerous research. Furthermore, the active component of EOs varied based on the regions where the plants were grown and the parts of the plant used for extraction, along with the extraction techniques employed, all these factors could influence the values (Martins et al., 2016; Somrani et al., 2020).

Combining two antimicrobial substances enhances the activity of combating pathogenic bacteria if they exert synergism. This research investigated that combined phage vB_LmoS-PLM9 and cinnamon or clove oils showed more effectiveness against *L. monocytogens* than individual ones. The usage of different concentrations of EOs alone varied the inhibition degrees against *L. monocytogenes*. Phage (MOI = 10) + C.b or C.c (0.03 %), and phage (MOI = 1) + clove oil (0.125 %) exhibited high efficacy in reducing the surviving cells and suppressing the resistant cell growth in broth. Similarly, phages and other antimicrobial agents can work together to inhibit the development of phage-resistant mutants (Ni et al., 2020). The precise mechanism of the anti-listerial activity of phage and EOs should be further researched.

Cinnamon or clove EOs have been researched in previous studies for their potential use in milk and dairy products (Brnawi et al., 2018; Cui et al., 2016; Elafify et al., 2022). Natural plant extracts are used as fragrance and flavor enhancers in the fluid milk industry due to the growing trend of introducing new aromas and flavors. Since ancient times, the dairy industry has employed plant EOs to flavor food and beverages. Milk drinks flavored with cinnamon, clove, and other spices are popular, especially in Spain and Latin American countries (Cava et al., 2007). However, using high volumes of essential oils might alter the aroma of food products, which may be unacceptable to some consumers. This study investigated the combining phage and C.b, C.c, or clove oils by using lower concentrations to control pathogenic *L. monocytogenes* in milk. Phage (MOI of 100) mixed with C.b or C.c (0.125 %) lowered cell numbers at 2 h and inhibited regrowth cells at 30 °C, while those decreased surviving cells at 1 d and arrived at less than detection limit at 4 or 5 d at 4 °C. In the treatment of phage (MOI 100) plus clove oil (0.5 %), the viable counts were reduced at 4 h but some resistant bacteria regrew at 24 h at 30 °C, whereas those were reduced at 1 d, and then gradually decreased until the end of 7 days trail. The first report demonstrated that combining phage with cinnamon or clove oils has bacteriostatic and bactericidal effects against *L. monocytogenes*

in milk. More research will be done on the antibacterial efficacy of combining phage and EOs against *L. monocytogenes*, especially in various solid foods.

4.5. Summary

This chapter examined the antibacterial activity of eight different EOs against *Listeria* spp. including *L. monocytogenes*. The most effective C.b and C.c had MIC and MBC values of 0.0625 % and 0.125 %, respectively, whereas the values for clove oil were 0.25 % and 0.5 %. Single and combined applications of phage vB_LmoS-PLM9 and C.b, C.c, or clove oils were investigated in broth and milk. Single treatment of phage reduced the viable cells after 2 h, but regrowth at 24 h of storage at 30 °C. The survival cell was inhibited when treated with phage in milk at 4 °C. The number of viable cells was inhibited or reduced in treatments with EO alone, depending on the concentrations used. However, mixed phage (MOI 10) and C.b/ C.c (0.03 %), and phage (MOI 1) and clove oil (0.125 %) decreased the viability of bacterial cells and prevented the regrowth cells *in vitro* at 30 °C. Mixing phage (MOI 100) and C.b/ C.c (0.125 %) or clove oil (0.5 %) reduced the survival cells and inhibited the resistant cell growth in milk, particularly at 4 °C. These findings suggested that phage plus EOs might be an effective method for controlling *L. monocytogenes* in milk.

Table 4.1. Anti-listerial activity of EOs at 10 % by disc diffusion assay at 30 ℃

No.	*Listeria* spp.	Zone diameter (Mean ± S.D. mm)			
		Cinnamon bark	Cinnamon cassia	Clove	Lemon grass
1	*L. monocytogenes* 193*	26.7±1.2^{c}	21.7±1.5^{b}	9.7±1.2^{a}	9.7±0.6^{a}
2	*L. monocytogenes* ATCC 15313^{T}	25.5±0.9^{c}	30.2±0.8^{d}	8.2±0.3^{a}	12.0±1.0^{b}
3	*L. innocua* ATCC 33090	32.0±1.0^{b}	32.7±0.6^{b}	10.3±0.6^{a}	9.7±0.6^{a}
4	*L. innocua* LIS31	24.3±0.6^{c}	28.2±0.8^{d}	7.7±0.6^{a}	9.7±0.6^{b}
5	*L. ivanovii* ATCC 19119	30.3±0.6^{b}	29.3±0.6^{b}	8.7±0.6^{a}	10.0±1.0^{a}
6	*L. seeligeri* ATCC 35967	31.3±0.6^{b}	33.7±2.5^{b}	11.7±1.5^{a}	11.7±1.2^{a}
7	*L. seeligeri* LIS34	30.3±0.6^{b}	33.8±01.9^{c}	10.7±0.6^{a}	10.7±0.6^{a}
8	*L. welshimeri* ATCC 35897	24.3±1.5^{b}	24.7±0.6^{b}	9.7±0.6^{a}	10.7±0.6^{a}
9	*L. welshimeri* LIS36	23.6±1.5^{b}	22.0±2.0^{b}	0	11.3±0.6^{a}
10	*L. grayi* ATCC 25401	20.0±1.0^{b}	25.7±1.5^{c}	9.8±0.8^{a}	10.7±1.2^{a}

No inhibition was observed in 10 % ginger, turmeric, basil, and lemon.

*Host strain of phage vB_LmoS-PLM9

$^{a–c}$ Different superscript letters indicate significant differences ($P < 0.05$) among EOs in each row

Table 4.2. MIC and MBC values of EOs

EOs	10^6 CFU/mL		10^8 CFU/mL	
	MIC (%)	MBC (%)	MIC (%)	MBC (%)
Cinnamon bark	0.0625	0.125	0.125	0.25
Cinnamon cassia	0.0625	0.125	0.125	0.25
Clove	0.25	0.5	0.5	1

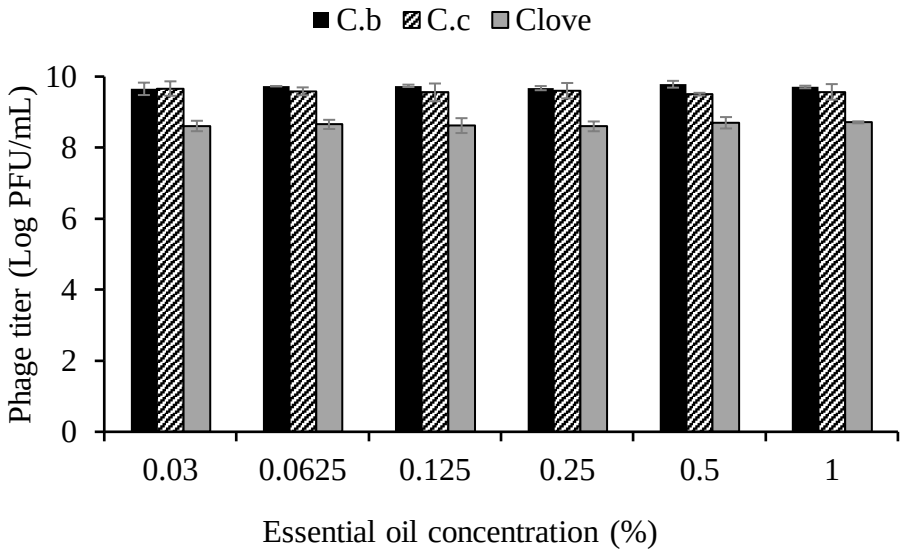


Fig. 4.1. Phage activity in treatments of C.b, C.c, and clove oils. Phage (5×10^9 PFU/mL) was utilized in the presence of cinnamon bark and cassia oils, while phage (5×10^8 PFU/mL) was employed in clove oil. Mean ± S.D. (n = 3).

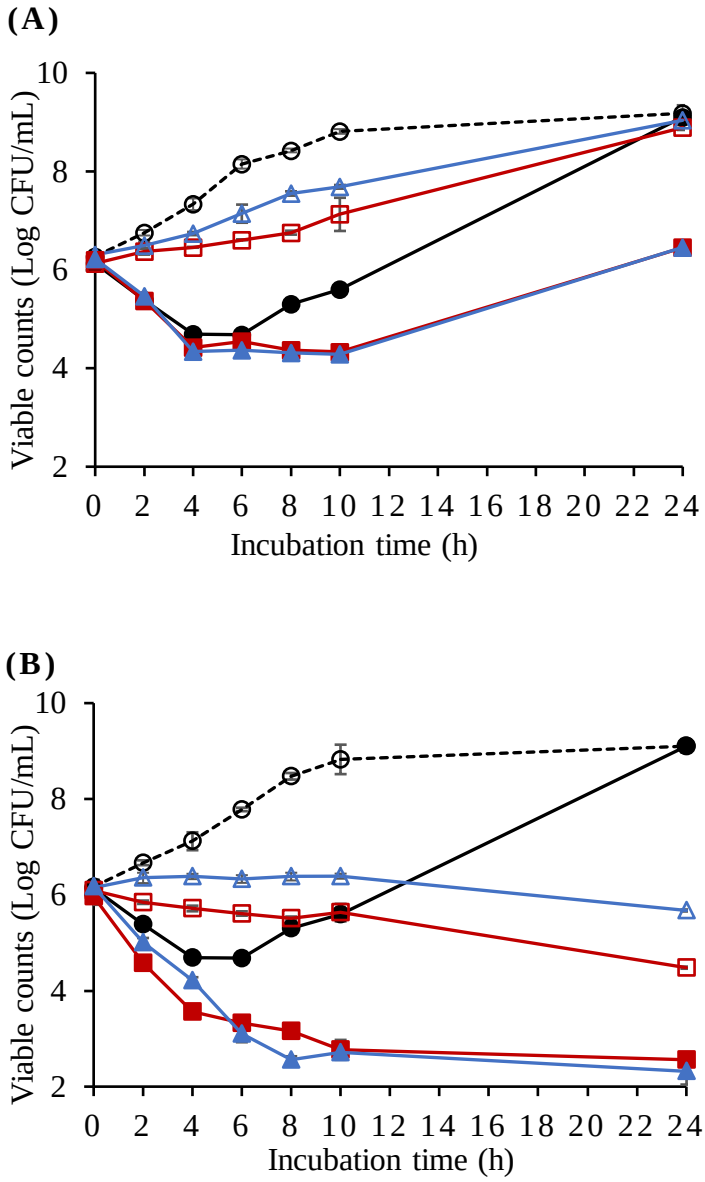


Fig. 4.2. Effects of phage vB_LmoS-PLM9 and/or C.b or C.c (A) 0.02% and (B) 0.03% against *L. monocytogenes* in broth at 30 ℃. Mean ± S.D. (n = 3).

(A) Symbols: ○, Control; ●, Phage (MOI=10); □, 0.02% C.b; △, 0.02% C.c; ■, Phage (MOI=10) + 0.02% C.b; ▲, Phage (MOI=10) + 0.02% C.c.

(B) Symbols: ○, Control; ●, Phage (MOI=10); □, 0.03% C.b; △, 0.03% C.c; ■, Phage (MOI=10) + 0.03% C.b; ▲, Phage (MOI=10) + 0.03% C.c.

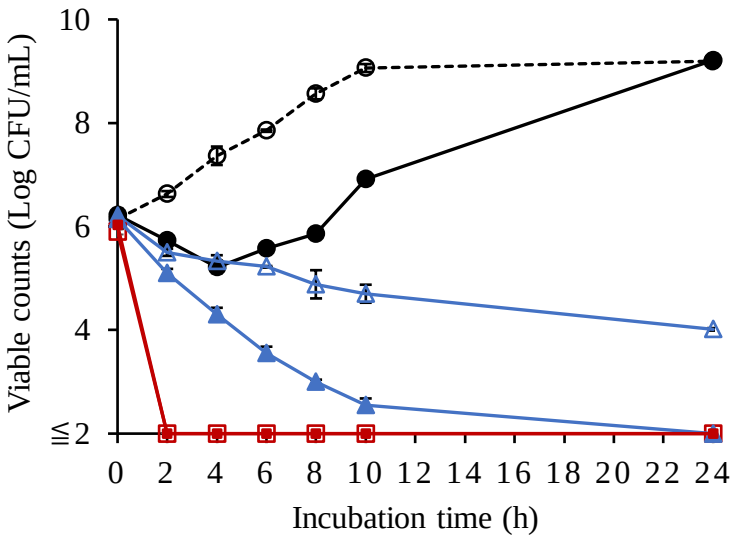


Fig. 4.3. Effects of phage vB_LmoS-PLM9 and/or clove oil against *L. monocytogenes* in broth at 30 ℃. Mean ± S.D. (n = 3).

Symbols: ○, Control; ●, Phage (MOI=1); △, 0.125% Clove oil; □, 0.25% Clove oil; ▲, Phage (MOI=1) + 0.125% Clove oil; ■, Phage (MOI=1) + 0.25% Clove oil.

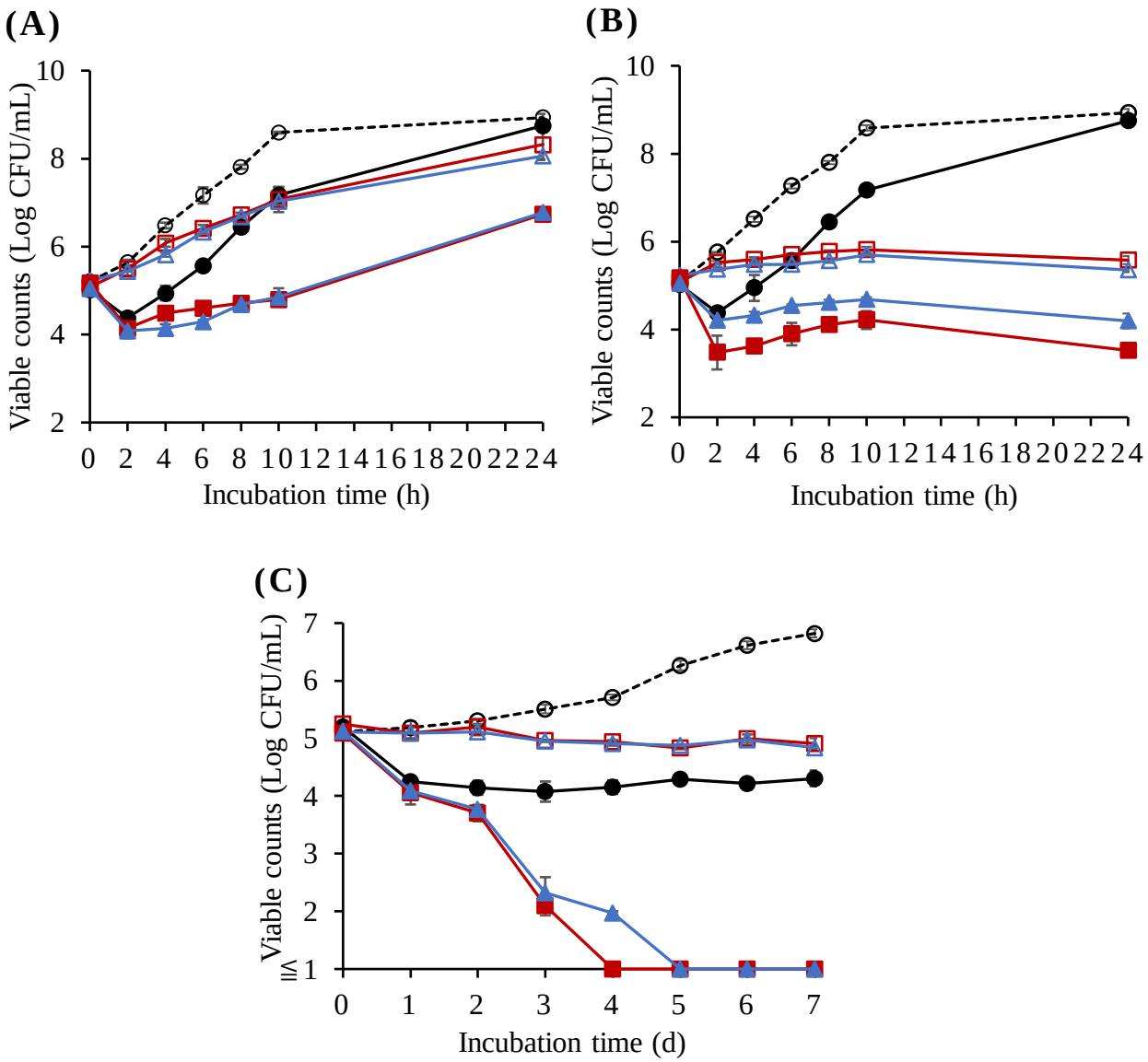


Fig. 4.4. Effects of phage vB_LmoS-PLM9 and/or C.b or C.c against *L. monocytogenes* in milk at (A and B) 30 ℃, and (C) 4 ℃. Mean ± S.D. (n = 3).

(A) Symbols: ○, Control; ●, Phage (MOI=100); □, 0.0625% C.b; △, 0.0625% C.c; ■, Phage (MOI=100) + 0.0625% C.b; ▲, Phage (MOI=100) + 0.0625% C.c.

(B and C) Symbols: ○, Control; ●, Phage (MOI=100); □, 0.125% C.b; △, 0.125% C.c; ■, Phage (MOI=100) + 0.125% C.b; ▲, Phage (MOI=100) + 0.125% C.c.

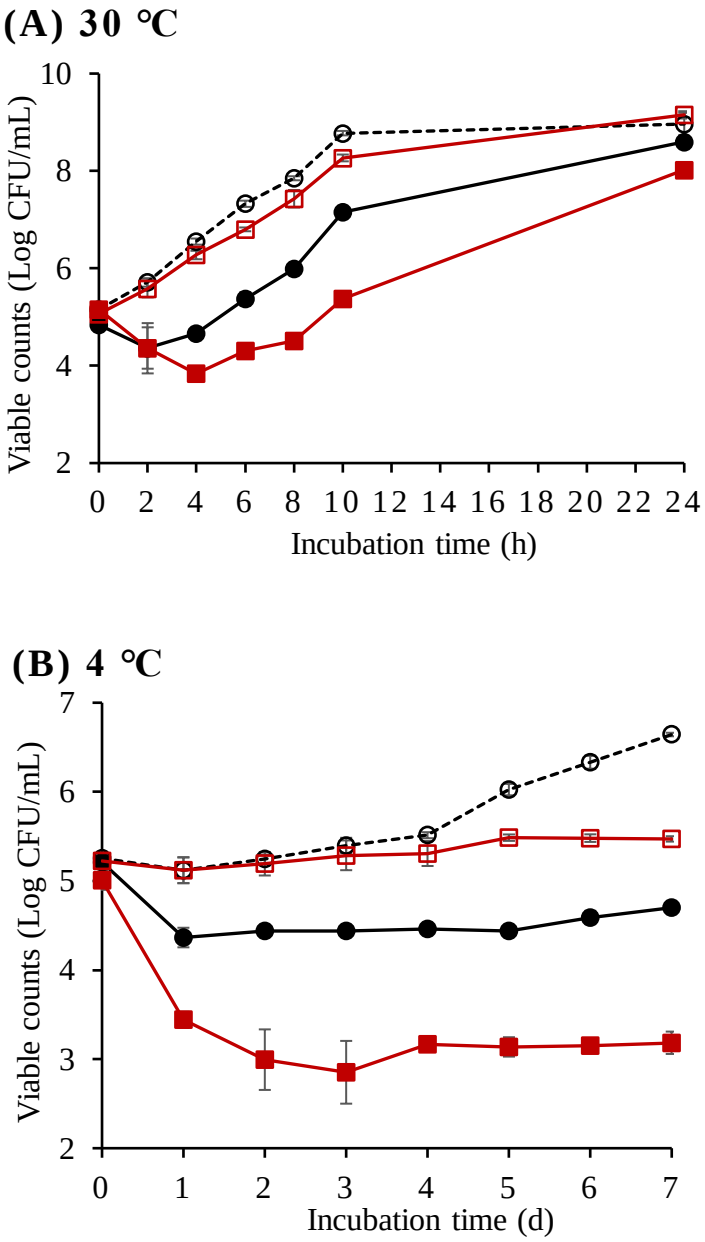


Fig. 4.5. Effects of phage vB_LmoS-PLM9 and/or clove oil against *L. monocytogenes* in milk at (A) 30 ℃, and (B) 4 ℃. Mean ± S.D. (n = 3).

Symbols: ○, Control; ●, Phage (MOI=100); □, 0.5% Clove oil; ■, Phage (MOI=100) + 0.5% Clove oil.

Chapter 5. Control of pathogenic *Listeria monocytogenes* on various solid foods and its biofilm by using bacteriophage vB_LmoS-PLM9 and/or cinnamon bark and clove oils

5.1. Introduction

Food contaminated by serious pathogens is a major risk to our socioeconomic and healthcare systems. The consumption of contaminated food causes 600 million illnesses and 420,000 deaths per year worldwide (WHO, 2022). *L. monocytogenes* is a ubiquitous environmental microorganism that can survive in high salt concentrations, low temperatures, and low pH. It can cause listeriosis outbreaks with a 20-30 % mortality rate, which are linked to the consumption of contaminated ready-to-eat foods, meat and meat products, milk and milk products, seafood, and vegetables (Duze et al., 2021; Hossain et al., 2022). Additionally, this bacterium can form biofilms, which spread harmful and spoiling microorganisms to food and food processing environments. Zhu et al. (2022) have reported that microbial biofilm formation is a severe concern in the food industry and causes over 60 % of foodborne outbreaks. Since food safety is one of the primary goals of the "One Health" approach, controlling the contamination and biofilm capability of *L. monocytogenes* is crucial in the food sector.

The use of promising natural and eco-friendly antimicrobial agents, such as bacteriophages, bacteriocin, enzymes, and compounds extracted from plants, has been reported recently in response to the serious worldwide public health issue of antibiotic resistance (Bai et al., 2023). Bacteriophages (phages) are viruses that infect specific target bacterial serotypes or strains. The ability of phage-host interactions ensures the target bacteria without disrupting the food's typical microbiota (Stone et al., 2020). Single wide-host range phage or phage cocktails have been employed as effective antimicrobial agents to combat *L. monocytogenes* contamination and associated biofilm in food or food processing environments (Akhtar et al., 2017; Byun et al., 2022; Soni

and Nannapaneni, 2010). However, most previous research revealed that using phages to eradicate a high population of biofilm was unsuccessful due to the host's development of resistance to the phage and the biofilms' ability to persist on phage-treated biotic and abiotic surfaces (Byun et al., 2022).

Essential oils and their components have been demonstrated as promising natural antimicrobial agents to inhibit foodborne pathogens including *L. monocytogenes* (Hossain et al., 2022; dos Santos et al., 2022; Zhang et al., 2020; Zhu et al., 2022). Cinnamon oil is the most common spice extracted from cinnamon bark (*Cinnamomum zeylanicium*), which exhibits antibacterial and antioxidant properties (Żukowska and Durczyńska, 2024). It has been used as a preservative to extend the microbial shelf life of foods (Van Haute et al., 2017). It also effectively prevents biofilm growth of pathogenic and spoilage bacteria due to its active components, cinnamaldehyde, cinnamate, and cinnamic acid (Vasconcelos et al., 2018; Liu et al., 2020a; Zhu et al., 2022). Clove oil (*Eugenia caryophyllius*) is a non-toxic, biodegradable, and broad-spectrum antimicrobial agent. Its main component, eugenol, is effective for bacteriostasis and anti-biofilm (Hossain et al., 2022; Zhang et al., 2020).

The combination of natural antimicrobial agents from plants and microorganisms can minimize effective concentrations, costs, and sensory effects if they work synergistically (Corrêa et al., 2021; Hossain et al., 2022; Moon et al., 2020). Combining phage and cinnamon or clove oil had significant benefits in suppressing the survivability of *L. monocytogenes* in milk as described in Chapter 4 (Maung et al., 2024), however, more research is required to utilize phage and essential oil in diverse foods for advanced applications in the food sector. Therefore, this chapter investigated the biocontrol of pathogenic *L. monocytogenes* on solid food matrices and its biofilms using single and combined phage vB_LmoS-PLM9 and cinnamon or clove oil.

5.2. Materials and methods

5.2.1. Preparation of *L. monocytogenes* culture

The pathogenic serotype 4b strain, *Listeria monocytogenes* no.193 was used in this experiment. This bacterium was isolated from minced pork and well-characterized in our Laboratory (Chapter 2). The bacterial cell was prepared by streaking the frozen stock (−80 °C) onto a TSA plate for 24 h and cultured in 5 mL BHI broth overnight at 30 °C. The fresh culture was used for further trials.

5.2.2. Preparation of bacteriophage vB_LmoS-PLM9

The isolated *Listeria* phage vB_LmoS-PLM9 was used in this study. The potential phage vB_LmoS-PLM9 was isolated from chopped chicken meat using the target host *L. monocytogenes* no.193. The phenotypic and genotypic characterization of this phage was described in Chapter 3. The fresh phage stock solution was prepared using the described propagation method in Chapter 4. In brief, 100 µL phage suspension (10^4 PFU/mL) was mixed with 100 µL host culture in 4 mL of top agar with 1 mmol/L $CaCl_2$, plated on TSA, and maintained at 30 °C for 24 h. The SM buffer (5 mL) was added to dense-plaque assay plates and left at room temperature (25 °C) overnight with shaking. The phage suspension was centrifuged, and then the supernatant was filtered and kept as phage stock (10^{10} PFU/mL) at 4 °C until use.

5.2.3. Preparation of EOs

As documented in Chapter 4, cinnamon bark (cinnamon) and clove essential oils purchased from iHerb (NowFoods, Japan) were dissolved in 0.5 % (v/v) Tween 80 to prepare 4 % stock. Working solutions were prepared by diluting the stock solution with BHI broth or 0.5 % Tween 80.

5.2.4. Determination of viability of *L. monocytogenes* treated with phage and/or cinnamon oil on ham, boneless skinless chicken meat, smoked salmon, and natural cheese

The food models such as ham, boneless skinless chicken meat, smoked salmon, and natural cheese purchased from supermarkets in Fukuoka were used in this experiment. For the treatments on ham, chicken meat, and natural cheese, phage (5×10^8 PFU/mL) and cinnamon oil (0.5 or 1 %) were applied. The concentrations of phage (10^9 PFU/mL) and cinnamon oil (0.125 or 0.25 %) were used to apply on smoked salmon. Briefly, samples were cut into 2 cm × 2 cm pieces and exposed to ultraviolet (UV) radiation for 30 min to reduce surface contamination. The samples were artificially contaminated with *L. monocytogenes* suspension (100 µL) to achieve 10^4 CFU/piece. After 30 min, 100 µL of phage and/or cinnamon suspension was spread to the surface of the contaminated samples and then stored at 30 °C for 24 h and 4 °C for 5 days. Sterilized broth was expended for the control sample. At 2, 4, 6, and 24 h at 30 °C and every single day at 4 °C, samples were taken, homogenized in 10 mL of PBS, serially diluted in PBS, and overlaid on CHROMagar™ *Listeria* (CHROMagar, Paris, France). The viable cells were counted after incubation at 30 °C for 24 h.

5.2.5. Determination of viability of *L. monocytogenes* treated with phage and/or cinnamon oil on ham and salmon fillet

In the application of phage and/or clove oil on ham and salmon fillets, treatments of phage (10^9 PFU/mL) and clove oil (1 or 2 %) were used. The samples were prepared as described in Section 5.2.4, and the viable cell numbers were detected at 30 °C for 2, 4, 6, 24 h, and 4 °C every day for 5 d using CHROMagar.

5.2.6. Determination of viability of *L. monocytogenes* treated with phage and/or cinnamon oil in mixed salad and coleslaw

In these food trials, the effectiveness of phage and/or essential oil against *L. monocytogenes* in mixed salad and coleslaw was performed at a low temperature of 4 °C. For single treatment, phage (10^8 PFU/mL), cinnamon (1 %), and clove (2 %) were applied. For phage plus essential oils treatments, phage (10^8 PFU/mL) and half concentrations of cinnamon (0.5 %) or clove (1 %) were formulated. Sample preparation was performed using the procedure by Lewis et al. (2019). Briefly, samples were prepared by weighing 10 g each in a sterilized stomacher bag and keeping them under UV for 30 min. After that, 100 µL diluted bacterial suspension (approximately 10^6 CFU/mL) was contaminated in the prepared sample. One milliliter of phage suspension was diluted in SM buffer, 1 mL of cinnamon or clove oil solution was diluted in 0.5 % Tween 80 for single treatments, whereas 500 µL of phage and 500 µL of essential oil for combined treatments were added to the contaminated samples. The SM buffer and 0.5 % Tween 80 were added as a control. All the prepared samples with or without treatments were kept in zip-lock bags and maintained at 4 °C for 5 d. Samples were extracted every day, homogenized in 9 mL of PBS, and diluted with PBS, and then cell viability was determined on CHROMagar.

5.2.7. Determination of biofilm of *L. monocytogenes* after the treatment with phage and/or cinnamon and clove oils

The biofilm development assay was carried out in a 96-well polystyrene microplate (Violamo, Japan) following the procedure reported by Miyamoto et al. (2011). *L. monocytogenes* 193 was cultivated in BHI broth overnight, shaking at 120 rpm and 30 °C. The overnight culture was diluted with broth to produce an OD_{660} of 0.7. Two hundred microliters of diluted suspensions were inoculated to each well of microtiter plates and incubated at 30 °C for 24 h to form biofilm.

Time-kill experiment of single and combined phage and cinnamon or clove oil

against *L. monocytogenes* biofilms' removal was carried out as described by Duc et al. (2023). Following the formation of biofilms, the planktonic cells were removed by pipetting, and the wells were cleaned twice with sterile water before being treated with phage (5×10^9 PFU/mL) and cinnamon (0.125 %) or clove oil (0.25 %). For the control, the broth was inoculated instead of the treatment. After incubating at 30 °C for 0, 2, 4, 6, and 24 h, the supernatants were gently removed, and the wells were raised twice with sterile water. After adding 200 µL of PBS to each well, biofilm cells were collected by scratching the surface with pipette tips and continuously resuspending. The obtained suspensions were diluted with PBS, and viable counts were examined by the conventional plating method on CHROMagar.

5.2.8. Statistical analysis

Data were calculated as mean ± standard deviation from triplicate experiments. The student's t-test was used to identify significant differences ($P < 0.05$) between the control and treatment groups.

5.3. Results

5.3.1. Effects of phage and/or cinnamon oil on the viability of pathogenic *L. monocytogenes* on ham, boneless skinless chicken meat, smoked salmon, and natural cheese

The applications on ham are shown in Fig. 5.1. At 30 °C, the viable cells were reduced by approximately 1 log at 2 h and 2.3 log at 24 h incubation in the phage-treated sample. Survival cells decreased when cinnamon oil was treated alone compared to the control group. However, the combined therapy with phage and cinnamon oil was more effective than the single treatment. *L. monocytogenes* viable counts were lowered by approximately 2.5 log at 6 h, and cell growth was inhibited by 4.2 log at 24 h in the presence of phage and 1 % cinnamon oil (Fig. 5.1A). At 4 °C, a single phage treatment lowered around 1.3 log at 1 d, whereas cinnamon oil (0.5 or 1 %) alone had no

discernible impact. The combination of phage and cinnamon oil (1 %) reduced surviving cells by 2.3 log at 1 d and continuously inhibited by suppressing the regrowth cells under initial cell counts (Fig. 5.1B).

The applications on boneless skinless chicken meat are displayed in Fig. 5.2. At 30 °C, viable cells were reduced by 1 and 0.7 log after 6 h of phage and cinnamon oil treatment alone, respectively. However, phage combined with 0.5 or 1 % cinnamon oil lowered viable numbers by about 1.3 log and 2.3 log, respectively, after 6 and 24 h of incubation compared to the growth of the control sample. The combined treatment of phage and 1 % cinnamon oil immediately reduced approximately 1 log after 2 h (Fig. 5.2A). Figure 5.2B also shows that the combined phage and cinnamon oil treatments were more effective than single treatments at 4 °C. The viable cells were reduced by approximately 1.2 and 1.4 log at 1 d in combined phage and 0.5 or 1 % cinnamon oil. The survival cell counts were decreased by approximately 2.9 log in the combined phage and 0.5 % cinnamon oil after 5 d storage, whereas they were lowered and reached under a detection level in the combined phage and 1 % cinnamon oil.

The effects on smoked salmon at 30 °C are shown in Fig. 5.3. The viability of *L. monocytogenes* was reduced by approximately 1 log after 2 h incubation in phage treatment. Cinnamon oil treatment did not affect viable counts at 0.125 or 0.25 %. No additional effects were observed on the viability of *L. monocytogenes* treated with combined phage and cinnamon oil.

Figure 5.4 indicates the efficiency of phage and/or cinnamon oil against *L. monocytogenes* on natural cheese at 4 °C. The viable counts of *L. monocytogenes* in the phage-treated sample were reduced immediately compared to the control, but not in the 0.5 or 1 % cinnamon oil-treated sample. The surviving cells were decreased by 1.8 log at 2 d in combining phage and 0.5 % cinnamon oil treatment, and by 2 log at 1 d in combining phage and 1 % cinnamon oil, demonstrating that the combination treatments were more effective than the single ones.

5.3.2. Effects of phage and/or clove oil on the viability of pathogenic *L. monocytogenes* on ham and salmon fillet

The applications of phage and/or clove oil on ham at 30 °C, the phage-treated ham sample indicated that the viable cells were reduced by about 1.1 log and 1.7 log after 2 and 24 h of incubation compared to the control. Although the viable counts of *L. monocytogenes* were not significantly reduced in clove oil-treated samples at 2 h, they were reduced by 1.6 log and 1.9 log in 1 and 2 % clove oil-treated samples at 24 h. The survival cells were reduced at a maximum of 2.3 log at 6 h and 3.6 log at 24 h in combined treatment with phage and 2 % clove oil, whereas they were decreased by 1.6 log at 6 h and 2.8 log at 24 h in the combined phage and 1 % clove oil (Fig. 5.5A). At 4 °C, phage treatment reduced the viable cells by 1.5 log at 1 d and continuously inhibited cell growth until 5 d. Clove oil therapy with 2 % inhibited bacterial cell development for 1 to 3 d. Nevertheless, combined treatments were more effective than single ones. When *L. monocytogenes* was treated with phage and clove oil (1 or 2 %), their vitality steadily declined and nearly reached the lowest detection limit at 4 and 5 d (Fig. 5.5B).

On salmon fillet, the viable cells were reduced by 1.1, 0.6, and 0.9 log in the single treatment of phage, 1 %, and 2 % of clove oil after 4 h at 30 °C. These results indicated lower effects than the viability of *L. monocytogenes* reduced by 1.3 and 1.5 log in phage combined with 1 and 2 % clove oil after 4 h incubation (Fig. 5.6A). At 4 °C, a reduction of viable cells was observed by 1.1 log in treatment of combined phage and 2 % clove oil at 2 d, however, no significant effects were found in other therapies (Fig. 5.6B).

5.3.3. Effects of phage and/or cinnamon and clove oils on the viability of pathogenic *L. monocytogenes* in mixed salad and coleslaw

In the mixed salad trial, the sample treated with phage alone decreased small numbers of cells at 3 d. The viable cells were immediately reduced by approximately 1

log CFU/g in the 2 % clove oil treatment, but it had no effect in the 1 % cinnamon oil treatment. The combined phage and 0.5 % cinnamon oil reduced the surviving cells by about 1 log at 1 d and continuously inhibited the regrowth of cells. Combined phage and clove oil (2 %) had the greatest impact, reducing viable counts by 1.5 log CFU/g at 2 d and steadily falling to nearly an undetectable level after 4 d (Fig. 5.7A).

Similar results showed that combination treatments were more beneficial than individual ones in the coleslaw food trial at 4 °C. Combined phage and half concentration of cinnamon oil (0.5 %) treatment reduced survival cells by about 1 log at 1 d and 1.5 log at 2 d, then continuously prevented regrowth cells, which were more effective than 1 % cinnamon oil treatment alone. In 2 % clove oil treatment, survival cells were reduced only by about 1 log at 1 d and inhibited the regrowth of cells over a 5-d period. However, combining phage and half concentration of clove oil (1 %) was more effective by reducing the viable counts to 2.3 log CFU/g at 2 d and limiting the resistance cell populations till the end of the experiment (Fig. 5.7B).

5.3.4. Effects of phage and/or cinnamon oil on removal of *L. monocytogenes* biofilm

The effect of phage vB_LmoS-PLM9 and/or cinnamon oil on the removal of biofilms is shown in Fig. 5.8A. The biofilm cells were reduced by approximately 1.5 log CFU/well after 2 h incubation in the phage treatment alone. The treatment of cinnamon oil (0.125 %) alone did not show an obvious effect until 6 h but it reduced by 1.8 log at 24 h when compared with the control. In phage plus 0.25 % cinnamon oil, biofilm cells were decreased by 1.5 log at 2 h, 2 log at 4 h, and 4.4 log at 24 h compared to the biofilm cells well without treatment.

5.3.5. Effects of phage and/or clove oil on removal of *L. monocytogenes* biofilm

Fig. 5.8B illustrates the effects of phage vB_LmoS-PLM9 and clove oil in an individual or combination on removing *L. monocytogenes* biofilms. The biofilm cells were decreased by approximately 1.4 log CFU/well after 2 h and continuously inhibited

after 24 h in phage treatment alone, while those were not observed in 0.25 % clove oil alone. The combination of phage and 0.25 % clove oil was more effective than the treatment alone, which decreased the biofilm cells by approximately 3.2 log after 4 h compared to the initial cell counts and suppressed the regrowth cell populations.

5.4. Discussion

Listeria monocytogenes contamination in various foods and their biofilm formation capability are serious food safety problems for consumers and the food industry. The development of natural alternative antimicrobial agents has become a top focus in research since foodborne bacteria are resistant to antibiotics. According to this purpose, our earlier study in Chapter 4 isolated a potential phage vB_LmoS-PLM9, evaluated the effective essential oils including cinnamon and clove oils, and determined their effects in single and combined therapy against *L. monocytogenes* in milk. However, we still need to do more research to control this pathogen which may lead to the food safety approach. Therefore, this study aimed to investigate the biocontrol of pathogenic *L. monocytogenes* in various solid food matrices such as ham, salmon, chicken meat, natural cheese, mixed salad, and coleslaw, and its biofilms using phage, cinnamon, and clove oil alone or in combination.

Controlling *L. monocytogenes* on the ham food model using commercial phages such as Listex™ P100 and ListShield™ (Gutiérrez et al., 2017) and essential oils including cinnamon oil (dos Santos et al., 2022) have been reported previously. Using high concentrations (3, 5, and 10 %) of cinnamon oil could inhibit the growth of *L. monocytogenes* on ham, however, it may not be acceptable for some consumers due to sensory factors (dos Santos et al., 2022). To reduce sensory impacts using a lower dosage of essential oils, a combination of two antibacterial agents is increasing in interest. Furthermore, there was no report of combining phage and essential oil therapy against *L. monocytogenes* on ham. In this study, ham treated with phage alone reduced the viable counts of *L. monocytogenes* at 2 h but some resistance cells grew back after

24 h at 30 °C. At 4 °C, the phage treatment reduced viable cells at 1 d and inhibited until 5 d. Single cinnamon or clove oil treatments showed their effects relied on tested concentrations. Although we did not find the surviving cells reduction in treatments of cinnamon oil (0.5 or 1 %) and clove oil (1 %) alone, the viable cells were decreased when treated with clove oil (2 %) at 4 °C. However, the combined phage and cinnamon or clove oil reduced *L. monocytogenes* vitality and limited the populations of resistant cells compared with treatment alone, especially at 4 °C storage. These results suggested that the phage combined with essential oil treatment could be a solution to suppress the phage resistance mutants and lessen the sensory effects of essential oil by using lower concentrations on ham.

The phage and cinnamon or clove oils were also applied to the smoked salmon. The viable cells were reduced at 2 h in the phage-treated sample, while they did not show any effects in the cinnamon oil-treated sample. The combined phage and cinnamon oil did not observe any additional effects to inhibit the *L. monocytogenes*. Moosavi-Nasab et al. (2019) reported similar findings, stating that cinnamon oil had no antibacterial impact on vacuum- or modified atmosphere-packaged salmon. Van Haute et al. (2017) also found no antimicrobial effectiveness when cinnamon oil was applied to salmon. And, only a few viable cells were reduced in the treatment of 2 % clove oil interaction with phage. These results may be due to the abundance of lipids in salmon, resulting in the distribution of active chemicals of essential oils to the salmon lipid phase. Other variables may be necessary to activate the antibacterial activity of essential oil and phage on salmon.

Chicken meat is the most consumable and important vehicle for public health concerns worldwide. Applications of phage against *Salmonella, E. coli,* and *C. perfringens* were previously reported in our laboratory (Duc et al., 2020; Noor Mohammadi et al., 2022a; Zhao et al., 2023), however, it has not been studied yet against *L. monocytogenes* using specific *Listeria* phage. Plant-based essential oils and

their compounds including cinnamon and clove oils alone against *L. monocytogenes* in chicken meats were documented in other studies (Mahfuzul Hoque et al., 2008; Noori et al., 2018; Shahbazi et al., 2018; Raeisi et al., 2016; Yousefi et al., 2020). Only one report observed a combination of commercial phage (SALMONELEX) and essential oils against *Salmonella* in chicken meat (Moon et al., 2020). This study examined using phage and cinnamon oil alone or in combination to combat *L. monocytogenes* in boneless skinless chicken meat. The maximum viable counts were reduced after 6 h at 30 °C and 3 d at 4 °C in phage-treated samples. The treatment of 1 % cinnamon oil was more effective at a low temperature (4 °C) than storage at 30 °C. However, bacterial cell reduction was not observed in the treatment of 0.5 % cinnamon oil in both temperatures. Nonetheless, the combined therapy with phage and cinnamon oil indicated better effectiveness than the single treatment, especially at 4 °C. The survival cell counts were reduced immediately at 1 d in both combined treatments. And they were continuously decreased in phage combined with 0.5 % cinnamon oil while reduced to a lower detection level in phage combined with 1 % cinnamon oil at 5 d trial. These findings suggested that combined phage and cinnamon oil could be an effective and promising anti-listerial treatment for chicken meat.

A listeriosis outbreak was caused by contaminated natural cheese, as reported in Japan in 2001 (Makino et al., 2005). Cheese contains rich nutrients, which makes bacteria and fungi more likely to become contaminated. Khorshidian et al. (2018) reported that several essential oils can be used as natural antimicrobial agents to combat foodborne pathogens in cheese and extend the shelf-life. However, negative effects on organoleptic properties were observed if they applied high concentrations of essential oils. Therefore, this study investigated phage plus lower concentrations of cinnamon oil against *L. monocytogenes* in natural cheese at 4 °C. Phage alone instantly reduced viable cell counts, however, cinnamon oil (0.5 or 1 %) alone had no effect. The combined treatment of phage and cinnamon oil immediately reduced the survival cells

and inhibited the resistance cell populations until 5 d. These results may be helpful for consumer safety without any sensory impacts.

Similar results were observed when we applied the phage and/or cinnamon or clove oil in mixed salad and coleslaw food models. At 4 °C storage for 5 d, phage and half concentrations of cinnamon (0.5 %) or clove (1 %) oil treatments were more effective than single ones. Similar results were reported by Lewis et al. (2019) that the combined use of commercial phage (P100) and Nisaplin was more effective than single use in coleslaw stored at 4 °C during a 10-d period. Most of the results in this study indicated that the combined phage and essential oil therapy was more effective than the phage or essential alone. This may be due to phage and active components of essential oils may exert synergism to inhibit bacterial cells and prevent the resistance cell populations. Leistner and Gorris. (1995) also documented that hurdle technology should be employed often to remove or control pathogens by combining multiple preservation methods.

Biofilm formation is a severe problem in the food industry and is difficult to prevent. Many studies are approaching to inhibit or eradicate the *L. monocytogenes* biofilms by using phages or phage cocktails (Byun et al., 2022; Soni and Nannapaneni, 2010) and various essential oils such as *Cinnamon zeylanicum* and *Thymbra capitata* (Maggio et al., 2023), oregano (Tahric et al., 2023), lavender (Han et al., 2022), garlic (Liu et al., 2024), and clove oil (Zhang et al., 2020). Furthermore, a combination of two antimicrobial agents is being developed to provide greater results in combating pathogenic biofilms. Nisin and essential oil components such as thymol and eugenol (Hossain et al., 2022), nisin and plant extracts (Santativongchai et al., 2023) against *L. monocytogenes* biofilms, as well as bacteriophage and carvacrol against *Pseudomonas syringae* and *actinidiae* biofilms (Ni et al., 2020) have been effectively employed. The results in this study are in agreement with those previous reports for the removal of preformed biofilms. The phage alone with high MOI also showed a reduction of

preformed biofilm cells. The use of sub-inhibitory concentrations of cinnamon or clove oil did not show obvious effects in single therapies, however, those combined with phage had better effectiveness in destroying preformed biofilms. These preliminary findings in controlling the preformed pathogenic *L. monocytogenes* biofilms using phage interacted with cinnamon or clove oil could be useful in the ongoing investigation of controlling biofilms in the food industry.

5.5. Summary

This chapter investigated the effects of single and combined phage and cinnamon or clove oil against pathogenic *L. monocytogenes* in various solid food matrices and their biofilm removal. The treatments of the phage plus cinnamon oil (0.5 or 1 %) or clove oil (1 or 2 %) reduced the survival cell counts of *L. monocytogenes* on ham but not salmon. The combined phage and 1 % cinnamon oil treatment reduced the number of surviving cells in chicken meat more than the individual treatments, especially at 4 °C storage. In natural cheese at 4 °C, phage alone and phage combined with cinnamon oil treatments reduced the viable cell counts, however, cinnamon oil (0.5 or 1 %) alone did not show any effects. In mixed salad and coleslaw trials at a low temperature, phage + 1 % clove oil and phage + 0.5 % cinnamon oil treatments were more effective than others. On the other hand, the combined treatments of phage and 0.125 % cinnamon oil or 0.25 % clove oil have shown high effectiveness in inhibiting preformed biofilms compared to individual treatments. These results suggested that combined phage and cinnamon or clove oil is a promising candidate to control *L. monocytogenes* in various foods and their biofilms.

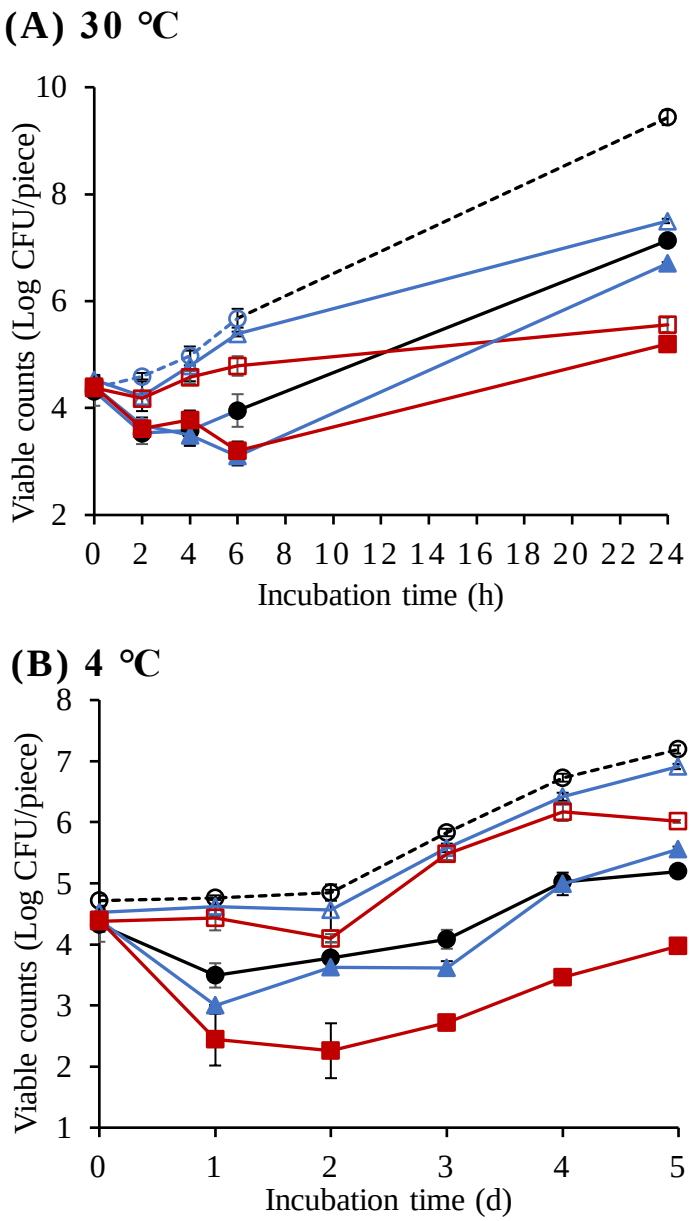


Fig. 5.1. Effects of phage vB_LmoS-PLM9 and cinnamon bark oil alone or in combination on the viability of *L. monocytogenes* on ham at (A) 30 ℃, and (B) 4 ℃. Mean ± S.D. (n = 3).

Symbols: ○, Control; ●, Phage (5×10^8 PFU/mL); △, 0.5 % C.b; □, 1 % C.b; ▲, Phage (5×10^8 PFU/mL) + 0.5 % C.b; ■, Phage (5×10^8 PFU/mL) + 1 % C.b.

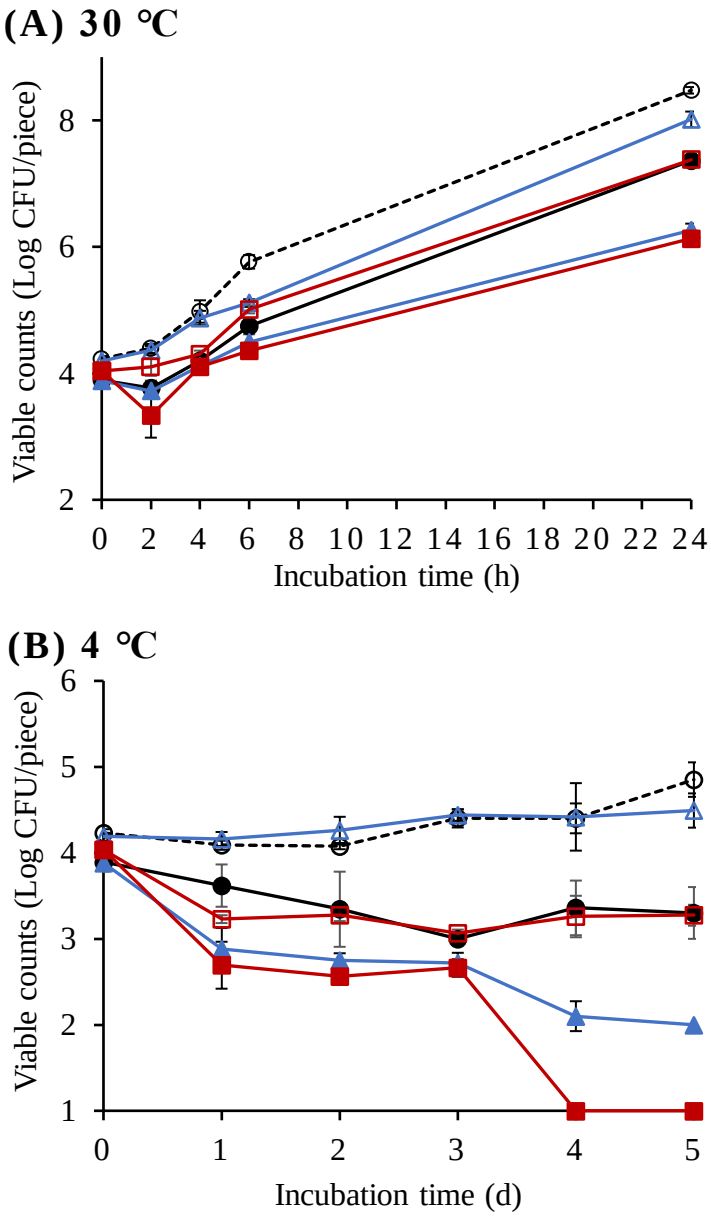


Fig. 5.2. Effects of phage vB_LmoS-PLM9 and cinnamon bark oil alone or in combination on the viability of *L. monocytogenes* on boneless skinless chicken meat at (A) 30 ℃, and (B) 4 ℃. Mean ± S.D. (n = 3).

Symbols: ○, Control; ●, Phage (5×10^8 PFU/mL); △, 0.5 % C.b; □, 1 % C.b; ▲, Phage (5×10^8 PFU/mL) + 0.5 % C.b; ■, Phage (5×10^8 PFU/mL) + 1 % C.b.

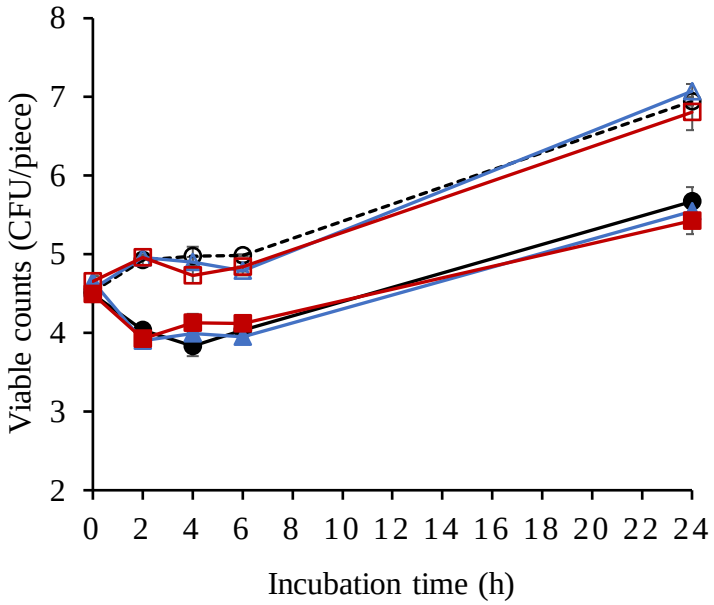


Fig. 5.3. Effects of phage vB_LmoS-PLM9 and cinnamon bark oil alone or in combination on the viability of *L. monocytogenes o*n smoked salmon at 30 °C. Mean ± S.D. (n = 3).

Symbols: ○, Control; ●, Phage (10^9 PFU/mL); △, 0.125 % C.b; □, 0.25 % C.b; ▲, Phage (10^9 PFU/mL) + 0.125 % C.b; ■, Phage (10^9 PFU/mL) + 0.25 % C.b.

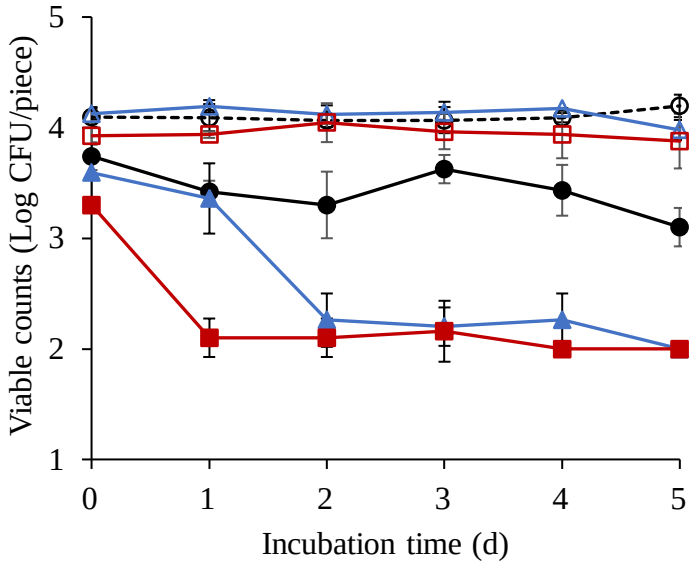


Fig. 5.4. Effects of phage vB_LmoS-PLM9 and cinnamon bark oil alone or in combination on the viability of *L. monocytogenes* on natural cheese at 4 ℃. Mean ± S.D. (n = 3).

Symbols: ○, Control; ●, Phage (5×10^8 PFU/mL); △, 0.5 % C.b; □, 1 % C.b; ▲, Phage (5×10^8 PFU/mL) + 0.5 % C.b; ■, Phage (5×10^8 PFU/mL) + 1 % C.b.

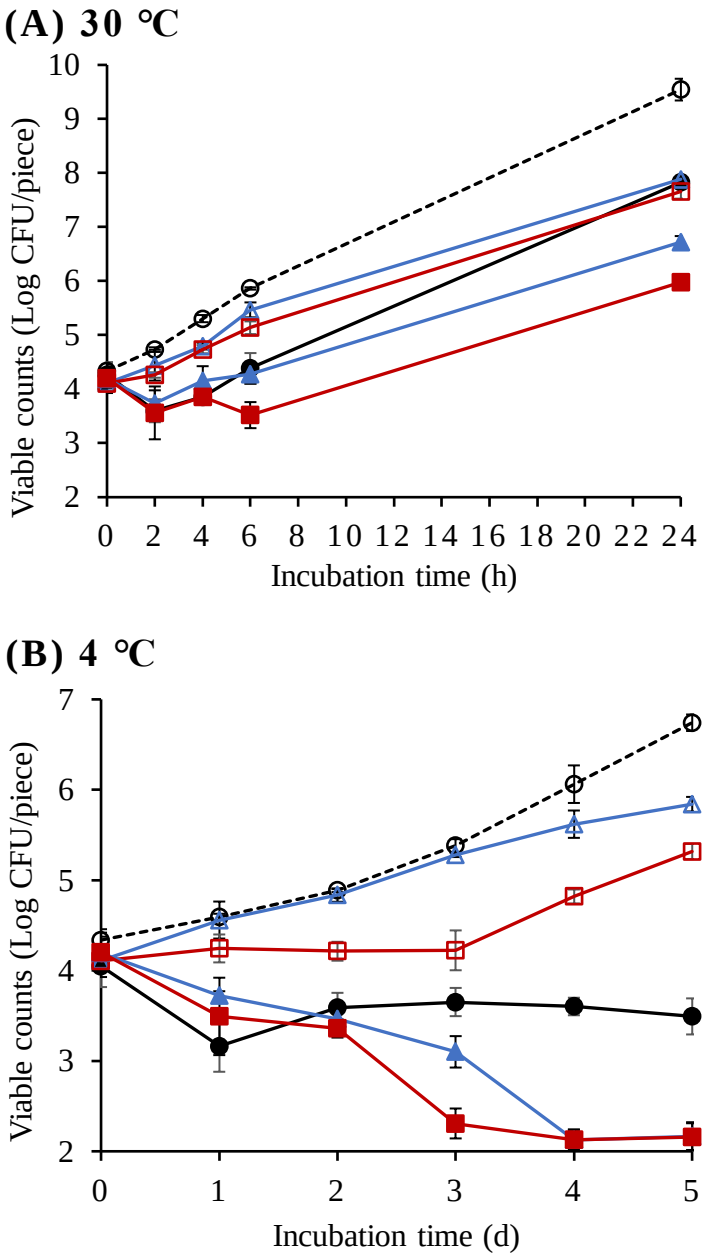


Fig. 5.5. Effects of phage vB_LmoS-PLM9 and clove oil alone or in combination on the viability of *L. monocytogenes* on ham at (A) 30 ℃, and (B) 4 ℃. Mean ± S.D. (n = 3).

Symbols: ○, Control; ●, Phage (10^9 PFU/mL); △, 1 % Clove oil; □, 2 % Clove oil; ▲, Phage (10^9 PFU/mL) + 1 % Clove oil; ■, Phage (10^9 PFU/mL) + 2 % Clove oil.

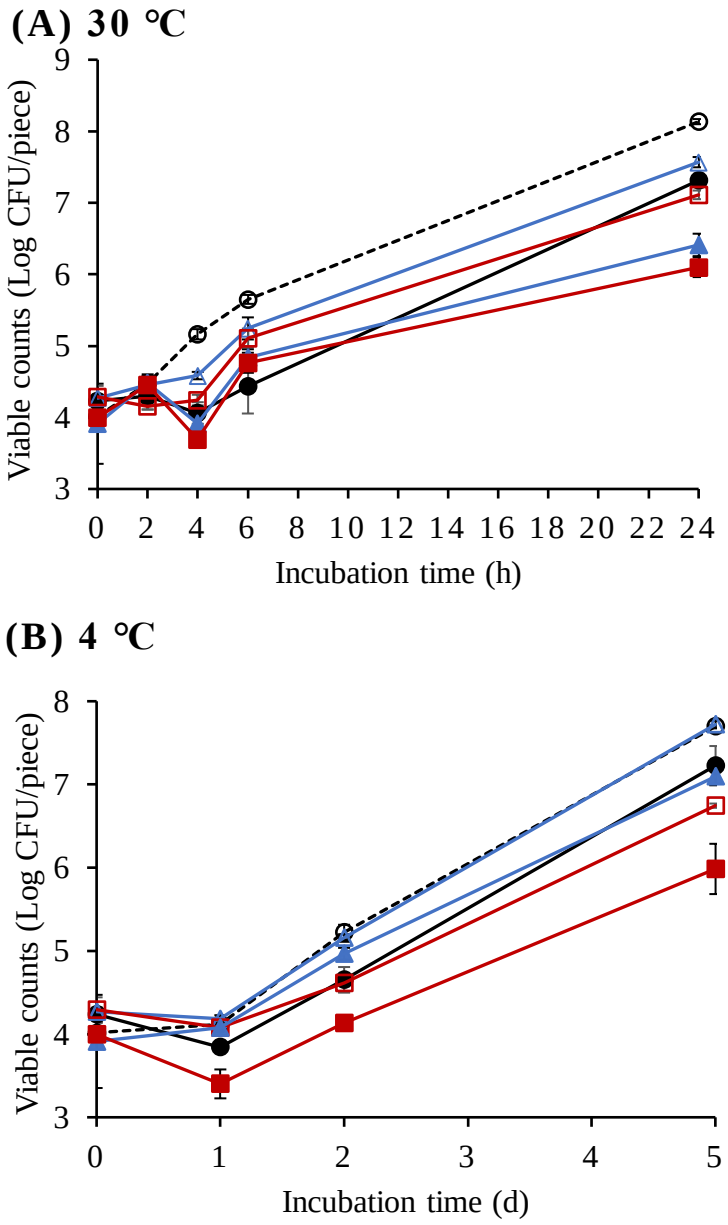


Fig. 5.6. Effects of phage vB_LmoS-PLM9 and clove oil alone or in combination on the viability of *L. monocytogenes* on salmon fillet at (A) 30 ℃, and (B) 4 ℃. Mean ± S.D. (n = 3).

Symbols: ○, Control; ●, Phage (10^9 PFU/mL); △, 1 % Clove oil; □, 2 % Clove oil; ▲, Phage (10^9 PFU/mL) + 1 % Clove oil; ■, Phage (10^9 PFU/mL) + 2 % Clove oil.

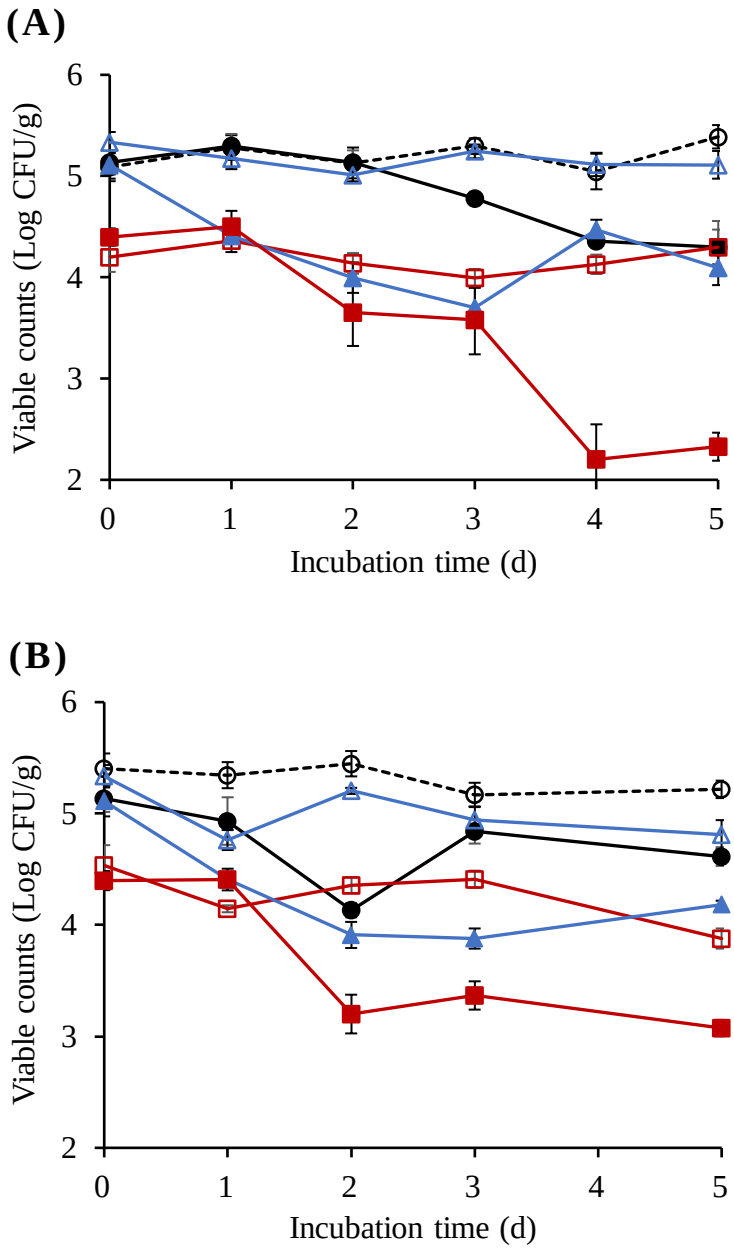


Fig. 5.7. Effects of phage vB_LmoS-PLM9 and cinnamon bark or clove oil alone or in combination on the viability of *L. monocytogenes* at a low temperature, 4 ℃ storage (A) mixed salad, and (B) coleslaw. Mean ± S.D. (n = 3).

Symbols: ○, Control; ●, Phage (10^8 PFU/mL); △, 1 % C.b; □, 2 % Clove oil; ▲, Phage (10^8 PFU/mL) + 0.5 % C.b; ■, Phage (10^8 PFU/mL) + 1 % Clove oil.

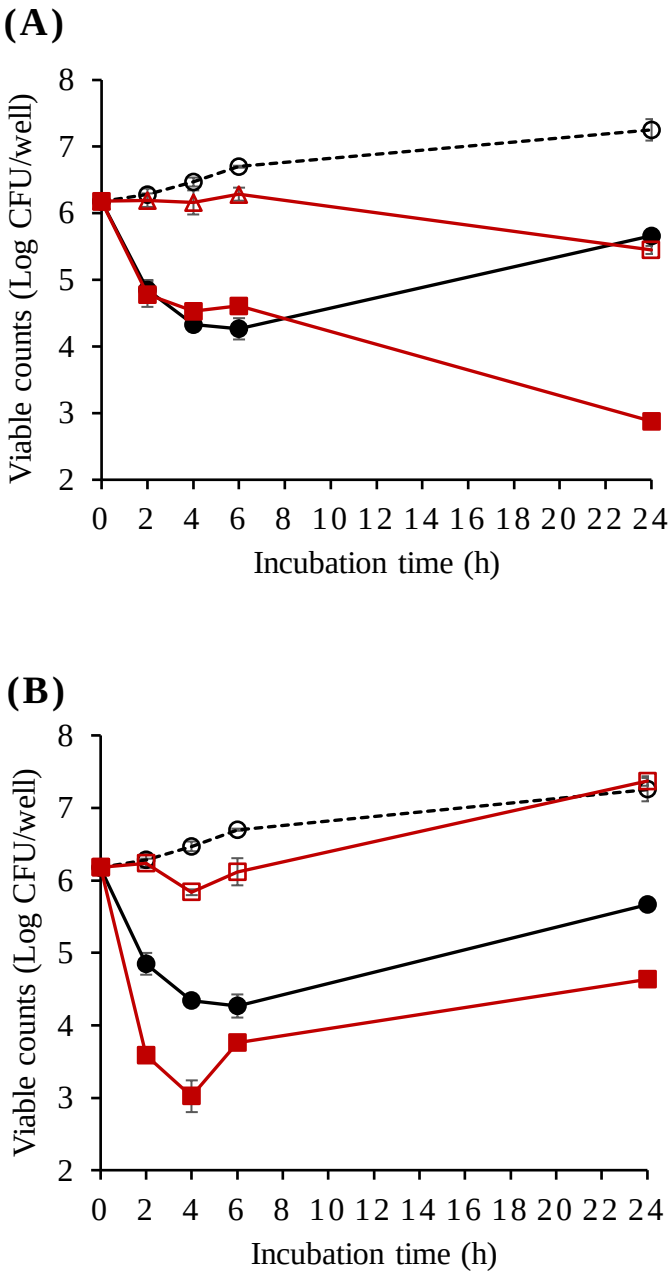


Fig. 5.8. (A) Effects of phage vB_LmoS-PLM9 and cinnamon bark oil alone or in combination on removing 24 h-preformed biofilms at 30 ℃. Mean ± S.D. (n = 3).

Symbols: ○, Control; ●, Phage (5×10^9 PFU/mL); □, 0.125% C.b; ■, Phage (5×10^9 PFU/mL) + 0.125% C.b.

(B) Effects of phage vB_LmoS-PLM9 and clove oil alone or in combination on removing 24 h-preformed biofilms at 30 ℃. Mean ± S.D. (n = 3).

Symbols: ○, Control; ●, Phage (5×10^9 PFU/mL); □, 0.25% Clove oil; ■, Phage (5×10^9 PFU/mL) + 0.25% Clove oil.

Chapter 6. Conclusions

Food safety has become an important public health concern worldwide due to the emergence of microbial foodborne pathogens. *L. monocytogenes* is a severe food poisoning pathogen, widely disseminated in our environment, which may easily infect food. It causes several listeriosis outbreaks in humans and animals, with high mortality rates due to consuming contaminated foods. Additionally, controlling biofilms in this pathogen on food and food-contact surfaces is essential since biofilm-associated infections have been linked to over 65 % of serious health problems. Due to increasing antimicrobial resistance, developing promising alternative antimicrobial agents to combat foodborne pathogens is critically necessary. Bacteriophage is a bacterial virus abundant in nature and possesses many advantages such as high host specificity, low cost, and no harmful effect on humans. According to previous studies, the bacteriophage is a potential antibacterial agent that can be used safely in foods. Plant-based essential oils have antimicrobial and antioxidant properties; therefore, they are used for bacteriostasis, bactericidal, and anti-biofilm in the food industry. Nevertheless, it is important to pay great attention to the combination of antibacterial agents derived from plants and microorganisms, as this may help to avoid the regrowth of resistant bacteria and require lower concentrations of each agent. Therefore, this study was conducted to characterize the antimicrobial-resistant *L. monocytogenes* isolated from food and investigate their biocontrol using specific bacteriophage and essential oils in a single or combination.

In Chapter 2, *L. monocytogenes* was isolated from 85 raw chicken and viscera samples collected from supermarkets in Fukuoka in 2022. Nine samples (10.6 %) were positive, and 17 strains were isolated. Characterization in serotyping indicated serotypes 1/2b (41.2 %), 3a (29.4 %), 3b (23.5 %), and 1/2a (5.9 %) respectively. All the isolates were identified and clustered by MALDI-TOF mass spectrometry. Antimicrobial susceptibility testing demonstrated the isolates were sensitive to several

antibiotics except cefoxitin, oxacillin, and fosfomycin. In the comparison of 5-year differences surveillance, the incidence of *L. monocytogenes* in 2022 was significantly ($P < 0.05$) lower than those in 2017 (24 %) and 2012 (52.9 %), and pathogenic serotype distributions decreased over time. Antimicrobial resistance (AMR) and multi-drug resistance rates of isolates in 2022 were reduced compared to those in 2017 and 2012. The virulence genes (*hlyA, plcA, clpC,* and *inlA*) were harbored in most isolates in 5-year intervals. The biofilm mass in 2022 isolates was significantly ($P < 0.05$) weaker than previous isolates. Although rates of detection from chicken meat and AMR of the isolates were not high, virulent *L. monocytogenes* contamination in foods still should pay attention.

Chapter 3 demonstrated the isolation and characterization of bacteriophages against *L. monocytogenes*. Eleven phages from raw chicken samples and 15 phages from Kyushu University's cattle farm samples were isolated. According to host range determination, most phages exhibited lytic activity against *L. monocytogenes*, *L. innocua, L. seeligeri, L. welshimeri,* and *L. grayi*. Characterization of three selected phages was performed. All the phages were stable at pH (4-10), and temperatures (4-50 °C), except for phage vB_LmoS-PLM34, which only survived up to 40 °C. The transmission electron microscopy of the broadest host range phage vB_LmoS-PLM34 showed an icosahedral head (diameter ~ 60 nm) and noncontractile tail (length ~ 230 nm) that belongs to the *Siphoviridae* family. The whole genomic DNA sequencing analysis demonstrated all 3 phages have no genes for tRNA, virulence, or antimicrobial resistance genes but endolysin genes. Phages vB_LmoS-PLM9 and vB_LmoS-PLM34 had N-acetylmuramoyl-l-alanine amidases, whereas phage vB_LmoS-PLM26 had peptidoglycan l-alanyl-d-glutamate endopeptidase endolysins. However, only phage vB_LmoS-PLM9 was a lytic phage since it did not have lysogenic-related genes. Its latent period (70 min) and burst size (51.2 ± 2.1 PFU/cell) were observed. These results

indicated that bacteriophages against *L. monocytogenes* were isolated and characterized phenotypically and genotypically.

In Chapter 4, eight essential oils including cinnamon bark, cinnamon cassia, clove, lemongrass, ginger, basil, turmeric, and lemon were evaluated for their antibacterial efficiency. Cinnamon bark and cinnamon cassia exhibited the strongest anti-listerial activities, followed by clove and lemongrass oils against *L. monocytogenes*, and various *Listeria* spp. were observed when tested at 10 %. The MIC and MBC values of both cinnamon bark and cassia oils were 0.0625 and 0.125 %, respectively, and those of clove oil were 0.25 and 0.5 %, respectively. The application of essential oils and/or phage vB_LmoS-PLM9 alone and in combination was investigated in broth and milk. The combined applications were highly effective compared to single ones. In broth at 30 °C, the survival cells of *L. monocytogenes* were decreased, and the regrowth of resistant cells was suppressed in the combined treatments with phage (MOI of 10) and both cinnamon oils (0.03 %), and phage (MOI of 1) and clove oil (0.125 %). The combined phage (MOI of 100) and cinnamon oil (0.125 %) showed high effectiveness in milk, particularly at 4 °C by reducing the viable counts under the lower detection level. Phage (MOI of 100) plus clove oil (0.5 %) decreased the surviving cells and inhibited the resistant cell growth in milk at 4 °C. These results suggested combining phage and essential oil is a potential approach for controlling *L. monocytogenes* in milk.

In Chapter 5, the biocontrol of pathogenic *L. monocytogenes* in various solid foods and their biofilm using phage vB_LmoS-PLM9 and cinnamon or clove oils were examined. Phage and cinnamon or clove oils showed higher effectiveness than single treatments, particularly at 4 °C. Phage (5×10^8 PFU/mL) and cinnamon bark (1 %) on ham, phage (5×10^8 PFU/mL), and cinnamon bark (0.5 %) on chicken meat, cheese, mixed salad, and coleslaw reduced the viable counts compared with the single treatment, but not in smoked salmon. The combined treatment of phage (5×10^8 PFU/mL) and cinnamon bark (1 %) decreased the viable cells less than the detection limit in chicken

meat after 4 d storage at 4 °C. In combined use of phage (10^9 PFU/mL) and clove oil (1 %) on ham, mixed salad, and coleslaw, viable counts decreased or reduced at 4 °C. Moreover, phage (5×10^9 PFU/mL) and cinnamon oil (0.125 %) or clove oil (0.25 %) decreased the viability of *L. monocytogenes* in the 24 h preformed biofilm at 30 °C. These results suggested that combined phage and cinnamon or clove oil might be a promising candidate for controlling pathogenic *L. monocytogenes* in the food industry.

In conclusion, this study investigated a 5-year difference surveillance on the occurrence, characterization, AMR, and pathogenicity of *L. monocytogenes* from raw chickens in Fukuoka, which will benefit the microbial risk assessment of the nation. Bacteriophages specific to *L. monocytogenes* were successfully isolated and characterized. Cinnamon bark, cinnamon cassia, and clove oils have been recognized as strong anti-listerial activities among 8 essential oils tested in this study. Moreover, combining phage and cinnamon or clove oils could be exploited as effective tools for controlling *L. monocytogenes* in various foods and their biofilms. Further studies will focus on the expression of endolysins as a promising biocontrol agent for controlling bacterial pathogens.

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