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Heterologous Biosynthesis of Lasso and LCI-Type Bacteriocins

Master thesis

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Thesis supervisor
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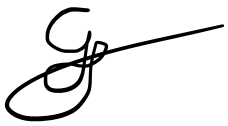
Author of the thesis: Gabija Smulkaitė 

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Abbreviations

AMP – antimicrobial peptide

DSMZ – German Collection of Microorganisms and Cell Cultures

GNAT - GCN5-related N-acetyltransferase

GNB – Gram-negative bacteria

GPB – Gram-positive bacteria

GRAS – substances generally recognized as safe by the United States Food and Drug Administration

LAB – lactic acid bacteria

Man-PTS – mannose phosphotransferase system

OD_{600} – optical density at 600 nm

PTLB – phage tail-like bacteriocin

RBP – receptor binding protein

RiPP – ribosomally synthesized and post-translationally modified peptide

RRE – RiPP recognition element

Introduction

Phytopathogenic bacteria are a major threat to global agriculture, causing extensive crop damage, significant economic losses, and negatively impacting the quantity and quality of agricultural production. These losses, in turn, pose challenges to food safety at the household, national, and global levels. Although the collection and comparison of standardized quantitative data on crop losses across different crops, agroecosystems, and geographic regions is difficult, estimates from 2019 indicate that among the five major crops, wheat, rice, maize, potatoes, and soybeans, diseases caused by 137 phytopathogenic organisms resulted in yield losses of approximately 10.2 %, 10.8 %, 8.5 %, 14.5 %, and 8.9 %, respectively (Savary et al., 2019). Traditional chemical pesticides pose environmental risks and contribute to the development of resistant bacterial strains, which results in the loss of their effectiveness (Rooney et al., 2020). This emphasizes the urgent need for the development of sustainable and ecologically responsible strategies to manage plant diseases effectively.

Bacteria naturally produce a wide range of bioactive compounds that enable them to compete with other microorganisms in their environment. These include broad-spectrum non-ribosomally synthesized antibiotics, lytic enzymes, exotoxins, secondary metabolites, and bacteriocins (Subramanian & Smith, 2015). Bacteriocins are a group of ribosomally synthesized antimicrobial peptides (AMPs) produced by bacteria (Kumariya et al., 2019). First described in 1925 by A. Gratia, interest in their production, functional roles, and potential applications has grown significantly in recent years. Due to their precise mode of action, bacteriocins are regarded as promising agents for targeted antimicrobial strategies, protecting beneficial microbial communities, particularly in the context of plant disease control. Of particular interest are lasso peptides and LCI-type bacteriocins.

LCI-type peptides are effective compounds with strong antimicrobial activity against plant pathogens like *Xanthomonas* and *Pseudomonas*. Lasso peptides are characterized by their threaded, lasso topology, providing them stability and resistance to proteolysis. Despite their potential, native production of these bacteriocins is often limited (Cheng & Hua, 2020). As a result, heterologous biosynthesis has emerged as a powerful tool to enhance the production of these AMPs. Given the increasing demand for narrow-spectrum AMPs that can be selectively targeted against phytopathogens without harming beneficial microbes, research into the heterologous production of structurally distinct bacteriocins is timely and strategically relevant.

Research aim: heterologously synthesize and characterize novel bacteriocins with antimicrobial activity against phytopathogenic bacteria.

Objectives:

1. Conduct heterologous gene expression of novel lasso peptide and LCI-type bacteriocins in *Escherichia coli* and *Lactococcus lactis* cells.
2. Confirm biosynthesis of bacteriocin peptides in heterologous hosts using SDS-PAGE.

3. Purify bacteriocin peptides for further characterization.
4. Evaluate the antibacterial activity of the synthesized bacteriocins against selected phytopathogenic bacteria.

1. Literature review

1.1. Bacteriocins and their sources

Antibiotics have transformed modern medicine, especially during the 20th century, by providing effective tools for treating bacterial infections (Gordon et al., 2005). However, the widespread and frequent use of these compounds has led to the rise of antibiotic-resistant bacteria (Nishie et al., 2012). This problem has encouraged researchers to explore natural alternatives for fighting these infections. One notable alternative is **antimicrobial peptides** (AMPs) – small, naturally occurring molecules synthesized by various organisms, such as fungi, plants, vertebrates, and bacteria (Sang & Blecha, 2008). Nevertheless, the most critical AMPs are those synthesized by bacteria. Bacterial AMPs can be categorized as ribosomally synthesized peptides, known as bacteriocins, and non-ribosomally synthesized peptides, which lack associated structural genes (Sang & Blecha, 2008; Ye et al., 2013; Chikindas et al., 2018). Bacteriocins present a compelling alternative to conventional antibiotics due to their narrower range of activity and minimized side effects compared to traditional antimicrobial agents.

Bacteriocins are a kind of ribosomally synthesized AMPs produced by bacteria, which can kill or inhibit bacterial strains closely related to or not related to the producer bacteria. However, due to specific immunity proteins, they will not harm the bacteria themselves (Yang et al., 2014). Bacteriocins can be synthesized by both Gram-positive bacteria (GPB) and Gram-negative bacteria (GNB), as well as some archaea (Karpíski & Szkaradkiewicz, 2016). These peptides demonstrate significant heterogeneity in size, structural configuration, and modes of action. Their biological activity is often characterized by high potency, primarily mediated through interactions with specific cell surface receptors. Yet, their spectrum of activity tends to be relatively narrow, as bacteriocins typically exhibit efficacy only against species that are phylogenetically related to the producer strain (J. W. Hegarty et al., 2016). Nonetheless, some bacteriocins have been identified as capable of acting not only against closely related bacteria but also across a broader spectrum (Simons et al., 2020).

Bacteriocin-producing bacteria can be isolated from diverse environmental and biological sources, such as aquatic ecosystems, soil, animal intestines, and the human gastrointestinal tract. The latter is a significant reservoir for bacteriocin-producing microorganisms (Darbandi et al., 2022). Gut-dwelling bacteria, for example, secrete AMPs to manage microbial competition within their communities. These peptides have garnered attention for their potential therapeutic and competitive advantages within the host microbiome. Nevertheless, soil remains the most extensively investigated natural source for bacteriocin-producing bacteria. Most of the soil-derived isolates belong to the genus *Bacillus*, whose AMPs have demonstrated promising utility in agricultural settings, particularly as biopesticides for sustainable pest control (Lv et al., 2017; Zimina et al., 2020).

Lactic acid bacteria (LAB), another critical group of bacteriocin producers, have been widely studied due to their extensive use in fermented food products. Their Generally Regarded as Safe (GRAS) status further enhances their applicability. LAB-derived AMPs encompass all three major classes of GPB bacteriocins, underscoring their versatility and relevance in food preservation and safety research (Trejo-González et al., 2021). Related to that, LAB bacteriocins have also been shown to display other biological functions such as antiviral activity, inhibition of biofilm formation, and anti-cancer activity (Pérez-Ramos et al., 2021). Although bacteriocins are extensively applied, documented instances of bacteria developing resistance to them remain relatively uncommon, which may be partly explained by their ability to act at very low concentrations and disrupt bacterial cells through pore formation in the membrane (Pérez-Ramos et al., 2021; Punia Bangar et al., 2022).

1.2. Classification of bacteriocins

The classification of bacteriocins has changed considerably as scientific knowledge about these biomolecules has expanded. The initial frameworks, established in the early 1990s, primarily categorized bacteriocins based on physicochemical traits, such as thermostability and molecular weight (Klaenhammer, 1993; Jack et al., 1995). However, as research progressed, new criteria were added to classification systems, including enzyme susceptibility, post-translational modifications, and distinct functional groups (K. Sharma et al., 2021). This shift in classification shows the complexity of categorizing bacteriocins, given their extensive diversity in structure, mechanisms of action, and genetic characteristics.

Currently, the most common approach to classifying bacteriocins is based on the cell wall type of the producing microorganism. This classification includes GPB and GNB bacteriocins, with some researchers also recognizing those produced by archaea, such as halocins. Beyond cell wall-based classification, bacteriocins can be categorized by structure, amino acid sequence, or physicochemical properties. Structurally, they are divided into cyclic forms, like “lasso peptides” with unique intramolecular bonds, and linear forms, such as lactococcin. Sequence-based classification relies on secondary structures (e.g., α-helices, β-sheets), while physicochemical classification groups bacteriocins by specific property values (Arnison et al., 2013; Zimina et al., 2020; Solis-Balandra & Sanchez-Salas, 2024). Further, the classification will be explained based on the cell wall type of the producing microorganism.

1.2.1. Gram-negative bacteria bacteriocins and their mechanisms of action

Bacteriocins produced by GNB tend to have a more limited range of activity, which restricts their potential applications when compared to those produced by GPB. This limitation arises from the fact that most of the bacteriocins identified in GNB are derived from *Escherichia coli* strains. Nonetheless, other genera from the Enterobacteriaceae family, such as *Pseudomonas* and *Klebsiella*,

also demonstrate the ability to produce active bacteriocins (Simons et al., 2020). GNB-derived bacteriocins are typically categorized into two main groups: colicins (30–80 kDa) and microcins (1-10 kDa) (Negash & Tsehai, 2020). However, there is a third type of phage tail-like bacteriocins (PTLBs), which is not yet fully characterized (Solis-Balandra & Sanchez-Salas, 2024).

Colicins are a class of AMPs mainly produced by *E. coli* that harbor a colicinogenic plasmid (Negash & Tsehai, 2020). They provide an important framework for investigating the structural and functional adaptations of bacteriocins (Zimina et al., 2020). These peptides are cytotoxic to closely related bacterial strains with specific outer membrane receptors but lack the immunity proteins needed to counteract such toxins. Similar bacteriocins, known as **colicin-like peptides**, are synthesized by other bacterial species but exhibit similar mechanisms of action and structural, functional properties (Tracanna et al., 2017).

Colicins are further classified into three groups based on their mechanisms of action against target cells: pore-forming, nuclease, and peptidoglycan-degrading (**Figure 1.1**) (Hahn-Löbmann et al., 2019). Their uptake by target cells is facilitated through receptors normally involved in nutrient transport. These include receptors for vitamin B12 (BtuB), iron-bound siderophores (FhuA, FepA, Cir, Fiu), and nucleosides (Tsx). Additionally, some colicins exploit porin proteins, which regulate the passive diffusion of sugars, phosphates, and amino acids across the outer membrane (Zimina et al., 2020).

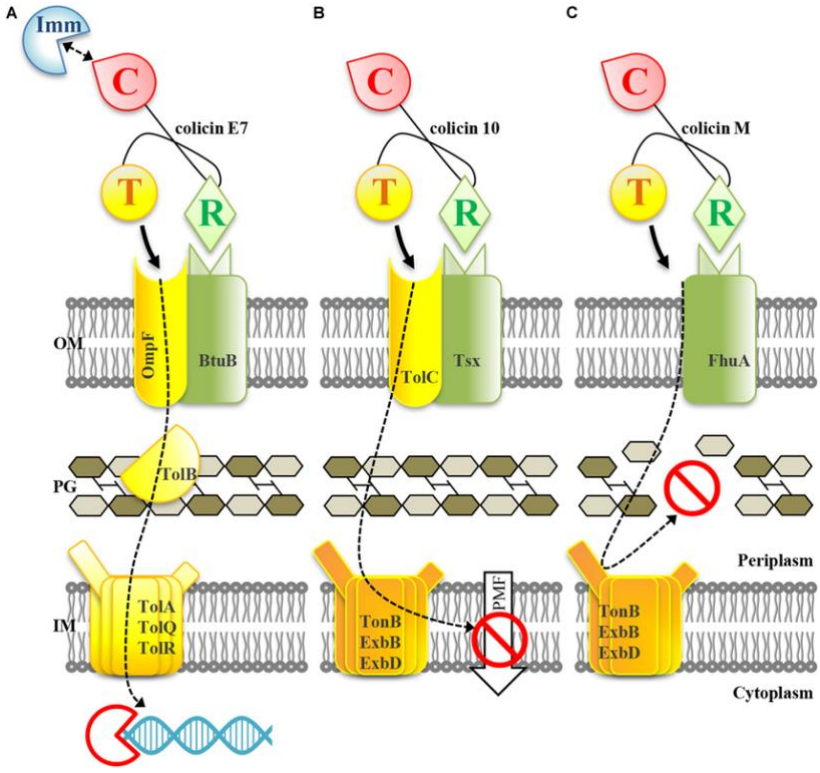

Figure 1.1. Mechanisms of antimicrobial action of colicins with nuclease (**A**), pore-forming (**B**), and murein synthesis inhibiting (**C**) activities (Hahn-Löbmann et al., 2019).

Microcins are small, highly stable peptides that exhibit resistance to proteases, extreme temperatures, and pH variations, making them exceptionally robust. They are primarily synthesized by bacteria species within the Enterobacteriaceae family. Structurally, microcins are either simple linear peptides or complex molecules resulting from significant post-translational modifications. These modifications enhance their chemical properties and expand their range of antimicrobial activity. Accordingly, microcins are categorized into two subclasses: Class I, which includes post-translationally modified microcins with molecular weights under 5 kDa, and Class II, which comprises unmodified or minimally modified microcins with molecular weights between 5–10 kDa (Zimina et al., 2020). These AMPs employ a variety of mechanisms to exert their antimicrobial effects. Some microcins disrupt the cytoplasmic membrane, causing depolarization, while others inhibit essential intracellular functions such as ATP synthase activity or mRNA translation (Simons et al., 2020).

Phage tail-like bacteriocins (20–100 kDa) exhibit cylindrical structures similar to those of bacteriophage tails, enabling them to perforate bacterial cell membranes and thereby induce cell death. The genes responsible for these bacteriocins are encoded within gene clusters in the bacterial genome, whose structural organization mirrors that of bacteriophage tails (Scholl, 2017). It is hypothesized that the evolutionary origin of these PTLBs may be traced back to a phage gene, potentially introduced into bacteria as a mobile genetic element. Consequently, this class of bacteriocins is divided into two types, R and F. R-type bacteriocins are evolutionarily related to the tails of bacteriophages in the Myoviridae family that can form similar sheathed structures, which feature a receptor-binding protein (RBP) complex at one end (Sun et al., 2018). In contrast, F-type bacteriocins, which are evolutionarily akin to phages of the Siphoviridae family, form unsheathed cylindrical structures but also possess RBP complexes. Although the precise mechanisms of action of PTLBs remain incompletely understood, it is believed that they initially attach to cell wall receptors via their RBP components, subsequently triggering rapid cell death (J. P. Hegarty et al., 2016). While R-type PTLBs are believed to kill by penetrating the cell envelope and disrupting membrane potential, the mechanism for F-type PTLBs remains less understood but may similarly involve interference with membrane gradients (Scholl, 2017).

1.2.2. Gram-positive bacteria bacteriocins and their mechanisms of action

Since LAB are widely used in the food industry due to their GRAS status, the most extensive research has focused on bacteriocins synthesized by LAB. Consequently, the classification of GPB bacteriocins is based primarily on LAB bacteriocins and can be applied to bacteriocins produced by other GPB genera (Acedo et al., 2018). Therefore, most GPB bacteriocins can be divided into three primary classes (Alvarez-Sieiro et al., 2016; Schofs et al., 2020).

Class I bacteriocins are small molecular weight peptides (< 10 kDa) that undergo post-translational modifications and contain unusual amino acids (Mokoena, 2017). These modified amino acids can form multi-ring structures, providing bacteriocins with structural stability under high temperatures, extreme pH conditions, or in the presence of proteolytic enzymes (Zimina et al., 2020). For instance, lantibiotics are classified as Class I bacteriocins and they employ a dual mechanism of action (**Figure 1.2**). They bind to lipid II, a hydrophobic carrier involved in transporting peptidoglycan monomers from the cytoplasm to the cell wall, thereby inhibiting cell wall biosynthesis and compromising bacterial viability. Additionally, lipid II acts as a docking molecule that facilitates membrane insertion and pore formation, further destabilizing the bacterial membrane (Hernández-González et al., 2021).

Class II bacteriocins are small (< 10 kDa), heat-resistant peptides with hydrophobic and/or amphiphilic regions. However, unlike Class I, these peptides are not post-translationally modified (Alvarez-Sieiro et al., 2016). Though the former is structurally stabilized by conserved disulfide bridges (Cui et al., 2012). The mannose phosphotransferase system (Man-PTS) serves as a primary target for non-lantibiotic bacteriocins (**Figure 1.2**). This system is utilized for sugar binding and its phosphorylation and contains a specific carbohydrate-protein complex composed of three proteins (AB, C, D) (Simons et al., 2020). Class II bacteriocins bind to MptC and MptD subunits which results in the irreversible opening of an intrinsic channel, allowing ions to diffuse freely across the membrane and ultimately causing the cell's death. Other class II bacteriocins disrupt the membranes of susceptible bacteria by forming pores or directly interacting with the bacterial membrane (**Figure 1.2**) (Hernández-González et al., 2021).

Class III bacteriocins are large proteins (> 30 kDa) that are either heat-sensitive (lytic) or heat-resistant (non-lytic) (Alvarez-Sieiro et al., 2016). The main difference between lytic and non-lytic bacteriocins is in their mechanism of action (**Figure 1.2**). Lytic bacteriocins act by lysing the cell wall of the target bacteria or by inhibiting the synthesis of DNA and proteins in the target bacteria (Hernández-González et al., 2021). In contrast, non-lytic bacteriocins exert their action by disturbing the glucose uptake by cells, starving them, and disturbing the membrane potential (Acedo et al., 2018). Class III bacteriocins also include PTLBs, which are described in more detail in section 1.2.1 (Ghequire & De Mot, 2015).

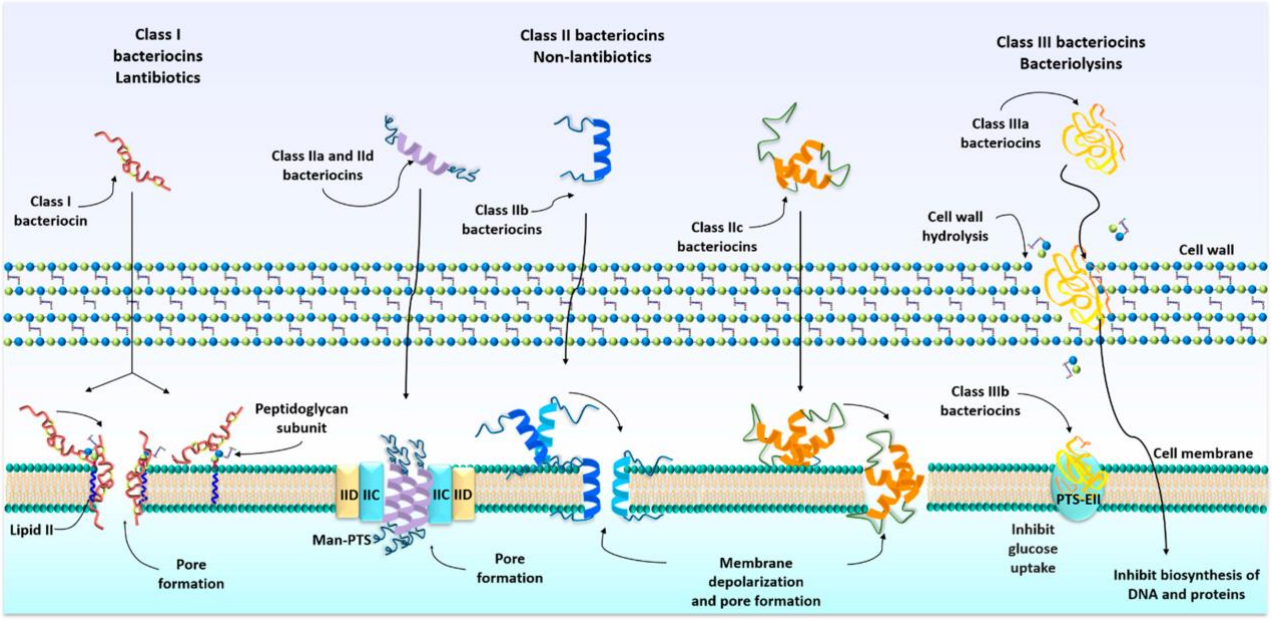

Figure 1.2. Mechanism of action of Gram-positive bacteria bacteriocins (Hernández-González et al., 2021).

To further demonstrate the classification of GPB bacteriocins, **Table 1.1** presents the subclasses within each of the three primary classes described above, highlighting their distinct characteristics.

Table 1.1. Classification and distinctive characteristics of Gram-positive bacteria bacteriocins (continued on page 14). Adopted from Alvarez-Sieiro et al., 2016; Zimina et al., 2020; Hernández-González et al., 2021

Class of bacteriocin	Subclasses	Distinctive characteristic
Class I	Lanthipeptides	Residues (methyl)lanthionine
	Cyclic peptides	Cycling from N-terminus to C-terminus
	Sactipeptides	Disulfide α-carbon bridges
	Linear azole-containing peptides (LAPs)	Thiazole and (methyl)oxazole rings, linear back bone
	Glycocins	Glycosylated residues
	Lasso peptides	Cyclization of an N-terminal amine into a γ-acid
	Lipolantins	N-terminal fatty acid and avionin fragment
	Botromycins	Macrocyclic amidine, decarboxylated C-terminal thiazole, β-methylated residues
	Lipolantins	N-terminal fatty acid and avionin fragment
Class II	Pediocin-like bacteriocins	Consensus YGNG-motif, at least one disulfide bridge

	Two peptide bacteriocins	Synergy of two peptides
	Leaderless bacteriocins	Lack of a leader peptide
	Other linear bacteriocins	Non-YGNG-like linear peptides
Class III	Bacteriolysins	Large lytic polypeptides
	Non-lytic bacteriocins	Large non-lytic polypeptides
	Phage tail-like bacteriocins	Multiprotein complex, a structure similar to a phage tail

1.3. Biosynthesis of bacteriocins

Bacteriocins are synthesized by ribosomes in a particular manner, rendering them biologically inactive until maturation occurs (Fernandes & Jobby, 2022). Their synthesis involves several stages: the production of a pre-bacteriocin, cleavage of the pre-peptide at a specific site to remove the leader sequence, and the subsequent transport of the pro-bacteriocin across the cell membrane. Notably, the leader peptide often serves as a protective mechanism for the producing cell, keeping the bacteriocin inactive until secretion. Additionally, the leader peptide plays a critical role in the maturation of class I bacteriocins (Simons et al., 2020). The genes encoding bacteriocin synthesis are organized within one or two operons containing various components, typically located on plasmids, chromosomal DNA, or transposons integrated into the chromosome (Fernandes & Jobby, 2022). Class I GPB bacteriocins are synthesized through the coordinated action of several genes arranged in clusters. These genes include:

- **Structural genes** that encode pre-bacteriocins with an N-terminal leader sequence and conserved glycine residues at the C-terminus, recognized by ABC transporters. These transporters are required for leader sequence processing and mature bacteriocin secretion.
- **Immunity genes** encode small proteins (51–154 amino acids) that protect the producing cell from its bacteriocin.
- **Genes for processing and secretion** encode proteins responsible for pre-bacteriocin cleavage, transport, and extracellular release.
- **Modification genes** coding for enzymes involved in post-translational modifications of bacteriocins.
- **Regulatory genes** encode proteins that regulate bacteriocin synthesis (Skaugen et al., 2003; Perez et al., 2018).

Bacteriocin production and secretion are regulated by a signal transduction system composed of an inducer peptide, a response regulator, and a sensor histidine kinase (**Figure 1.3**). Two models describe this induction. In the first, inducer peptide is produced in small amounts and accumulates with cell growth, eventually triggering bacteriocin gene expression. In the second, inducer peptide

levels remain low until environmental factors cause a temporary increase, leading to self-induction and activation of the entire gene cluster. Generally, these inducer peptides function in quorum sensing to regulate bacteriocin biosynthesis (B. R. Sharma et al., 2022).

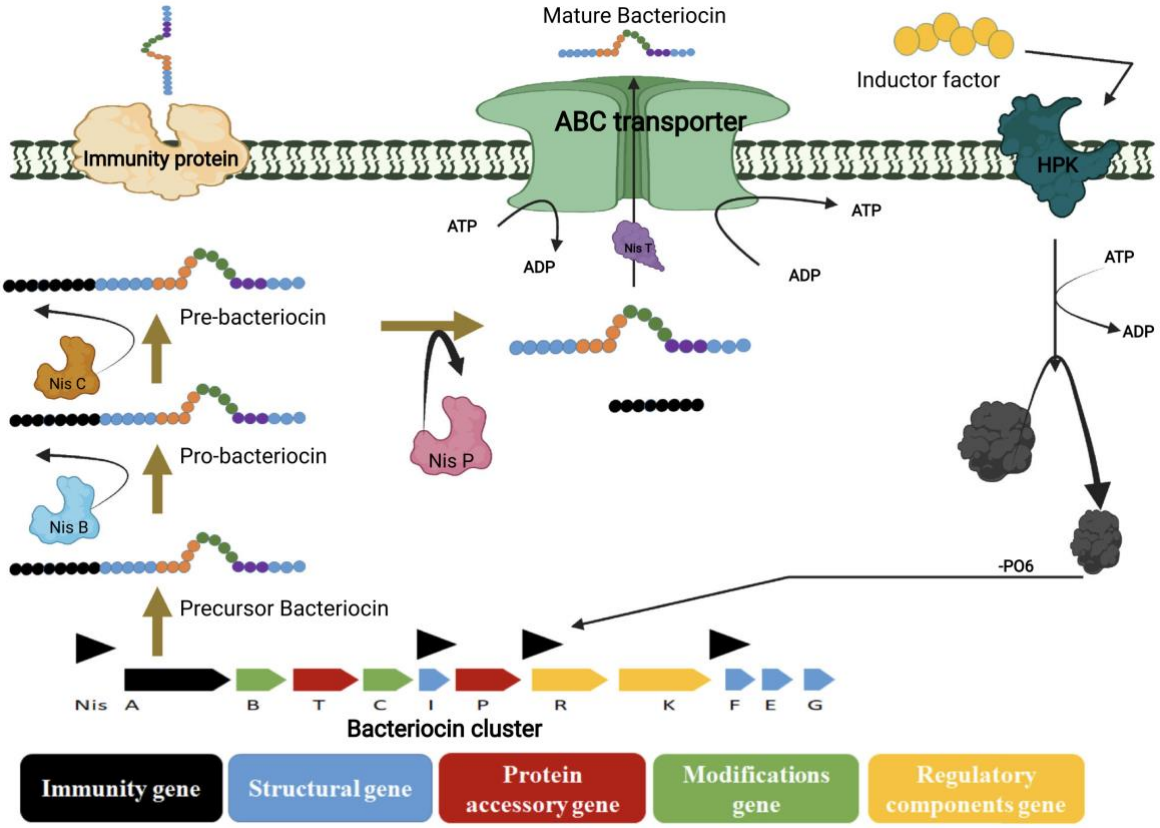

Figure 1.3. The biosynthesis of nisin through signal transduction systems, which consist of three components: the inductor peptide (IP), the response regulatory protein (RR), and the sensor histidine protein kinase (HPK) (B. R. Sharma et al., 2022).

Nisin is a well-studied example of class I GPB bacteriocin biosynthesis (**Figure 1.3**). The genes *nisA*, *nisB*, *nisC*, *nisP*, and *nisT* form a gene cluster encoding their synthesis. The *nisA* gene produces a precursor peptide (pre-nisin A), which is converted to active nisin A by the gene products of *nisB* and *nisC*. The mature bacteriocin is exported via NisT and NisP, followed by leader peptide cleavage (Perez et al., 2014). Similarly, class II GPB bacteriocins are synthesized as inactive pre-peptides with N-terminal leader sequences and a double-glycine proteolytic site, which acts as an induction factor for activating the necessary signal transduction system (Dimov et al., 2005). Accordingly, class II GPB bacteriocins do not rely on specific post-translational modification genes, with maturation typically coinciding with secretion (Simons et al., 2020).

1.4. LCI-type bacteriocins

AMPs have gained attention due to their structural diversity and potent activity against various pathogens, making them promising alternatives to traditional antibiotics. Among these, **LCI**, a 47-residue novel AMP derived from *Bacillus subtilis*, exhibits unique characteristics that differentiate it

from other AMPs (Gong et al., 2011). Previous studies have broadly categorized AMPs based on their secondary structures, such as α-helices and β-sheets, with many relying on disulfide bonds for stability (Gross & Morell, 1971; Peypoux et al., 1999). However, recent investigations into LCI reveal an unusual β-sheet structure that lacks disulfide bonds yet retains significant thermal stability and antibacterial activity, attributed to aromatic stacking interactions (**Figure 1.4**) (Zhu et al., 2001; Gong et al., 2011). Interestingly, the C-terminal region of LCI retains strong antimicrobial activity compared to the full-length peptide (Saikia et al., 2019). Additionally, LCI is highly stable under a range of pH conditions, resistant to protease degradation, and unaffected by UV radiation (Wang et al., 2021).

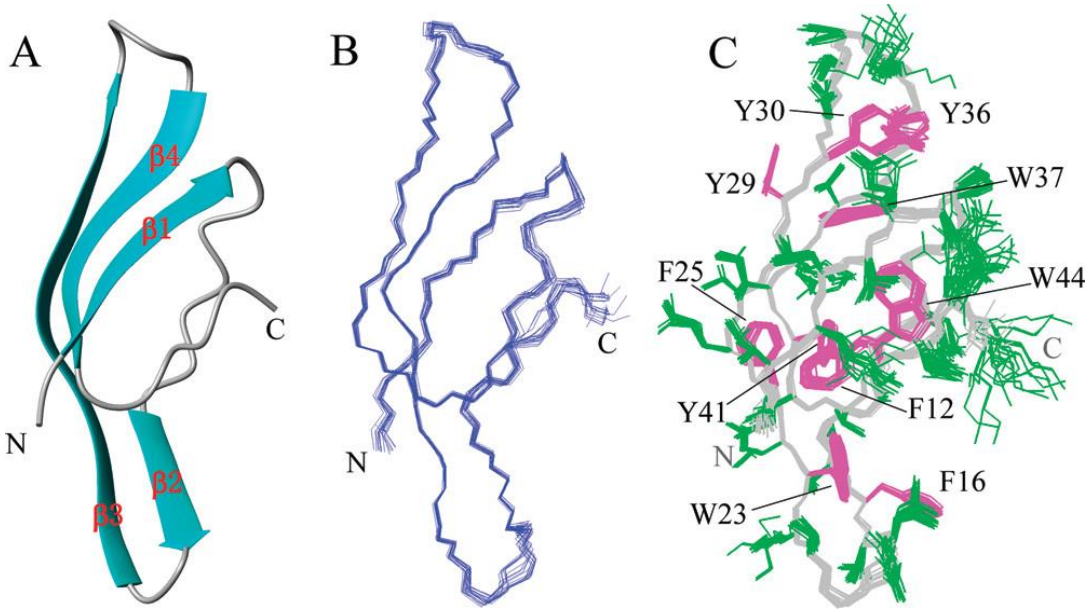

Figure 1.4. Nuclear magnetic resonance structure of LCI. (**A**) Ribbon representation of the average conformer. (**B**) Backbone atom superposition of 20 LCI conformers. (**C**) Side chains (green) in LCI, in which all aromatic rings are colored magenta (Gong et al., 2011).

LCI exhibits potent antimicrobial activity against various plant and human pathogens, including *Xanthomonas*, *Pseudomonas*, and methicillin-resistant *Staphylococcus aureus*. Although the exact mechanism of action is not fully understood, studies indicate that LCI interacts with membranes, binding specifically to negatively charged lipids (Gong et al., 2011; Saikia et al., 2019). It has been shown to penetrate the outer membrane of *E. coli* without causing significant permeability of the inner membrane, suggesting that membrane damage may not be the sole mechanism underlying its antimicrobial activity (Saikia et al., 2019).

1.5. Lasso peptides

Lasso peptides represent a growing class of bioactive ribosomally synthesized and post-translationally modified peptides (RiPPs) characterized by their distinctive lasso-shaped structure (Cheng & Hua, 2020). These peptides are primarily synthesized by Actinobacteria and certain

Proteobacteria (Arnison et al., 2013). They exhibit a wide range of potential bioactivities, including antimicrobial, antitumor, antiviral, and receptor-antagonistic effects (Duan et al., 2022).

The hallmark lasso structure is defined by a macrolactam ring at the N-terminus, which forms through an isopeptide bond between the α-amino group of the first amino acid residue and the side-chain carboxyl group of aspartate or glutamate located in positions 7–9. The C-terminus of the peptide threads through this macrolactam ring, creating the lasso-like conformation. This unique structure imparts exceptional stability to lasso peptides, making them highly resistant to proteases, heat, and denaturing agents (Duan et al., 2022). Based on the number and position of disulfide bridges, lasso peptides are classified into four distinct subclasses (**Figure 1.5**) (Cheng & Hua, 2020).

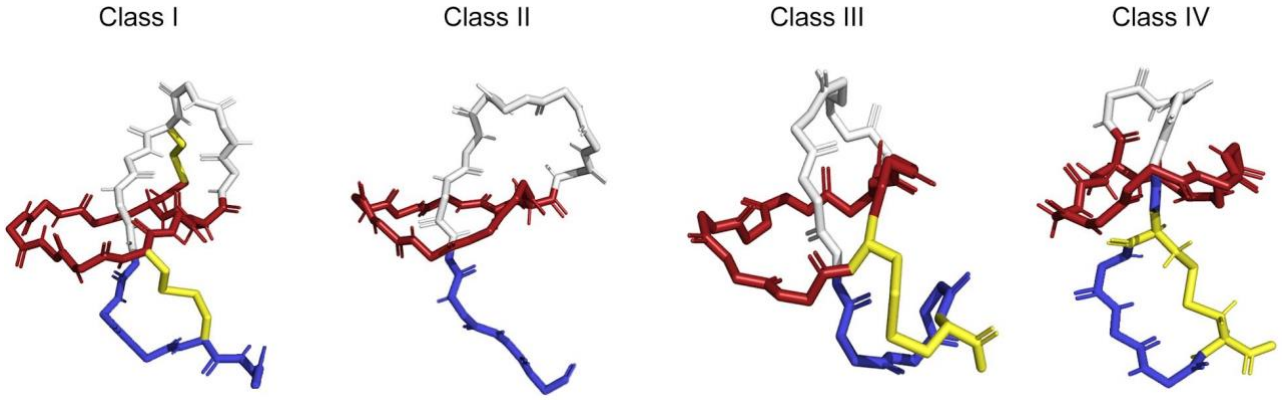

Figure 1.5. The representative structures of the four classes of lasso peptides are distinguished by specific visual markers: the macrolactam ring is highlighted in red, the C-terminal region in blue, and the disulfide bonds in yellow (Cheng & Hua, 2020).

Class I lasso peptides are characterized by two disulfide bonds, whereas class II peptides lack disulfide bonds entirely. In contrast, lasso peptides belonging to classes III and IV each contain a single disulfide bond, though the location of this bond varies (Arnison et al., 2013; Cheng & Hua, 2020). For class III peptides, the disulfide bond forms between the macrolactam ring and the C-terminal region of the peptide, while in class IV, the bond is localized within the C-terminal region itself (Cheng & Hua, 2020).

1.5.1. Biosynthesis of the lasso peptides

The biosynthesis of lasso peptides requires at least three essential genes. The *A* gene encodes the precursor peptide, the *B* gene encodes an ATP-dependent cysteine protease responsible for cleaving the precursor, and the *C* gene encodes an ATP-dependent macrolactam synthetase that facilitates the cyclization of the lasso peptide (Duan et al., 2022). Additional enzymes involved in post-translational modifications are often encoded close to the *A*, *B*, or *C* genes (Zong et al., 2018). Some gene clusters involved in lasso peptide biosynthesis may encode ABC transporters. The presence of these transporters suggests that the synthesized lasso peptides could possess antibacterial activity (Romano et al., 2018).

The biosynthesis of RiPPs utilizes iterative enzymatic catalysis to generate a wide range of structurally and functionally diverse molecules. Typically, these iterative enzymes act on linear precursor peptides by introducing multiple post-translational modifications before final folding into complex three-dimensional structures rather than modifying already folded, mature RiPPs. A notable example of functional divergence within this framework is a class of GCN5-related N-acetyltransferases (GNATs) that catalyse sequential acylation of two lysine residues located in the loop and ring regions of lasso peptides (Xiong et al., 2025).

The lasso peptide precursor (A) consists of an N-terminal leader peptide and a C-terminal core peptide region. The leader peptide plays a critical role in substrate recognition and interaction with the enzymes responsible for post-translational modifications, while the core peptide undergoes these modifications. The biosynthesis of lasso peptides is thought to occur in three (or in some cases, four) primary stages (**Figure 1.6**). Initially, the RiPP recognition element (RRE) identifies and binds to the core peptide region of the A precursor. This interaction serves as a critical regulatory step that initiates subsequent post-translational modifications (H.-N. Tan et al., 2024). Notably, the RRE and protease domains can be organized either as a single didomain protein, exemplified by McjB in the MccJ25 biosynthetic gene cluster, or as two distinct proteins, as observed with PadeB1 and PadeB2 in the paeninodin biosynthetic gene cluster (S. Zhu et al., 2016; H.-N. Tan et al., 2024). So, the B protein removes the leader peptide via proteolysis, thereby liberating the core peptide. Finally, the lasso cyclase (C) activates the carboxyl group of aspartate or glutamate in the core peptide as an AMP ester before catalyzing the formation of the macrolactam ring through condensation with the α-amino group. These steps collectively enable the formation of the characteristic lasso structure (Cheng & Hua, 2020). In the presence of an ABC transporter, the biosynthesis process includes an additional fourth step – exporting the lasso peptides from the cell (Romano et al., 2018).

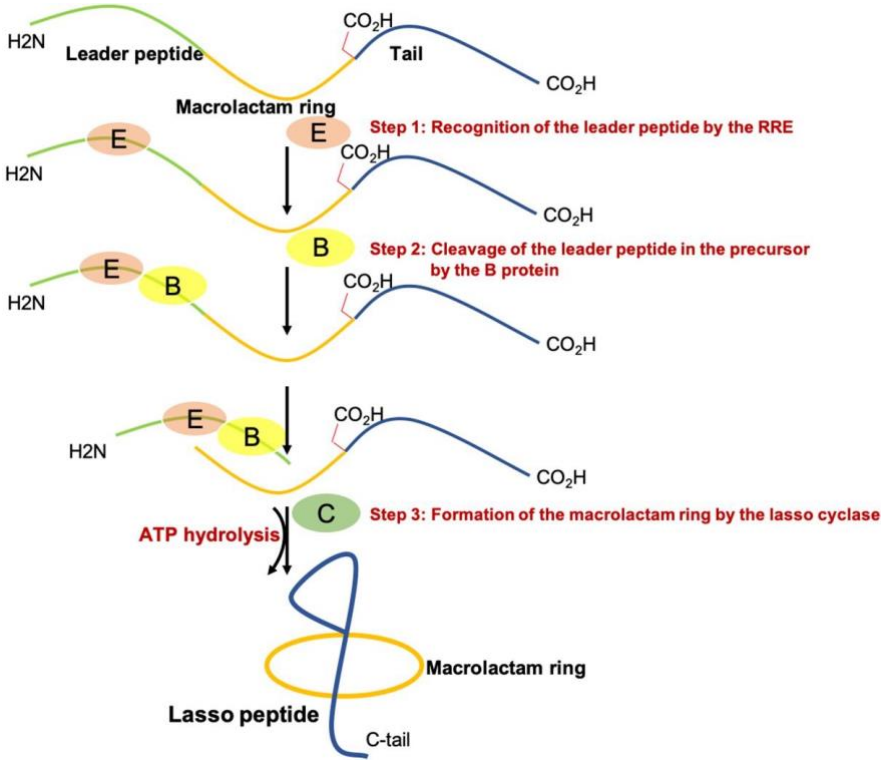

Figure 1.6. The proposed mechanism of lasso peptide biosynthesis involves three distinct stages. RRE – RiPP recognition element (Cheng & Hua, 2020).

1.5.2. Mechanisms of action of lasso peptides

Lasso peptides exhibit dual modes of action: enzyme inhibition or receptor antagonism. These mechanisms confer antibacterial activity on some lasso peptides, typically directed against GPB or GNB and often characterized by a narrow spectrum of activity (Arnison et al., 2013). Known targets of lasso peptides include disruption of protein homeostasis (e.g., lassomycin), inhibition of RNA synthesis (e.g., microcin J25), and interference with cell wall biosynthesis (e.g., siamycin-I and streptomonomicin) (S. Tan et al., 2019).

Two lasso peptides, siamycin-I and streptomonomicin, are specifically recognized for their ability to inhibit peptidoglycan biosynthesis. Siamycin-I demonstrates a unique mechanism because while most lipid II inhibitors lead to the accumulation of the cytoplasmic precursor, siamycin-I does not exhibit this effect. Instead, siamycin-I localizes specifically to the division septa of *S. aureus* and *B. subtilis*, distinguishing it from other antibacterial agents targeting lipid II (S. Tan et al., 2019). Another key target of lasso peptides is the ATP-dependent protease complex ClpC1P1P2 in *Mycobacterium tuberculosis*. This complex facilitates protein degradation by channeling substrates through ClpC1 ATP hydrolysis into the ClpP1P2 protease. Lassomycin increases ATP hydrolysis rates by 7–10 times. Notably, lassomycin binds to a site distinct from the substrate-binding region of the protease, supporting the hypothesis that it inhibits the translocation of protein substrates into the proteolytic complex (Gavrish et al., 2014).

1.6. Applications of bacteriocins

The application of bacteriocins is a significant strategy in addressing the issue of bacterial resistance, as bacteriocins exhibit specific antibacterial effects against closely related microbial species and thus hold great potential for inhibiting the growth of antibiotic-resistant bacteria. Bacteriocins offer several benefits as novel AMPs due to their diverse modes of action, their potential for bioengineering, low toxicity, anti-biofilm activity, sporicidal properties, and promising *in vitro* anti-cancer activity (Meade et al., 2020). However, a few factors limit bacteriocin use, including high production costs and limited knowledge regarding their toxicity. Usually, wild type bacteriocin producers generate low bacteriocin yields because production is a high-energy process for the cell and is thus tightly controlled (Sugrue et al., 2024). Also, compared to antibiotics, some bacteriocins are less temperature-labile and can withstand extreme pH (Gradisteanu Pircalabioru et al., 2021). Nevertheless, these challenges may be overcome through the development of bioengineered variants of natural bacteriocins with enhanced efficacy (Soltani et al., 2021). Since their discovery, bacteriocins have been established as promising antimicrobial compounds with potential applications in the food, health, veterinary, and agricultural sectors.

1.6.1. Bacteriocins in the food industry

The use of bacteriocins in the food industry offers a natural and effective alternative to chemical preservatives, addressing both consumer demands for minimally processed foods and the industry's need to combat spoilage and foodborne pathogens. Bacteriocins can contribute to food safety through multiple applications, including the direct incorporation of purified or semi-purified preparations, the use of ingredients pre-fermented with bacteriocin-producing strains, or the integration of such strains into starter cultures to facilitate *in situ* production during fermentation (Lahiri et al., 2022). Beyond antimicrobial protection, bacteriocins also enhance food quality and sensory attributes by promoting proteolysis and mitigating defects such as gas blowing in cheese (Negash & Tsehai, 2020; Putri et al., 2024). Moreover, their incorporation into bioactive packaging serves as an effective barrier against external contamination, thereby improving microbial safety and prolonging product shelf life.

Bacteriocins are widely regarded as safe (GRAS) by regulatory authorities, but nisin remains the only bacteriocin legally approved by regulatory agencies as a food preservative (Soltani et al., 2021). Nisin, the most extensively studied bacteriocin, is used in dairy products to control the growth of pathogens such as *Listeria monocytogenes* and *Clostridium botulinum*. Other bacteriocins, such as pediocin, have also shown activity against *L. monocytogenes* in ready-to-eat foods, while micocin is applied specifically to inhibit this pathogen in processed meat products. Unlike many traditional preservatives, bacteriocins are colourless, odorless, and tasteless, allowing them to maintain the sensory qualities of food products (Meade et al., 2020; Negash & Tsehai, 2020).

The incorporation of bacteriocins into food systems presents significant advantages, primarily due to their diverse mechanisms of action, including the disruption of bacterial membranes, inhibition of cell wall biosynthesis, and impairment of cellular respiration, which collectively make them highly effective against resistant bacterial strains. For example, BacTN635 and BacFL31, produced by *Lactobacillus plantarum* and *Enterococcus faecium*, respectively, have demonstrated activity against *Salmonella typhimurium* and *L. monocytogenes* in poultry and beef while simultaneously enhancing sensory attributes like colour and odor (Meade et al., 2020). Moreover, innovative encapsulation techniques, such as nisin encapsulated in liposomes or alginate-starch matrices, have significantly improved the stability and gradual release of bacteriocins. This improvement enables their use in complex food systems (e.g. cheddar cheese) without compromising the quality of the starter cultures or the food matrix (Soltani et al., 2021).

Besides direct food incorporation, bacteriocins are increasingly utilized in antimicrobial food packaging. This strategy involves embedding bacteriocins in biopolymer films, which release the peptides gradually during storage, effectively reducing spoilage and contamination risks (Soltani et al., 2021). Studies have shown that nisin-coated packaging films can inhibit the growth of heat-resistant spores in processed cheeses and control *L. monocytogenes* contamination in refrigerated foods. Similarly, encapsulated bacteriocins, such as plantaricin BM1 applied to ham surfaces, provide targeted inhibition of pathogens while overcoming distribution challenges within food matrices (Zhou et al., 2015). Additionally, bacteriocins are often employed alongside other preservation methods in hurdle technologies, which combine antimicrobial peptides with physical or chemical treatments to extend shelf life and improve food safety.

1.6.2. Bacteriocins in medicine

The growing prevalence of antimicrobial resistance has underscored the need for alternative therapeutic agents, and bacteriocins have emerged as promising candidates for treating infectious diseases. Their specific modes of action, high efficacy at low concentrations, and minimal impact on non-target microbiota make them particularly suitable for medical applications. For example, nisin F has been shown to effectively treat *S. aureus*-induced lung infections in rat models, while mersacidin successfully eradicated MRSA colonization in a rhinitis mouse model. Similarly, lactocin has demonstrated efficacy against systemic *S. aureus* infections in murine studies (Meade et al., 2020). Narrow-spectrum bacteriocins such as turicin CD, produced by *Bacillus thuringiensis*, have exhibited potent activity against *Clostridioides difficile* infections, with comparable efficacy to vancomycin and metronidazole but without significant disruption of the gut microbiota (Soltani et al., 2021). Pediocin PA-1 has proven effective against *L. monocytogenes* infections in mice, preserving commensal microbial communities, while engineered bacteriocins like Ent35-MccV, a fusion of

enterocin CRL35 and microcin V, have demonstrated activity against clinically relevant pathogens, including enterohemorrhagic *E. coli* and *L. monocytogenes* (Meade et al., 2020; Soltani et al., 2021).

Despite promising *in vitro* results, some bacteriocins face challenges in clinical application. For instance, colicins have shown potential for treating *E. coli*-induced urinary tract infections, however, their clinical efficacy remains underexplored. Similarly, piocins, produced by *Pseudomonas aeruginosa*, have been investigated for the treatment of cystic fibrosis-related lung infections, but their success in clinical settings has been limited, potentially due to immune interactions and the complex nature of *P. aeruginosa* infections (Meade et al., 2020).

In addition to their antimicrobial capabilities, bacteriocins have demonstrated potential as anticancer agents. Certain bacteriocins, including microcin E492, colicins, and nisin, exhibit cytotoxic activity against malignant human cell lines by inducing apoptosis or depolarizing cell membranes, leading to altered permeability (Negash & Tsehai, 2020; Soltani et al., 2021). These mechanisms emphasize their potential to serve as adjunctive therapies in oncology. For instance, nisin has shown efficacy in reducing tumour growth in preclinical studies (Niamah et al., 2024).

The use of bacteriocins in medicine is still in its early days, and several hurdles need to be overcome before they can be widely used in clinical settings. Some of the main challenges include poor bioavailability, limited solubility under physiological conditions, sensitivity to proteolytic enzymes, high production costs, and a lack of thorough evaluations for potential toxicity (Meade et al., 2020; Soltani et al., 2021). That said, despite these obstacles, bacteriocins show real promise in the fight against antibiotic-resistant bacteria (Meade et al., 2020; Gradisteanu Pircalabioru et al., 2021). Their ability to target specific bacteria while being low in toxicity and impressively stable makes them an exciting alternative to conventional antibiotics (Gradisteanu Pircalabioru et al., 2021).

1.6.3. Bacteriocins in veterinary

Bacteriocins have shown great promise in veterinary medicine, as well as alternatives to traditional antibiotics, addressing the growing concern over antimicrobial resistance in livestock production. In poultry farming, bacteriocins like Divercin AS7 have been effective in controlling pathogenic bacterial strains such as *Campylobacter*, with additional applications in the management of *Salmonella enterica Typhimurium* and *Clostridium perfringens* in both chickens and pigs. Similarly, nisin and lacticin 3147 have been successfully used in the treatment of mastitis caused by *S. aureus* and *Streptococcus agalactiae* in dairy cows, offering a natural and effective solution to bacterial infections in livestock (Soltani et al., 2021).

In pig farming, the partially purified fraction of pediocin PA-1 has demonstrated significant improvements in the growth performance of broilers infected with *Clostridium perfringens*, indicating its potential to enhance health in livestock. Additionally, gassericin A, a circular bacteriocin

produced by *Lactobacillus gasseri* LA39, has shown potential as an antibiotic alternative for managing diarrhea in mammals. It's thought that gassericin A acts by binding to the plasma membrane of intestinal epithelial cells and promoting fluid absorption (Soltani et al., 2021). Further studies have also explored the use of bacteriocins for controlling *Campylobacter jejuni* in chickens and *Streptococcus suis* in pigs (Soltani et al., 2021; Sugrue et al., 2024).

1.6.4. Bacteriocins in agriculture

Bacteriocins hold significant promise as eco-friendly and sustainable tools in agriculture. Their applications range from biocontrol agents to plant growth-promoting biostimulants, helping to reduce the dependence on chemical pesticides and fertilizers. At the same time, they offer a natural way to improve crop productivity and make plants more resilient to stress (Nazari & Smith, 2020; Fischer et al., 2024; Reuben & Torres, 2024; Greer et al., 2025). These peptides are particularly valued for their specificity, targeting pathogens without adversely affecting beneficial microorganisms or the environment.

One of the most studied bacteriocins in agriculture is thuricin 17, produced by *B. thuringiensis* NEB17 (Gray et al., 2006). It has demonstrated potential in promoting plant growth by increasing leaf area and biomass in crops such as maize, soybeans, and canola. Additionally, thuricin 17 has been shown to enhance root development under adverse conditions, including salinity and temperature stress (Ahmad et al., 2017). Studies have also revealed its role in mitigating abiotic stress, such as water deficit, by improving plants' physiological responses. Beyond these effects, bacteriocins contribute to plant health by inducing systemic defense mechanisms. For example, thuricin 17 enhances phenolic compound production and activates antioxidant enzymes, bolstering plant resistance to pathogens (Nazari & Smith, 2020). Soybean plants treated with thuricin 17 and inoculated with nitrogen-fixing bacteria *Bradyrhizobium japonicum* exhibited increased biomass and reduced drought impact. Other bacteriocins, such as Bac-GM17, have also been shown to improve seed germination and early seedling development in crops like tomatoes and melons (Fischer et al., 2024).

In the context of biocontrol, bacteriocins have been used effectively to combat plant pathogens, reducing disease prevalence and improving crop health. *B. subtilis* strains producing Bac IH7 and Bac 14B bacteriocins have demonstrated efficacy against *Agrobacterium tumefaciens*, which causes crown gall disease, significantly reducing infection rates and tumor formation in greenhouse trials (Fischer et al., 2024). Similarly, tailocins produced by *Pseudomonas fluorescens* SF4 have shown activity against *Xanthomonas vesicatoria*, reducing bacterial spot symptoms on tomato leaves (Fischer et al., 2024; Reuben & Torres, 2024). Transgenic plants engineered to express bacteriocins, such as plantaricin and leucocin, have exhibited enhanced resistance to bacterial pathogens,

including *Clavibacter michiganensis* and *Pseudomonas syringae* (Fischer et al., 2024; Greer et al., 2025).

Despite these advancements, the application of bacteriocins in agriculture faces challenges, including their stability under field conditions, production costs, and potential for resistance development in target pathogens. Additionally, optimizing delivery methods, such as foliar sprays or seed treatments, remains a critical area for research (Ahmad et al., 2017). Nevertheless, ongoing studies continue to explore innovative solutions, such as encapsulation techniques to improve bacteriocin stability and compatibility with other biocontrol agents. In conclusion, bacteriocins offer a sustainable and effective approach to improving agricultural productivity and resilience. However, further research is needed to fully realize their potential and develop cost-effective, scalable applications in modern agricultural practices.

1.7. Overview of previous research on LCI and lasso peptide

During previous research, 63 bacterial isolates were obtained from soil samples collected in Lithuania's Dūkštai Oak Grove and screened for antibacterial activity against phytopathogenic bacteria. Among these, two of the most active strains, DM1.10 and AB3, were taxonomically identified as *Bacillus velezensis* and a novel *Streptomyces* species, respectively. Bioinformatic analysis using BAGEL4 and PRISM tools revealed gene clusters associated with bacteriocin biosynthesis. In the genome of *B. velezensis* DM1.10, 14 gene clusters were identified, including three linked to bacteriocin production. Similarly, genome analysis of *Streptomyces* sp. AB3 uncovered 41 gene clusters, four of which are involved in bacteriocin biosynthesis (Smulkaitė, 2023).

One gene cluster in *Streptomyces* sp. AB3 is predicted to encode a lasso peptide-type bacteriocin (**Figure 1.7A**). It is well established that the biosynthesis of lasso peptides requires three essential genes encoding the precursor peptide (A), a cysteine protease (B), and a lasso peptide cyclase (C) (Cheng & Hua, 2020). All genes necessary for lasso peptide biosynthesis were found in the identified *lasABCD* gene cluster (**Figure 1.7A**). In addition to these core genes, the cluster also includes a gene encoding acetyltransferase (D), responsible for a post-translational modification of the lasso peptide. However, the cluster lacks a gene encoding an ABC transporter, which is commonly found in lasso peptide biosynthetic gene clusters and is potentially involved in mediating the antibacterial activity of the synthesized lasso peptide (Cheng & Hua, 2020).

Sequence analysis of LasA using the BLASTp algorithm against the NCBI and UniProt databases revealed 26 % similarity to albusnodin, a class II lasso bacteriocin originally identified in *Streptomyces albus* DSM 41398 (Zong et al., 2018). Based on this homology, the leader, core peptide regions, and conserved residues were predicted (**Figure 1.7B**).

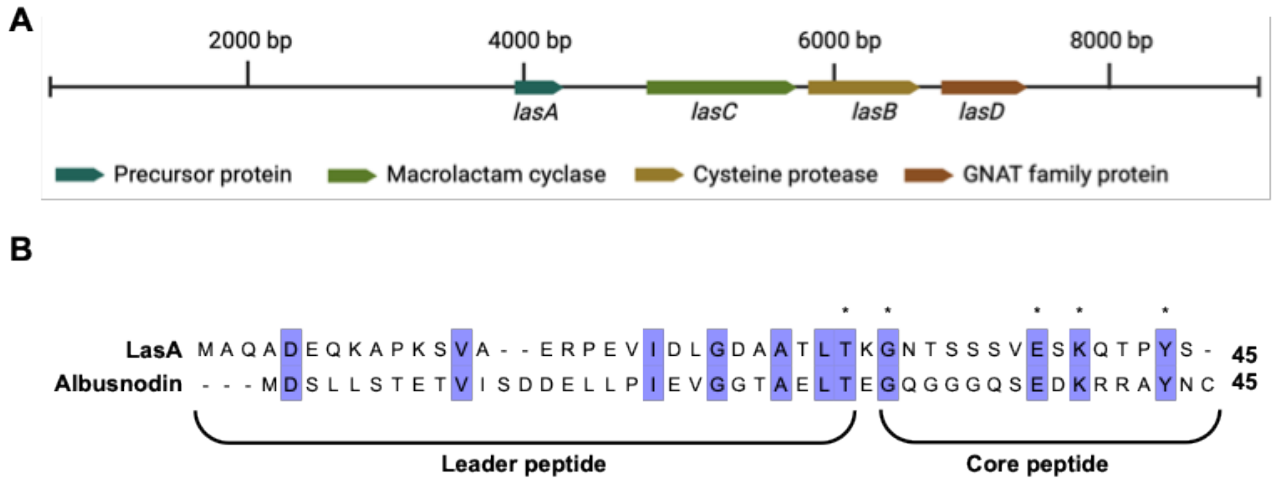

Figure 1.7. *Streptomyces* sp. AB3 is predicted to encode a lasso peptide-type bacteriocin. (**A**) The lasso peptide gene cluster. (**B**) Sequence alignment of LasA and albusnodin: conserved amino acids residues (*), Thr in the leader peptide, Gly-1 of the core peptide, Glu residue for isopeptide bond cyclization, Lys residue for acetylation, a highly conserved Tyr residue (Smulkaitė, 2023).

Based on the findings from the albusnodin study (Zong et al., 2018), the biosynthesis of lasso peptides involves precise recognition and modification of specific amino acid residues by dedicated enzymes encoded within the gene cluster. The cysteine protease (LasB) typically cleaves the precursor peptide between a universally conserved threonine residue located in the penultimate position of the leader peptide and a glycine residue at the N-terminus of the core region. This cleavage is essential for removing the leader sequence and enabling subsequent cyclization. The macrolactam synthetase (LasC) then catalyses the formation of an isopeptide bond between the α-amino group of the N-terminal glycine and the side-chain carboxyl group of a conserved glutamate residue located within the core sequence, generating the characteristic lasso ring structure. Additionally, the GNAT-family acetyltransferase (LasD) modifies one or more lysine residues, typically found in the loop and ring regions of the lasso peptide.

Following cloning of the lasso peptide gene cluster *lasABCD* into the pRSFDuet-1 expression vector and their expression in different *E. coli* cells, it was found that lasso peptide synthesis possibly takes place only in *E. coli* Arctic Express (DE3) strain. However, the synthesized lasso peptide does not exhibit antibacterial activity (Smulkaitė, 2023).

One gene cluster in *B. velezensis* DM1.10 is associated with the production of class II bacteriocin – an LCI-type bacteriocin (Smulkaitė, 2023). Our previous studies did not analyse this peptide. However, alongside the previously described lasso peptide, it was selected for investigation in the present work.

2. Materials and Methods

2.1. Materials

2.1.1. Bacterial strains and plasmids

For the evaluation of antibacterial activity, indicator strains of phytopathogenic bacteria from the German Collection of Microorganisms and Cell Cultures (DSMZ) were employed (**Table 2.1**). Two strains identified in previous research were used for the amplification of bacteriocin coding genes (**Table 2.1**).

Table 2.1. Bacterial strains

Species	**Strain**	**Obtained from**
Xanthomonas campestris	DSM 3586	DSMZ
Xanthomonas vesicatoria	DSM 22252	
Pseudomonas syringae	DSM 10604	
Pseudomonas syringae	DSM 50315	
Dickeya solani	DSM 28711	
Streptomyces scabiei	DSM 41658	
Pectobacterium carotovorum	DSM 30168	
Pectobacterium atrosepticum	DSM 18077	
Agrobacterium radiobacter	DSM 30147	
Streptomyces lavendulae subsp. *lavendulae*	DSM 40069	
Streptomyces lavendulae subsp. *grasserius*	DSM 40385	
Bacillus velezensis	DM1.10	Soil (Smulkaitė, 2023)
Streptomyces sp.	AB3	

The *Escherichia coli* DH5α (Novagen) and *E. coli* MC1061 (Mobitec) strains were used for cloning experiments. The following expression strains were used for heterologous protein biosynthesis: *E. coli* BL21 (DE3) (Novagen), *E. coli* Rosetta (DE3) (Novagen), and *Lactococcus lactis* NZ9000 (Mobitec). The pET15b (Novagen), pRSFDuet-1 (Novagen), pETDuet (Novagen), and pNZ8048 (Mobitec) plasmids were used for DNA cloning.

2.1.2. Media used for the cultivation of microorganisms

Soil-derived isolates were cultivated in Brain Heart Infusion (BHI) (OXOID) media at 30 °C with shaking at 200 rpm. Phytopathogenic bacteria strains were cultivated in Luria-Bertani (LB) (Carl Roth) media at 30 °C with shaking at 200 rpm. *E. coli* strains were grown in Luria-Bertani (LB) (Carl Roth) medium at 37 °C with shaking at 180 rpm. The LB medium was supplemented with the appropriate antibiotics: kanamycin (30 μg/mL), chloramphenicol (34 μg/mL), and ampicillin (50 μg/mL). *Lactococcus lactis* was grown in M17 (Biolab) medium supplemented with 0.5 % glucose (Carl Roth) at 30 °C without aeration. The growth media used in this study and their

compositions are detailed in **Table 2.2**. Solid LB, M17, and BHI media were prepared by adding 15 g/L agar (Biolab).

Table 2.2. Media used for cultivation of microorganisms

Name of the medium (manufacturer)	Composition of the medium
Luria-Bertani (Carl Roth)	10 g/L peptone 5 g/L yeast extract 10 g/L sodium chloride
Brain Heart Infusion (OXOID)	12.5 g/L brain infusion solids 5 g/L beef heart infusion solids 2 g/L glucose 5 g/L sodium chloride 10 g/L proteose peptone 2.5 g/L disodium phosphate
M17 (Biolab)	17.25 g/L peptone 5 g/L lactose 0.25 g/L magnesium sulphate 0.50 g/L ascorbic acid 19 g/L sodium glycerophosphate
ISP-2	4 g/L yeast extract (AppliChem) 10 g/L malt extract (Carl. Roth) 4 g/L glucose (Carl Roth)
SOC	5 g/L yeast extract (AppliChem) 20 g/L peptone (Merck) 0.5 g/L sodium chloride (AppliChem) 0.19 g/L potassium chloride (Merck) 2.4 g/L magnesium sulphate (Merck) 20 mL/L of 20 % glucose solution (Carl Roth)
GYT	100 mL/L glycerol (Carl Roth) 1.25 g/L yeast extract (AppliChem) 2.5 g/L tryptone (Merck)

2.2 Methods

2.2.1. Genomic DNA and plasmid DNA extraction

For genomic DNA extraction, bacterial cultures were grown for 18-24 hours in 5 mL of BHI medium at 30 °C with shaking at 200 rpm. Genomic DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

For plasmid DNA extraction from *E. coli*, bacterial cultures were grown for 12-18 hours in 5 mL LB medium with appropriate antibiotics at 37 °C with shaking at 180 rpm. For plasmid DNA isolation from *L. lactis*, bacterial cultures were grown for 24-32 hours in 10 mL M17 medium supplemented with 0.5 % glucose and 10 µg/ml chloramphenicol at 30 °C without aeration. Further plasmid DNA was extracted using the GeneJET Genomic Plasmid Miniprep Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Before the procedure, the collected *L. lactis* cell pellet was resuspended in resuspension buffer containing 2 mg/mL lysozyme and incubated for 30 minutes at 37 °C.

2.2.2. DNA purification and cloning

For DNA purification, the GeneJET PCR Purification Kit (Thermo Fisher Scientific) was used, while DNA extraction from agarose gel was performed using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific). Both procedures were carried out according to the manufacturer's protocols. DNA digestion was performed using FastDigest restriction enzymes (Thermo Fisher Scientific) following the manufacturer's instructions. DNA ligation was conducted using T4 DNA ligase (Thermo Fisher Scientific) or the NEBuilder HiFi DNA Assembly Master Mix (NEB) cloning kit according to the recommended protocol. The DNA concentration was measured with Nanodrop One Spectrophotometer (Thermo Scientific).

2.2.3. Amplification of bacteriocin genes by PCR

To amplify the *lasA*, *lasB*, *lasC,* and *lasD* genes, Phusion polymerase (Thermo Fisher Scientific) was used. Buffers and reagents were used according to the manufacturer's standard recommendations. The PCR mixture also included 3 % DMSO, and the initial denaturation was extended to 10 min. To amplify the LCI genes, Phusion polymerase (Thermo Fisher Scientific) was used. Buffers and reagents were used according to the manufacturer's standard recommendations. The primers used during PCR are listed in **Table 2.3**.

Table 2.3. Primers used for amplification of bacteriocin coding genes and the selection of transformants (continued on page 29)

Primer name	**Primer sequence 5‘-3‘**	**Restriction site**
LasA-NcoI.F	ATCGACCATGGCACAGGCCGATGAACAGAAGG	NcoI
LasA-BamHI.R	TAGCAGGATCCTAGCTGTACGGCGTCTGCTTGCT	BamHI
LasBCD_2-NdeI.F	TAGACATATGTCGGCGCGGGACCTCGCGAGC	NdeI
LasC-XhoI.R	AATTCCTCGAGTCACCGGGCCACCTCGCTGACTGGA	XhoI
LasB-NcoI-F	TTGAACCATGGGCTCCAGTCAGCGAGGTGGC	NcoI
LasB-HindIII.R	TTATGAAGCTTATCTGCGGATCGCTCACGGAGAGCT CCTCT	HindIII
LasD-NdeI.F	TCTAACATATGCTGCCCGAGTACCACCGATGGGAGA A	NdeI
LasD-XhoI.R	TAATCTCGAGCGGACCGCTACTTCATGGCGGAC	XhoI
LasD.HindIII.R	TCAGTCAAGCTTCGGACCGCTACTTCATGGCGGAC	HindIII
LCI1.F	GAAAACCTGTATTTTCAAGGTAACTTCAAAAAAGTG TTAACCGGTTCTGC	-
LCI2.F	GAAAACCTGTATTTTCAAGGTGCATCACCAACAGCA TCCGCATC	-
LCI3.F	GAAAACCTGTATTTTCAAGGTGCCATCAAACTCGTT CAAAGCCCTAACG	-
LCI.R	CTTTGTTAGCAGCCGGATCCTTATTTATCTACACTTTC ATAAATCCCTACCCAATACCC	-
pET-TEV.F	GGATCCGGCTGCTAACAAAGCCCGAAAG	-
pET-TEV.R	ACCTTGAAAATACAGGTTTTCACCGCTGCT	-
ACYCDuetUP1	GGATCTCGACGCTCTCCCT	-

T7 Terminator	GCTAGTTATTGCTCAGCGG	-
DuetDOWN1	GATTATGCGGCCGTGTACAA	-

2.2.4. Cloning of the *lasA*, *lasB*, *lasC,* and *lasD* genes in *E. coli*

The genes were amplified by PCR using *Sterptomyces* sp. AB3 genomic DNA as a template with the following primers (**Table 2.3**) and subsequently was digested with the restriction enzymes:

- *lasA* amplified with LasA-NcoI.F and LasA-BamHI.R and digested with NcoI and BamHI;
- *lasC* amplified with LasBCD_2-Nde.F and LasC-XhoI.R and digested with NdeI and XhoI;
- *lasB* amplified with LasB-NcoI.F and LasB-HindIII.R and digested with NcoI and HindIII;
- *lasD* amplified with LasD-NdeI.F and LasD-XhoI.R and digested with NdeI and XhoI.

After digestion of PCR products, *lasA* and *lasC* were cloned into the first and second multiple cloning sites, respectively, of the pRSFDuet-1 vector. Before cloning, the vector was digested with the same enzymes as the PCR products. *LasB* and *lasD* were cloned into the first and second multiple cloning sites, respectively, of the pETDuet-1 vector. Before cloning, the vector was digested with the same enzymes as the PCR products. DNA ligation was performed using T4 DNA ligase and transformed into *E. coli* DH5α strain using chemical transformation. The selection of transformants was performed using colony PCR using ACYCDuetUP1 and T7 terminator primers. In the end, new vectors pRSF-lasAC and pET-lasBD were successfully obtained from positive transformants.

2.2.5. Cloning of the the *lasA*, *lasB*, *lasC* and *lasD* genes in *L. lactis*

The *lasABCD* gene cluster was amplified by PCR using *Sterptomyces* sp. AB3 genomic DNA as a template with the LasA-NcoI.F and lasD.HindIII.R primers. PCR product and pNZ8048 plasmid were digested with NcoI and HindIII restriction enzymes. After digestion, *lasABCD* gene cluster was cloned into the pNZ8048 vector. DNA ligation was performed using T4 DNA ligase. Subsequently, the ligation mixtures were transformed into *E. coli* MC1061 strain through electrotransformation. The selection of transformants was performed using colony PCR using LasA-NcoI.F and lasD.HindIII.R primers. In the end, the new vector pNZ8048-lasABCD was successfully obtained from positive transformants.

2.2.6. Cloning of the *lci* genes

The genome of *Bacillus velezensis* DM1.10 encodes the *lci* gene, which was chosen to be synthesized in three variants of different lengths: LCI1, LCI2, and LCI3. These gene variants were individually amplified using PCR. For LCI1, LCI2, and LCI3 fragment amplification, the following primer pairs were used: LCI1.F and LCI.R, LCI2.F and LCI.R, LCI3.F and LCI.R, respectively. Next,

the plasmid pET15b-His-TEV-Geo6, encoding a 6×His tag and a TEV protease recognition site, was amplified using primers pET15b-TEV.F and pET15b-TEV.R. The amplified plasmid was subsequently purified, digested with the restriction enzyme DpnI, and ligated with the *lci* gene variants LCI1, LCI2, and LCI3 using the NEBuilder HiFi DNA Assembly Master Mix cloning kit according to the manufacturer's protocol. The ligation mixture was transformed into *E. coli* DH5α cells via electrotransformation. The selection of transformants was performed using colony PCR using ACYCDuetUp1 and T7 terminator primers. In the end, new constructs pET15b-His-TEV-LCI1, pET15b-His-TEV-LCI2, and pET15b-His-TEV-LCI3 were successfully obtained from positive transformants.

2.2.7. DNA electrophoresis in agarose gel

DNA electrophoresis was performed in a 1 % agarose gel containing 0.5 μg/mL ethidium bromide (AppliChem). When it was necessary to excise specific PCR products or plasmid DNA from the gel, Midori Green (Nippon Genetics) stain was used instead. To evaluate PCR product size, the GeneRuler DNA ladder (Thermo Fisher Scientific) was used. The electrophoresis system was filled with 1×TAE buffer (Thermo Fisher Scientific), and the electrophoresis was run at 120 V for 20–30 minutes. Visualization of gels containing ethidium bromide was performed using the MiniBIS Pro transilluminator (DNR Bio-Imaging System), whereas gels with Midori Green were visualized using the FastGene FAS-Nano gel documentation system (Nippon Genetics).

2.2.8. Preparation of competent cells

For the preparation of chemically competent cells, *E. coli* DH5α, BL21 (DE3), Rosetta (DE3) strains were grown overnight in liquid LB medium at 37 °C with aeration. In the morning, 1 % inoculum from the overnight culture was transferred into 100 mL of fresh LB medium and incubated until the optical density at 600 nm (OD_{600}) reached 0.3–0.4. Once the desired OD_{600} was achieved, the cells were cooled at 0 °C for 30 minutes. The cultures were then centrifuged at 1200×g for 10 minutes at 4 °C, after which the supernatant was discarded, and the cells were resuspended in 30 mL of cold 100 mM $CaCl_2$ solution. The suspension was incubated for 30 minutes at 0 °C, followed by centrifugation at 1000×g for 20 minutes at 4 °C. After removing the supernatant, the cells were resuspended in 1.5 mL of cold 100 mM $CaCl_2$ solution. After 3 hours of incubation, 1 mL of 50 % glycerol was added, and the cells were stored at –75 °C.

For the preparation of electrocompetent cells, *E. coli* MC1061 was grown overnight in liquid LB medium at 37 °C with aeration. In the morning, 7.5 mL of overnight culture was transferred into 150 mL fresh LB medium and incubated under the same conditions until OD_{600} reached 0.4. Once the desired OD_{600} was reached, the cells were cooled at 0 °C, followed by centrifugation at 1000×g for 15 minutes at 4 °C. The resulting cell pellets were washed with 150 mL of sterile distilled water and

centrifuged at 1000×g for 20 minutes at 4 °C. Then, cell pellets were washed with 75 mL of cold sterile 10 % glycerol solution and centrifuged at 1000×g for 20 minutes at 4 °C. This washing step was repeated with 15 mL of cold sterile 10 % glycerol solution. The pellet was then resuspended in 0.3 mL of GYT medium and aliquoted into tubes in 40 µL portions. The prepared electrocompetent cells were stored at –70 °C.

For the preparation of electrocompetent *L. lactis* cells, the NICE® Expression System for *Lactococcus lactis* (Mobitec) protocol was followed. On the first day, 5 mL of M17 medium supplemented with 0.5 M sucrose, 2.5 % glycine, and 0.5 % glucose was inoculated with *L. lactis* and incubated overnight at 30 °C without aeration. On the second day, a 1:100 dilution of the overnight pre-culture was used to inoculate 50 mL of fresh M17 medium supplemented with 0.5 M sucrose, 2.5 % glycine, and 0.5 % glucose, followed by incubation at 30 °C under the same conditions. On the third day, 50 mL of the fully grown culture was added to 400 mL of fresh M17 medium supplemented with 0.5 M sucrose, 2.5 % glycine, 0.5 % glucose, and incubated until an OD_{600} of 0.2–0.3 was reached. The cells were then harvested by centrifugation at 6000×g for 20 minutes at 4 °C. The resulting pellet was washed with 400 mL of cold 0.5 M sucrose containing 10 % glycerol, followed by centrifugation at 6000×g for 20 minutes. Next, the cells were resuspended in 200 mL of cold 0.5 M sucrose, 10 % glycerol, and 50 mM EDTA, and the suspension was kept on ice for 15 minutes before another centrifugation step. A second wash was performed using 100 mL of 0.5 M sucrose with 10 % glycerol, and cells were pelleted once more by centrifugation. Finally, the cells were resuspended in 4 mL of cold 0.5 M sucrose and 10 % glycerol. Aliquots of 40 µL were stored at –80 °C.

2.2.9. Chemical and electroporation transformation

For chemical transformation of *E. coli* cells, up to 5 µL of DNA was mixed with 50 µL of chemically competent *E. coli* cells and incubated on ice (0 °C) for 30 minutes. The mixture was then subjected to a heat shock at 42 °C in a water bath for 30 seconds, then immediately transferred to ice (0 °C), and incubated for an additional 5 minutes. Following transformation, the cells were recovered by adding 1 mL of SOC medium and incubated at 37 °C with aeration for approximately 1-1.5 hours. After recovery, 100 µL of the transformed cells were plated on LB agar supplemented with the appropriate antibiotic and incubated overnight at 37 °C. Positive transformants containing the desired construct were subsequently inoculated into a liquid LB medium supplemented with the appropriate antibiotic and grown overnight at 37 °C with shaking at 180 rpm. 200 µL of 50 % glycerol was added to the 800 µL overnight culture, and the cells were stored at -75 °C.

For electroporation transformation of *E. coli* cells, 1-2 µL of purified and clean DNA was added to cold (0 °C) electrocompetent *E. coli* cells and transferred to a pre-chilled electroporation cuvette. Electroporation was performed using the following parameters: 1800 V/cm, 5 ms. After

electroporation, the cells were recovered by adding 1 mL of SOC medium and incubated at 37 °C for 1 hour with aeration. Following recovery, 100 µL of the transformed cells were plated on LB agar supplemented with the appropriate antibiotic and incubated overnight at 37 °C. Positive transformants carrying the constructed plasmid DNA were inoculated into liquid LB medium with the appropriate antibiotic and grown overnight at 37 °C with shaking at 180 rpm. 200 µL of 50 % glycerol was added to the 800 µL overnight culture, and the cells were stored at -75 °C.

For electroporation transformation of *L. lactis* cells, 40 µL of electrocompetent *L. lactis* cells were mixed with 1-2 µL of purified and clean DNA and transferred to a pre-chilled electroporation cuvette. Electroporation was performed with the following parameters: 2000 V/cm, 5 ms. Immediately after electroporation, 1 mL of M17 medium supplemented with 0.5 % glucose, 20 mM $MgCl_2$, and 2 mM $CaCl_2$ was added to the cuvette. The cuvette was kept on ice for 5 minutes, followed by incubation at 30 °C for 1-1.5 hours without aeration. Following recovery, 10 µL, 100 µL, and 900 µL of the transformed culture were plated on M17 agar supplemented with appropriate antibiotics and 0.5 % glucose. The plates were incubated at 30 °C for 1–2 days until colonies appeared. Positive transformants carrying the constructed plasmid DNA were inoculated into liquid M17 medium supplemented with the appropriate antibiotic and 0.5 % glucose and grown at 30 °C without aeration. 200 µL of 50 % glycerol was added to the 800 µL overnight culture, and the cells were stored at -75 °C.

2.2.10. Biosynthesis of recombinant protein

E. coli expression strains BL21 (DE3) and Rosetta (DE3) harboring the pET15b-TEV-LCI1, pET15b-TEV-LCI2, pET15b-TEV-LCI3, pRSF-lasAC, and pET-lasBD constructs were cultivated in liquid LB medium supplemented with the appropriate antibiotics at 37 °C with shaking at 180 rpm. The cells were grown until the OD_{600} reached 0.4-0.5. Upon reaching the desired OD_{600}, the culture was divided in half: in one flask, gene expression was induced with 1 mM IPTG, while the other flask served as an uninduced control. Following induction, the cells were incubated for an additional 3-4 hours.

For the nisin induction of gene expression in *L. lactis*, the NICE® Expression System for *Lactococcus lactis* (Mobitec) protocol was followed. *L. lactis* NZ9000 was grown overnight in 10 mL M17 medium supplemented with the appropriate antibiotic and 0.5 % glucose. The following day, the culture was diluted 1:25 into 50 mL fresh M17 medium supplemented with the appropriate antibiotic and 0.5 % glucose. The cultures were incubated until they reached an OD_{600} of 0.4-0.5. Upon reaching the desired OD_{600}, the culture was divided in half: in one flask, gene expression was induced with 5 ng/mL and 20 ng/mL nisin, while the other flask served as an uninduced control. Both cultures were incubated for 5 hours at 30 °C.

After induction, cells were harvested by centrifugation at 10000×g for 10 minutes. Cell pellets were disrupted using glass beads. Disrupted cells and supernatant were subsequently analysed by Tricine-SDS-PAGE. In addition, antibacterial activity was also assessed using the spot-on-lawn assay.

2.2.11. Cell disruption using glass beads

After induction, the harvested cells were washed three times with distilled water and resuspended in 20 mM sodium phosphate buffer, pH 7.4. The samples were then mixed with approximately 100 µL of glass beads. The mixtures were vortexed thoroughly, and the cells were disrupted using a bead beater device set to maximum speed for 4 minutes. After cell disruption, the samples were centrifuged at 12300×g for 5 minutes, and the supernatant containing the soluble protein fraction was transferred into fresh 1.5 mL tubes. The insoluble protein fraction was first washed twice with 20 mM sodium phosphate buffer, pH 7.4. After each wash, the supernatant was discarded, and the pellets were centrifuged at 12300×g for 3 minutes. Following the second wash, 400 µL of 8 M urea was added to the pellets, thoroughly vortexed, and centrifuged again at 12300×g for 5 minutes. The insoluble proteins solubilized in urea remained in the supernatant. The samples were collected for subsequent analysis by Tricine-SDS-PAGE protein electrophoresis and for the evaluation of antibacterial activity using the spot-on-lawn assay.

2.2.12. Cell disruption by ultrasound

E. coli cells collected by centrifugation were resuspended in 20 mM sodium phosphate buffer containing 0.5 M NaCl, pH 7.4. Further cells were disrupted using ultrasonic homogenization with a Vibra Cell sonicator (SONICS). The sonication was performed for 15 minutes using a pulse setting of 5 seconds on/10 seconds off, at 36 % amplitude. After disruption, the lysate was centrifuged at 15000×g for 15 minutes at 4 °C to separate the soluble and insoluble protein fractions. Proteins in the insoluble fraction were resuspended and solubilized in 0.5 M NaCl, 25 mM sodium phosphate buffer supplemented with 6 M urea, pH 7.4, followed by a second centrifugation step under the same conditions. The resulting supernatant containing solubilized proteins from the insoluble fraction was collected for subsequent purification by chromatography.

2.2.13. Imobilized metal (Ni^{2+}) affinity chromatography (IMAC)

The purification of the LCI bacteriocin was performed using Ni^{2+} affinity chromatography from soluble and insoluble protein fractions of *E. coli* cell lysates. The procedure was performed using a HisTrap 1 mL column (GE). Protein chromatography was performed using the BioLogic DuoFlow medium-pressure chromatography system (Bio-rad). Before the sample application, the column was equilibrated with 25 mM sodium phosphate, 500 mM NaCl, pH 7.4. The protein sample was suspended in the same buffer and then loaded onto the column. Following the sample application, the

column was washed with the equilibration buffer. Elution of the 6×His tag protein from the column was performed using 25 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4.

2.2.14. Desalting

After Ni^{2+} affinity chromatography, protein samples were desalted using gel filtration chromatography with a HiTrap 5 mL column (GE) and the BioLogic DuoFlow system (Bio-Rad). Desalting was performed with 50 mM sodium phosphate, 100 mM NaCl, pH 7.4.

2.2.15. TEV protease cleavage

Cleavage of the N-terminal 6×His tag was performed enzymatically using TEV protease. TEV protease (4.13 mg/mL) was added to the desalted protein solution in a ratio of 1:29, and the reaction mixture was supplemented with DTT to a final concentration of 1 mM. The mixture was incubated at room temperature (~22 °C) for approximately 18 hours. The TEV protease used in this study was heterologously expressed in *E. coli* and purified in-house.

2.2.16. Ion exchange chromatography

Sample purification was performed using a UNO S-1 1 mL column (Bio-Rad) and the BioLogic DuoFlow system (Bio-Rad). The column was first equilibrated with 20 mM sodium phosphate, pH 7.4. The sample was loaded onto the column and washed with the same buffer. Elution was performed using 20 mM sodium phosphate, 500 mM NaCl, pH 7.4.

2.2.17. Tricine-SDS-PAGE protein electrophoresis

Tricine-SDS-PAGE protein electrophoresis was performed following the protocol described by Schägger (2006). Protein separation was carried out in an SDS-polyacrylamide gel consisting of a 4 % stacking gel, a 10 % separating gel, and a 16 % separating gel. Alongside the samples, the PageRuler Unstained Low Range Protein Ladder (Thermo Fisher Scientific) was loaded as a molecular weight standard. Following electrophoresis, the gels were fixed for 30 minutes in a fixing solution containing 20 % propanol, 10 % acetic acid, and 70 % deionized water. The gels were then washed three times for 10 minutes each in deionized water. Protein bands were visualized using the PageBlue Protein Staining Solution (Thermo Fisher Scientific), following the manufacturer's instructions.

Additionally, gels were stained using the short silver nitrate staining method described by Chevallet et al. (2006). After electrophoresis, the gels were fixed for 30 minutes in a fixing solution containing 20 % propanol, 10 % acetic acid, and 70 % deionized water. The fixed gels were then washed twice with 20 % ethanol for 20 minutes each, followed by two washes in deionized water for 10 minutes each. To sensitize the gels, they were dipped in 0.8 mM sodium thiosulfate for one minute and subsequently rinsed twice with deionized water, one minute per wash. Silver impregnation was

performed using a 12 mM silver nitrate solution, after which the gels were rinsed with deionized water for 10 seconds. Finally, gels were developed in a developer solution (3 % potassium carbonate, 250 µl formalin, 125 µl 10 % sodium thiosulfate per liter). The gels were gently agitated in this solution until protein bands became visible or for no longer than 45 minutes. Stained gels were visualized and analysed using the MiniBIS Pro transilluminator (DNR Bio-Imaging System).

2.2.18. Antibacterial activity evaluation

To assess antibacterial activity, bacterial strains were cultivated in a liquid LB medium for 24 hours at 30 °C with shaking at 200 rpm. The molten LB agar was inoculated with the bacterial suspensions at the following concentrations: 1 % (v/v) for *Xanthomonas campestris* DSM 3586, *X. vesicatoria* DSM 22252, and *Agrobacterium radiobacter* DSM 30147; and 5 % (v/v) for *Pseudomonas syringae* DSM 10604, *P. syringae* DSM 50315, *Dickeya solani* DSM 28711, *Pectobacterium carotovorum* DSM 30168, and *P. atrosepticum* DSM 18077. The inoculated medium was mixed thoroughly and poured into sterile Petri dishes. After cell disruption with glass beads, 10 µL of the resulting sample was spotted onto the surface of the solidified agar. After the droplets dried, the plates were incubated at 30 °C for up to 72 hours. Following incubation, zones of inhibition of the indicator strains' growth were evaluated.

Streptomyces scabies DSM 41658, *Streptomyces lavendulae* subsp. *lavendulae* DSM 40069, *Streptomyces lavendulae* subsp. *grasserius* DSM 40385 bacterial strains were cultivated in a liquid ISP-2 medium for 24 hours at 30 °C with shaking at 180 rpm. Following incubation, 100 µL of the overnight culture was evenly spread onto solid ISP-2 agar plates using a sterile Q-tip. After cell disruption with glass beads, 10 µL of the resulting sample was spotted onto the surface of the agar. After the droplets dried, the plates were incubated at 30 °C for up to 72 hours. Following incubation, zones of inhibition of the indicator strains' growth were evaluated.

3. Results and discussion

3.1. Heterologous gene expression of lasso peptide in *E. coli* cells

To synthesize bacteriocins *in vivo*, heterologous expression of the lasso peptide gene cluster *lasABCD* (**Figure 1.7A**) has been previously attempted in *E. coli* cells. The whole *lasABCD* gene cluster has been cloned into pRSFDuet-1 expression vector and expression performed in *E. coli* BL21 (DE3), Rosetta (DE3), C41 (DE3), and Arctic Express (DE3) host strains. As a result, low-level peptide biosynthesis was observed only in the *E. coli* Arctic Express (DE3) strain. However, no antibacterial activity of the produced bacteriocin was detected against any of the nine tested phytopathogenic bacteria strains (Smulkaitė, 2023).

To further investigate and potentially overcome these limitations, the present study aimed to improve the biosynthesis level of the *lasABCD* genes in *E. coli* cells. To achieve heterologous biosynthesis of mature lasso peptide in *E. coli* cells, it was planned to clone *lasA* and *lasC* genes into pRSFDuet-1 expression vector, and *lasB* and *lasD* genes into pETDuet-1 expression vector, where the T7 promoter would control every gene separately. This resulted in vectors pRSF-lasAC and pET-lasBD (**Figure 3.1**). These vectors were transformed, and gene expression was induced in *E. coli* BL21 (DE3) and *E. coli* Rosetta (DE3) cells. The *E. coli* BL21 (DE3) strain is well-known and commonly used for recombinant protein expression. The *E. coli* Rosetta (DE3) expression strain is distinguished by enhanced protein expression due to the presence of the pRARE plasmid, which encodes tRNAs for codons that are rarely found in *E. coli* cells.

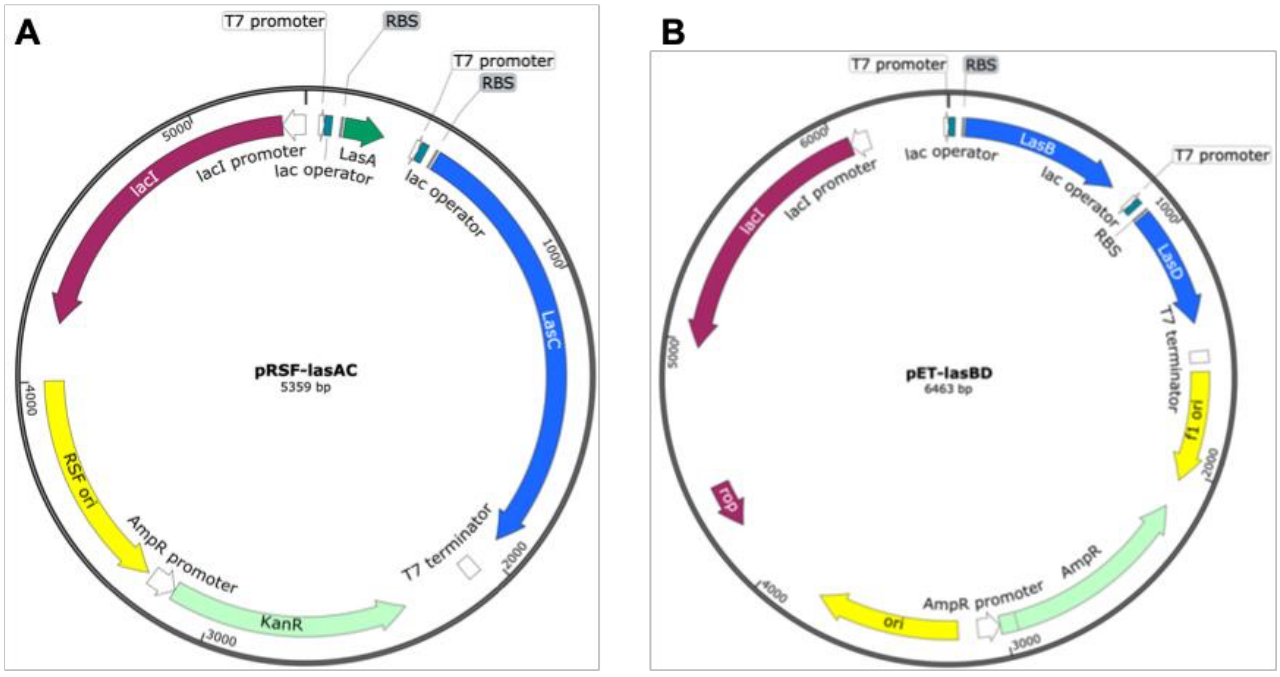

Figure 3.1. Plasmid constructs used for the heterologous expression of the *lasABCD* gene cluster in *E. coli* cells. (**A**) Plasmid pRSF-lasAC carries the genes encoding the lasso peptide precursor (*lasA*) and the macrolactam synthetase (*lasC*). (**B**) Plasmid pET-lasBD carries the genes encoding the cysteine protease (*lasB*) and the GNAT-family acyltransferase (*lasD*).

The induction of *lasABCD* gene expression was performed with a 1 mM final concentration of IPTG. Four hours after the induction, *E. coli* cells were harvested and analysed by Tricine-SDS-PAGE to assess protein synthesis (**Figure 3.2)**. In Tricine-SDS-PAGE gels, soluble and insoluble fractions of induced and uninduced cells were analysed. After electrophoresis, gels were stained with PAGE Blue (**Figure 3.2A**) and silver staining (**Figure 3.2B**). When comparing the induced and uninduced cell samples on the PAGE Blue-stained gel, no distinct peptide bands corresponding to the theoretical molecular weights of LasA (4.7 kDa), the cleaved leader peptide (3.1 kDa), or the mature lasso peptide (1.6 kDa) were observed in either the soluble or insoluble fractions. Similarly, protein bands corresponding to LasB (23.3 kDa) and LasC (52.3 kDa) were not visible. However, in the insoluble fractions of both induced *E. coli* BL21 (DE3) and *E. coli* Rosetta (DE3) samples, a protein band near 18 kDa that was more intense than in the uninduced cell sample was observed, consistent with the expected molecular size of LasD (18.3 kDa). Notably, in the silver-stained gel, a brighter peptide band near 5 kDa was visible in the soluble fractions of both induced *E. coli* BL21 (DE3) and *E. coli* Rosetta (DE3) samples, more intense than in the corresponding uninduced controls. This band likely corresponds to the lasso peptide with a leader sequence (4.7 kDa), suggesting that at least partial expression of the lasso peptide precursor occurred in the soluble fraction of both *E. coli* strains. However, silver-staining gel above 5 kDa was over-saturated with staining, and proteins LasB, LasC, and LasD could not be observed.

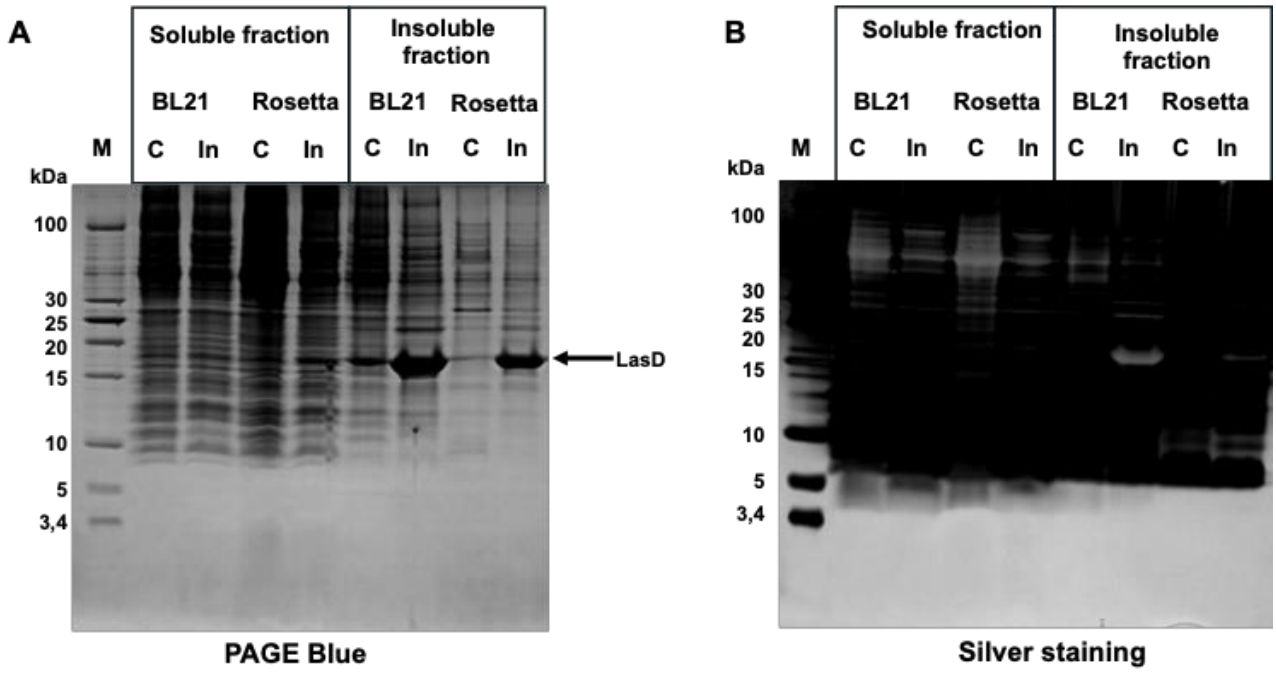

Figure 3.2. Tricine-SDS-PAGE analysis of *E. coli* cells after *lasABCD* gene expression. **M** – PageRuler Unstained Low Range Protein Ladder, **C** – uninduced cells, **In** – induced cells.

The absence of detectable peptide bands corresponding to the mature lasso peptide, despite the presence of a band potentially representing the precursor LasA, suggests that post-translational

modifications of the peptide were incomplete or inefficient in the *E. coli* expression system. This is likely due to insufficient expression or functional activity of the putative biosynthetic enzymes encoded by the *lasBCD* genes. The *lasB* gene encodes a cysteine protease required for cleavage of the leader sequence from the precursor peptide, a critical step in lasso peptide maturation. Additionally, *lasC* encodes a macrolactam synthetase responsible for cyclizing the peptide, forming the characteristic lasso structure (Duan et al., 2022). The *lasD* gene encodes a GNAT-family acyltransferase that modifies lysine residues in the loop and ring regions through sequential acylation (Xiong et al., 2025). Failure to detect LasB, LasC, and LasD proteins in Tricine-SDS-PAGE gels suggests that these enzymes were either not expressed or not functionally active in *E. coli* cells. As a result, the precursor peptide likely remained unprocessed, explaining the absence of a mature lasso peptide.

3.2. Heterologous gene expression of lasso peptide in *L. lactis* cells

As the active bacteriocin could not be successfully synthesized in *E. coli* cells, the *L. lactis* expression system was chosen as an alternative. Native lasso peptide is encoded in *Streptomyces* sp. AB3, a Gram-positive bacterium. As a Gram-positive organism, *L. lactis* may provide a more physiologically and biochemically compatible environment for proper peptide folding and maturation compared to *E. coli*. This host-specific compatibility is particularly important in the context of lasso peptides, many of which originate from high-GC content Gram-positive bacteria such as *Streptomyces* and *Bacillus*. While *E. coli* has proven suitable for the heterologous expression of several lasso peptides derived from Gram-negative Proteobacteria, its performance with lasso peptides of Gram-positive origin has frequently been limited due to poor expression yields or incomplete peptide maturation. In contrast, Gram-positive hosts such as *Streptomyces coelicolor*, *S. lividans*, and *Bacillus subtilis* have demonstrated higher compatibility and success in expressing complex or post-translationally modified lasso peptides, including albusnodin, citrulassin, and sviceucin (Cheng & Hua, 2020). Thus, the selection of *L. lactis* for heterologous expression is consistent with the need to replicate the native biosynthetic environment and enhance the likelihood of producing a functional, bioactive lasso peptide.

To achieve heterologous biosynthesis of the lasso peptide in *L. lactis*, the whole *lasABCD* gene cluster was cloned into pNZ8048 expression vector, resulting in vector pNZ8048-lasABCD (**Figure 3.3**). *L. lactis* NZ9000 is a widely used heterologous expression host engineered from the nisin-negative MG1363 strain. It carries the chromosomally integrated regulatory genes *nisR* and *nisK*, which enable controlled expression of target genes placed under the nisin-inducible Pnis promoter. Gene expression is induced by the addition of sub-inhibitory concentrations of nisin (typically 0.1–5 ng/mL). The commonly used expression vector pNZ8048 is compatible with this system and allows

for gene insertion at the NcoI site, which overlaps the ATG start codon, enabling seamless fusion of the target gene to the promoter for tightly regulated expression in *L. lactis* NZ9000 (Song et al., 2017; Frelet-Barrand, 2022).

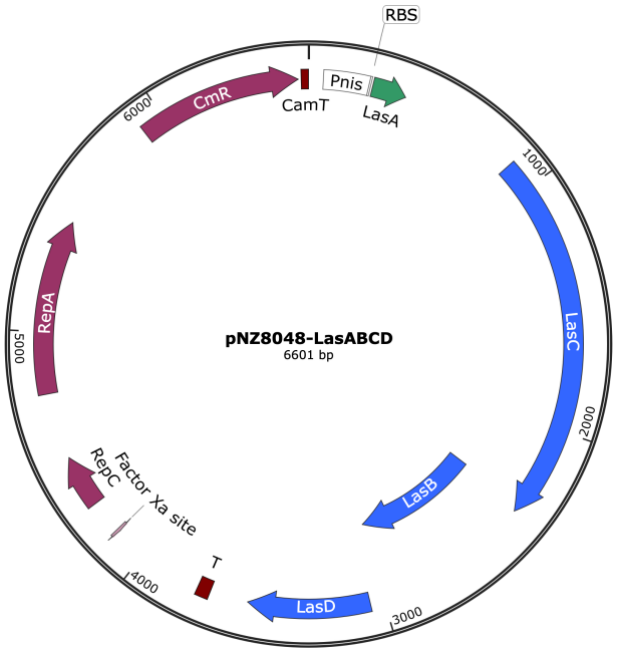

Figure 3.3. Plasmid construct used for the heterologous expression of the *lasABCD* gene cluster in *L. lactis* NZ9000. The plasmid pNZ8048-LasABCD carries the complete *lasABCD* gene cluster responsible for lasso peptide biosynthesis. The genes include *lasA* (precursor peptide), *lasB* (cysteine protease), *lasC* (macrolactam synthetase), and *lasD* (GNAT-family acyltransferase).

The induction of *lasABCD* gene expression was performed with 5 ng/mL and 20 ng/mL of nisin. Five hours after induction, *L. lactis* cells were harvested and analysed by Tricine-SDS-PAGE to assess protein synthesis (**Figure 3.4**). In Tricine-SDS-PAGE gels, soluble and insoluble fractions of induced and uninduced cells were analysed. After electrophoresis, gels were stained with PAGE Blue (**Figure 3.4A**) and silver staining (**Figure 3.4B**). However, analysis showed that no protein and peptide bands corresponding to LasA peptide or other maturation proteins were observed. These results suggest that the heterologous expression of the lasso peptide or maturation proteins in *L. lactis* was unsuccessful.

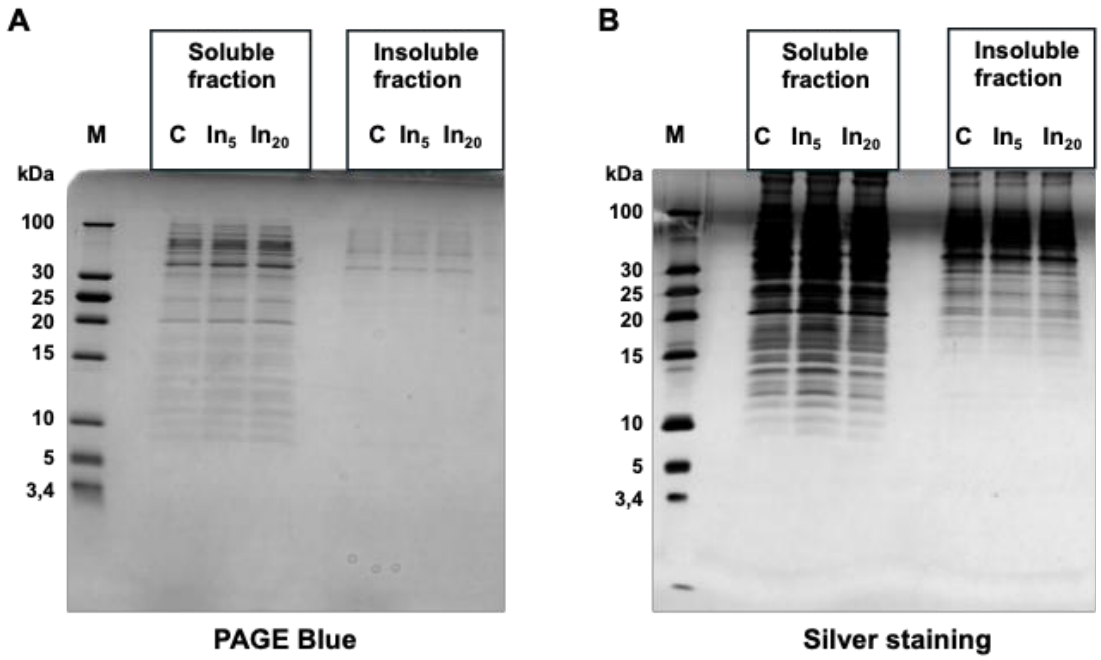

Figure 3.4. Tricine-SDS-PAGE analysis of *L. lactis* cells after *lasABCD* gene expression. **M** – PageRuler Unstained Low Range Protein Ladder, **C** – uninduced cells, **In$_5$** – induced cells with 5 ng/mL nisin, **In$_{20}$** – induced cells with 20 ng/mL nisin.

In the *L. lactis* expression system, no visible bands corresponding to the target lasso peptide or biosynthetic proteins were observed. The absence of detectable peptides or biosynthetic enzymes in *L. lactis* suggests that a possible limitation lies in the transcriptional or translational efficiency of the introduced genes. Although the Pnis promoter is well-established for inducible expression in *L. lactis*, heterologous gene clusters, especially those derived from high-GC content organisms like *Streptomyces*, may suffer from codon usage incompatibilities, leading to inefficient translation (Parvathy et al., 2022). Thus, the undetectable expression of the lasso peptide and its maturation proteins may reflect a complex interplay of transcriptional, translational, and post-translational limitations specific to the *L. lactis* expression system.

3.3. Heterologous gene expression of LCI-type bacteriocin in *E. coli* cells

The aim was to synthesize an active LCI-type bacteriocin in *E. coli* cells through heterologous biosynthesis. In previous studies, this peptide was identified in the *Bacillus velezensis* DM1.10 genome as a class II bacteriocin (Smulkaitė, 2023). The amino acid sequence analysis was performed using the BLASTp algorithm in the NCBI and UniProt databases. The amino acid sequence of the LCI-type bacteriocin showed 93.5 % similarity to the antimicrobial peptide LCI (UniProt accession number P82243) produced by *Bacillus subtilis* A014 (**Figure 3.5**). LCI is a β-structured antimicrobial peptide composed of 47 amino acids and carries a hydrophobic core formed by valine, tryptophan, and tyrosine residues (Gong et al., 2011; Iqbal et al., 2023). However, it is important to note that the LCI from *B. subtilis* A014 sequence lacks the N-terminal region, and its amino acid sequence exhibits

several variations in the C-terminal end when compared to the LCI-type bacteriocin from *B. velezensis* DM1.10. Therefore, a more detailed investigation of this peptide is needed to reveal its properties and potential functional implications.

Figure 3.5. Sequence alignment of LCI core peptides encoded in *Bacillus velezensis* DM1.10 (**A**) and *Bacillus subtilis* A014 (**B**). Letters in purple indicate identical amino acids at the same position. A dash (–) denotes a gap introduced by the alignment algorithm to maximize sequence similarity.

This non-modified bacteriocin was chosen to be synthesized in three variants (**Figure 3.6**): **LCI1** – whole peptide precursor with the signal peptide, which is encoded in *lci* gene in the genome of *B. velezensis* DM1.10, **LCI2** – precursor without the signal peptide, **LCI3** – mature bacteriocin without the signal peptide and N-terminal end (similar to native LCI produced by *B. subtilis* A014). The signal peptide in LCI-type bacteriocins directs the peptide to the appropriate secretion pathway. For these bacteriocins to become active, the signal peptide must be accurately and efficiently cleaved (Simons et al., 2020). Therefore, LCI1 was used as a control to evaluate the impact of signal peptide and its cleavage site position on bacteriocin activity.

Figure 3.6. Different LCI-type bacteriocin variants for expression. Numbers indicate the position of amino acids in the sequence.

To purify the LCI1, LCI2, and LCI3 peptides, their respective genes *lci1*, *lci2,* and *lci3* were cloned into the pET15b expression vector, which already contained DNA sequence encoding a 6×His tag and a TEV protease recognition site (**Figure 3.7**). The 6×His tag was included to facilitate protein purification, while the TEV protease cleavage site was introduced to allow removal of the 6×His tag after the purification. The resulting constructs pET15b-His-TEV-LCI1, pET15b-His-TEV-LCI2, and pET15b-His-TEV-LCI3 were transformed into *E. coli* BL21 (DE3) cells for peptide biosynthesis.

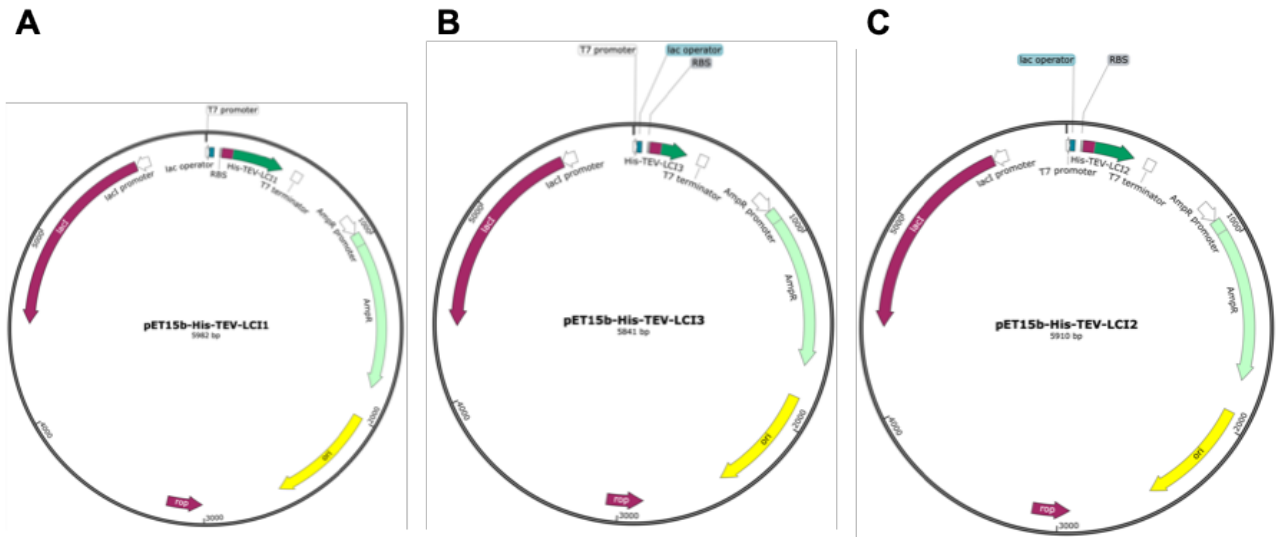

Figure 3.7. Plasmid constructs used for the heterologous expression of different LCI-type bacteriocin variants in *E. coli* cells: (**A**) pET15b-His-TEV-LCI1; (**B**) pET15b-His-TEV-LCI2; (**C**) pET15b-His-TEV-LCI3. Each construct contains a multiple cloning site with sequences encoding an N-terminal 6×His tag and a TEV protease recognition site (His-TEV), followed by the respective gene encoding the LCI variant.

The induction of *lci* gene expression was performed with a 1 mM final concentration of IPTG. Three hours after induction, *E. coli* cells were harvested and analysed by Tricine-SDS-PAGE to assess peptide synthesis (**Figure 3.8)**. In Tricine-SDS-PAGE gels, soluble (**Figure 3.8A**) and insoluble (**Figure 3.8B**) fractions of induced and uninduced cells were analysed. When comparing the uninduced and induced cell samples of the soluble fraction, distinct peptide bands close to the expected theoretical molecular weights are visible in the induced samples. Similarly, when comparing the uninduced and induced cell samples of the insoluble fraction, LCI1 and LCI2 peptide bands close to the expected theoretical molecular weights are visible in the induced samples. The predicted molecular weights of the recombinant peptides are 12 kDa for His-TEV-LCI1, 9.7 kDa for His-TEV-LCI2, and 7.5 kDa for His-TEV-LCI3. However, the observed molecular weights do not precisely correspond to the predicted molecular masses. This difference may be attributed to the β-sheet-rich secondary structures within the LCI-type bacteriocins. Such structural features can persist under denaturing SDS-PAGE conditions (Nielsen et al., 2007), affecting the peptide's migration through the gel matrix. As a result, peptides with stable β-sheet conformations may display reduced mobility and appear at slightly higher apparent molecular weights than their actual mass.

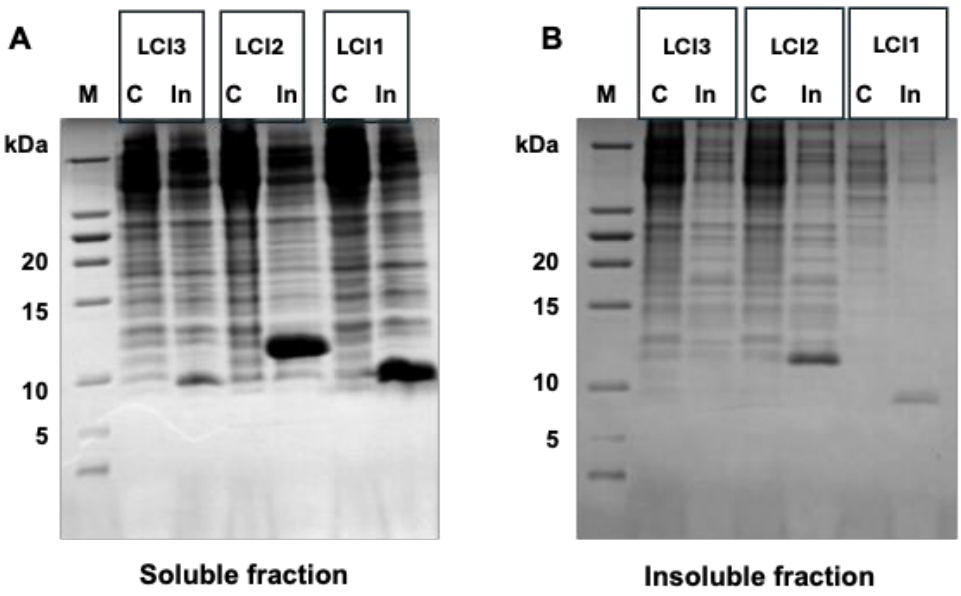

Figure 3.8. Tricine-SDS-PAGE analysis of *E. coli* BL21 (DE3) cells after *lci1, lci2* and *lci3* gene expression. **M** – PageRuler Unstained Low Range Protein Ladder, **C** - uninduced cells, **In** – induced cells.

Bright and well-defined peptide bands of LCI1 and LCI2 indicate successful expression of these peptides. Given the lack of a bright LCI3 band and the preliminary focus on successfully expressed variants, further experimental work with LCI3 was not performed.

3.4. Purification of LCI-type bacteriocin using protein chromatography

After successful gene expression, the next aim was to purify LCI1 and LCI2 peptides. Following the induction, cells were disrupted by ultrasonication, and the peptides were purified using Ni^{2+} affinity chromatography. Subsequently, elution fractions obtained from the Ni^{2+} column were desalted via gel filtration chromatography and further digested with TEV protease to cleave the 6×His tag. Finally, peptides without a 6×His tag were purified using ion exchange chromatography. After each purification step, samples were analysed by Tricine-SDS-PAGE (**Figure 3.9**).

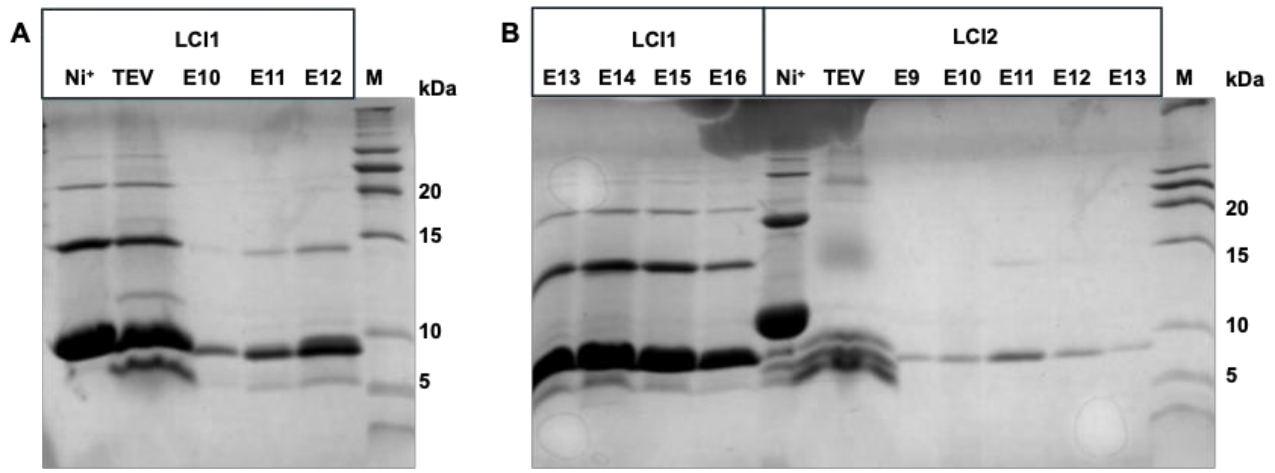

Figure 3.9. Tricine-SDS-PAGE analysis of purified LCI1 and LCI2. **Ni^{+}** – purified LCI after Ni^{+} affinity chromatography, **TEV** – 6×His tag cleavage with TEV protease, **E** – elution fractions after ion exchange chromatography, **M** – PageRuler Unstained Low Range Protein Ladder.

For LCI1, a distinct peptide band at approximately 10 kDa is visible after the first purification step using Ni^{2+}-affinity chromatography, corresponding to the His-TEV-LCI1 theoretical molecular weight of 12.1 kDa. Also, cleavage of the 6×His tag (2.3 kDa) by TEV protease was confirmed, resulting in a peptide band of 9.8 kDa, corresponding to the expected size of the LCI1 peptide. The subsequent ion-exchange chromatography step further purified the cleaved LCI1, as indicated by a clear peptide band at the same molecular weight. LCI2 followed an analogous purification process. After Ni^{2+}-affinity chromatography, a peptide band was detected near 9.7 kDa, corresponding to the predicted size of the His-TEV-LCI2. Also, cleavage of the 6×His tag (2.3 kDa) by TEV protease was confirmed, resulting in a peptide band of 7.4 kDa, consistent with the theoretical molecular weight of LCI2 peptide, although signs of proteolytic degradation were also observed. Despite this, ion-exchange chromatography confirmed the presence of the cleaved LCI2 peptide. Overall, while LCI1 was recovered in higher yield, although with lower purity, LCI2 exhibited higher purity but at a lower concentration.

3.5. Evaluation of antibacterial activity

To assess the antimicrobial activity of the cell lysate after lasso peptide biosynthesis, it was analysed using a spot-on-lawn assay. The antibacterial activity was tested against four phytopathogenic bacteria: *Xanthomonas vesicatoria* DSM 22252, *Streptomyces lavendulae* subsp. *lavendulae* DSM 40069, *Streptomyces lavendulae* subsp. *grasserius* DSM 40385, and *Streptomyces scabies* DSM 41658. However, no activity was observed.

To assess the antimicrobial activity of LCI-type bacteriocin, first, the cell lysates after induction were analysed using a spot-on-lawn assay. For the assay, 7 phytopathogenic bacteria were used: *Agrobacterium radiobacter* DSM 30147, *Pseudomonas syringae* DSM 50315, *Dickeya solani* DSM 28711, *Pseudomonas syringae* DSM 10604, *Xanthomonas campestris* DSM 3586,

Pectobacterium carotovorum DSM 30168, *Pectobacterium atrosepticum* DSM 18077. However, no antimicrobial activity of His-TEV-LCI1 and His-TEV-LCI2 was detected, probably due to the presence of the N-terminal 6×His tag, which may interfere with the bacteriocin's structural integrity essential for antimicrobial function. Further, the elution fractions of synthesized and purified LCI1 and LCI2 after ion exchange chromatography were tested using a spot-on-lawn assay against *P. syringae* DSM 10604. However, no activity was detected in any of the purified elution fractions (**Figure 3.10**).

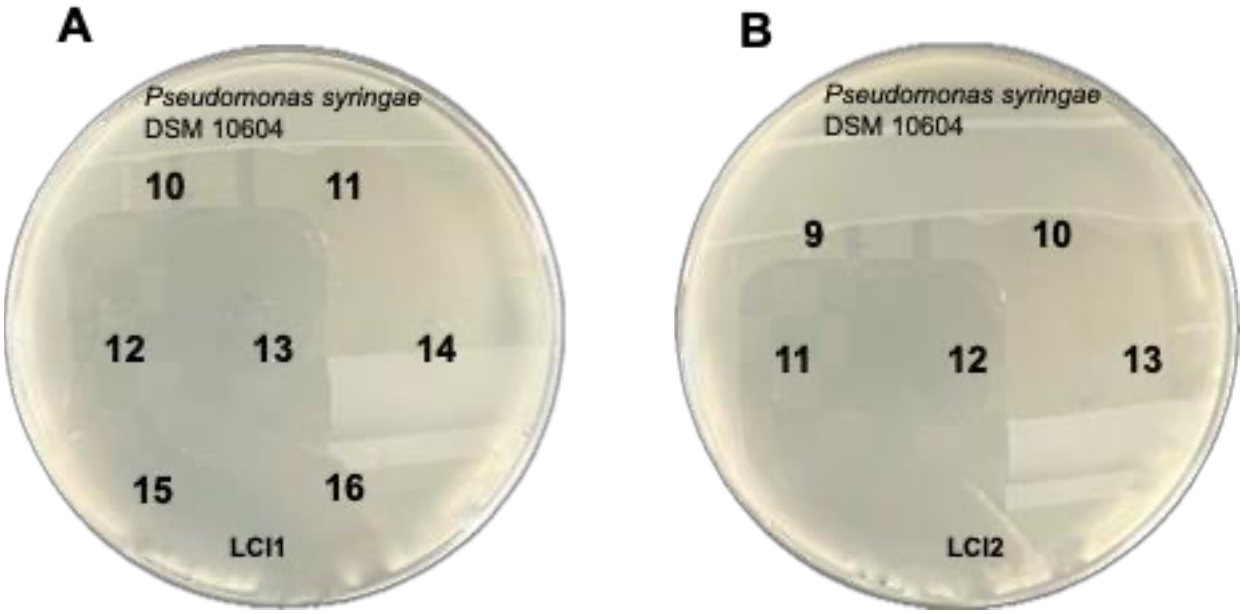

Figure 3.10. Evaluation of antibacterial activity of LCI1 (**A**) and LCI2 (**B**) after ion exchange chromatography against *Pseudomonas syringae* DSM 10604. Numbers indicate elution fractions after ion exchange chromatography.

It was predicted that purified LCI1 would not be active due to the presence of the signal peptide, which may hinder proper peptide folding or prevent the formation of the mature bacteriocin structure necessary for antimicrobial activity. Yet, the absence of antimicrobial activity of the purified LCI2 bacteriocin, which lacked the signal peptide, may be due to insufficient peptide concentration or partial degradation during purification, which can impair its functional activity.

3.8. Discussion

This study aimed to heterologously synthesize and characterize novel lasso and LCI-type bacteriocins with antimicrobial activity against phytopathogenic bacteria. While the cloning and transformation of all designed expression constructs were successfully achieved, the outcomes of heterologous expression varied significantly depending on both the bacteriocin type and the host system used.

The expression of the *lasABCD* gene cluster in *E. coli* strains resulted in only partial expression, as evidenced by weak peptide bands corresponding to the lasso precursor (LasA) and one of the maturation proteins LasD. No detectable mature peptide or associated antimicrobial activity was

observed. A similar outcome was obtained when the *lasABCD* gene cluster was expressed in *L. lactis* cells, despite its closer phylogenetic similarity to the native *Streptomyces* host. These findings suggest that the expression and functional maturation of lasso peptides derived from high-GC Gram-positive bacteria require host-specific biosynthetic compatibility that was not adequately met in *E. coli* or *L. lactis* host strains. In the future, it is worth considering *Streptomyces coelicolor* or *Streptomyces lividans* as heterologous hosts for heterologous expression of lasso peptide or try to obtain them by isolation from natural hosts (Cheng & Hua, 2020).

In contrast, LCI-type bacteriocin variants LCI1 and LCI2 were successfully expressed in *E. coli* BL21 (DE3), purified using Ni^{2+}-affinity chromatography, TEV cleavage, and ion-exchange chromatography. While peptide bands corresponding to expected molecular weights confirmed successful expression and purification, antimicrobial assays revealed no inhibitory activity against phytopathogenic strains. The lack of activity could be attributed to residual signal peptides (in LCI1), low peptide concentrations, or proteolytic degradation during purification.

Conclusions

1. Expression vectors pRSF-lasAC and pET-lasBD were successfully constructed and transformed into *Escherichia coli* BL21 (DE3) and *Escherichia coli* Rosetta (DE3) strains, while the constructed pNZ8048-lasABCD vector was transformed into *Lactococcus lactis* NZ9000 for heterologous expression of the lasso peptide biosynthetic genes.
2. Expression vectors pET15b-His-TEV-LCI1, pET15b-His-TEV-LCI2, and pET15b-His-TEV-LCI3 were successfully constructed and transformed into *E. coli* BL21 (DE3) for heterologous expression of LCI-type bacteriocin variants.
3. Based on Tricine-SDS-PAGE analysis, the biosynthesis of lasso peptide in *E. coli* and *L. lactis* cells was unsuccessful.
4. Based on Tricine-SDS-PAGE analysis, LCI1, LCI2, and LCI3 peptide variants were successfully synthesized in *E. coli* BL21 (DE3) cells.
5. LCI1 and LCI2 peptides were successfully purified using protein chromatography, whereas purification of the LCI3 variant was not performed due to low yield.
6. The cell lysates obtained after lasso peptide expression did not show antimicrobial activity against the tested phytopathogenic bacteria. Purified LCI1 and LCI2 peptides did not demonstrate antibacterial activity either, probably due to the low concentration of purified peptides.

Author's Personal Contribution

The author was involved in all stages of the research presented in this thesis. The research idea and objectives were jointly developed in collaboration with the thesis supervisor. The author contributed to the conceptualization of the study by helping to define the experimental strategy for the heterologous expression and evaluation of bacteriocins. The author performed all experimental work and laboratory analyses. The author visualized results in figures and tables and prepared the original draft. The thesis supervisor provided supervision and methodological guidance and contributed to the review and editing of the final work.

Dissemination of Results

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VILNIUS UNIVERSITY
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Gabija Smulkaitė

Master's Thesis

Heterologous Biosynthesis of Lasso and LCI-Type Bacteriocins

Abstract

Bacteriocins are a diverse group of ribosomally synthesized antimicrobial peptides produced by bacteria to inhibit the growth of competing microorganisms. These compounds have gained attention as promising alternatives to traditional chemical products due to their high specificity, low toxicity, and potential applications in agriculture, food preservation, and medicine. Among the various bacteriocins, lasso peptides and LCI-type bacteriocins represent structurally unique subgroups. Lasso peptides are characterized by a threaded lasso topology that provides resistance to heat and proteolysis, while LCI-type peptides are small, heat-stable antimicrobials. Despite their potential, the native production of these bacteriocins is often limited by low yields and the difficulty of cultivating producer strains under laboratory conditions.

The aim of this research was to heterologously synthesize and characterize novel bacteriocins with antimicrobial activity against phytopathogenic bacteria. To achieve this, objectives were pursued: the heterologous expression of lasso peptide and LCI-type bacteriocins in *Escherichia coli* and *Lactococcus lactis* host strains; verification of bacteriocin production through Tricine-SDS-PAGE analysis; purification of the expressed LCI peptides using Ni^{2+}-affinity and ion-exchange chromatography; and evaluation of the antibacterial activity of the synthesized peptides against selected phytopathogenic bacterial strains.

The results showed successful heterologous expression of LCI-type bacteriocins LCI1, LCI2, and LCI3 in *E. coli* BL21 (DE3), as confirmed by Tricine-SDS-PAGE. LCI1 and LCI2 peptides were subsequently purified using Ni^{2+}-affinity and ion-exchange chromatography, while the purification of LCI3 was not performed. In contrast, heterologous expression of the lasso peptide in both *E. coli* and *L. lactis* cells was unsuccessful. Antimicrobial activity assays revealed no inhibitory effect of either the lasso peptide cell lysates or the purified LCI peptides against selected phytopathogenic bacteria, which may be attributed to insufficient LCI peptide concentrations or incomplete post-translational modifications of the lasso peptide in the heterologous host.

VILNIAUS UNIVERSITETAS
GYVYBĖS MOKSLŲ CENTRAS

Gabija Smulkaitė

Magistro baigiamasis darbas

Heterologinė Laso ir LCI Tipo Bakteriocinų Biosintezė

Santrauka

Bakteriocinai yra ribosomų sintetinama antimikrobinių peptidų grupė, kurią produkuoja bakterijos siekdamos slopinti konkurencingų mikroorganizmų augimą. Šios medžiagos sulaukė didelio dėmesio kaip perspektyvios alternatyvos tradicinėms cheminėms priemonėms dėl jų didelio specifiškumo, mažo toksiškumo ir galimo pritaikymo žemės ūkio, maisto pramonės ir medicinos srityse. Vieni iš struktūriškai unikalių bakteriocinų yra laso peptidai ir LCI tipo bakteriocinai. Laso peptidai pasižymi laso formos struktūra, kuri suteikia jiems atsparumą karščiui ir proteolizei, o LCI tipo peptidai yra maži, termostabilūs antimikrobiniai junginiai. Nepaisant jų potencialo, natūraliai šie bakteriocinai dažnai yra produkuojami mažais kiekiais.

Šio darbo tikslas buvo heterologiškai susintetinti ir charakterizuoti naujus bakteriocinus, turinčius antimikrobinį aktyvumą prieš fitopatogenines bakterijas. Šio darbo uždaviniai: laso peptido ir LCI tipo bakteriocinų heterologinė genų raiška *Escherichia coli* ir *Lactococcus lactis* ląstelėse; bakteriocinų sintezės patvirtinimas naudojant Tricininę-SDS-PAGE analizę; susintetintų LCI peptidų gryninimas Ni^{2+} afininės ir jonų mainų chromatografijos būdu; bei susintetintų peptidų antibakterinio aktyvumo įvertinimas prieš atrinktas fitopatogeninių bakterijų rūšis.

Tricininė-SDS-PAGE analizė parodė sėkmingą LCI tipo bakteriocinų LCI1, LCI2 ir LCI3 heterologinę raišką *E. coli* BL21 (DE3) ląstelėse. LCI1 ir LCI2 peptidai buvo toliau sėkmingai išgryninti naudojant Ni^{2+} afininę ir jonų mainų chromatografiją, o LCI3 gryninimas nebuvo atliktas. Priešingai, laso peptido heterologinė raiška *E. coli* ir *L. lactis* ląstelėse nevyko. Antimikrobiniu aktyvumu fitopatogeninėms bakterijoms nepasižymėjo nei laso peptido ląstelių lizatas, nei išgrynintas LCI peptidai, tikėtina, dėl per mažos LCI koncentracijos arba nepilnai vykstančių laso peptido potransliacinių modifikacijų heterologiniame šeimininke.

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