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Corso di dottorato di ricerca in: Alimenti e Salute Umana
Ph.D. course in Food and Human Health

Cycle XXXV

Analysis of undescribed bacteriocins gene clusters and exploration of their regulation mechanism

Dottorando

Dott. Francesco Salini

Supervisore

Prof. Giuseppe Comi

Co-supervisore

Prof. Lucilla Iacumin
Prof. Leon M.T. Dicks

Anno 2023

Analysis of undescribed bacteriocins gene clusters and exploration of their regulation mechanism

A Ph.D. dissertation presented by
Francesco Salini

to the
University of Udine

for the degree of Ph.D. in the subject of
Food and Human Health (Cycle XXXV)

Department of Agricultural, Food,
Environmental and Animal Science

UNIVERSITY OF UDINE
Italy
January 2023

Coordinator:	Prof. Umberto Baccarani, Ph.D. Department of Medicine, University of Udine, Italy
Supervisors:	Prof. Giuseppe Comi, Department of Agricultural, Food, Environmental and Animal Science University of Udine, Italy
Co-Supervisors:	Prof. Lucilla Iacumin, Ph.D. Department of Agricultural, Food, Environmental and Animal Science University of Udine, Italy
	Prof. Leon Dicks, Ph.D. Department of Microbiology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa
Reviewers:	Prof. Mariantonietta Succi, Ph.D. Department of Agriculture, Environmental and Food Sciences, University of Molise
	Prof. Fausto Gardini, Ph.D. Department of Agricultural and Food Sciences, University of Bologna, Cesena, Italy

ABSTRACT

Bacteriocins are a heterogeneous group of small, ribosomal-synthesized antimicrobial peptides produced by bacteria capable of inhibiting closely related bacteria. These peptides are often active in the nanomolar range. Their bactericidal proprieties at low concentrations allow room for their application as a preservative agent. This makes them highly valuable in the food and medical industries. In this study, we set out to isolate and characterize novel antimicrobials from *Lacticaseibacillus casei* strains. An unknown biosynthetic gene cluster belonging to the pediocin-like genic organization was identified using in silico prediction tools. This thesis expands the large body of research and knowledge in bacteriocins.

The first research chapter examines bacteriocins' current and potential applications with a particular focus on class IIa, highlighting old and newer identification and analysis methods available.

Chapter two used conventional bacteriocin culture-based screening approaches combined with the whole genome in silico screening and peptide characterization to discover new antimicrobial candidates in the genus *Lactilactobacillus*. This resulted in the discovery of two potentially novel bacteriocins, whose amino acid sequence and molecular mass are unknown due to the inability to generate enough peptides for in depth physicochemical characterization. Antimicrobial activity against relevant pathogens was proven with specifically designed fusion-protein-based expression systems.

The third research chapter sought to identify and understand these novel bacteriocins' regulation modes, narrowing the evaluation of their full potential applicability conditions. Four variations of these peptides were identified with an extensive homology-based investigation in well-established databases. In all variants, the candidate putative genes for the production of these bacteriocins were present. However, bacteriocin production were not detectable from the bacterial strains using conventional methods. Chapter four experimentally confirms the hypothesis proposed in the previous research chapter related to the peculiar genetic organization and the involvement of the dedicated protease of these novel bacteriocins.

The final research chapter related to the novel discovered peptides sought to advance these bacteriocins towards potential applications in food manufacturing. The last chapter investigates the mode of regulation of the bacteriocin thermophilin 13, which was addressed with a bioinformatics approach.

This thesis aims to generate interest in bacteriocin discovery and application in academia and industry. Furthermore, the studies provided direction for the future development of these novel peptides within this field and demonstrated the efficacy of bacteriocin use in food.

ACKNOWLEDGMENT

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

- My supervisors, Prof. Giuseppe Comi, Prof. Lucilla Iacumin and Prof. Leon Dicks, for their support and for allowing me to work on this project.
- Colleagues and personnel at the Department of Agricultural, Food, Environmental and Animal Sciences at the University of Udine and the Department of Microbiology at Stellenbosch University, South Africa.

This thesis is dedicated to loved ones (wife, parent and friends). Thank you for your endless love, prayers, support, and encouragement.

PREFACE

This thesis is presented as a compilation of 7 chapters. Each chapter is introduced separately and is written according to the American Society for Microbiology style, except Chapter 6, which is written according to the style of Microorganisms (MDPI).

The following work is currently under a joint patent between the University of Udine and Stellenbosch University. The patent was filed on the 15 August 2022 with patent application number 2211905.1. Agreement - SU-AGR-2019-54-T1844 contains details of the collaboration.

Chapter 1	**General Introduction**
Chapter 2	Caseicin FS-X and Caseicin FS-Y: The first class IIa bacteriocins produced by *Lacticaseibacillus casei*
Chapter 3	Genetic Diversity of Caseicin gene clusters and evaluation of their mode of regulation
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CHAPTER 1

General Introduction

INTRODUCTION

In recent decades different strategies aimed to raise consumer awareness and sensibility about the health-related to the food consumed. With concerns surrounding allergies, behavioral changes, and carcinogenic effects, many consumers are opting for fresh and natural foods with fewer synthetic additives (Balciunas et al., 2013). In the globalized world, pathogens bacteria can easily be disseminated through international travel and trade. As a result, foodborne pathogens have been a significant threat to public health, especially for new-borns, adolescents, the elderly, and pregnant women (Zhang et al., 2017). Additionally, the misuse of non-therapeutic antibiotics in animal agriculture has led to the development of antibiotic-resistant bacteria, further complicating infection treatment. Besides the research investment in identifying novel therapeutic agents, there is no new class of antibiotics except oxazolidinones and lipopeptides, which have entered clinical practice over two decades (Fair & Tor, 2014). Antibiotic-resistant pathogens in food have further intensified the difficulties of infection treatment. These issues, combined with a demand for minimally processed food, resulted in increased research focusing on naturally occurring metabolites produced by bacteria to inhibit the growth of undesirable contaminants and food-borne pathogens (De Vuyst & Vandamme, 1994). To address these challenges, researchers have turned to naturally occurring metabolites produced by bacteria to inhibit the growth of undesirable contaminants and food-borne pathogens Therefore ,valid alternative can be the implementation of bacteriocins usage, which are antimicrobial peptides produced by many bacteria, including lactic acid bacteria (LAB), normally acting against closely related species, among which some spoilage bacteria and disease-causing pathogens are present. Current EU legislation (Commission Regulation (EU) No 1129/2011) modified the conditions for chemical preservatives. Therefore, biological conservation using bio-protective cultures as a possible approach to achieve food safety and shelf-life control has gained increasing attention (Patrovský et al., 2016).In addition, several intrinsic and extrinsic factors are directed to promote the preservation of the final product, the most important of which include water activity (Aw), temperature, preservatives, acidity (pH), competitive microorganisms, and redox potential (Eh). These extrinsic and intrinsic factors are limited when applied singularly (Blackburn, 2006). Biopreservation is defined as the extension of shelf-life and the increase in food safety by using controlled microorganisms or their metabolites (Zapaśnik, Sokołowska & Bryła, 2022). In this regard, bacteria are already intensively used in fermented food products. Beneficial bacteria are generally selected in this process to control spoilage and render pathogen inactive. In fermentation, the raw materials are converted by microorganisms (bacteria, yeast and mold) to products that have acceptable qualities of food. Microorganisms belonging to the genera *Bifidobacterium*, *Lactococcus*, *Leuconostoc*, *Streptococcus, Pediococcus* and in according with the new proposed classification of the genus Lactobacillus into *25 genera of Lactobacillus delbrueckii group, Paralactobacillus, Acetilactobacillus, Agrilactobacillus, Amylolactobacillus, Apilactobacillus, Bombilactobacillus, Companilactobacillus, Dellaglioa, Fructilactobacillus, Furfurilactobacillus, Holzapfelia, Lacticaseibacillus, Lactiplantibacillus, Lapidilactobacillus, Latilactobacillus, Lentilactobacillus, Levilactobacillus, Liquorilactobacillus, Ligilactobacillus, Limosilactobacillus, Loigolactobacillus, Paucilactobacillus, Schleiferilactobacillus, Secundilactobacillus* are involved in these fermentations and are also part of normal human intestinal microflora, exerting a positive effect on human health (Zheng et al., 2020).

Lactic acid bacteria (LAB) and their bacteriocins

Lactic acid bacteria (LAB) are a diverse group of microorganisms found in various ecological niches including foods, the oral cavity, the urogenital tract and the gastrointestinal tract of humans, animals, and insects (Van Belkum & Stiles, 2000). This family detain a notable industrial application and economic value, especially bacteriocinogenic strains, in the food industry as starter cultures for the fermentation of raw milk, meat and vegetable products, which further aids in the exclusion of specific foodborne pathogens. Antimicrobial activity of lactic acid bacteria is mainly based on the production of metabolites such as lactic acid, organic acids, hydroperoxide and bacteriocins (Mokoena, 2017; Chen et al., 2022).

Additionally, many LAB are generally recognized as safe (GRAS) for ingestion since humans and animals have consumed these organisms for centuries without any adverse effect, which is one of the prerequisites for their use as natural preservatives in foods and feeds and as antimicrobials in the treatment of infections (Sewalt et al., 2016; Kouhounde et al., 2022). These organisms alter raw food products' flavour, texture and appearance desirably to bestow unique aroma, flavour, taste, texture and other sensory properties (Tamang et al., 2020; Voidarou et al., 2021). LAB, frequently found in food, constitute an important portion of our gut microbiota. Probiotic strains, bacteriocin producers in the gastrointestinal GI, have a competitive advantage over other strains. Bacteriocins have potential in modulating the gut microbiota through antimicrobial action and immune modulation (Dobson et al., 2012; Anjana & Tiwari, 2022).
Production and accumulation of these compounds in the gut depend on the producer strains, bioavailability, and physical conditions influenced from the surrounding environment (Garcia-Gutierrez et al., 2019).

Bacteriocins, generally consist of 10–50 amino acids, and their ability to kill bacteria depends on their interaction with bacterial membranes and cell walls. More precisely, the antimicrobial action is mainly due to the electrostatic interaction with the negatively charged phosphate groups present on the walls of the target cell, which allow the initial anchoring of the bacteriocin; hence, the subsequent formation of pores on the wall with a consequent entry of the bacteriocin leads to the death of the target cell (Hernández-González et al., 2021). The first bacteriocin was nisin, described in 1928, and it is the deeper studied lantibiotic (Chatterjee et al., 1992; Shin et al., 2016). Since the discovery of nisin the production of bacteriocins by a variety of Gram-positive bacteria has been reported, and with sequenced genomes becoming more readily available, the identification in Gram-negative and Gram-positive of putative bacteriocin is increasing rapidly (Arias & Murray, 2009). The term 'bacteriocins' was proposed to group all such entities, proteinaceous molecules with bactericidal or bacteriostatic activity against other strains of the same species (LWOFF, 1953).

Many lactic acid bacteria (LAB) have the ability to produce ribosomally synthesized antimicrobial peptides or proteins and are topic of interest since they are safe, active in a nanomolar range, heat stable, and readily digested by gastric enzymes (Negash & Tsehai, 2020). In this respect, food preservation through in situ production of bacteriocins by LAB introduced into the food system would be the most logical approach. However, there is a need to understand the relationship between bacterial growth and bacteriocin production in various types of food system due to the effectiveness of bacteriocins against various pathogens and/or spoilage bacteria, many alternative applications in the pharmaceutical and food industries are studied. A bacteriocin alone in a food is not likely to ensure satisfactory safety. This is of

particular significance with regards to the nature of the Gram-negative and Gram-positive pathogenic bacteria cell walls that are differently susceptible to the bacteriocins classes.

A combination of preservation methods works synergistically or at least provides greater protection than a single method alone (Martinez & De Martinis, 2005). The application of bacteriocins or bacteriocin-producing LAB strains in food has the potential used as part of the hurdle technology. Although the nature of bacteriocins, their application in combination with other treatments within the optimization of the production process also increases their effectiveness (Deegan et al., 2006).

Till date, applications of LAB-bacteriocins have been mostly limited to the food sector (Perez, Zendo & Sonomoto, 2014; Juturu & Wu, 2018), and only the lantibiotic nisin (E234) and, more recently pediocin PA-1/AcH, marketed as Nisaplin and Alta™ 2341, respectively, are currently approved and commercialized as a food preservative by the Food and Drug Administration (FDA)(Sidhu & Nehra, 2019).

Bacteriocin classes

Nomenclature of bacteriocins produced by Gram-positive based initially on the species, then on the genus of the producer strain. Furthermore, the classification was divided into four classes of bacteriocins (Klaenhammer, 1993a). Over the years many attempts were directed to draft an adequate and consistent classification, although it is not easy to identify a classification that includes all the existing bacteriocins. Actual classification of bacteriocins is based on their chemical nature and on their spectrum and mode of action mainly divided into two primary classes, i.e., lantibiotics (class I) and unmodified bacteriocins (class II), a third class is also contemplated, in which structure are reported (Cotter, Hill & Ross, 2005). In **Table 1.1** a summary of bacteriocins classification showing the evolution in time was reported.

Table 1.1: Evolution of the bacteriocins classification from Gram-Positive bacteria.

Class	Klaenhammer *et al* (1993b)	Nes et al., (1996)	Franz et al. (2007)	Zimina et al. (2020)
I	Lantibiotics	Lantibiotics	Class I, Lantibiotic enterocins Class II enterocins	Lantibiotics Lipolantins Thiopeptides Botromycins Linear azole-containing peptides Sactibiotics (sactipeptides) Lasso peptides Cyclic bacteriocins with a "head-to-tail" connection Glycocins
IIa	Pediocin-like or '*Listeria*' active with YGNGVXC motif near N terminus and GG leader peptide	Pediocin - like or '*Listeria*' active with YGNGVXC motif near N terminus and GG leader peptide	Cystibiotics with two disulphides bridges with YGNGVXC motif near N terminus	YGNG-motif containing bacteriocins
IIb	Two-compounds peptides with GG leader peptide	Two compound peptides with GG	II.2. Enterocins synthesized	Linear two-peptide bacteriocins

		leader peptides	without a leader peptide	
IIc	Thiol-activated peptides with GG leader peptide	Bacteriocins secreted by signal peptide pathway	II.3. Other linear nonpediocin-like enterocins	Leaderless bacteriocins
IId	-	-	Thiolbiotics with one or no cysteine residues Two-component peptides with GG leader peptide	Other linear bacteriocins
IIe	-	-	-	-
IIf	-	-	Atypical bacteriocins	-
III	Larger, heat labile protein	Larger, heat labile protein	Larger, heat labile protein	Bacteriolysins Non-lytic bacteriocins Tailocins
IV	Protein complexes	-	-	-

The ribosomally produced post-translationally modified peptides (RiPPs) of class I are further sub-grouped according to the type of endured post-translational modification. These modifications include lanthionine residues, head-to-tail cyclization, sulphur linkages, heterocycles, glycosylation and macrolactam rings (Hegemann et al., 2015).

Bacteriocins represented in class II are also ribosomally synthesised, but have limited or no post-transcriptional modifications, and never exceed a mass of 10 kDa. The class II is further divided into sub-classes depending on the bacteriocin's mode of action, genetic and biochemical characteristics (Acedo et al., 2018). Finally, class III bacteriocins have relatively higher molecular weights (>10 kDa) and are heat labile (Alvarez-Sieiro et al., 2016).

The similarity and diversity of the class IIa

Class IIa, or pediocin-like bacteriocins, are a subgroup of class II bacteriocins produced by LAB, which are defined by their high anti-*Listeria* activity and conserved N-terminal YGNGV motif or "pediocin box" (Lohans & Vederas, 2012). This conserved motif, with a typical β-turn structure, is easily exposed and recognized by a putative membrane MptC protein `receptor', a component of the Mannose Phosphotransferase System, which allows correct positioning of the bacteriocin on the membrane surface, necessary for activity towards *Listeria* spp., but also other similar bacteria such as Enterococcus, *Carnobacterium, Lacticaseibacillus, Leuconostoc, Pediococcus* and *Clostridium* (Kjos et al., 2010). In addition to the pediocin box motif, at least one disulfide bridge and an amphipathic α-helix with an overall cationic charge despite the low sequence homology in the C- terminus are present (Drider et al., 2006). All pediocins-like group are synthesised as a precursor peptide having a leader component that keeps the peptide inactive, which is generally characterised by the conservative Gly-Gly motif, which is removed during the maturation and secreted in their actual active form. Based on their similarity, a generic structural conformation is proposed in **Figure 1.1**.

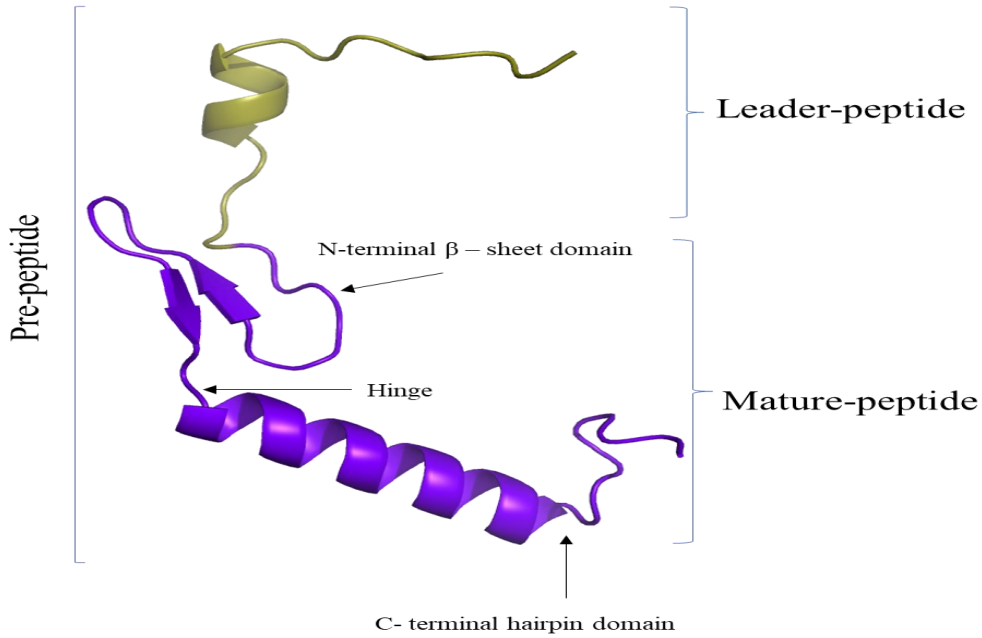

Figure 1.1: Illustration of the proposed domain structure of pediocin-like bacteriocins.

Class II bacteriocins have an amphiphilic helical structure, which allows them to insert into the membrane of the target cell, leading to depolarisation and death. The antimicrobial action is mainly due to the electrostatic interaction with the negatively charged phosphate groups present on the walls of the target cell, which allow the initial anchoring of the bacteriocin; hence, the subsequent formation of pores on the wall with a consequent entry of the bacteriocin leads to the death of the target cell (Anjana & Tiwari, 2022). Strain sensitivity related to bacteriocin is correlated with the expression level of the receptor/target protein and mutations of the target Man-PTS (Kjos, Nes & Diep, 2009). These more sensitive Man-PTS appear in a phylogenetic cluster termed Group-I characterized by three distinct regions: α, β and γ.

Region α is localized in the N-terminal part of subunit IIC and it contains a conserved GGQGxxG or GG[D/K]FxxxG sequence, where x indicates any amino acid. Region β is localized in the C-terminal part of subunit IIC, DP[I/L/V]GDI[I/L][D/E/N]xY sequence. Region γ is localized in subunit IID and is subject to a variation of 35–40 amino acids, which are absent in the IID components from the other groups (Tymoszewska, Diep & Aleksandrzak-Piekarczyk, 2018). **Figure 1.2** shows a schematic representation of the class IIa mode of action.

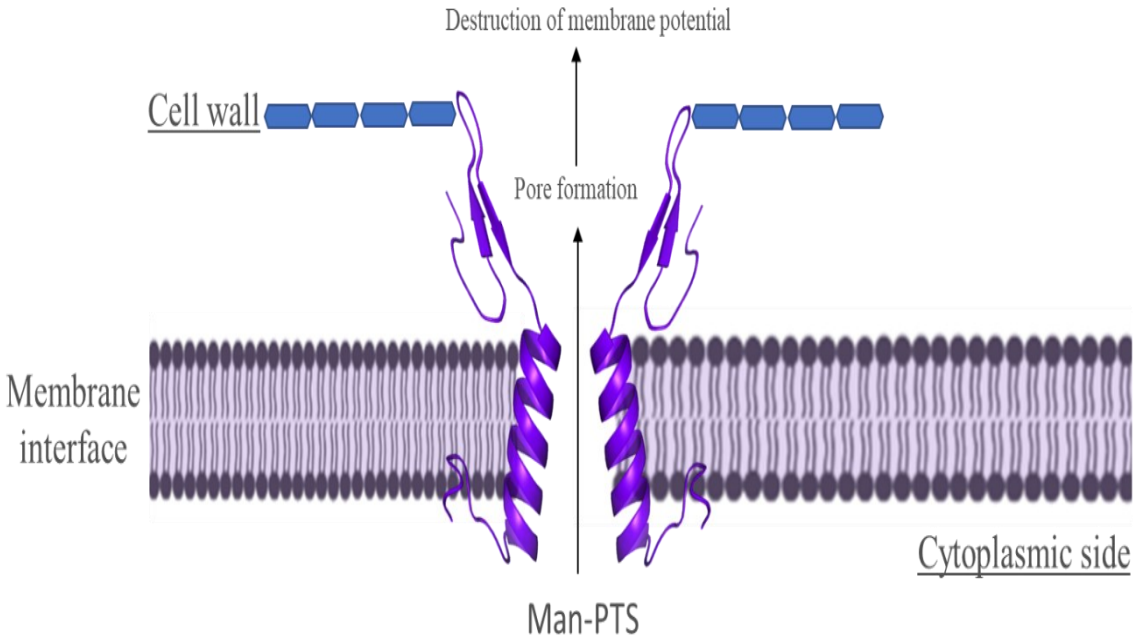

Figure 1.2: Simplified mechanism of action of class IIa bacteriocins on Gram-positive bacteria.

Great efforts have been made to identify the sequence of bacteriocins that LAB can produce and further recognize the bases of their antibacterial activity. Nonetheless, Class IIa is one of the largest groups of bacteriocins and includes a range of small peptides. Besides their high similarity in the conservative motifs, the variation in their aminoacid sequence is associated with a somewhat different inhibitory spectrum. In **Table 1.2** are listed the most representative sequence of class IIa bacteriocins reported by Zhang et al. (2022).

Table 1.2: Differences in aminoacid sequence including leader (when available) and core peptide of class II bacteriocins. The leader peptides are in bold and bacteriocins with variation in the typical Gly-Gly motif that relate them with the general secretion pathway (Sec) for secretion are underlined (Foulquié Moreno et al., 2006). Enterocin P, bacteriocin 31 and Enterocin SE-K4 are reported as an example of bacteriocins without the double-glycine motif in their leaders which are exported by sec-dependent translocation system (Tomita et al., 1996; Cintas et al., 1997; Cui et al., 2012)

Name of Bacteriocins	Sequence	Producer Species	Strain
Bacteriocin 31	**MKKKLVICGIIGIGFTALGTNVEAATAT**YYGNGLYCNKQKCWVDWNKASREIGKIIVNGWVQHGPWAPR	*Enterococcus faecalis*	YI717
Bacteriocin B2	**MNSVKELNVKEMKQLHGG**VNYGNGVSCSKTKCSVNNGQAFQERYTAGINSFVSGVASGAGSIGRRP	*Carnobacterium piscicola*	LV17B
Bacteriocin BM1	**MKSVKELNKKEMQQINGG**AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH	*Carnobacterium piscicola*	LV17B
Bacteriocin L-1077	TNYGNGVGVPDAIMAGIIKLIFIFNIRQGYNFGKKAT	*Ligilactobacillus salivarius*	1077
Bacteriocin T8	MKKKVLKHCVILGILGTCLAGIGTGIKVDAATYYGN<u>GLYCNKEKCWVDWNQAKGEIGKIIVNGWVNHGPW</u><u>APRR</u>	*Enterococcus faecium*	T8
Bavaricin A	KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANXATGXNAGG	*Latilactobacillus sakei*	MI401
Bavaricin MN	TKYYGNGVYCNSKKCWVDWGQAAGGIGQTVVXGWLGGAIPGK	*Lactobacillus bavaricus*	MN

Bifidocin B	KYYGNGVTCGLHDCRVDRGKATCGIINNGGMWGD IG	*Bifidobacterium bifidum*	NCFB 1454
Carnobacteriocin B2	**MNSVKELNVKEMKQLHGG**VNYGNGVSCSKTKCS VNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	*Carnobacterium piscicola*	LV17
Carnobacteriocin BM1	**MKSVKELNKKEMQQIIGG**AISYGNGVYCNKEKC WVNKAENKQAITGIVIGGWASSLAGMGH	*Carnobacterium piscicola*	LV17B
Curvacin A	**MNNVKELSMTELQTITGG**ARSYGNGVYCNNKKC WVNRGEATQSIIGGMISGWASGLAGM	*Latilactobacillus curvatus*	LTH1174
Divercin V41	**MKNLKEGSYTAVNTDELKSINGG**TKYYGNGVYC NSKKCWVDWGQASGCIGQTVVGGWLGGAIPGKC	*Carnobacterium divergens*	V41
Enterocin A	**MKHLKILSIKETQLIYGG**TTHSGKYYGNGVYCTK NKCTVDWAKATTCIAGMSIGGFLGGAIPGKC	*Enterococcus faecium*	CTCA 92/T136
Enterocin CRL35	**MKKLTSKEMAQVVGG**KYYGNGVSCNKKGCSVD WGKAIGIIGNNSAANLATGGAAGWKS	*Enterococcus faecium*	CRL 35
Enterocin HF	**MEKLTVKEMSQVVGG**KYYGNGVSCNKKGCSVD WGKAIGIIGNNAAANLTTGGKAGWKG	*Enterococcus faecium*	M3K31
Enterocin P	MRKKLFSLALIGIFGLVVTNFGTKVDAATRSYGNGV YCNNSKCWVNWGEAKENIAGIVISGWASGLAGMG H	*Enterococcus faecium*	P13
Enterocin SE-K4	MKKKLVKGLVICGMIGIGFTALGTNVEAATYYGNG VYCNKQKCWVDWSRARSEIIDRGVKAYVNGFTKV LG	*Enterococcus faecalis*	K-4
Lactococcin MMFII	TSYGNGVHCNKSKCWIDVSELETYKAGTVSNPKDIL W	*Lactococcus lactis*	MMFII
Leucocin A	**MMNMKPTESYEQLDNSALEQVVGG**KYYGNGVH CTKSGCSVNWGEAFSAGVHRLANGGNGFW	*Leuconostoc gelidum*	UAL 187
Leucocin C	KNYGNGVHCTKKGCSVDWGYAWTNIANNSVMNG LTGGNAGWHN	*Leuconostoc mesenteroides*	TA33a
Mesentericin Y105	**MTNMKSVEAYQQLDNQNLKKVVGG**KYYGNGVH CTKSGCSVNWGEAASAGIHRLANGGNGFW	*Leuconostoc mesenteroides*	Y105
Mundticin	KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLAT GGAAGWSK	*Enterococcus mundtii*	ATO6
Mundticin KS	**MKKLTAKEMSQVVGG**KYYGNGVSCNKKGCSVD WGKAIGIIGNNSAANLATGGAAGWKS	*Enterococcus mundtii*	NFRI 7393
Pediocin PA-1	**MKKIEKLTEKEMANIIGG**KYYGNGVTCGKHSCSV DWGKATTCIINNGAMAWATGGHQGNHKC	*Pediococcus acidilactici*	PAC 1.0
Piscicocin VIa	KYYGNGVSCNKNGCTVDWSKAIGIIGNNAAANLTT GGAAGWNKG	*Carnobacterium piscicola*	V1
Piscicolin 126	**MKTVKELSVKEMQLTTGG**KYYGNGVSCNKNGCT VDWSKAIGIIGNNAAANLTTGGAAGWNKG	*Carnobacterium piscicola*	JG126
Plantaricin 423	**MMKKIEKLTEKEMANIIGG**KYYGNGVTCGKHSCS VNWGQAFSCSVSHLANFGHGKC	*Lactiplantibacillus plantarum*	423
Plantaricin C19	KYYGNGLSCSKKGCTVNWGQAFSCGVNRVATAGH GK	*Lactiplantibacillus plantarum*	C19
Plantaricin PL-1	VIADKYYGNGVSCGKHTCTVDWGEAFSCSVSHLAN FGHGKC	*Lactiplantibacillus plantarum*	LPL-1
Sakacin A	**MNNVKELSMTELQTITGG**ARSYGNGVYCNNKKC WVNRGEATQSIIGGMISGWASGLAGM	*Latilactobacillus sakei*	706
Sakacin G	**MKNTRSLTIQEIKSITGG**KYYGNGVSCNSHGCSVN WGQAWTCGVNHLANGGHGGVC	*Latilactobacillus sakei*	2512
Sakacin P	**MEKFIELSLKEVTAITGG**KYYGNGVHCGKHSCTV DWGTAIGNIGNNAAANWATGGNAGWNK	*Latilactobacillus sakei*	MI401

Biosynthesis and Genetics

Production and export of class IIa bacteriocins require several genes: bacteriocin structural genes, genetic determinants involved in immunity, and transport (ABC-transporter). An accessory protein can also be present to facilitate membrane translocation. The accessory protein ensures correct disulfide bond formation for class IIa bacteriocins with more than three cysteine residues (Oppegård et al., 2015). The relevant bacteriocin genes are mostly plasmid-encoded but can also be located on the chromosome or transposons. The gene clusters are most often arranged in operons and can be located in one integral operon or over correlated operons, where one operon carries the structural and immunity gene, a second operon carries the gene coding for secretion, and a third operon carries genes involved in the regulation of bacteriocin production (Mathiesen et al., 2005; Todorov, 2009). These syntheses of class IIa bacteriocins is typically regulated by a quorum sensing (QS) system that consists of three components: an inducing peptide (IF), a membrane-associated histidine protein kinase (HPK), and a cytoplasmic response regulator (RR) (Diep et al., 2001).

The IF serves as an indicator of the cell-density. The secreted pheromone binds to the HPK, activating the RR, and triggering the expression of all operons needed for bacteriocin synthesis (Diep et al., 2000; Straume et al., 2007).

Class IIa bacteriocins show a general conservation of gene arrangement. However, some unusual organizations of biosynthetic gene clusters were also characterised and generally correlated to different transcription regulation systems. Co-culture-based regulation, auto-inducing peptide, acetate, temperature, and divalent cation regulation are reported as independent triggers of the QS (Hugas et al., 2002; Nilsson et al., 2002; Kleerebezem, 2004; Meng et al., 2021; Kareb & Aïder, 2020). **Figure 1.3** shows the genic organization differences between the most diverse Class IIa operon.

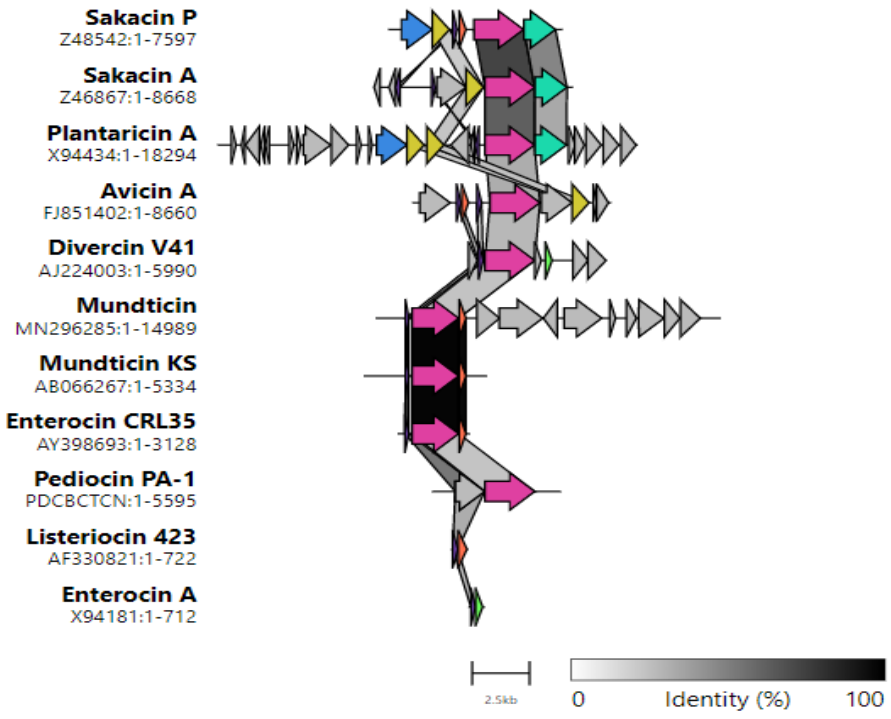

Figure 1.3: Class IIa bacteriocin biosynthetic gene clusters comparison. Relative, accession number and length in nucleotides sequence are also reported. Arrows indicates the operon reading frame (ORF) found in the different operons.

Conventional and non-conventional methods for bacteriocin detection

The selection of bacteriocin-producing bacteria is an easily accessible practices without the need for special equipment or expertise. The most popular assays directed to the identification of antimicrobial capability are spot-on-lawn assay, disc diffusion test, microtiter plate assay, and agar well diffusion assay (Pingitore et al., 2007).

When a positive result for antimicrobial capability is pointed out, and there is evidence of their proteinaceous nature, an initial characterization of bacteriocins stability to various factors, such as pH, temperature, and proteolytic enzymes are needed in order to evaluate their behaviour (Zhao et al., 2016). Furthermore, a proteinaceous compound requires a few steps of purification in order to discover their aminoacid sequence identity, which is quite long and time-consuming. Among the most popular purification techniques, the ammonium sulphate precipitation ion-exchange chromatography and reversed-phase chromatography in combination are the most used (Larsen, Vogensen & Josephsen, 1993; Holck et al., 1994; Vijay Simha et al., 2012). Without knowing the characteristics of the peptide, an optimization in salts, buffers, solvents and sorbents is required for the optimal recovery rate of bacteriocins, considering that there is no purification technique suitable for all classes of bacteriocins (Kaškonienė et al., 2017).

For the fast screening of the presence or absence of bacteriocins, the use of solvent extraction or precipitation are faster and cheaper procedure, such as Isopropanol, Methanol/chloroform, Chloroform, Acetone and nonpolar XAD Amberlite resin (Sawa et al., 2013; Ramu et al., 2017; Zou et al., 2018; Xad et al., 2020). Finally, the pre-purified peptide is further processed to define it the amino acid composition by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) an LTQ linear ion trap mass spectrometry coupled to HPLC (Ge et al., 2016). However, multi-steps purification methodologies in combination are generally embodied.

Most studies successfully isolate peptides with inhibitory effects using in vitro methods, which are still applied in assessing the diversity of novel antimicrobial inhibitors produced by culturable microorganisms (Soomro et al., 2007). However, these methods usually do not discriminate the inhibition caused by bacteriocins, low-molecular-weight antibiotics, bacteriophages, lytic enzymes, and metabolic by-products (Azevedo et al., 2015). Therefore, the limitation of in vitro methods is related to the incubation conditions, medium composition, stress conditions, and the presence of target cells, which can lead to false-negative results (Delgado et al., 2007; Settanni et al., 2008).

Besides the variety of strategies by which novel bacteriocin can be detected, they can be divided into traditional as culture-based approaches and, more recently, with the support of vast quantities of DNA sequence data from metagenomics-based projects from varying environments across the globe *in silico*-based strategies (Barh et al., 2020). The increasing availability of genomic data means it is becoming easier to identify bacteriocins encoded within genomes. Notably, after the first reported discovery through directed genome mining in early 2000 (Kodani et al., 2004; Goto et al., 2010), these approaches simplify the management and interpretation of the amount of data obtained from the sequencing genetic initial and consent and increase accuracy in predicting protein sequence of interest and their record.

In recent decades, *in silico* screening, searching for potential new bacteriocins within bacterial genomes has become increasingly popular (Egan et al., 2018; Zou et al., 2018).

Identifying novel bacteriocin involves classical microbiology screening associated with protein purification techniques for extensive collections of strains. Based on the sequence analysis of bacteriocins, there appear to be conservative motifs between the different classes, influencing the classification itself (Srinivasan et al., 2013).

Specific Bioinformatic screening tools such as BAGEL4, antiSMASH, RODEO, and RippMiner can now process vast amounts of genomic data to search for antimicrobial operons, allowing researchers to identify, within the genome provided, a starting point for evaluating the clusters coding for bacteriocins production (Agrawal et al., 2017; Blin et al., 2017; Van Heel et al., 2018; Walker et al., 2020).
Starting from genomic or amino acid sequences, the main advances are a significant reduction in the time in comparison to the traditional screening method and subsequently the costs embroiled to the use of laboratory materials. However, the presence of bacteriocin genes in a strain is no often translated into biological antimicrobial activity (Russell & Truman, 2020). Antimicrobial genome-mining tools have been closing the gap between the large number of predicted biosynthetic gene cluster (BGC) encoding bacteriocins, including ribosomally synthesized, post-translationally modified peptides (RiPPs) and also polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) (Tietz et al., 2017). Furthermore, most of the precursor proteins are completely unknown and awaiting verification within *in vitro* methods.

Improving Bacteriocin by Genetic Engineering

The commercial availability of bacteriocins is still limited due to the low yield of production due to the susceptibility of bacteriocins producer strains to various culture conditions, such as the composition of the medium, pH, temperature, and growth kinetics of the microorganisms (Sidooski et al., 2019). Optimizing fermentation conditions is a complex approach with a high impact on production costs but is critically essential for high-performance bacteriocin production at a commercial scale. In many cases, bacteria's optimal growth does not reflect the optimal productivity of bacteriocins by producers strains. Moreover, bacteriocin-producing LAB need complex nutrition to grow, which also gives rise to the difficulties related to their purification (Li et al., 2002). In this regard, the effects of various media composition and culture conditions on the yield of bacteriocins are proposed to efficiently use these compounds to overcome the low yield and the high production costs (Abbasiliasi et al., 2017).
Besides fermentation conditions, bacteriocin production can be increased by genetic approaches either by engineering the producer cells or using various heterologous expression systems, allowing the production in genetically customized bacteria and host yeast cells (Kumar et al., 2011).
The use of synthetic genes in heterologous expression systems offers a number of advantages over native systems through facilitating the control of bacteriocin gene expression or achieving higher production levels (Borrero et al., 2012; Ahmad et al., 2014). *Escherichia coli* is the organism of choice for the production of recombinant proteins and due to its well-established cell factory properties has become the most popular expression platform (Rosano & Ceccarelli, 2014). In addition to recombinant *E. coli* strains, other bacterial expression hosts include strains of *Streptococcus thermophilus*, *Lactococcus lactis* subsp. lactis and *Enterococcus faecalis* demonstrated their ability to express pediocin (Coderre & Somkuti, 1999; Mesa-Pereira et al., 2017). Heterologous expression in *E. coli* systems is currently utilized to produce bacteriocins because *E. coli* strains have relatively clear genetic backgrounds that are convenient to control the gene expression and attain low culture costs with higher production of interest proteins. Accordingly, the growing knowledge of the genetics and biosynthesis of class II bacteriocins has enabled researchers to quickly bridge the gap between the discovery of bacteriocin genes and their *in vitro* production, resulting in an increase of methodology to express these genes in a new host heterologously. The combination of *in silico*-based strategy within heterologous expression and in vivo techniques is the key to developing further applications and more advanced systems leading in understanding the mode of production and regulation of antimicrobials, novel or not, in the vision of a more cost-effective production of bacteriocins.

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CHAPTER 2

Caseicin FS-X and Caseicin FS-Y: The first class IIa bacteriocins produced by *Lacticaseibacillus casei*

ABSTRACT

This study aimed to identify novel bacteriocins and define their potential antimicrobial activity. Bacteriocin mining of the entire genome of *Lacticaseibacillus casei* UD 2202 and *Lacticaseibacillus casei* UD 1001 were used as a template to screen the presence of bacteriocins Biosynthetic gene clusters. Two novel class IIa peptides during an *in-silico* study were detected and named Caseicin FS-X and Caseicin FS-Y. Heterologous expression systems using fluorescent protein as a gene fusion partner in *E. coli* BL21 (DE3) were developed to produce recombinant Caseicin FS-X and Caseicin FS-Y. The ability of NisP protease to cleave the nisin leader from 6xHis-tag-GFP-Nisin leader-Caseicin FS-X and 6xHis-tag-GFP-Nisin leader-Caseicin FS-Y was tested. Using a method that *in-silico* predicted the protein and then performing its heterologous expression by fusion protein approach in *E. coli* BL21 (DH3) allowed to isolate and test the efficacy of the heterologous expressed mature peptide Caseicin FS-X and Caseicin FS-Y. These new bacteriocins belonging to class IIa are the first isolated from *Lacticaseibacillus casei* spp. with bactericidal action against *Listeria monocytogenes* and a wide range of Gram (+) species. In addition, our findings report the first application of a lantipeptides protease involved in the cleavage of a class IIa bacteriocins, which notably possesses any lanthionine ring, considered a prerequisite for the capability of explicating the catalytic function of this peptide.

KEYWORDS: Bacteriocin class IIa, NisP, GFP, Fusion protein; Heterologous expression, Caseicin, *Lacticaseibacillus casei*

INTRODUCTION

Bacteriocins have long been used in food preservation but are becoming increasingly important in the treatment of bacterial infections. and their application may replace or reduce the usage of antibiotics to fight pathogens in the bacteria antibiotic-resistance era. However, various bacteriocins have been unknowingly consumed for centuries through foods such as meat and dairy products since these foods contain lactic acid bacteria and are normally found in the gastrointestinal tract of humans and animals. Lactic acid bacteria are also associated with probiotics and are defined as "live microorganisms, which, when consumed in adequate amounts, could provide a health benefit to the host" (Dobson et al., 2012; Van Zyl, Deane & Dicks, 2020). Bacteriocins are among the new bio-protection strategies that must be considered for the war to pathogens, possibly in combination with other technologies that enhance their antimicrobial effect (Caniça et al., 2019). Bacteriocins produced by LAB are divided into two primary classes, i.e., lantibiotics (class I) and unmodified bacteriocins (class II) (Cotter, Hill & Ross, 2005; Zimina et al., 2020). Lantibiotics contain lanthionine and dehydrated amino acids that forms lanthionine bridges. Conversely, class II bacteriocins consist only of unmodified peptides or peptides with minor changes (e.g., sulfide bridges and cyclisation). Furthermore, class II bacteriocins are classified into four sub-classes, i.e., pediocin-like (class IIa), two-peptide (class IIb), cyclic (class IIc), and linear non-pediocin-like (class IId) (Cotter, Ross & Hill, 2013). The bacteriocins in this class are called "non-lantibiotic" or "unmodified peptides". They can be defined as small, low molecular weight, thermostable peptides with a leader peptide and a core peptide in the sequence. They are thermostable small peptide compose by leader and core peptide. Class IIa bacteriocins have highly conserved "YGNGV" and "CXXXXCXV" sequence motifs in the N-terminal (Eijsink et al., 1998; Ennahar et al., 2000; Gálvez et al., 2007). They bind to the mannose phosphotransferase system (man-PTS) and induce pore formation which leads to target cell death (Diep et al., 2007; Opsata, Nes & Holo, 2010; Guo et al., 2020). The effective production of these bacteriocins depends on several other associated proteins (Fimland, Eijsink & Nissen-Meyer, 2002; Johnsen et al., 2004). A generic operon organisation, associated with the ABC transporter, must be produced by cells to transport the bacteriocin outside the cell, and an immunity protein is also required to protect the producing strain from being killed by its own bacteriocin (Drider et al., 2006).

The regulation of these class IIa operons can prove a challenging hurdle to overcome during top-down screening assays. This makes purification from the native producer strains a challenging task with inconsistent production yields because of yet to be documented factors that influence the native production rate.

To date, only Nisin (Nisaplin, Danisco) and pediocin PA1 (MicrogardTM, ALTA 2431, Quest) have been commercialised as food preservatives (Vijay Simha et al., 2012; Da Costa et al., 2019). However, other LAB bacteriocins offered promising perspectives to be used as biopreservatives. Examples are enterocin AS-48 (Sánchez-Hidalgo et al., 2011) and lacticin 3147 (Suda et al., 2012; Chen & Narbad, 2018). To date, approximately fifty class IIa bacteriocins have been isolated from isolated from *Lactobacillus*, *Enterococcus, Pediococcus, Carnobacterium, Leuconostoc, Streptococcus* and *Weissella* (Yildirim & Johnson, 1998; Nicolas, Lapointe & Lavoie, 2011; Cui et al., 2012). Class IIa bacteriocins have also been described in non-LAB such as *Bifidobacterium bifidum* (Yildirim, Winters & Johnson,

1999), *Bifidobacterium infantis* (Cheikhyoussef et al., 2010), *Bacillus coagulans* (Yildirim & Johnson, 1998) and *Listeria innocua* (Kalmokoff et al., 2001).

However, there are many more class IIa bacteriocin and other antimicrobial peptides in online sequence databases like NCBI, which could offer alternatives or improvements to Nisin and pediocin PA1. Many of these operons are identified by way of genome mining and they will undoubtedly be subject to undescribed regulatory mechanisms rendering their production and therefore discovery hindered.

To our knowledge, no class IIa bacteriocins have been described for *Lacticaseibacillus casei* (*Lcb*). Here we describe two novels and seemingly silent class IIa bacteriocins named Caseicin FS-X and Caseicin FS-Y identified from the whole genomes of *Lcb.*UD 2202 and *Lcb.*UD 1001. This work shows the identification, cloning, and heterologous expression of the Caseicin FS-X and Caseicin FS-Y after genome mining of the producer strain *Lcb.*UD 2202 and *Lcb.*UD 1001.

MATERIALS AND METHODS

In silico genome screening

The genome sequence of *Lcb.*UD 2202 and *Lcb.*UD 1001 (Iacumin et al., 2015, Colautti, 2023) has been screened for putative bacteriocin genes using the BAGEL4 bacteriocin mining software (Van Heel et al., 2018). Coding sequences were identified using the NCBI database (https://blast.ncbi.nlm.nih.gov) and blastx and tblastn software. Putative bacteriocin genes within the respective genomes were annotated using the CLC Main Workbench – QIAGEN Bioinformatics software (CLC bio, Aarhus, Denmark). Sequence alignment was done using Muscle WS (Edgar, 2004) and displayed by the Tree Of Life (iTOL) v5 online tool (Letunic & Bork, 2021). Database Bactibase (Hammami et al., 2007) and LABioicin (Kassaa et al., 2019) supported the class IIa bacteriocins comparison analysis. The genome sequences of *L. casei* UD 2202 and UD 1001 (Iacumin et al., 2015, Colautti, 2023) were mined for putative bacteriocin genes using Bagel 4 software (Van Heel et al., 2018). Coding sequences in the NCBI database (https://blast.ncbi.nlm.nih.gov) were identified using and blastx and tblastn software. Putative bacteriocin genes within the respective genomes were annotated using the CLC Main Workbench – QIAGEN Bioinformatics software (CLC bio, Aarhus, Denmark). Sequence alignment was done using Muscle WS (Edgar, 2004) and displayed by the Tree Of Life (iTOL) v4 online tool. Database Bactibase (Hammami et al., 2007) and LABioicin (Kassaa et al., 2019) were used in bacteriocin comparison analyses.

Bacterial strains and culture conditions

Lactococcus lactis QU2 (Nisin producer) and *Lcb.*strains UD 2202 and UD 1001 were cultured in De Man, Rogosa and Sharpe (MRS) broth (Merck-Millipore, USA). *Escherichia coli* BL21 (DE3) was cultured in Luria Bertani (LB) broth (Merck-Millipore), supplemented with 1.2% (w/v) agar, and recombinant strains of *E. coli* BL21 (DE3) in Terrific broth (Merck-Millipore), supplemented with 50 µg/mL Kanamycin (Merck-Millipore). All strains were incubated at 37°C for 24 h.

DNA amplification and cloning

DNA was isolated from pure cultures of *Lactococcus lactis* QU2, *Lcb.*UD 2202 and *Lcb.casei* UD 1001 using the ZR Fungal/Bacterial DNA MiniPrep kit according to the manufacturer's instructions. Oligonucleotides were designed using the CLC main workbench program (CLC bio, Aarhus, Denmark).

DNA concentration was determined using BioDrop μLite+ (Cambridge, UK). Polymerase chain reaction mixtures were compiled according to Q5 high-fidelity PCR DNA polymerase (NEB) instructions. Thermocycler GeneAmp PCR, model 9700 (ABI, Foster City, CA), were set up with the following ramp rate specifications: initial denaturation at 98 °C for 10 minutes followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at the respective primer temperatures (worked out using NEBs TM calculator; http://tmcalculator.neb.com) for 15 s, elongation at 72 °C (time dependent on fragment size) and final extension at 72 °C for 2 minutes.T4 DNA ligase and restriction enzymes (PstI-HF®, HindIII-HF®) were from New England Biolabs (NEB, Ipswich, MA, USA) and used according to the manufacturer's instructions.

Plasmid DNA extractions were performed using the PureYield™ Plasmid Miniprep System (Promega, Madison, WI, USA). Electrophoresis gel was run at 100 V, using an EphortecTM 3000V power pack (Triad Scientific, Manasquan USA) with TBE (5:1) as an electrophoresis buffer. Gene sequencing was performed by the Central Analytical Facility (CAF), Stellenbosch University.

Construction of caseicin expressing and NisP protease expressing strains

The pRSF-GFP construct published by Vermeulen et al. (2020) was used as a template. In brief, the N-terminal of the GFP gene, *mgfp5,* was fused to a hexa-histidine tag and cloned into pRSFDuet-1 downstream of the T7 promoter. The *cas-x* gene was amplified by PCR using *Lcb casei* UD 2202 as a template, excluding the bacteriocin leader sequence. Additional restriction enzyme sequence PstI/HindIII were inserted in amplicon sequence allowing the N-terminus of the gene encoding mature bacteriocin Caseicin-X to fuse to the C-terminus of *mgfp5*. The cleavage sequence of the WELQut protease (*SplB* gene of *Staphylococcus aureus;* Thermo-Fisher Scientific, USA) was introduced between *mgfp5* and mature bacteriocin genes. This allowed for post-translational cleavage of the mature peptide with WELQut protease. This approach was used by applying pRSF-GFP plasmid digested with PstI/HindIII and ligated using T4 ligase according to the manufacturer's instructions with the inserted gene encoding mature Caseicin FS-X (*cas-x* gene without leader), obtaining plasmid pRSF-GFP-Wcas-x. Fusion PCR techniques were used to add nisin leader amplified from *L. lactis* genomic DNA to *cas-x* and *cas-y* amplicons encoding mature Caseicin FS-X and Caseicin FS-Y from *Lcb.* UD 2202 and *Lcb.*UD 1001, respectively. Restriction enzyme site *PstI*/*Hind*III were appropriately added into the primers sets. Inserting fused genes into a linearised pRSF-GFP with PstI/HindIII enzyme, we gain plasmid pRSF-GFP-Nislcas-x and pRSF-GFP-Nislcas-y. Plasmid pRSF-NisP8xHis, described by Van Staden et al. (2019), was digested with HindIII and the mCherry gene previously amplified and inserted to obtain the pRSF _Nisp_Mcherry8xHis construct. Plasmids, primer sets and strains used in this study are listed in **Table 2.1**.

Table 2.1: Plasmids, bacterial strains and primers used in this study.

	Description, characteristics or sequence (5'→3') forward primer, reverse primer	**Source or reference**
Plasmid		
pRSF-GFP	Shuttle vector,Kan*	Vermeulen et al. (2020)
pRSF-NisP8xHis	Shuttle vector,Kan*	Van Staden et al. (2019)
pRSF-GFP-Wcas-x	Vector producer 6xHis-tag-GFP-WELQ-Caseicin FS-X	This work
pRSF-GFP-Nislcas-x	Vector producer 6xHis-tag-GFP-Nisin leader peptide-Caseicin FS-X	This work

pRSF-GFP-Nislcas-y	Vector producer 6xHis-tag-GFP-Nisin leader peptide-Caseicin FS-Y		This work
pRSF-Nisp-Mcherry8xHis	Vector producer NisP-Mcherry-8xHis-tag peptide		This work
Strain			
***Lactococcus lactis* QU2**	Nisin producer strain		Van Staden et al. (2019)
***Lacticaseibacillus casei* UD 2202**	Strain under study with *cas-x* gene		This work
***Lacticaseibacillus casei* UD 1001**	Strain under study with *cas-y* gene		This work
***E. coli* BL21 (DE3)**	Expression host		
Primer		Tm (°C)	
GFPNisLeader_Pst	GGAACTGCAGATGAGTACAAAAGA	57	Van Staden et al. (2019)
Rev_NisLeaderOri_Caseicin	CATAGTATTTGCGTGGTGATG	57	This work
FWNispL_casx-y	CAGGTGCATCACCACGCAAATACTATGGTAATGGTGT	57	This work
PstCFbactFwd	GAACTGCAGAAATACTATGGTAATGGTG	56	This work
Hind_REV_casx-y	GCAAAGCTTACTTGATGCCAGAATTC	56	This work
Fwd_McherryNisP_Hind	GACAAGCTTTGGCAATCATCAAAGAATT	60	This work
Rev_McherryNisP_Hind	GTCAAGCTTTATATAATTCATCCATACCAC	60	This work
pRSFMCS1_F	GGATCTCGACGCTCTCCCT	63	Van Staden et al. (2019)
pRSFMCS1_R	GATTATGCGGCCGTGTACAA	63	Van Staden et al. (2019)

* Kan, kanamycin resistance

Then, by sequencing the correct ligation of the amplicon of interest with the corrective vector, each plasmid was transformed independently into chemically competent *E. coli* BL21 (DE3) cells and plated onto BHI agar with additional kanamycin$_{50\mu g/mL}$ and incubated overnight at 37 °C. Single colonies were isolated and used in subsequent expression experiments.

Expression of fusion proteins in *E. coli* BL21(DE3)

Sterile tubes of 10 ml BHI broth added with 50 µg/mL kanamycin were inoculated with fresh transformant cells of *E. coli* BL21 containing plasmid overnight at 37 °C in constant agitation. Subsequently, *E. coli* expressing protein of interest were used to inoculate (1.0%, v/v) 500 mL of Terrific broth supplemented with 50 µg/mL kanamycin and incubated at 37 °C under constant aeration. At an O.D.$_{600}$ of 0.6, protein expression was induced using 0.1 mM IPTG. The cultures were then incubated at 18 °C for 48 h in an orbital shaker at 160 rpm.

Ni-NTA Purification of novel Caseicin and NisP protease

All fused proteins were expressed according to a modified protocol (Shi et al., 2011). The induced cells were collected by centrifugation at 8000 x g for 20 minutes at 4 °C. The supernatant was discarded, and the cell pellet was resuspended in 15 mL/g of SB buffer (Tris 50 mM, NaCl 500 mM, pH 8.0). Cells

resuspensions were supplemented with 1 mg/ml of lysozyme (Merck-Millipore, USA) and incubated with stirring at 25 °C for 45 minutes. After incubation, the lysed cells were subjected to sonication (50% amplitude, 2 seconds pulse, 2-second pause, 6 minutes) using Omni Ruptor 400 (Ultrasound Homogenizer, Omni International Inc., Kennesaw, GA). RNaseI and DNaseI (BioLabs, New England) were added to a final concentration of 10 µg/ml and 5 µg/ml, respectively and then incubated at room temperature for 15 minutes. The cell lysate was centrifuged for 90 minutes at 20,000 x g at 4 °C. The cell-free supernatant was collected. Imidazole (Merck-Millipore, USA) was added to the cell-free supernatant at a final concentration of 10 mM. According to the instructions, fusion proteins were purified with immobilised metal affinity chromatography (IMAC) using the super-flow resin Ni-NTA (Qiagen, Germany). The super-flow Ni-NTA resin was balanced in SB10 buffer (SB buffer containing 10mM Imidazole) and then added directly to the cell-free supernatant. The ÄKTA purifier system (Amersham, Biosciences) was used for IMAC purification according to the following program: 5 column volumes (CV) SB10 (2% B buffer where A is SB and B is SB500), washed with 10 CV of SB20 (4% B buffer), elution occurred in approximately 40 mL of SB500 (100% B buffer). Eluted proteins were detected at 254 nm and 280 nm, respectively. Subsequently, luted His-tagged proteins were desalted using size exclusion chromatography. The ÄKTA purifier system was used in conjunction with Sephadex G25 resin packed into a chromatography column (GE Healthcare Technologies) for exchanging the sample in SB500 buffer to WELQut cut buffer (Table 2.1) at a flow rate of 1 mL/min. For the purified fusion protein GPF-Nisleader-cas-x, GPF-Nisleader-cas-y and Nisp_Mcherry Imidazole desalting was accomplished using DEAE Sepharose Fast Flow (GE Healthcare, USA). Elution was performed using 50 mM Tris pH 8.3 (buffer C) and 50 mM Tris 1M NaCl pH 7.5 (buffer D). Using the FPLC system, a flow rate of 2 ml/min, the peptides of interest detected by A_{220} were collected manually with 20% of buffer D corresponding to 50 mM Tris and 200 mM NaCl. Buffers used in all purification steps are reported in **Table 2.2**. Fusion protein concentrations were determined using a BCA protein assay Sigma-Aldrich (Milan, Italy).

Table 2.2: Buffers used in IMAC purification and WELQut cleavage**.**

Purification step	Buffer	Chemical Composition
Ni-NTA	SB	50 mM Tris, 500 mM NaCl pH 8.0
Ni-NTA	SB500	50 mM Tris, 500 mM NaCl, 500 mM Imidazole, pH 8.0
DEAE Sepharose Fast Flow	buffer C	50 mM Tris, pH 8.3
DEAE Sepharose Fast Flow	buffer D	50 mM 1 M NaCl, pH 7.5
Sephadex G25	WELQut buffer	100 mM Tris, pH 8.0

Bacteriocin Cleavage and MIC determination

In order to release the bacteriocin Caseicin FS from the GFP-system, complex obtained from the engineered *E. coli,* and make it active, a preliminary step of cleavage was necessary.

In the case in which the GFP-system was GFP-Nisleader, a reaction mixture containing 8×His-Tagged NisP_Mcherry and the purified GPF-Nisleader-Caseicin FS at different ratios (1/9, 2/8, 3/7, 4/6 and 5/5) were mixed and incubated at 4, 16, 30 and 37 °C for 16 h. 8×His-Tagged NisP_Mcherry is the proteolytic enzyme, which recognizes the cleaving site GASPR↓IT. This cleaving site was used to replace the original leader of Caseicin FS core peptide.

Viceversa, in the case of the use of the system GFP-WELQ, the reaction mixture containing purified GFP-WELQ-Caseicin FS and WELQut was incubated at different concentration ratios (1:100, 1:50, 1:25, 1:5 (v/v) for 50 µL of final volume at 26, 30 and 37 °C for 16 h. To confirm the effectiveness of the cleavage reaction and the release of the active bacteriocins (Caseicin FS-X and Caseicin FS-Y), their antimicrobial activity was determined by using the well-agar diffusion assay, as described by (Balouiri, Sadiki & Ibnsouda, 2016) using Brain Heart Infusion (BHI) soft agar 0.8% (w/v) agar (Merck-Millipore). Briefly, overnight cultures of *L. monocytogenes* EGD-e were inoculated at approximately 1 x 10^7 cfu/ml into 45 ml of soft BHI agar (0.7% agar).

Secondly the addition of WELQut or NisP_Mcherry enzyme, bacteriocins activity and size were also analysed using two Tricine-SDS-PAGE electrophoresis gels at 12% (stacking gel) and 4% (running gel) of acrylamide:bis-acrylamide 29:1 (Sigma-Aldrich, Poole, UK), one resolved according to the protocol described in (Schägger, 2006) and the second used for the overlay techniques according to (Gilbreth & Somkuti, 2005).

The cleaved bacteriocin was diluted 1:6 with 75% Acetonitrile and incubated for 30 min under agitation (150 rpm) at 26 °C. The top layer containing the bacteriocin was spin down at 3500 rpm for 4 min and then freeze-dried. After that, a resuspension of concentrated bacteriocin in water suitable for HPLC was performed and loaded onto a Poroshell 120 EC-C18 HPLC column (120 Å, 4 µm, 4.6 mm × 150 mm, Agilent) and eluted with a linear gradient created with 0.1% (v/v) trifluoracetic acid (TFA) in analytically pure water (eluent A) and 0.1% (v/v) TFA in acetonitrile (eluent B). The flow rate was set at 1.3 mL/min, and the elution program utilised was as follows: 10% eluent A from 0 to 3 min (initial conditions), 3–30 min linear gradient from 10 to 90% eluent B. Separation was performed on an Agilent 1260 Infinity II LC system. Peaks detected were collected during elution and tested for antimicrobial activity using the well-agar diffusion assay described elsewhere.

Active fractions containing pure bacteriocins were lyophilised, and the powder obtained was weighed with analytical balance XP26 (Mettler-Toledo, USA). Purified bacteriocins were diluted in different aliquots with sterile Milli-Q water at a defined concentration to evaluate the minimum inhibitory concentration (MICs) by well-agar diffusion test. The inhibition halo was highlighted by adding 10 µl resazurin dye solution (0.015 %) to all wells after incubation for 24 h at 37 °C.

Scanning electron microscopy (SEM)

For SEM analysis, a single colony from a pure culture of *L. monocytogenes* EGD-e was inoculated in 10 ml of sterile BHI broth and incubated at 37 °C with shaking (120 rpm). After 24 hours of incubation, aliquots of 50 µl were added with 50 µl of sterile BHI broth (1:1). Coverslip treated with UV light for 30 min was used as support to prepare the sample for scanning electron microscopy. A final volume of 100 µl of diluted *L. monocytogenes* EGD-e culture (approximately 107 CFU/ml) was added to the coverslip top surface previously positioned in a sterile petri dish (35 × 15 mm). A total of 100 µl of cleaved peptide in 50 mM Tris and 200 mM NaCl buffer were added to the coverslip. Subsequently, the coverslips were incubated at 26 °C overnight for each treatment. After this incubation period, cells treated were fixed with 4 % paraformaldehyde (PFA) in PBS (pH 7.2) for 16 h at 4 °C. Coverslips were stained with 2 % OsO4 for 30 mins, washed 3x with dH20, dehydrated in a graded ethanol series (20, 50, 70, 90, 100 v/v) for 5 min each and sputter coated with 50 nm Gold/Palladium. SEM was conducted using a ThermoFisher Apreo FESEM at a beam strength of 2kV and a current of 20 nA.

RESULTS

Lactic acid bacteria are known to produce various antimicrobial compounds with different modes of regulation and production. Although many genes relevant to antimicrobial compound biosynthesis have been identified in LAB spp., the isolation, purification, identification and characterisation of antimicrobial compounds have yet to be accomplished for all species belonging to this genus. However, *Lcb. casei* UD 2202 and *Lcb. casei* UD 1001 do not show antimicrobial properties. The in silico bacteriocins analysis of the genomes revealed unknown pediocin-like biosynthetic gene clusters named CAS-X and CAS-Y. Additionally, the manual annotation of these areas of interest revealed a gene collection having an unambiguous relation with the class IIa operons systems, as shown in **Figure 2.1**.

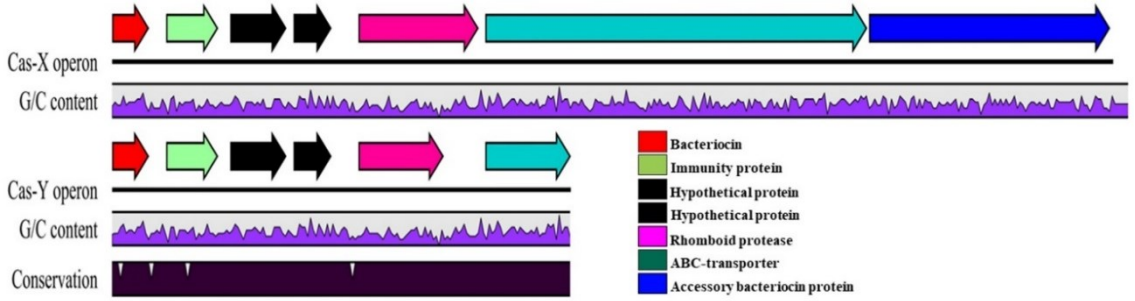

Figure 2.1: Schematic representation of the alignment of genes clusters CAS-X and CAS-Y detected. Different colours indicate the other gene functions *in silico* predicted to be responsible for the biosynthesis of Caseicin FS-X and Caseicin FS-Y bacteriocin.

Investigation of aminoacidic sequence homology connected to the antimicrobial properties of class IIa bacteriocins and in correlation with the novel Caseicin FS-X and Caseicin FS-Y bacteriocins were perform using multialigment approach, as shown in **Figure 2.2**.

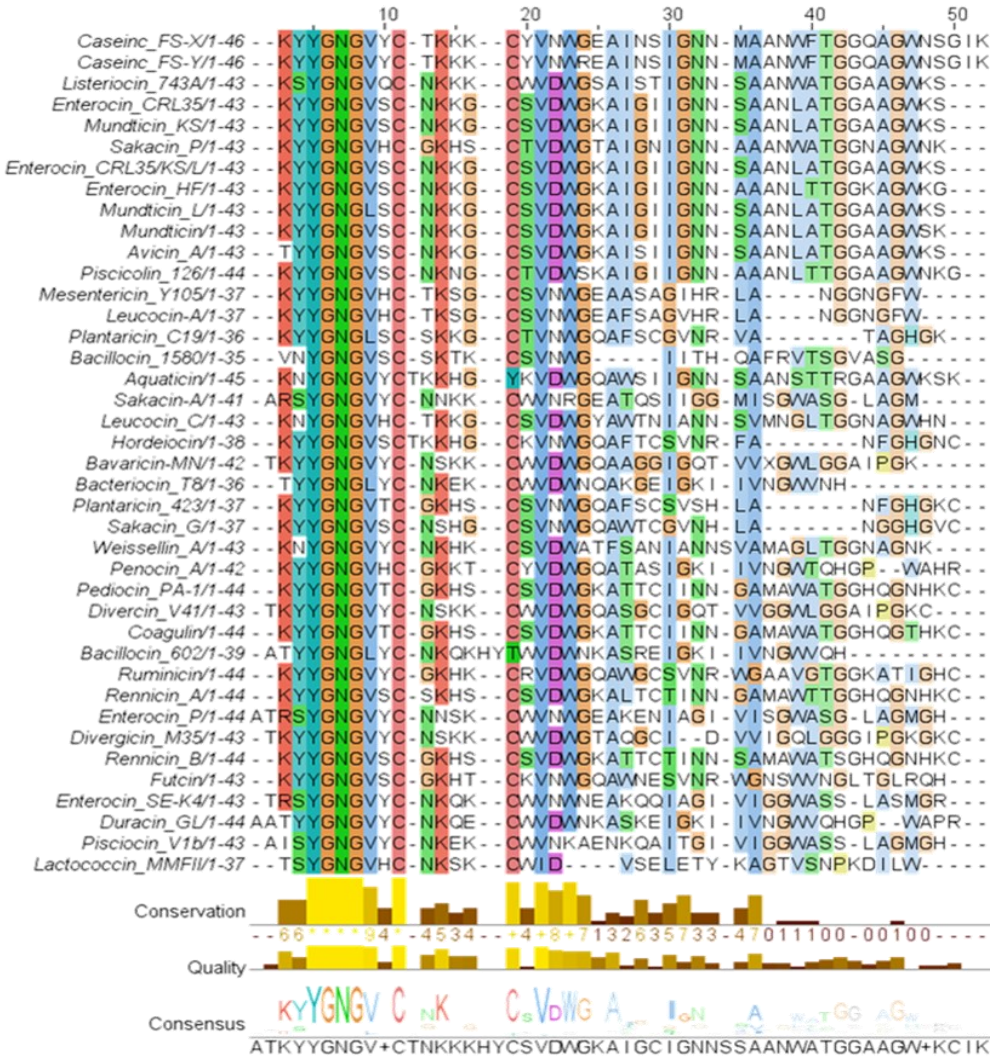

Figure 2.2: Alignment of class IIa bacteriocins. These analyses are based on primary sequence comparisons of the mature peptides. Conservation scores are presented as histograms based on the number of shared properties between the residues of each column. Consensus is displayed as a normalised logo diagram. Residues are coloured according to Clustalx color code in Jalview.

The most similar bacteriocins to Caseicins' were Listeriocin 743A and Sakacin P. In this regard, a more representative data visualization is available in **Figure 2.3.**

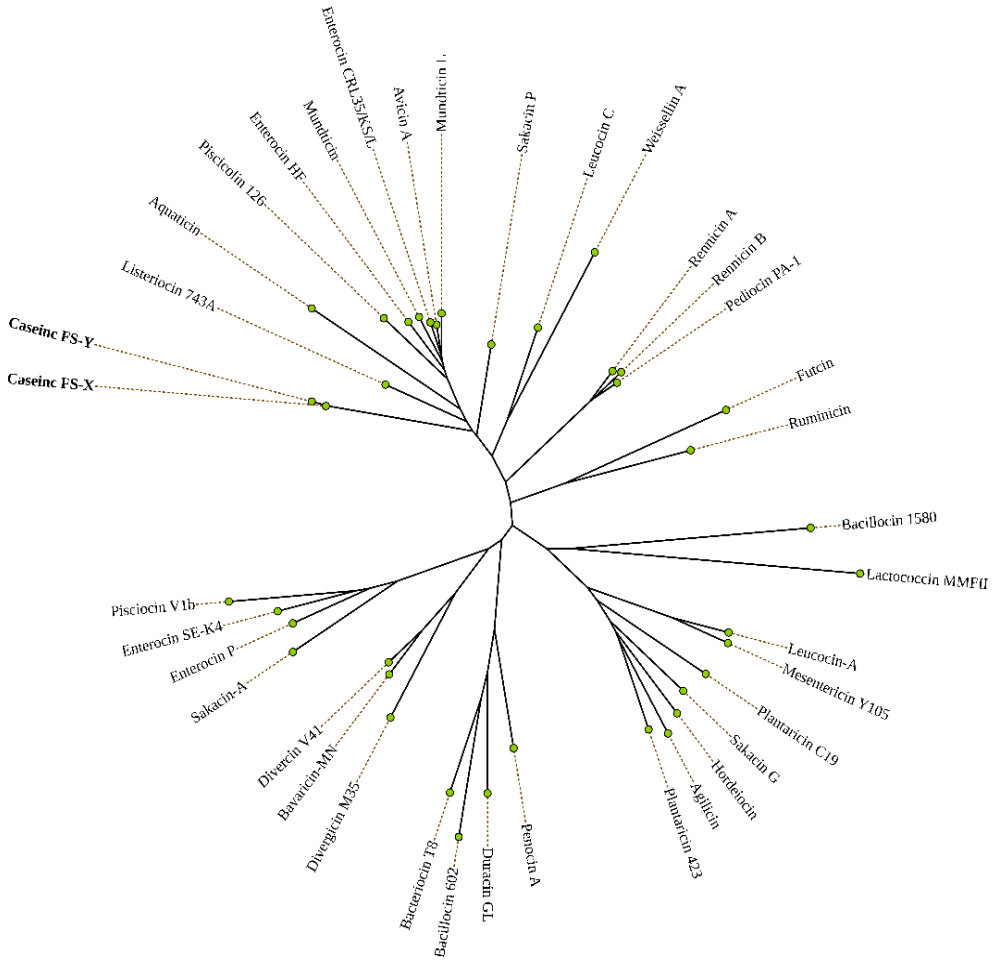

Figure 2.3: Cladogram representation alignment of mature class IIa bacteriocins including Caseicin FS-X and Caseicin FS-Y.

Both the *cas-x* and *cas-y* gene consists in 210 bp and contain a protein-coding region of 69 amino acids. Both the peptides have five glycine amino acids in the leader peptide, which differs from the other bacteriocins of class IIa and IIb, which are characterized by a double glycine cleavage site in their leader peptide. The mature peptide sequence of both Caseicin FS-X and Caseicin FS-Y is composed of 46 amino acids, including the anti-listeria or Pediocin-like conservative amino acid domain YGNGV (N-terminal consensus sequence 'Tyr-Gly-Asn-Gly-Val') and "CXXXXCXV" sequence motif. Interestingly, Caseicin FS-X and Caseicin FS-Y aminoacidic sequences differ at position 7 by having either Valine (V) or Alanine (A), at position 17 by having either Isoleucine (I) or Valine (V), and at position 42 by having either Glycine (G) or Arginine (A). Both class IIa bacteriocins, Caseicin FS-X and Caseicin FS-Y, are novel, as shown in **Figure 2.4**, which depicts the relationship.
Despite that, *cas-x* and *cas-y* genes were PCR confirmed, and the correctness of gene insertion through restriction enzyme-based techniques was verified by cloned gene sequencing.

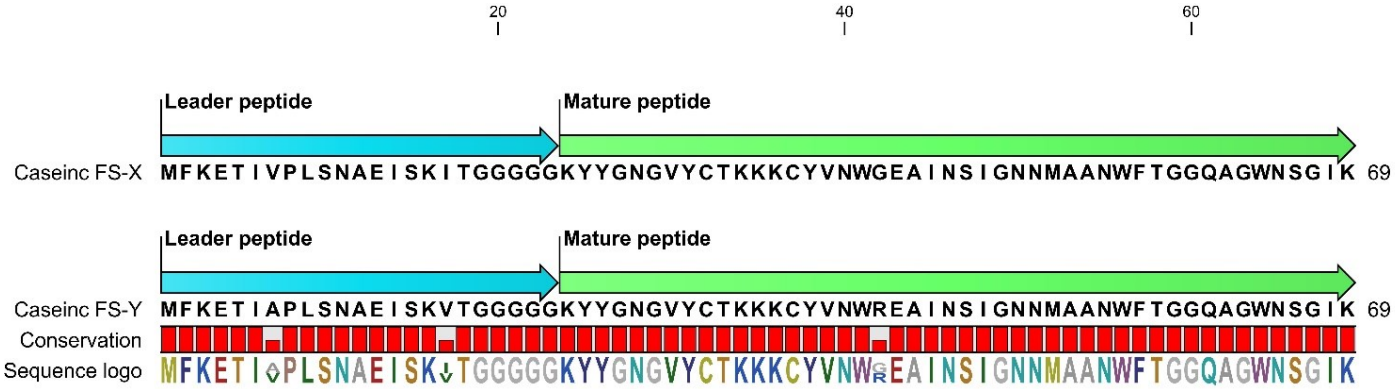

Figure 2.4: Alignment of Caseicin FS-X and Caseicin FS-Y highlighting the differences between the two antimicrobial peptides and representing the leader peptide and the mature peptide.

Expression plasmids system and detection of putative peptides:

The mature Caseicin FS-X and Caseicin FS-Y production was achieved in *E. coli* BL21 (DE3) through a "plug and play" expression system under the control of the inducible T7 promoter. Plasmids pRSF-GFP-Wcas-x, pRSF-GFP-Nislcas-x and pRSF-GFP-Nislcas-y included the WELQ cleavage sites and the entire Nisin leader, respectively. In addition, a portion of NisP protease, including the specific lantibiotic S8 domain fused with Red Fluorescent Protein (RFP), was also successfully obtained through heterologous protein expression in *E. coli* BL21 (DE3) under the T7 promoter in vector pRSF-Nisp-Mcherry8xHis. Plasmid maps are listed in **Figure 2.5**.

The overexpressed fusion peptides encoded by the different plasmids during the heterologous expression were detected from the cell lysate of *E. coli* BL21 (DE3) cells by Tricine-SDS-PAGE gel also determining the fusion proteins' total theoretical mass and purity after protein purification. Furthermore, the absent antimicrobial activity was detected in the absence of cleavage from 6xHis-tag-GFP-WELQ-Caseicin FS-X, 6xHis-tag-GFP-Nisin leader-Caseicin FS-X, 6xHis-tag-GFP-Nisin leader-Caseicin FS-Y, NisP-Mcherry-8xHis-tag fluorescent peptides complex and commercially available WELQut protease.

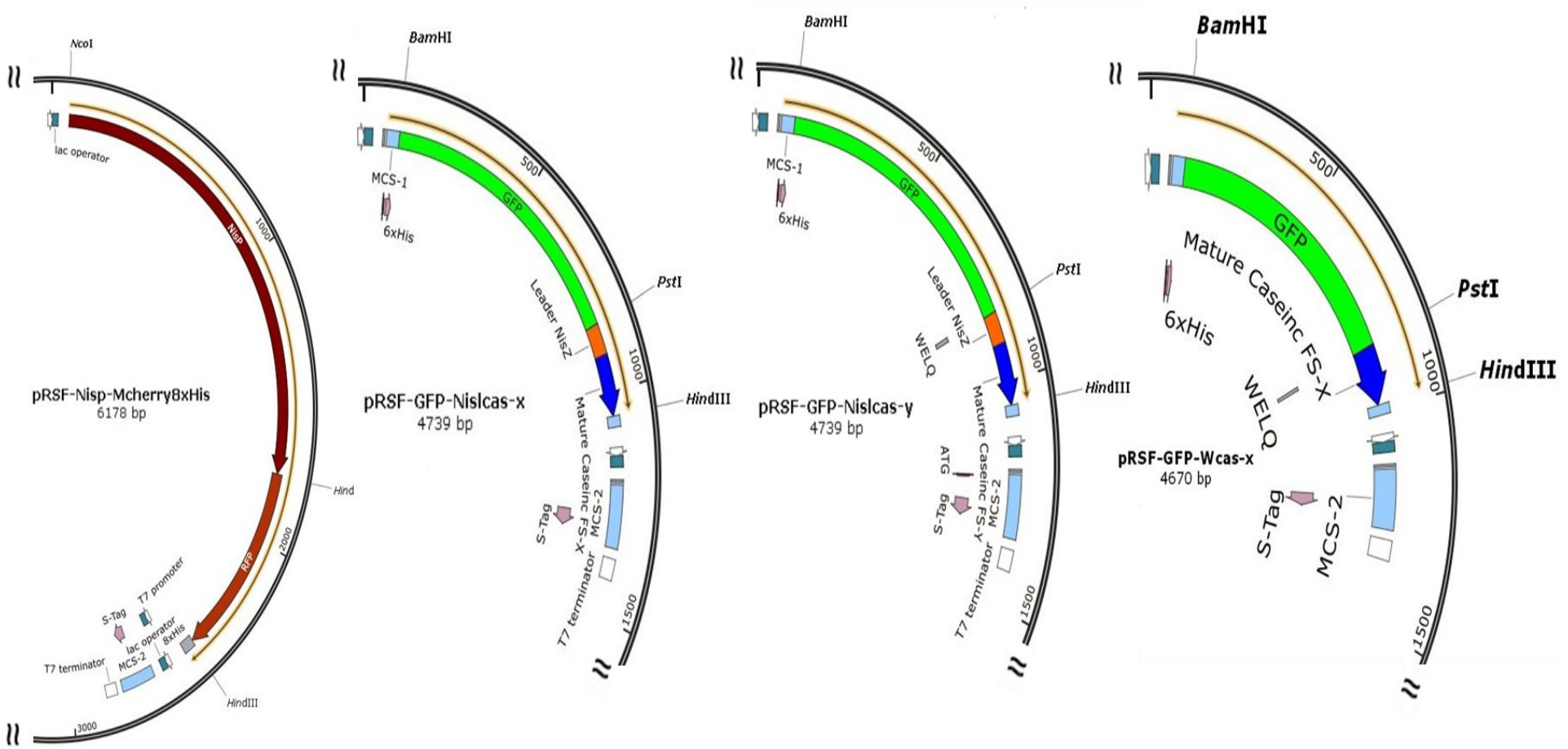

Figure 2.5: Schematic plasmid maps showing the features present in the expression vectors designed for production in *E. coli* BL21 of the fusion proteins covered in the present study.

Finally, the addition of NisP-Mcherry-8xHis-tag and WELQut proteases results in an appreciable inhibition zone due to the cleavage of Casein FS-X and Caseicin FS-Y bacteriocins. The mature Caseicin FS-X and Caseicin FS-Y liberation were evaluated by detecting antimicrobial activity against *Listeria monocytogenes* EDG-e before and after proteolytic cleavage. As support of cleavage Caseicin FS-X and Caseicin FS-Y and the ascribable antimicrobial activity well-agar diffusion assay, overlayed Tricine-SDS-PAGE and treatment of *Listeria monocytogenes* EDG-e by SEM analysis was confirmed. **Figure 2.6** summarises these results.

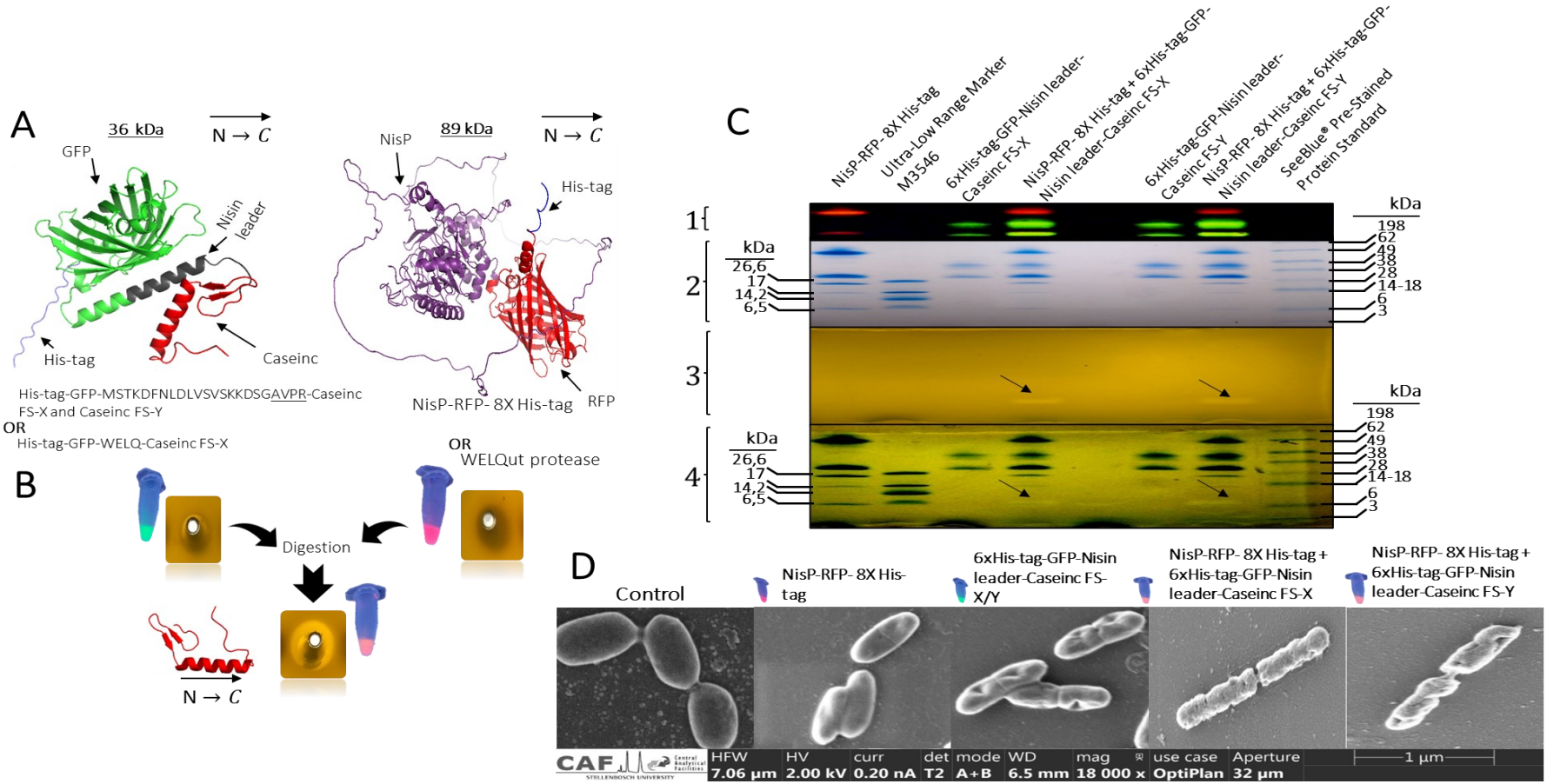

Figure 2.6: Antimicrobial propriety against *L. monocytogenes* EDG-e as target strain of liberated Mature Caseicin FS X/Y after cleavage. (A) 3D folding prediction of aimed fusion protein using Alphafold command line (Ghani, 2021; Ronneberger et al., 2021). (B) Well-agar diffusion assay before and after protease addition. (C) Tricin-SDS-PAGE gels are displayed in four different representations, as described in the following: 1. Fluorescent image of SDS PAGE gel. 2. Destained SDS PAGE gel. 3. SDS-PAGE gel overlaid with BHI soft agar inoculated with *L. monocytogenes* EGD-e as a target strain. 4. Desteined gel over a gel overlaid SDS-PAGE with *L. monocytogenes* EGD-e. (D) SEM images of the effect before and after cleavage liberating mature Caseicin FS X/Y compared to the untreated control cells of *L. monocytogenes* EGD-e.

Once the functionality of Caseicin FS-X and Caseicin FS-Y was confirmed, cleavage optimisation was performed, aiming to maximise the bacteriocin liberation; these results are shown in **Figure 2.7**.

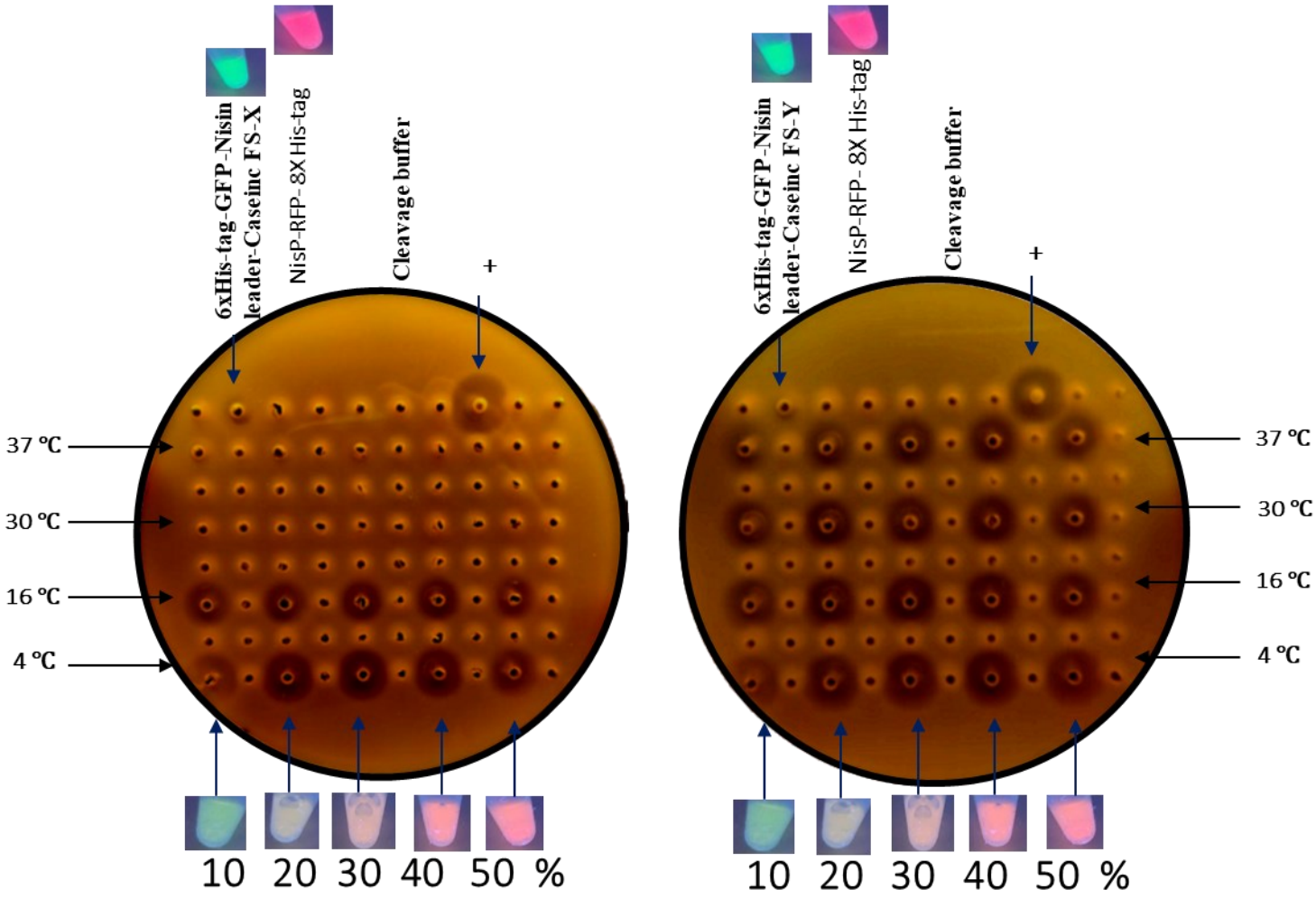

Figure 2.7: Cleavage optimisation of 6xHis-tag-GFP-Nisin leader-Caseicin FS-X (left) and 6xHis-tag-GFP-Nisin leader-Caseicin FS-Y (right) adding NisP-RFP-8X His-tag protease in different ratios, all conditions were then analysed after 24 h of incubation at different temperature. Cell free pH adjusted supernatant from strain *Lactiplantibacillus. plantarum* 423 producers of bacteriocin Plantarocin 423 was used as a positive control.

Starting from a 1 L of heterologous expression following the optimal cleavage parameter and the further purification steps performed in 1 mg of pure Caseicin FS-X and Caseicin FS-Y production. Furthermore, MIC results were performed from the HPLC pure Caseicin FS-X and Caseicin FS-Y, as shown in **Figure 2.8**.

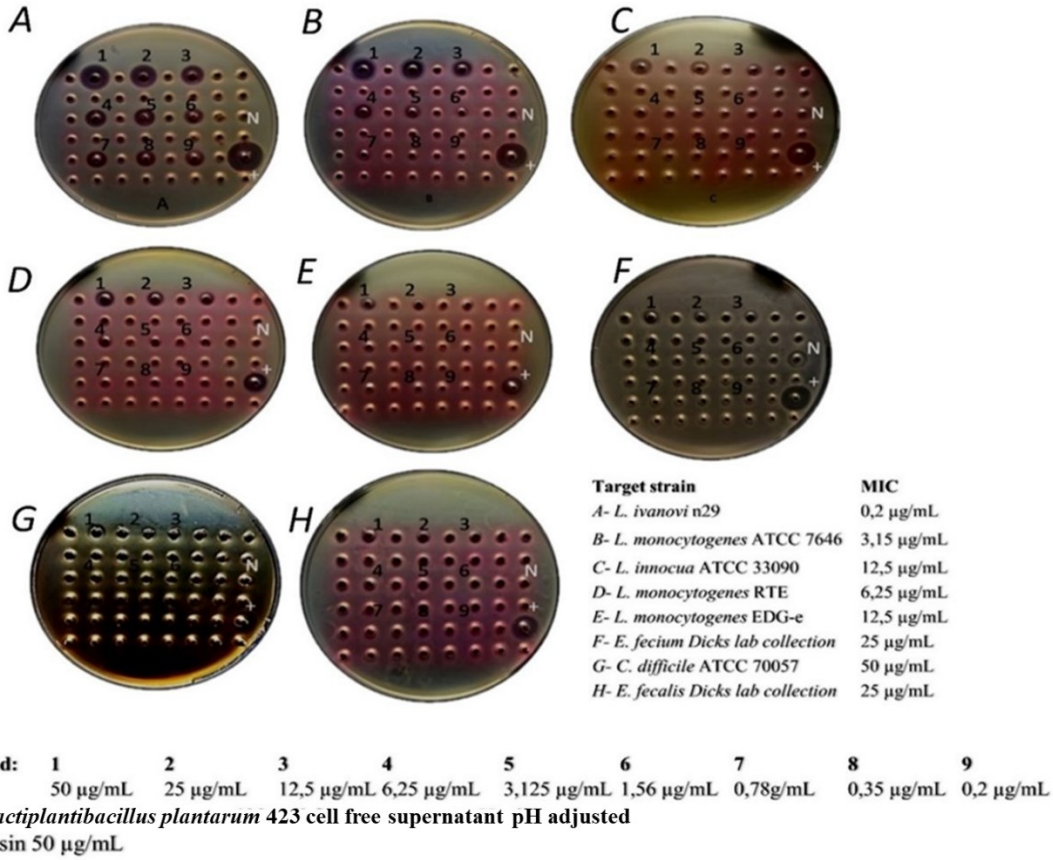

Figure 2.8: Minimum Inhibitory Concentration (MIC) of HPLC purified Caseicin FS-X against the test microorganisms. MIC were consistent also for Caseicin FS-Y for the same target strains.

DISCUSSION

In this study, we provide strong evidence that the *in silico* detected genes, *cas-x* and *cas-y,* are codifying two undescribed class IIa bacteriocins, which according to nomenclature, the suffix "cin" were used in defining the name Caseicin FS-X and Caseicin FS-Y (Montville & Kaiser, 1993). Despite that, *Lcb. casei* UD 2202 and *Lcb. casei* UD 1001 have not been considered bacteriocin producers because no related antimicrobial activity has been detected in their culture supernatant. After genome mining analysis of the entire *Lcb. case*i UD 2202 and *Lcb. casei* UD 1001 genomes, two biosynthetic gene clusters (BGCs) encoding a double-glycine leader motif and highly conserved "YGNGV" and "CXXXXCXV" domain sequences associated with pediocin-like bacteriocins were observed. Furthermore, based on the high similarity in the nucleotidic sequence of *cas-x* and *cas-y* and as indicated by the predicted transcription results, the nucleotide sequences differ in 3 amino acids in the final peptide backbone. Therefore, these bacteriocins were considered as novel and a natural co-evolution variant.

Moreover, the mature Caseicin FS is composed of 46 aminoacids with a single disulfide bridge that includes four amino acid residues designed between the 2 Cys residues (C_9TKKKC_{14}). Additionally, the prediction of the tridimensional structure reflects the typical pediocin-like conformation, characterised by a cationic and highly conserved N-terminal region. Together with a less conserved hydrophobic/amphiphilic C-terminal region hydrophobic or amphiphilic α-helical-structure which has been proposed to interact with the hydrophobic core of target-cell membranes, also called barrel-stave poration complex (Ojcius & Young, 1991). Despite that, the difference in position 19 Glycine (G) or Arginine (A) influences the net charge of this cationic peptide, with the potential variation of their

antimicrobial activity (Nes & Holo, 2000). Common peculiarity between the mature Caseicin FS-X/Y and the rest of the pediocin-like bacteriocins is the unique five glycine motif, the length of the core peptide (46 aminoacids) and lastly, the unique operon organisation that is missing the two-component systems, composed of a histidine kinase (HK) and response regulator (RR) (Jung et al., 2012).

However, other different antimicrobial peptides were isolated from *Lacticaseibacillus casei* spp, including bacteriocin LiN333 (Ullah et al., 2017), bacteriocin lactocin 705 (Vignolo et al., 1995; Cuozzo et al., 2000), bacteriocin caseicin 80 (Müller & Radler, 1993), bacteriocin LSEI_2163 (Kuo, Liu & Lin, 2013) and bacteriocin caseicin TN-2 (Kiran et al., 2012). There was no evidence that these as mentioned bacteriocins were previously associated with class IIa due to preliminary characterisations of the aminoacidic sequences and/or belonging to other antimicrobial peptides from previous studies. For this reason, this article reports the first evidence of class IIa bacteriocins isolated from *Lacticaseibacillus casei* spp. Furthermore, data mining served as a valuable tool in identifying new bacteriocins within this study (Cui et al., 2021). However, in the absence of concrete bacteriocin production from the strains under analysis, a suitable expression system was necessary to confirm their action. Despite this, the genes encoding these regulatory proteins are normally organised within a gene cluster containing several other bacteriocin biosynthetic genes, which were also identified for both Caseicin FS-X and Caseicin FS-Y. The mechanism of regulation of Caseicin FS X/Y due to the undetected bacteriocin production requires a deeper investigation. Therefore, genome mining and the heterologous protein expression combined fulfilled in the production of Caseicin FS X/Y from *Lcb. casei* UD 2202 and *Lcb. casei* UD 1001 allowing their confirmation as antimicrobial compounds. The elucidation of the regulatory mechanism of bacteriocins has led to the development of plasmid-based expression systems, to significantly improve the production of biologically active bacteriocins and reduce tedious bacteriocin purification steps (Yang et al., 2007; Chen et al., 2012). Thus, the heterologous expression system named His-tagged GFP-bacteriocin fusion proteins described by (Vermeulen, Van Staden & Dicks, 2020), which successfully produced Plantaricin 423 and Mundticin ST4SA, was chosen as an initial attempt to produce Caseicin FS-X. This system's advantage applies a fluorescent protein as a fusion partner, which provides constant tracking during fermentation, extraction, purification, and analysis of the heterologous fused peptide of interest. As a result of the low solubility, size and toxic effect on recombinant *E. coli* of class IIa bacteriocin systems, the His-tagged-bacteriocin was found not to meet the expectations of large-scale production entirely (Moon, Pyun & June, 2006). According to Vermeulen et al. (2020), the limiting factor observed in the involvement of the commercially available WELQut protease responsible for the liberation of active Caseicin FS-X had an inefficient cleavage rate. This resulted in missed maximisation in bacteriocin production, which could be time-consuming and costly. Despite that, this method was more successful in confirming the liberation and antimicrobial activity of Caseicin FS-X. From these observations in the current study, the effectiveness of heterologously expressed nisin protease NisP known to cleave precursor lanthipeptides (Montalbán-López et al., 2018; Van Staden et al., 2019) was demonstrated. NisP is a specific membrane-anchored subtilisin-like serine peptidase (Pfam entry Peptidase S8) and plays a protagonist role in the last step of the nisin maturation process (Xu et al., 2014). The engineered and/or natural variations of nisin induce a low cleavage efficiency generally assumed due to the incorrect formation of (methyl)-lanthionine rings, which is considered a prerequisite for an efficient cleavage (Lagedroste, Smits & Schmitt, 2017; Reiners et al., 2020). In contrast, the cleavage patterns observed in the nisin mutants reported by Montalbán-López et al. (2018) show that the presence of lanthionine is not essential for the cleavage, which is the case of the mature class IIa bacteriocins, characterised by one or more disulfide bridges. According to our aim in facilitating cleavage of a heterologous expressed GFP-bacteriocin was fulfilled using a self-produced protease NisP.

Thereafter, to minimise possible cleavage issues, we replaced the leader of Caseicin FS with a nisin leader. Then, to obtain the best cleavage condition, bacteriocin's optimisation occurred, which in our case results indicated that temperature and elution buffer pH was the determining factor. However, the difference in cleavage efficiency between Caseicin FS-X and Caseicin FS-Y suggests that the variation in position 19 Glycine (G) or Arginine (A), which introduces an additional positive charged residue in Caseicin FS-Y, increases the temperature range of NisP action. The addition of the mCherry gene in the pRSF-NisP8xHis vector explored the potential increase of NisP conservability due to loss of cleavage efficiency after storage condition at -20°C. A truncated NisP, heterologously purified daily, with or without fused with the RFP protein, gave the best results in bacteriocin release suggesting that the stability of the protease used in this method might be the cause of restricting downstream applications.

To improve the application of NisP8xHis and NisP-Mcherry-8xHis labelling. Further investigations were needed to optimize the storage conditions to increase interest in large-scale production. To our knowledge, this is the first report to show the possible use of a lanthipeptides protease able to exert its functionality in combination with class IIa peptides. This is a remarkable finding considering the sensibility to trypsin, a widely used peptidase and the limited applicability of the less expensive chemical cleavage approach due to the requirements of this type of reaction being non-performing when applied to the class II bacteriocin (Beaulieu et al., 2007). Due to the MIC concentration explicating activity against different target strains, Caseicin FSX/Y are related in terms of poration potency to others pediocin-like bacteriocins. This data is also strongly supported by the clear cell membrane disruption (Bédard et al., 2018; Soltani et al., 2022).

Additionally, the yield of HPLC-pure Caseicin was ten times more than the similar result reported in studies which used a protease to liberate the subclass IIa bacteriocin (Moon, Pyun & June, 2006). Although this method requires optimisation, it offers promising new applications for producing recombinant and active peptides as well as defining the effectiveness of antimicrobial properties of new candidate proteinaceous compounds.

CONCLUSION

To this end, the accumulation of metagenomics studies provides a vast body of information, which allows the comparison of data obtained by conventional microbiology techniques and simultaneously boosts the development of new technology. Extensive applications of predictional homology-based genomic tools are available to interpret the genomic data. The common knowledge is that the bacteriocins produced by LAB or other microbes that are Generally Recognized As Safe (GRAS), which seems evident from their ubiquitous presence, must play an important ecological role. Furthermore, with these results, it can be inferred that using *in silico* approaches in finding novel protein functions within an expression vectors-based system successfully confirmed the antimicrobial properties of Caseicin FS-X and the variant Caseicin FS-Y. To the best of our knowledge, these are the first pediocin-like bacteriocins isolated from *Lacticaseibacillus casei* spp. Additionally, the first application of the lantibiotics protease NisP in association with a whole class IIa bacteriocin is described. Further investigation is underway to evaluate the mechanism of regulation of Caseicin FS-X/Y as well the chemical and physical properties to evaluate the possible applications in the food industry and in human health.

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CHAPTER 3

Genetic diversity of Caseicin gene clusters and evaluation of their mode of regulation.

ABSTRACT

This research aimed to find the distribution of the novel operon regulation Caseicin FS leading to insight into the elucidation of the mode of regulation of this biological preservative that inhibits harmful bacteria. In silico approach four biosynthetic genes cluster named CAS-X, CAS-Y, CAS-J and CAS-Z. A comprehensive comparison highlighting the implication of genetic variation of their gene's loci may be the cause of the impossibility of explicating the production of these antimicrobial peptides. Different assays were performed to activate the bacteriocin expression with ineffective results. On the other hand, the functionality of the most diverse operons variant CAS-X and CAS-Y with adapted nisin-controlled expression (NICE) plasmid systems, expressed in *Lactococcus lactis* model stains was successfully evaluated. These results indicate that the production of Caseicin FS-X is achieved by gene organization found in CAS-X operon, which differs from the others by having a gene codifying for an entire ABC transporter and one for an accessory protein. The transformed bacteria *Lactococcus lactis* produced enough Caseicin FS-X to inhibit the growth of *L. monocytogenes* EDG-e.

KEYWORDS: Bacteriocin, operon, *in silico,* heterologous genes expression

INTRODUCTION

Antagonism toward pathogens and spoilage bacteria found protagonist bacteria, yeast, and mould is due to the direct competition for nutrients and/or production of antimicrobial metabolites, such as organic acids, hydrogen peroxide, and bacteriocins (Singh, 2018). Bacteriocins are ribosomally synthesized peptides with antibacterial activity toward closely related strains (Atassi & Servin, 2010). They are a heterogenous group concerning taxonomy, mode of action, inhibitory spectrum, and protein structure. Exploration of bacteriocins as novel antimicrobials, alone or in combination with established bioperservation methods, position them as a potential tool in support to the era of sustainable food productions and antibiotic resistance.

Bacteriocins classification was revised a few times in the following year, offering a more inclusive and detailed description of all characterize classes. The newest and most detailed classification so far considers four classes of bacteriocins: (a) class I – ribosomally synthesized post-translationally modified small peptides (RiPPs); (b) class II – unmodified peptides up to 10 kDa; (c) class III – large proteins; and (d) class IV – circular proteins (Meade, Slattery & Garvey, 2020). However, this classification is bound to continuously evolve, since bioinformatics and analytics methods gather more information (Lozo, Topisirovic & Kojic, 2021). Bacteriocin production is influenced by several environmental factors, which are also intrinsic characteristics of food, such as pH, temperature, NaCl, sugars, and ethanol concentrations (Nilsson et al., 2002). Understanding the influence of food-related intrinsic factors on the induction of bacteriocins is essential for the effective commercial application of bacteriocins. Despite their remarkable potential the limiting factor on large-scale are the costs of production (Sidooski et al., 2019). Bacteriocin preparations produced *ex situ*, obtained by cultivation of the producer strain or through plasmid-based technique using transformed strains in an industrial fermenter followed by adequate recovery, require the improvement of industrial plants taking into consideration the production costs, bacteriocin yield and purity (Kumar et al., 2012). Currently, the most studied bacteriocins due to their bactericidal power at low concentrations and stability are nisin A/Z and pediocin PA-1; they are also the only bacteriocins approved as food additives and commercially used worldwide (Anastasiadou et al., 2008; Bharti et al., 2015).

Therefore, bacteriocins' mode of regulation plays an important role in understanding the multiplicity and diversity of novel and already known bacteriocins, which remains an area of investigation, attracting many researchers (Cavera et al., 2015). Many studies have focused on the bacteriocins genome mining approach, which could guide the identification of antimicrobial peptides and reveal the organization and diversity of the biosynthetic machinery required for bacteriocin production (Azevedo et al., 2015). Genes required for bacteriocin production are typically organized in operon structures found in chromosomal DNA, plasmid and transposons (insertion sequences (IS) or inverted repeats (IR)) suggesting the intra and inter-species phylogenetic dissemination of bacteriocins with potential evolution from ancestors on mobile genetic elements (Lahiri et al., 2022). In this study, a comprehensive sequence analysis of Caseicin FS variant genes was first conducted to understand the possible mechanism of regulation of Caseicin bacteriocin. Then, this study aimed to provide a preliminary evaluation of the effective ability of these biosynthetic gene clusters (BGCs) in terms of bacteriocin production.

MATERIAL AND METHODS

Detection of bacteriocin production by agar well diffusion assay

Cell-free, pH adjusted (6.5) supernatant for each strain containing plasmid and at different times was used to determine the bacteriocin production using the agar well diffusion method (Yu et al., 2020). Briefly 100 µl were spotted in wells obtained in soft BHI media supplemented with the antibiotic Chloramphenicol (Cm) at a concentration of 7.5 µg/ml inoculated with about 10^6 CFU/ml of fresh overnight culture of *Listeria monocytogenes* EDG-e used as a target strain. Cell free supernatant of *Lactiplantibacillus plantarum* 423, producer of bacteriocin (Plantaricin 423), was used as a positive control. To determine the nature of the inhibitory compounds for cell-free culture supernatants was treated with the enzyme proteinase K (Sigma) at a final concentration of 1 mg/ml. All samples were incubated at 37 °C for 1 h.

Bacteriocin detection and extraction

Amberlite XAD16N from MRS agar

XAD-16 beads were activated by treating with 80% isopropanol containing 0.1% (v/v) trifluoroacetic acid (TFA) and added to different solid media autoclaved. Overnight broth culture was added to 5 g/100ml activated XAD-16 beads, spread-plated onto XAD-16-treated agar in petri dishes with a diameter of 135 mm containing MRS agar media, and incubated at 37 °C for 24/48/72 h. Beads were collected from the plates and washed with sterile deionised water to remove the cells. Water was removed from beads by vacuum suction. The beads were washed with 150 ml 30% (v/v) ethanol for 15 min at 4 °C on an orbital shaker (100 rpm). Ethanol was removed by vacuum suction and the beads were washed with sterile deionized water. Compounds were liberated from the beads using 70% (v/v) isopropanol containing 0.1% (v/v) TFA (isopropanol-TFA). The eluent was filtered through a 0.45 µM cellulose nitrate filter and the isopropanol removed by using a rotary evaporator (RotaVapor® R-114, Büchi).

Salting-out

Strains were added in 1 L of MRS culture and incubated at 37 °C for 48 h and then the CFS was obtained by centrifugation at 2057 × g for 10 min at 4 °C. Next, the samples were salted out by adding 80% saturation ammonium sulphate to the CFS and stirring for 12 h at 4 °C. The crude bacteriocin was pelleted by centrifugation at 14000 g for 1 h, resuspension of the protein pellet, and dissolved in t in PBS buffer. For removal of the high salt concentration, the Waters SepPak C18 column was used with different concentrations of acetonitrile (20,40,60,80 %). All fraction was freeze-dried and resuspended in 1 ml of analytically pure water added with 0.1% (v/v) TFA.

Evaluation of different environmental factors activating the bacteriocin production

Strains *Lcb. casei* UD2202 and *Lcb. casei* UD1001 were evaluated for the capability of the bacteriocin production under different growth conditions as described in **Table 3.1**. treated with the following growth conditions.

Table 3.1: Trials under different conditions to evaluate the bacteriocin production capability.

Trial	Medium	Sugars	pH	Temperature	Time	NaCl	Acetic acid	Metabolism	Co-culture
1	MRS broth	Dextrose 20g/l	5.6-5.9	26, 30, 37, 42 °C	24, 48, 72 h	0%	/	Aerobic and anaerobic	/
2	M17 broth	Mannitol 20g/l, Sucrase 20g/l. Lactose 20g/l. Mannose 20g/l. Rhamnose 20 g/l. Glucose 20 g/l. Xylose 20g/l. Glycerol 1%	4, 5, 6, 7	26, 30, 37°C	24, 48, 72 h	0%	/	Aerobic and anaerobic	/
3	MRS broth	Dextrose 20g/l	4, 5, 6, 7	26, 30, 37°C	24, 48, 72 h	0%	/	Aerobic and anaerobic	/
4	M17 broth	Mannitol 20g/l, Sucrase 20g/l.Lactose 20g/l.Mannose 20g/l.Rhamnose 20 g/l.Glucose 20 g/l.Xylose 20g/l.Glycerol 1%	6.9 ± 0.2	37°C 48°c	24 h 72 h	0%	/	Aerobic and anaerobic	/
5	MRS broth	Dextrose 20g/l	5.6-5.9	37°C 48°c	24 h 72 h	0.1,0.2, 0.5 M Nacl %	/	Aerobic and anaerobic	/
6	MRS broth	Dextrose 20g/l	5.6-5.9	37°C 48°c	24 h 72 h	/	0.05, 0.1, 0.02, 0.5, 0.7, 0.1%	Aerobic	/
7	MRS broth	Dextrose 20g/l	5.6-5.9	30 °C	24, 48 h	/	/	Aerobic and anaerobic	*L. monocytogenes* EGD-e
8	BHI broth	Dextrose 2g/l	7,2 - 7,6	30 °C	24, 48 h	/	/	Aerobic and anaerobic	*L. monocytogenes* EGD-e

In each trial the different broths were inoculated with a single colony of the strains no older than 48 h which were streaked from a pure culture previously revitalised in MRS broth (Biolab, Merck, South Africa) or in the case of *L. monocytogenes* EGD-e in BHI broth (Biolab, Merck, South Africa) for 24 h at 37°C. At the end of the period of growth at the different conditions, the production of bacteriocin was evaluated by agar well diffusion assay.

Bacteriocin mining and *in silico* genome analysis

The genome sequence of *Lcb. casei* UD2202 and *Lcb. casei* UD1001 has been screened for the presence of putative bacteriocin genes using the BAGEL4 (Van Heel et al., 2018) and antiSMASH (Blin et al., 2019) bacteriocin mining software. The BLAST function was used to identify any genetic relation with *Lcb. casei* UD2202 and *Lcb. casei* UD1001 bacteriocin area of interest (AOI). Coding sequences were identified using the NCBI database (https://blast.ncbi.nlm.nih.gov) and blastx and tblastn software. Putative bacteriocin genes within the respective genomes were annotated using the CLC Main Workbench – QIAGEN Bioinformatics software (CLC bio, Aarhus, Denmark).
Sequence alignment was done using Muscle WS (Edgar, 2004) and displayed by Tree Of Life (iTOL) v4 online tool (Letunic & Bork, 2019). Comparative analyses of genomic datasets were performed using Operon-mapper (Taboada et al., 2018) and PRISM 4 (Skinnider et al., 2020). Identified genes were visualised using (Gilchrist et al., 2021). Database Bactibase (Hammami et al., 2007) and LABioicin (Kassaa et al., 2019) supported the class IIa bacteriocins comparison analysis. Operon representation was analysed with Clinker & Clustermap.js (Gilchrist & Chooi, 2021) and protein 3D prediction was obtained with Aphafold2 (Mirdita et al., 2022).

Construction of pNZ8048 based plasmid containing Caseicin biosynthetic cluster

Procedures including DNA extraction, *E. coli* transformations, and *E. coli* plasmid isolations were performed by using standard techniques (Ausubel et al., 1992). Restriction enzyme digest analysis and electrophoretic purification of DNA were performed on 1 % agarose gels at 10V/cm in TBE buffer using the EphortecTM 3000V (Triad Scientific, Manasquan United States) apparatus. DNA fragments were recovered from gel excisions using the ZymocleanTM gel DNA recovery kit (Zymo Research Corporation, Irvine, CA, USA). The construction plasmids were based on the pNZ8048 Lc. lactis NICE system high copy number plasmid (Mobitech, Goettingen, Germany). The vector contains the cat gene for chloramphenicol (Cm) resistance, the nisA gene promoter region (PnisA), a multiple cloning site (MCS), replication genes (repC and repA) for replication in LAB/E. coli and the termination (T) sequence of the *Lc. lactis* pepN gene (Mierau & Kleerebezem, 2005). Primer sets used (Table 3.2) were designed with the CLC main workbench program (CLC bio,Aarhus, Denmark) and supplied by Inqaba Biotechnical Industries (Pretoria, South Africa). DNA amplification was conducted for 35 cycles, with initial denaturation at 95 °C for 1.30 min, primer annealing for 210 sec at 67 °C and primer extension for 1 min at 72 °C using the high-fidelity Q5 DNA polymerase PCR (NEB) enzyme with the GeneAmp PCR system 9700 (ABI, Foster City, CA) PCR machine.
Reagents such as DNA ligase T4 and restriction enzymes (RE) were supplied by New England Biolabs (NEB, Ipswich, MA, USA) and used according to the manufacturers. Plasmid extraction and transformation protocols used are reported in NICE Expression System for *Lactococcus lactis* (Mierau & Kleerebezem, 2005). **Table 3.2** collects the primer used to obtain pNZ_CAS-X and pNZ_CAS-Y plasmid.

Table 3.2: Description, characteristics or sequence (5'→3') forward primer, reverse primer.

Primer name	Primer sequence (5'→3')	Tm (◦C)	Source or reference
Construct pNZ_CAS-X			
BglII_FO_FW	CTAAGATCTGATCATACTGTAGCCCAACG	65	This work
Kpnl _FO_REV	CGTGGTACCTCATTTGTTGGTTAGTGGTG	65	This work
Construct pNZ_CAS-Y			
BglII_FO_FW	CTAAGATCTGATCATACTGTAGCCCAACG	65	This work
BgIII_Y_REV	ATCAGATCTATGCAATCACAGAACCTTG		This work
BgIII_Y_FW	GAACTGCAGAAATACTATGGTAATGGTG	64	This work
Kpnl _FO_REV	CGTGGTACCTCATTTGTTGGTTAGTGGTG		This work

Bacterial strains, plasmids and culture media

Bacterial strains and general cloning plasmids are listed in Table 3.3. All subcloning experiments were done in *E. coli* MC1061 (Mobitec, Göttingen, Germany). *Escherichia coli* strain were grown in Luria–Bertani (LB), brain heart infusion (BHI) broth or solid agar (1.5% w/v) (Biolab Diagnostics, Midrand, South Africa) and incubated at 37 °C with rotary shaking at 200 rpm. The LAB strains *Lcb. casei* UD2202, *Lcb. casei* UD1001 and *Lactiplantibacillus plantarum* 423 were grown as static cultures at 30 °C in MRS broth (Biolab Diagnostics) or on MRS agar plates. *Lc. lactis* pNZ9000 was grown at 30 °C in M17 broth (Biolab Diagnostics) without shaking, or on agar plates supplemented with 0.5% (w/v) glucose. *E. coli* strain containing plasmids (**Table 3.3**) were cultured in LB or BHI medium supplemented with 10 µg/ml Cm. *L. monocytogenes* EGD-e was grown in BHI media supplemented with 7.5 µg/ml Cm and incubated at 37 °C on an orbital shaker (200 rpm).

Table 3.3: Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
	Strains	
E. coli		
MC1061	Host strain used for subcloning with Lc. lactis derived pNZ8048 vector; recA positive strain	Mobitec, Göttingen, Germany
Lacticaseibacillus casei		
UD2202	Strains with genes related to Caseicin FS-X production; originally isolated from hard cheese	This study.
UD1001	Strains with genes related to Caseicin FS-Y production; originally isolated from human faeces	This study
Lactococcus lactis		

pNZ9000	Standard host strain for nisin regulated gene expression; harbours the nisR and nisK nisin regulatory genes integrated into the pepN gene locus	Mobitec, Göttingen, Germany
	Plasmids	
pNZ8048	Broad-host range vector; E. coli Shuttle vector; LAB expression vector containing nisin A inducible-promoter (PnisA); CmR	Mobitec, Göttingen, Germany;
pNZ_CAS-X	Plasmid carrying CAS-X operon	This study
pNZ_CAS-Y	Plasmid carrying CAS-X operon	This study

CmR: chloramphenicol resistance.

RESULTS

Environmental factors activating the bacteriocin production

Lcb. casei UD1001 and *Lcb. casei* UD2202 strains were able to grow in all conditions tested. However, no antimicrobial activity was detected.

Identification of the distribution of Caseicin operon

A total of six LAB isolates with similarities to the bacteriocin Caseicin FS have been identified in the NCBI database (Table 3. 4). In addition to the recent identification of the new bacteriocins Caseicin FS-X and Caseicin FS-Y, other variants named Caseicin FS-J and Caseicin FS-Z were found, reflecting differences in the genes encoding the bacteriocin, but also an increment in variability compared to the previously identified CAS-X and CAS-Y operons. However, compared to all other bacteriocin gene clusters, operons CAS-X, CAS-Y, CAS-J and CAS-Z, are characterized by unique gene organization, highlighting their evolutionary relationship.

Table 3.4: Summary of the strains and genomic distribution of the Caseicin operons.

Strain	GenBank Accession	Bacteriocin	Genetic organization
Lacticaseibacillus casei UD2202	-	Caseicin FS-X	CAS-X
Lacticaseibacillus zeae CECT 9104	LS991421.1	Caseicin FS-X	
Lacticaseibacillus casei UD1001	-	Caseicin FS-Y	CAS-Y
Lacticaseibacillus casei FBL6	CP074377.1	Caseicin FS-J	CAS-J
Lacticaseibacillus casei N	CP077759.1	Caseicin FS-J	
Lacticaseibacillus chiayiensis FBL7	CP074378.1	Caseicin FS-Z	CAS-Z

Additionally, Cytochrome d ubiquinol oxidase subunit, ammonium transporter and NAD(P)-binding domain-containing protein was present upstream and downstream of the bacteriocin genes in *Lcb. casei* UD2202, *Lcb. zeae* CECT 9104, *Lcb. casei* UD1001, *Lcb. casei* FBL6 and *Lcb. casei* N. The single exception is *Lcb. chiayiensis* FBL7, carrier of operon CAS-Z, which has a different scenario upstream and downstream. Such genes have not been found in other microbial operons associated with bacteriocin production. In fact, according to the literature, the fundamental genes correlated to bacteriocin production were the genes codifying bacteriocin, immunity protein, and ABC-transporter. Moreover, complementary factors in the mechanism of bacteriocin production found in native bacteriocin producer's bacteria were transmembrane histidine kinase (HK) and a transcriptional regulator (RR), which are both missing in all the strains which were the subject of the study. It was initially hypothesized that cytochrome d ubiquinol oxidase subunit, ammonium transporter and NAD(P)-binding domain-containing could influence the production of the bacteriocin. However, there is no evidence of these genes' involvement in bacteriocin regulation. The schematic representation of the operons for the bacteriocins predicted in each of the strains is shown in **Figure 3.1**.

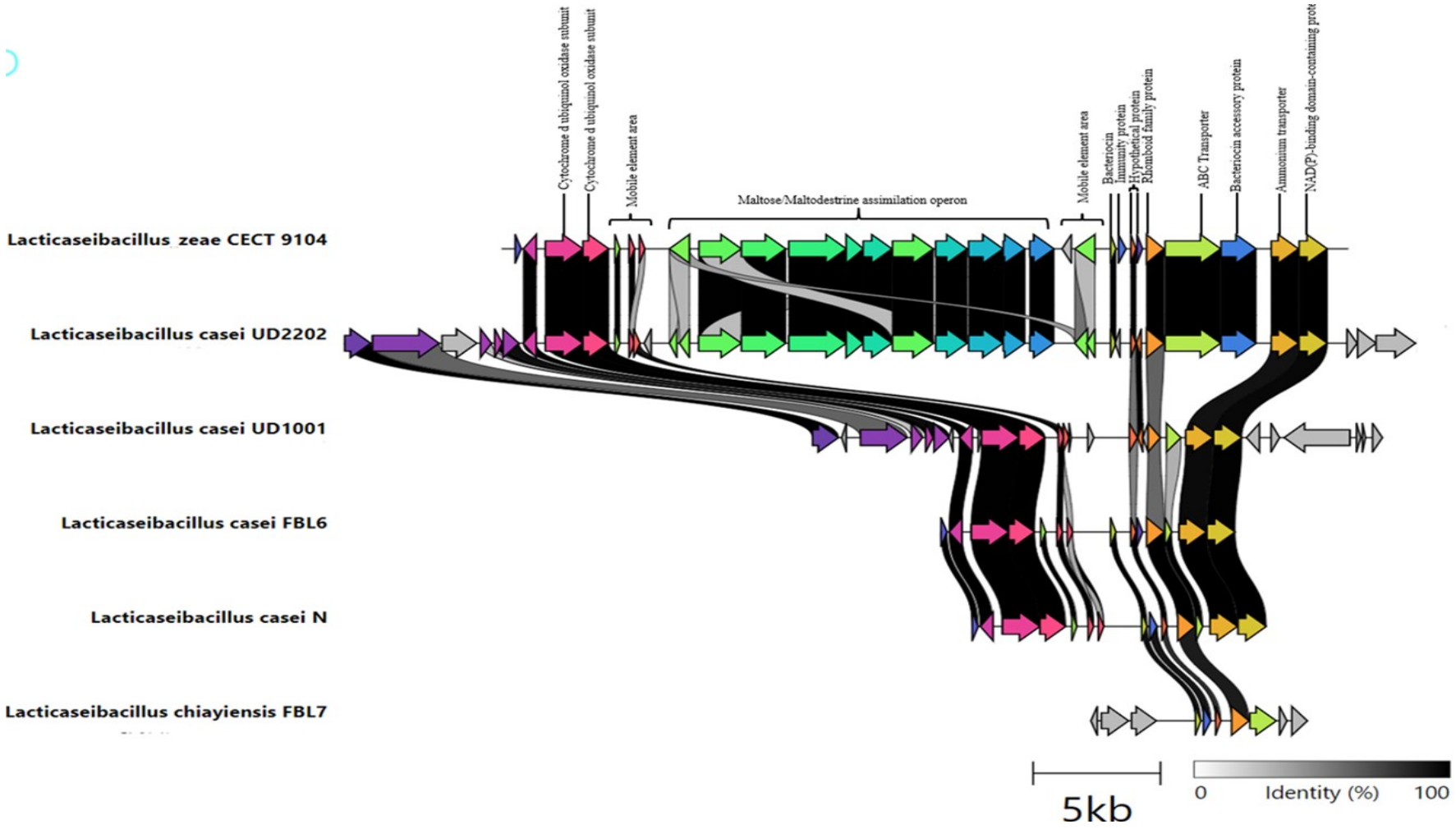


Figure 3.1: A schematic representation of the Caseicin FS gene clusters of operons detected in each strains investigated.

Another consideration is the presence only upstream of the CAS-X operon found in *Lcb. casei* UD2202 and *Lcb. zea*e CECT 9104, of an insertion related to the assimilation of Maltose/maltodextrin system. DNA sequence analysis shows that of all four operon includes the genes that encode a structural bacteriocin (*cas*) and its immunity protein (*im*), two hypothetical proteins (*hyp1* and *hyp2*), a Rhomboid protease (*rhop*), a dedicated ABC transporter (*trcs*) and in the case of CAS-X operon, an accessory bacteriocin protein (*accs*). Similarity and identity in nucleotidic sequence and reciprocal transcription in aminoacidic sequence of all operon variants are reported in **Table 3.5**.

Table 3.5: Summary of genes function in all Caseicin FS variant operon with the differences, average of the percentage of similarity and identity are also shown.

GENE FUNCTION	*Lcb. casei* UD2202[1]				*Lcb. casei* UD1001				*Lcb. casei* FBL6[2]				*L. chiayiensis* FBL7				% identity[×]	% similarity[×]
	gene (bp)		protein (aa)		gene (bp)		protein (aa)		gene (bp)		protein (aa)		gene (bp)		protein (aa)			
Bacteriocin	*casx*	210	casx	69	*casy*	210	casy	69	*casj*	210	casj	69	*casz*	210	casz	69	94,44	97,1
Immunity protein	*imx*	297	imx	98	*imy*	297	imy	98	*imj*	297	imj	98	*imz*	297	imz	98	95,92	97,45
Hypotetical protein	*hyp1x*	204	hyp1x	67	*hyp1y*	204	hyp1y	67	*hyp1j*	204	hyp1j	67	*hyp1z*	204	hyp1z	67	92,53	95,52
Hypotetical protein	*hyp2x*	216	hyp2x	71	*hyp2y*	216	hyp2y	71	*hyp2j*	219	hyp2j	72	*hyp2z*	225	hyp2z	74	77,29	81,25
Rhomboid family protein	*rhopx*	687	rhopx	228	*rhopx*	486	rhopy	161	*rhopj*	687	rhopj	228	*rhopz*	687	rhopz	228	79,53	87,135
ABC transporter	*trcsx*	2187	trcsx	728	*tcrsy*	587	tcrsy	188	*tcrsj*	255	tcrsj	84	*tcrsz*	870	tcrsz	289	31,1	32,65
Accessory bacteriocin protein	*accs*	1380	accs	459	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[×] average of the value calculated with www.bioinformatics.org

[1]*Lacticaseibacillus casei* UD 2202 and *Lacticaseibacillus* zeae CECT 9104 (LS991421-1) share the same operon organization

[2]*Lacticaseibacillus casei* FBL6 (CP074377.1) and *Lacticaseibacillus casei* N (CP077759.1) share the same operon organization

As previously described in Chapter 2, Caseicin FS-X and Caseicin FS-Y share the most distant amino acid chain related to the class IIa bacteriocins characterised by a conservative YGNGV motif which depicts the relationship between peptides belonging to this class. The same consideration is valid for variant Caseicin FS-J and Caseicin FS-Z bacteriocin due to their similarity. Each Caseicin FS variant contains 69 amino acids, with a disulfide bridge in their N-terminus due to the univariate presence of the amino acid cysteine (Cys) in positions 9 and 14 in the mature peptides. Differences in sequences found are reported in the alignment shown in **Figure 3.2**.

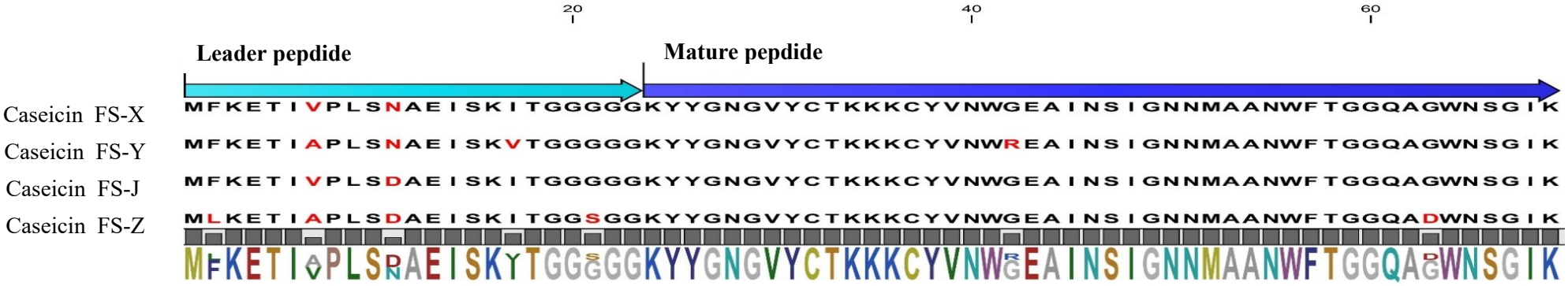


Figure 3.2: Aminoacidic sequence alignment of all Caseicin FS variants.

Refined operon prediction shows more than one putative structural gene involved in all versions of CAS operon, a high degree of conservation except for the dissimilarities in structure related to the ABC transporter possibly associated with Caseicin FS differences resulted mainly located in the leader peptide. **Figure 3.3** summarises the final alignment of all CAS operons.

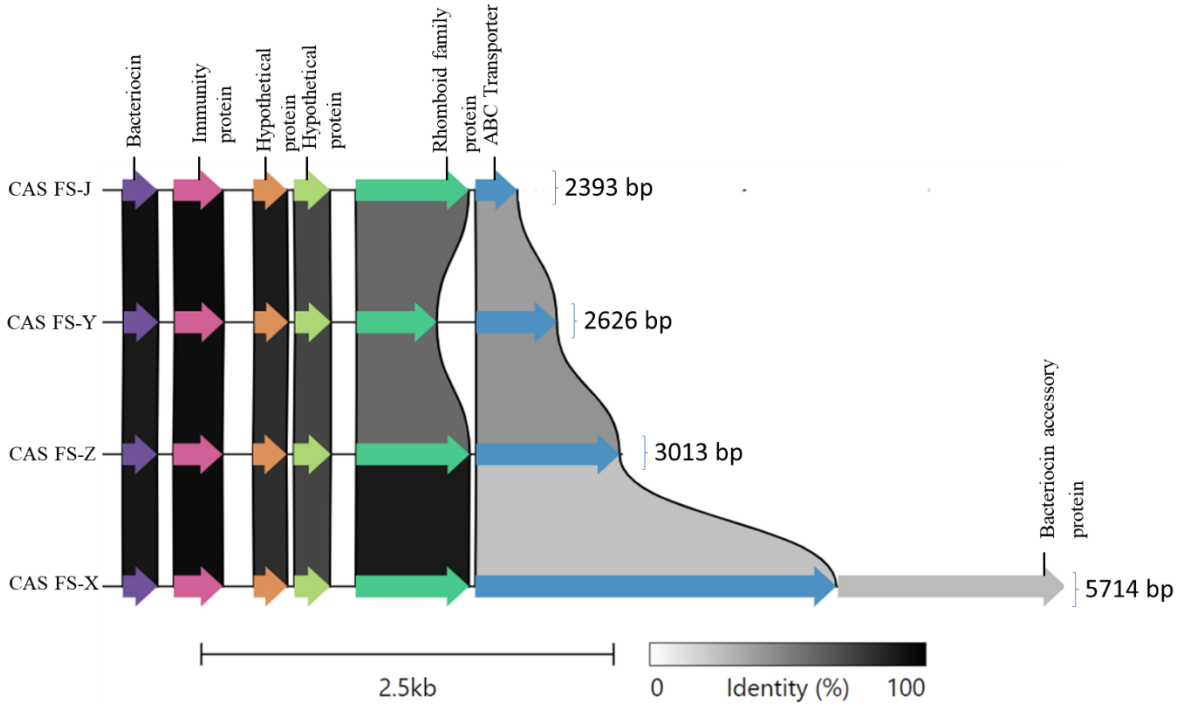


Figure 3.3: Alignment of Caseicin Biosynthetic gene clusters

Cloning of pNZ_CAS-X and pNZ_CAS-Y in a model strains *Lc. lactis* for producing Caseicin bacteriocin

The PCR results confirmed the presence in the genome and length of the two operons tested. Complete digestion of plasmid pNZ8048 and amplicons with BglII-KnpI resulted in a successful ligation and transformation of plasmid pNZ_CAS-X and pNZ_CAS-Y, which were designed as reported **in Figure 3.4.**

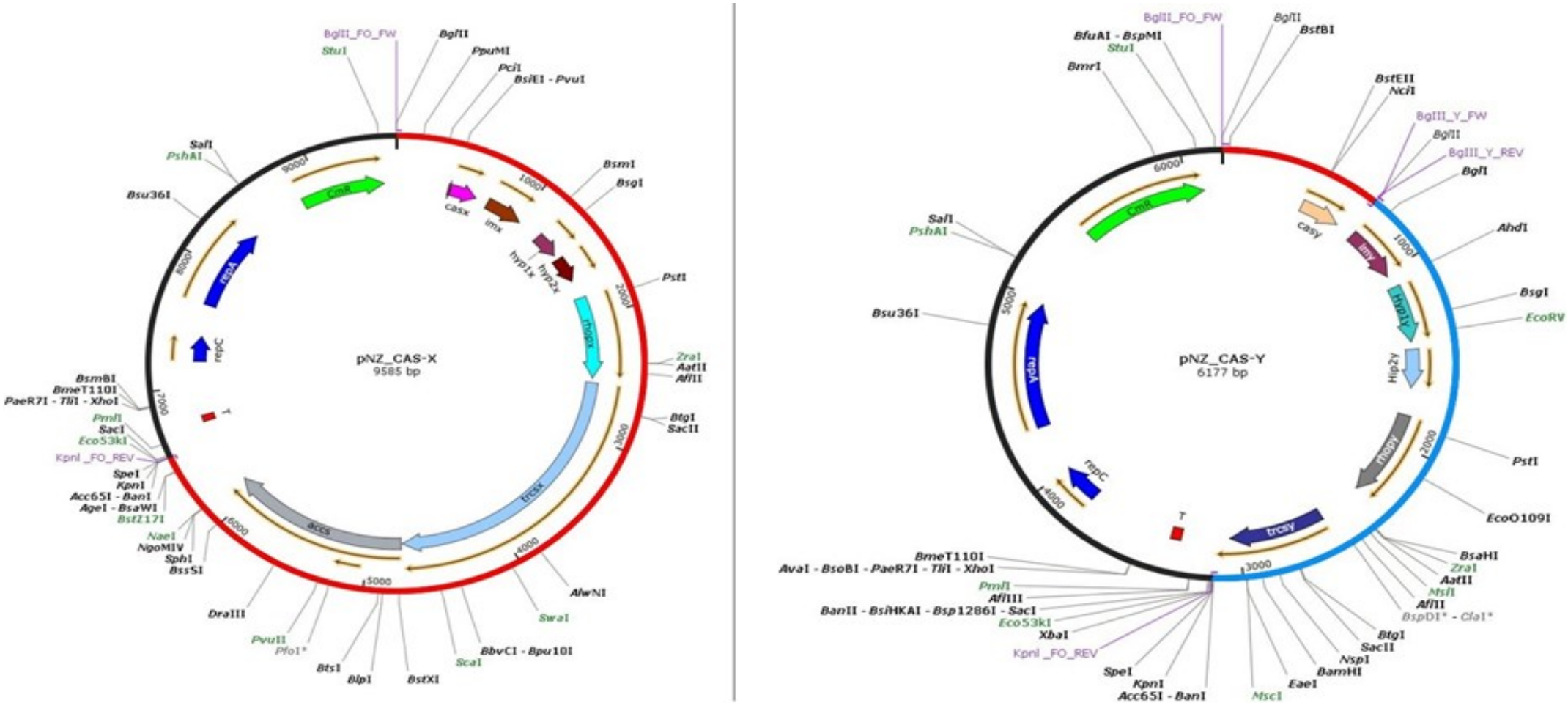
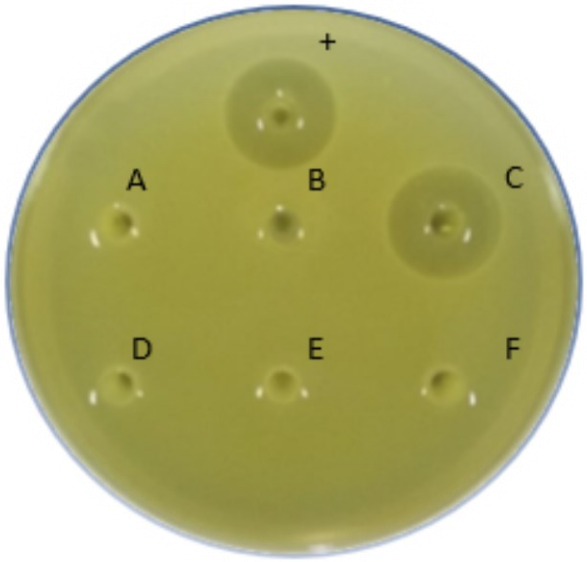


Figure 3.4: Schematic representation of the final vector design for the integration of operon CAS-X and CAS-Y operon. Relevant features are indicated, including restriction sites used for cloning; the *E. coli*/LAB *repA* and *repC* replication genes; the chloramphenicol acetyltransferase (*cat*) gene conferring resistance to chloramphenicol; the nisin-inducible *PnisA* promoter was removed by digestion.

Host colonies of *E. coli* MC1061 and *Lc. lactis* pNZ9000 before and after transformation with plasmid pNZ_CAS-X, pNZ_CAS-Y and pNZ8048 (used as a control) were incubated following the growing condition reported in the NICE manual—expression System. Antimicrobial activity was detected only after 48 – 72 h incubation in Lc. lactis pNZ9000 carrying plasmid pNZ_CAS-X, as shown in **Figure 3.5**.



Legend: +: Supernatant cell free pH adjusted from *Lactiplantibacillus plantarum* 423 strain (Plantaricin 423 producer); A: *Lactococcus lactis* PN9000; B: *Lactococcus lactis* PN9000-pNZ8048; C: *Lactococcus lactis* PN9000-pNZ_CAS-X; D: Supernatant cell free pH adjusted from *Lactiplantibacillus plantarum* 423 strain + Protease K (1 mg/ml); E: *Lactococcus lactis* PN9000-pNZ8048 + Protease K (1 mg/ml); F: *Lactococcus lactis* PN9000- pNZ_CAS-X + Protease K (1 mg/ml).

Figure 3.5: Inhibition of *Listeria monocytogenes* EDG-e by agar well diffusion assay. A 100 µL supernatant cell free pH adjusted volume was added to each well.

DISCUSSION

It is estimated that most LAB can produce at least one bacteriocin within a wide range of modes of action towards different target antagonists but related strains (Cotter, Hill & Ross, 2005). Most frequently, four genes are required for the production of class IIa bacteriocins, including a bacteriocin structural gene encoding a precursor, an immunity gene, genes encoding an ATP-binding cassette transporter accessory protein for extracellular translocation of bacteriocin (Drider et al., 2006). However, a simplest or more complex genome organization is also possible due to the different modes of regulation inducing bacteriocin production (Chanos & Mygind, 2016). Heterogeneity in amino acid sequence and related genes organization of class II bacteriocin is a not surprising phenomenon related to the evolution within and between species of bacteria. Based on our *in-silico* study, operons CAS-X, CAS-Y, CAS-J and CAS-Z share a unique and undescribed organization with unreferenced functionality also in the databases. During gDNA extraction of *Lcb. casei* UD1001 and *Lcb. casei* UD2202, any plasmid was detected, result in accordance with the authors Kim et al. (2022), which analyzed the *Lcb. casei* FBL6, *Lcb. chiayiensis* FBL7 genomes excluding the correlation of bacteriocins production with a small circular extrachromosomal DNA.

The possible implication as an induction factor for the two hypothetical proteins identified in all these operon variants, due to their short aminoacidic chain indicating the possible regulation of pheromone-based quorum sensing QS mechanism, was hypothesized. This assertion was supported by the results of the bioinformatics tools, which correlated the four operons under study to Sakacin P gene cluster (spp

locus). It is composed by a sakacin P precursor *SppA*, an immunity protein *SpiA*, an ABC transporter protein *SppT*, and an accessory protein *SppE*, which are highly regulated by QS mechanism, characterized by induction factor *SppIP*, histidine kinase (HPK) *SppK*, and a response regulator (RR) (Tichaczek, Vogel & Hammes, 1994; Hühne et al., 1996). However, in our case evidence collected through a comparative analysis upstream and downstream of all CAS loci did not reveal any association with the three-component regulatory system or other regulatory mechanisms. An interesting result was the presence in all operons of a Rhomboid protease classified as a rhomboid-like protease, whose role in bacteria remains unknown and is the first time the presence of these proteases in a bacteriocin loci is observed (Rather, 2013). It is intriguing to fully understand whether rhomboid proteases' (ubiquitous in bacteria but also in eukaria cells) functions are involved in the bacteriocin production quality control of polytopic membrane proteins in cooperation with other processive proteases, as reported by Began et al. (2020). In particular, this could be a double protease system involving Rhomboid protease and ABC transporter, which in all class IIa bacteriocins, is characterized by the presence of an intermembrane C39 protease responsible for the cleavage and secretion of the active mature peptide.

Focusing on the operons, ABC transporter genes resulted in the most variable, which differences in translation are referrable to the transmembrane domain (IPR011527) and ATP-binding domain, whereas the C39 motif was conserved in all genes loci (Wilkens, 2015).

Only the CAS-X operon possesses the gene *trcsx,* whose transcript resulted in a complete ABC transporter composed of 728 aa, including the C39 protease motif, transmembrane domain and ATP-binding domain. These profound variations in an aminoacidic sequence have never been reported in all class IIa biosynthetic gene clusters (BGCs) and could be the cause of a loss in enzyme dual-function functionality. **Figure 3.6** summarized with a 3D visualization the differences between the dedicated ABC transporters.

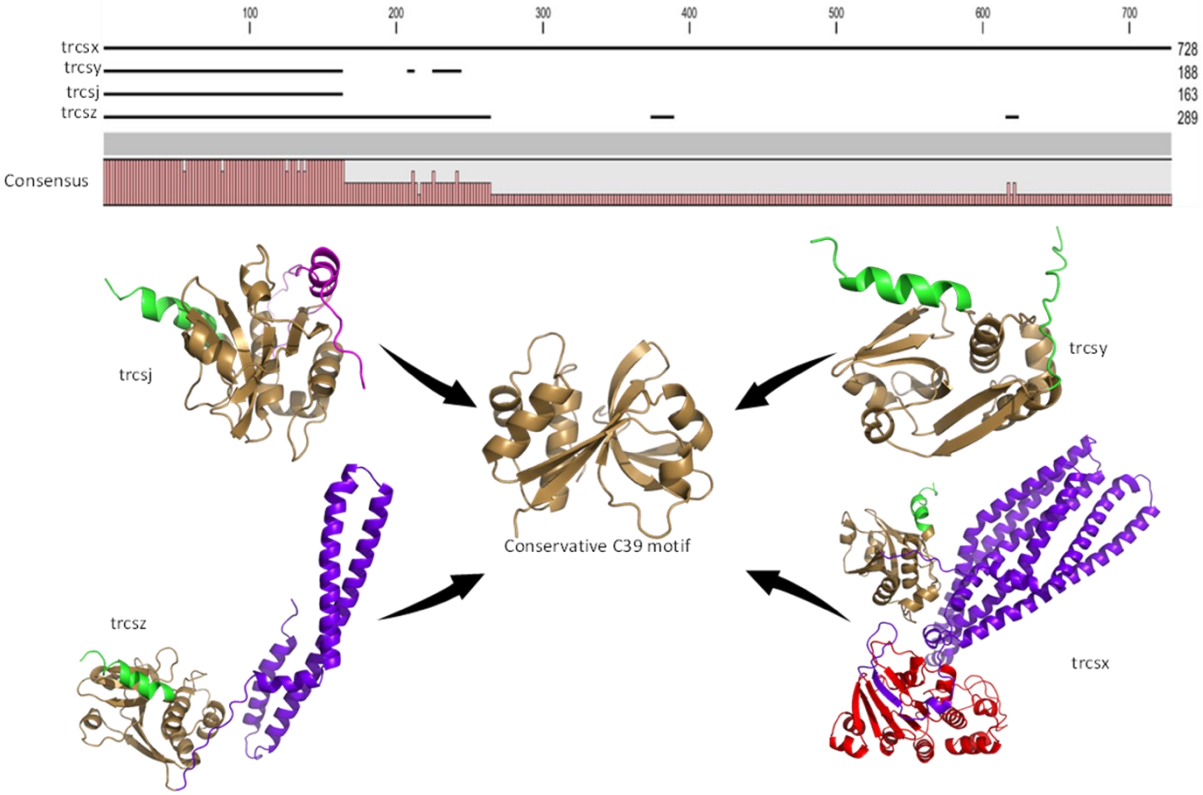


Figure 3.6: Alignment and Predicted 3D structures for monomeric trsx, trsy, trsj, trsz found respectively in operon CAS-X, CAS-Y, CAS-J and CAS-Z. Legend: gold (C39 motif), purple (Transmembrane domain), red (ATP-binding domain).

The secondary structure of peptides member of class II bacteriocins are characterized by one or more internal disulfide bonds shaping the correct folding.
The presence of accessory protein in pediocin-like bacteriocins still has an undefined function. However, Oppegård et al. (2015) showed the involvement of the accessory protein in forming the correct disulfide bonds in pediocin PA-1(Oppegård et al., 2015). Noteworthy, only in operon CAS-X is a dedicated accessory protein (accs) was present. Protein accs do not contain a CxxC motif, typical for the pediocin-like with only one disulfide bond, which is the case of all Caseicin FS variants. These results may indicate that accs proteins can play an important, but not essential, unidentified role in secreting Caseicin FS bacteriocins, due to the absence of the accessory protein in the genetic organizations of CAS-Y, CAS-J and CAS-Z operons.

By aligning the entire area of interest (AOI) upstream of the genes loci related to Caseicin FS production, a singular and identical transposable element was highlighted in CAS-Y, CAS-J and CAS-Z. Differently, upstream of CAS-X genes loci, five transposases were identified. Between them, the operon related to the assimilation of maltose/maltodextrin was located. As many transposons, encoding additional functions such as antibiotic resistance and virulence factors, their dissemination among species (carried by plasmids and viruses) contributed to the sharing of the bacterial gene pool (Curcio & Derbyshire, 2020). Some elements' transposition can result in flanking DNA's transduction, providing yet another means of rearranging host genes (Muñoz-lópez & García-pérez, 2010). *Lcb. casei* UD2202 and *Lcb. zeae* CECT 9104 share the CAS-X genes loci as well as an uncharacterized DDE-transposons system, generally associated with the 'cut-and-paste' mechanism. The DDE motif is responsible for excising the transposon from the donor and its integration into the target (Nesmelova & Hackett, 2010). A possible consequence of the apparent nucleotidic insertion is the downregulation of the entire bacteriocin operon CAS-X.

On the contrary, only one residual transposase is missing in the DDE motif and is not supposed to influence the transcription of CAS-Y, CAS-J and CAS-Z operons.
In silico interpretation of the deletion, in *Lcb. casei* UD2202 and *Lcb. zeae* CECT 9104 strain, of the mobile element, including the hypothetical insert, is proposed in **Figure 3.7**. The entire AOI of *Lcb. casei* UD2202 and *Lcb. casei* UD1001 was used as a model for this visual reconstruction, which shows that the two AOI appear specular after the elimination of the insertion of the maltose/maltodextrin operon.

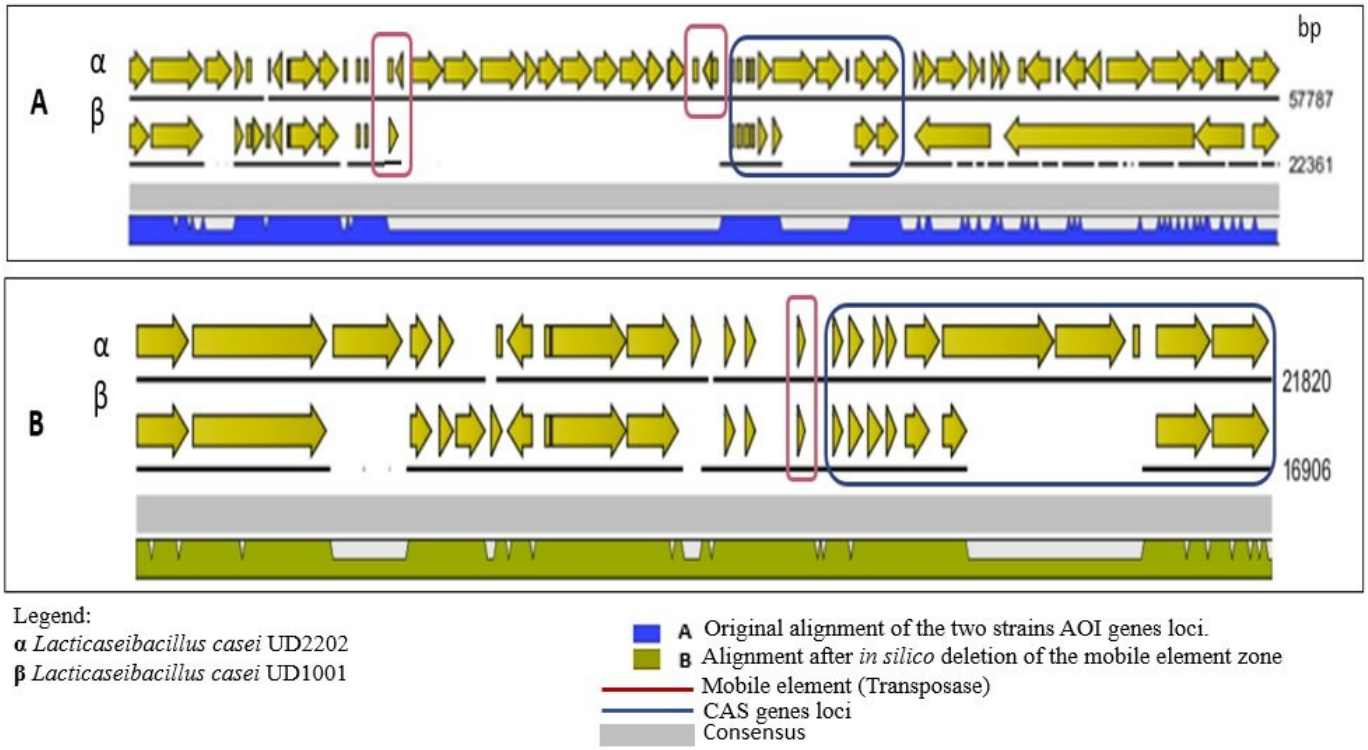


Figure 3.7: Schematic visualisation of the cut-and-paste insertion sequence deletion results in the same upstream genomic sequence between Caseicin operon variants. Operons CAS-Y and CAS-Z follow case β.

Concerning the bacteriocin production of wild strains, *Lcb. casei* UD1001 and *Lcb. casei* UD2202, none of the environmental factors tested resulted effective for activating the bacteriocin production. Many bacteriocins are likely to be produced in defined media and only to obtain appreciable amounts, the composition of such media may require some optimization (Møretrø et al., 2000). These results suggest that these strains are not bacteriocins producers. Nevertheless, metabolic regulation may still be crucial for production, and further systematic and well-controlled studies are required. According to the *in-silico* study, as mentioned before, the CAS-X operon can be inhibited by the mobile element, and operon CAS-Y is missing in the transmembrane and ATP-binding domains subunits in the ABC-transporter. However, authors Kim et al. (2022) reported the strains *Lcb. casei* FBL6 and *Lcb. chiayiensis* FBL7 as Sakacin P producers able to explicate their antimicrobial activities against *L. monocytogenes* ATCC 19111, *B. cereus* ATCC 21772, *E. coli* O157:H7 ATCC 43894, *E. coli* O1:K1:H7 ATCC 11775, *S. Enteritidis* ATCC 4931 and *L. ivanovii* ATCC 19119 (Kim et al., 2022).
In contrast with their findings, a deeper study demonstrates that *Lcb. casei* FBL6 and *Lcb. chiayiensis* FBL7 are carriers of the gene's loci variants CAS-J and CAS-Z, which also have a naturally truncated ABC transporter. In addition, class IIa bacteriocins can differ in their antimicrobial against Gram-positive bacteria. However, there is no evidence of Gram-negative bacteria being sensitive to these peptides, making it difficult to correlate bacterial inhibition to Cas FS-J and Cas FS-Z production.

The genes loci for Caseicin FS-X and Caseicin FS-Y were used in model strains *Lc. lactis*. The modified NICE system combined with *Lc. lactis* has already succeeded in expression study involving integral membrane and whole operon (Kunji, Slotboom & Poolman, 2003; Brian et al., 2000).
Successful cloning of the complete CAS-X and CAS-Y operons confirmed the organization of the genes found in strains *Lcb. casei* UD2202 and *Lcb. zeae* CECT 9104, being able to transcribe and secrete the bacteriocin of interest. This also supports the observation that the evolution of the strains *Lcb. casei*

UD2202 and *Lcb. zeae* CECT 9104 implying the specific insertion of mobile element and the presence of a naturally truncate ABC transporter could lead to a loss in bacteriocin production.

CONCLUSION

Bacteriocin mining and *in silico* genome analysis reveal the distribution and differences in different LAB strains of the undescribed biosynthetic genes cluster of Casein FS, a novel bacteriocin belonging to the class. The novel bacteriocin Caseicin FS is present in four different variants, including the genes governing the mode of regulation and secretion of these peptides. Besides differences extrapolating the observations, mobile elements may be the main effect of a down-regulation of operon CAS-X, which has been proven in a host plasmid-based system without the presence of transposase is able to express the ability to produce Caseicin FS-X. In addition, the discrepancy between the typical structure of bacteriocin and the lack of accessory bacteriocin protein could explain the witch ability in terms of bacteriocin production is still underway topic of investigation. Furthermore, this study provides novel information about a new bacteriocin system, especially the presence of a Rhomboid protease involved in antimicrobial peptide production, which function is still unknown.

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CHAPTER 4

Insight into processing and export of peptide Caseicin FS using the native protease

ABSTRACT

Bacteriocins have several modes of regulation, related to their structure and modifications, essential for the peptide to be released and made active its antimicrobial capabilities. Many non-lantibiotic bacteriocins of lactic acid bacteria are produced as precursors, which have N-terminal leader peptides that share similarities in amino acid sequence and contain a conserved processing site of two glycine residues. A dedicated ATP-binding cassette (ABC) transporter is responsible for the proteolytic cleavage of the leader peptides and subsequent translocation of the bacteriocins across the cytoplasmic membrane. The production of bacteriocins as recombinant proteins has been an implemented technology for decades. Extensive set of expression vectors and strains are already available aiming to make antimicrobial peptides more available for food or pharma applications. A plasmidic vector is an easily customized component when the goal is to engineer bacteria in order to produce a heterologous compound in industrially significant amounts. However, the application of native protease meets the necessity to increase efficiency and specificity in cleavage when required. In this study, we aimed to elucidate the effectiveness of cleavage properties of the two dedicated ABC transporters found in the gene's loci of the variants Caseicin FS-X and Caseicin FS-Y. The co-expression of each protease with the related Caseicin was evaluated to investigate the role of ABC transporters found in operons CAS-X and CAS-Y. We report the lack of enzymatic activity of the ABC transporter from *Lcb. casei* UD1001, whose aminoacid backbone includes only the C39 protease domain. In contrast, the effectiveness of the ABC transporter of *Lcb. casei* UD2202 in the liberation and secretion of mature Caseicin FS-X was confirmed. These findings confirm the hypothesis of the non-functioning bacteriocin loci with naturally truncated ABC transporter of *Lcb. casei* UD1001 is unable to complete the antimicrobial peptide's maturation and secretion.

KEYWORDS: ABC transporter, secretion, bacteriocin maturation, co-expression

INTRODUCTION

Bacteriocins have been classified into two major groups: class I, in which peptides undergo post-translational modifications, and class II peptides, which remain unmodified (Cotter, Ross & Hill, 2013). Most non-lantibiotics and some lantibiotics have leader peptides of the so-called double-glycine type. The double-glycine-type leader peptides guide secretion through the cytoplasmic membrane by ABC transporters (Van Belkum, Worobo & Stiles, 1997). Based on their structure and the signal sequence of their cognitive substrates, bacteriocin secretion transporters in gram-positive bacteria are divided into NisT-type transporters, SunT-type transporter, sec-dependent pathway and other uncharacterised transporters (Zheng & Sonomoto, 2018).

SunT group proteins contain an additional N-terminal protease to form an integrated bifunctional transporter, named AMS protein, sometimes referred to as Type 1 secretion systems (Håvarstein, Diep & Nes, 1995; Thomas, Holland & Schmitt, 2014). Based on structural analysis, SunT transporters have been demonstrated to consist of three major regions: an N-terminal peptidase domain (PEP) for leader peptide cleavage, a highly conserved C-terminal ATP binding cassette for ATP hydrolysis, and a less conserved hydrophobic transmembrane domain (TMD) inserted across cell membranes leading to the export of the substrate. As a general organization model, the structure of class II bacteriocins is represented by an N-terminus domain with β fold-like structure and a C-terminus domain composed of 1 or 2 α-helix structures, which continue with a C- terminus sequence, stabilized by one or more disulphide bridge (R−S−S−R′). This linkage is also called an SS-bond formed by the coupling of two thiol groups in two cysteine residues building blocks to create the molecular architecture of the secondary and tertiary structure of proteins (Wiedemann et al., 2020). Interconnection within ABC transporters, accessory protein and the number of disulphide bridges were described in the pediocin-like group (Oppegård et al., 2015). Specific peptide cleavage sites Gly-Gly in governate by endopeptidases of family C39 (IPR005074), representing the N-terminal domains of larger proteins (ABC transporters). This group of sequences, defined by the cysteine peptidase domain, belong to the MEROPS peptidase family C39 (clan CA) and have been proposed to be essential for substrate recognition and processing pediocin-like bacteriocins (Rawlings & Barrett, 1995; Wu & Tai, 2004; Rawlings et al., 2018). The biosynthesis and secretion of Class II bacteriocins are synthesized as prebacteriocins where leader peptide N-terminus end with a Gly-Gly motif, cleaved off specifically during the extra-cellular transfer of the molecule through the membrane. Functions of the leader peptide has been described as a dual function in the biosynthesis process to maintain the inactive state and to trigger a recognition signal for the molecule orientation toward the specific ABC transporter (Ditu et al., 2016).

After an *in-silico* evaluation was noted that bacteriocins Caseicin FS-X, Caseicin FS-Y, Caseicin FS-J and Caseicin FS-Z share an atypical -Gly-Gly-Gly-Gly-Gly/Ser- motif in combination with a unique operons' organization, which mainly differ in the length of the respective ABC transporters, whereas the C39 peptide motif is the only conservative component. This work investigated the functionality of Caseicin FS-X and Caseicin FS-Y ABC transporter specifically found in *Lcb*. UD2202 and *Lcb.* UD 1001 to elucidate their role in bacteriocin processing machinery.

MATERIAL AND METHODS

Comparison of the transporter trcsx and trcsy: *In silico* analysis

The putative open reading frame (ORF) *trcsx* and *trcsy* found in operons CAS-X and CAS-Y respectively, codifies trcsx and trcsy ABC transporters which where the query for the similarity searches at the level of amino acid sequences, which were made by BlastP programs (Boratyn et al., 2013), at NCBI database (Altschul et al., 1997) using the default setting. The multiple sequence alignment (MSA) Clust Omega tool was used to generate alignments (Edgar, 2004) and data sets visualisation was elaborated with the Interactive tree of life (iTOL) tool (Letunic & Bork, 2021). A transmembrane region and protein motif sequence were determined using DeepCoil, MembranFold and HMMER version 3.3.2 web server (Potter et al., 2018; Ludwiczak et al., 2019; Gutierrez et al., 2022).

Bacterial strains and culture media

All cloning experiments were done in chemically competent *E. coli* BL21 (DE3) strain grown in, brain heart infusion (BHI) broth or solid agar (1.5% w/v) (Biolab Diagnostics, Midrand, South Africa) and incubated at 37 °C with rotary shaking at 200 rpm. The LAB strains *Lacticaseibacillus casei* UD2202, *Lacticaseibacillus casei* UD1001 were grown as static cultures at 30 °C in MRS broth (Biolab Diagnostics) or on MRS agar plates. *Escherichia coli* BL21 (DE3) containing plasmids were cultured in LB or BHI medium and supplemented with kanamycin 50 µg/mL or chloramphenicol 25 µg/mL as selective antibiotics for pRSF and pACYC-constructs, respectively for the selection and maintenance of plasmids. *Listeria monocytogenes* EGD-e was grown in BHI media supplemented with 7.5 µg/ml Cm and incubated at 37 °C on an orbital shaker (200 rpm).

Plasmid design and cloning of leaderless bacteriocins with and without GFP and ABC transporter

Procedures described by Sambrook et al. (1989) were followed for all DNA studies and manipulations. DNA extract from *Lacticaseibacillus casei* UD2202 and *Lacticaseibacillus casei* UD1001 was used as a template to obtain amplicon of genes *casx, casy, trcsx* and *trcsy*. Genomic DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep kit from Zymo research (USA, CA). All PCRs were performed using the Q5 high fidelity polymerase (NEB) according to the manufacturer's instructions, together with the GeneAmp PCR system 9700 from Applied Biosystems (ABI, Foster City, CA) according to the manufacturer's instructions. The annealing temperatures of the primers were determined using the NEBs TM calculator (http://tmcalculator.neb.com). Restriction enzyme digest analysis and electrophoretic purification of DNA were performed on 1 % agarose gels at 10V/cm in TBE buffer using the EphortecTM 3000V (Triad Scientific, Manasquan United States) apparatus. Restriction enzymes and T4 DNA ligase were sourced from New England Biolabs (NEB, Ipswich, MA, USA) and used in cloning procedures according to instructions as described by the manufacturer. All restriction enzymes, DNase, RNAse, Q5 polymerase and T4 ligase were from New England Biolabs (USA, MA). PureYield plasmid extraction Miniprep kits were from Promega (USA, WI). DNA fragments were excised from agarose gels and purified using the Zymoclean™ gel DNA Recovery kit (Zymo Research, USA, CA). Confirmation of inserted genes in the plasmid was performed by sequencing at the Central Analytical Facility (CAF) at the University of Stellenbosch.

The pRSFDuet-1 (Novagen) and pACYCDuet-1 (Novagen) vectors were used for the β–D–1-thiogalactopyranoside (IPTG) induction of the heterologous expression system in this study. According

to the authors (Van Staden et al., 2019; Vermeulen, Van Staden & Dicks, 2020) the N-terminal His6-tag vectors pRSF-GFP or C-terminal His8-tag and pRSF _Nisp_Mcherry8xHis were used.

Both multiple cloning sites were involved in constructing plasmids used for the heterologous co-expression system. A description of the final plasmids and primer sets is provided in **Table 4.1**. Single colonies were selected, inoculated in LB broth supplemented with the respective antibiotics, and incubated overnight at 37 °C. Plasmid DNA was isolated and used for sequencing reactions, cloning and transformation.

Table 4.1: Bacterial strains, plasmids and primer used in the investigation of the role of the ABC transporter.

Description, characteristics, or sequence (5'→3') forward primer, reverse primer		**Source or reference**
Plasmid		
pRSF-GFP	Shuttle vector, Kan*	Vermeulen et al. (2020)
pRSF _Nisp_Mcherry8xHis	Shuttle vector, Kan*	Van Staden et al. (2019)
pRSF Duet-1	Vector with the IPTG inducible PT7, Km* and cloning site for N-terminal His-tag fusion.	Novagen Africa, Bloemfontein, South Africa
pACYC Duet-1	Vector with the IPTG inducible PT7, Cm* and cloning site for N-terminal His-tag fusion.	Novagen Africa, Bloemfontein, South Africa
pRSF-GFP- Precaseicin_X	Vector producer 6xHis-tag-GFP-Precaseicin FS-X (*casx*)	This work
pRSF-GFP- Precaseicin_Y	Vector producer 6xHis-tag-GFP-Precaseicin FS-Y (*casy*)	This work
pRSF-C39MOTIF	Vector producer C39 MOTIF-Mcherry-8xHis-tag peptide	This work
pRSF-C39X	Vector producer 6xHis ABC transporter (*trcsx*)	This work
pRSF-C39Y	Vector producer 6xHis ABC transporter (*trcsy*)	This work
pRSF_GFP_Precaseicin_X_ABC6	Vector producer of *casx* and *trcsx* genes	This work
pRSF_GFP_Precaseicin_Y_ABC3	Vector producer of *casy* and *trcsy* genes	This work
pACYC_Precaseicin_X_ABC6	Vector producer of *casx* and *trcsx* genes	This work

pACYC_Precaseicin_X	Vector producer of *casx*		
pACYC_Precaseicin_Y_ABC3	Vector producer of casy and trcsy genes		This work
pACYC_Precaseicin_Y	Vector producer of *casy*		
Strain			
Lacticaseibacillus casei UD 2202	Strain under study with *cas-x trcsx* genes		This work
Lacticaseibacillus casei UD 1001	Strain under study with *cas-y trcsy* genes		This work
E. coli BL21 (DE3)	Expression host		
Primer		**Tm (◦C)**	
Pst_precaseicin_FW	GGAACTGCAGATGAGTACAAAAGA	56	This work
Hind_REV_casx-y	GCAAAGCTTACTTGATGCCAGAATTC	56	This work
NCOL_PRSF FO3	ACTGCCATGGTCAAAGAAACAATTGCACC	64	This work
NCOL_PRSF_FO6	AGTCCATGGTCAAAGAAACGATCGTACC	64	This work
ABC3_KNPI_RV	GCAAAGCTTACTTGATGCCAGAATTC	64	This work
ABC6_KNPI_RV	GACAAGCTTTGGCAATCATCAAAGAATT	64	This work
BglI_FW_C39-2	GCCAGATCTGATGCTTAATATTAAATATGGATTCG	64	This work
Ncol_C39_MOTIF_FW	AGTCCATGGTGAATATTAAATATGGATTCG	61	This work
Hind_C39_MOTIF_REV	GTCAAGCTTTATATAATTCATCCATACCAC	61	This work
pRSFMCS1_F	GGATCTCGACGCTCTCCCT	63	Van Staden et al. (2019)
pRSFMCS1_R	GATTATGCGGCCGTGTACAA	63	Van Staden et al. (2019)

* Kan, kanamycin resistance

* Cm: chloramphenicol resistance

His-tag protein expression and purification

Protein expression and purification of His-tagged proteins were performed according to Van Staden et al. 2019 (Van Staden et al., 2019), with minor modifications. Overnight cultures of *E. coli* BL21 (DE3), which expressed the vectors pRSF-GFP, pRSF-GFP-Precaseicin_X, pRSF-GFP- Precaseicin_Y, pRSF-C39MOTIF, pRSF-C39X, pRSF-C39Y,pACYC _GFP_Precaseicin_X_ABC6,

pACYC_GFP_Precaseicin_Y_ABC3, and pRSF-C39Yconstructs separately, were used to inoculate (1.0% v/v) flasks containing 400 mL terrific broth supplemented with kanamycin 50 µg/mL. The cultures were then incubated at 37 °C under agitation (160 rpm) until an OD_{600} of 0.6-0.8 was reached. Thereafter, protein co-expression was induced by the addition of 0.1mM thio-B-D-galactopyranoside (IPTG) and incubated at 26 °C for 48 hours.
Cells were harvested (8000 x g, 20 min, 4 °C) and resuspended with proportion of 5 mL/g of wet weight of pellet in start buffer (SB, Tris 50 mM, NaCl 500 mM, pH 8.0). The start buffer was supplemented with lysozyme 1 mg/mL (Merck-Millipore, USA), DNase 1 U/mL (BioLabs, New England), and RNase 6 U/mL (BioLabs, New England). The cells suspension was incubated on ice for an hour and disrupted by sonication on ice (3 times at 70 % power output, 50 % pulses for 3 min) using Omni Ruptor 400 (Ultrasound Homogenizer, Omni International Inc., Kennesaw, GA). The lysed samples were centrifuged at 10000 x g for 20 minutes at 4 °C and the supernatant was collected and stored at -20 °C.

Protein purification was achieved using the ÄKTA purifier system (Amersham, Biosciences) system using prepacked immobilised metal affinity chromatography (IMAC) using the super-flow resin Ni-NTA (Qiagen, Germany). The supernatant from the soluble fractions was adjusted to a final imidazole concentration of 20 mM and loaded onto resin Ni-NTA columns pre-equilibrated with SB (Tris 50 mM, NaCl 500 mM, pH 8.0). and SB20. After loading, columns were washed with SB20 (Tris 50 mM, 20 mM Imidazole, NaCl 500 mM, pH 8.0). to remove non-specifically bound proteins. The His-tagged fusion proteins were eluted using SB500 (Tris 50 mM, 500 mM Imidazole, NaCl 500 mM, pH 8.0). Imidazole desalting was accomplished using DEAE Sepharose Fast Flow (GE Healthcare, USA). Elution was performed using 50 mM Tris pH 8.3- and 50-mM Tris 1M NaCl pH 7.5. Using the FPLC system, a flow rate of 2 ml/min, the peptides of interest detected by A220 were collected manually with 20 % of buffer corresponding to 50 mM Tris and 200 mM NaCl. Purified proteins were stored at -20 °C until further use.

Production and bacteriocin recovery in co-expression plasmid-based system

Overnight cultures of *E. coli* BL21 (DE3), which expressed the vector pACYC GFP_Precaseicin_X_ABC6, pACYC _GFP_Precaseicin_Y_ABC3, pACYC_Precaseicin_X and pACYC_Precaseicin_Y, separately, were used to inoculate (1.0% v/v) flasks containing 400 mL of terrific broth supplemented with chloramphenicol 25 µg/mL until an OD600 of 0.1 was reached. Thereafter, each proteins' expression was induced by adding 0.1 mM thio-B-D-galactopyranoside (IPTG) and incubated at 26 °C for 48 hours, after which cells were harvested (8000 x g, 20 min, 4 °C).

Cell-free supernatant (CFS) was added to previously activated-XAD-16 beads (10 g/100 ml) and placed on an orbital shaker (50 rpm) at 4 °C for 24 h. Beads were collected and washed with double-distilled water wash steps. Peptides bound to beads were eluted using 80 % Iso-TFA (vol/vol) and filtered through a 45 µM cellulose acetate filter. Isopropanol was removed using rotary evaporation (RotaVapor®, Buchi). The activation steps are described as follows: first, XAD-16 beads (Sigma-Aldrich, Germany) were washed using 80% isopropanol (Merck-Millipore, USA) containing 0.1 % trifluoroacetic acid (Iso-TFA) (vol/vol/vol) (Sigma-Aldrich, Germany) and then rinsed with double-distilled water before autoclavation at 121 °C for 15 min.

The resulting supernatant corresponds to the crude antimicrobial extract (CE). It was 10-times diluted in 50 mM Sodium Phosphate buffer (pH 6.8) and loaded in prepacked SP Sepharose Fast Flow resin

(GE Healthcare, USA) equilibrate with the same buffer with 0.15 M NaCl (Sigma-Aldrich, Germany) that was used to equilibrate the column. The proteins were eluted using 50 mM Sodium Phosphate buffer (pH 6.8) with 1 M NaCl. The elution was loaded onto a C18 Sep-Pak column (Waters, USA) and was washed with 5 column volume of Milli-Q water. Compound bound to C18 Sep-Pak column were eluted with 80% of Acetonitrile- Milli-Q water (Merck-Millipore, USA). The final elution was frozen at -80˚C, freeze-dried and stored at -20˚C until further use.

Peptides determination and detection of antimicrobial activities

Purified proteins were analysed using two Tricine-SDS-PAGE electrophoresis gels at 10 or 12% (stacking gel) and 4% (running gel) of acrylamide: bis-acrylamide 29:1 (Sigma-Aldrich, Poole, UK), resolved according to the protocol described in (Schägger, 2006) or according to (Gilbreth & Somkuti, 2005) when gel overlay technique was used.

Detection of antimicrobial activities was tested using the agar well diffusion assay. Wells were made using a sterilized 96-well PCR plates placed into melted 1 % BHI agar inoculated with *L. monocytogenes* EGD-e. The differently expressed GFP-Precaseicin and ABC transporter heterologously expressed and His-tag purified were added in ratio 1/1, 1/10, 1/100 and incubated 16-20 hours at 26 °C, 30 °C and 37 °C. Subsequently, 100 µl of each assay was loaded into the wells. Plates were incubated at 30 °C overnight until clear visible zones were observed.

Heterologous expression of Caseicin FS-X cleaved with NisP protease

The heterologous express Caseicin FS-X, which methodology of production and purification are described in Chapter 2, was used as a positive control to compare the size of Caseicin FS-X produce in co-expression as described and discussed in this Chapter.

RESULTS

ABC transporter characterization

The protein sequence of ABC transporters trcsx and trcsy are structured in 728 and 188 aa, respectively. Both share from position 13 to 150 the peptidase family C39 (pfam03412) subfamily with 100 % identity found in operon CAS-X and Cas-Y. The multidomain membrane proteins that utilise energy from ATP binding and hydrolysis to export substrates out of the membrane were found only in trcsx. Multi-alignment analysis performed into the NCBI database confirmed the spread of the ABC transporter between genera. However, the entire aa sequence of protein trcsx and trcsy, as is shown in **Figure 4.1**, resulted in having a conserved feature of this peptidase in database.

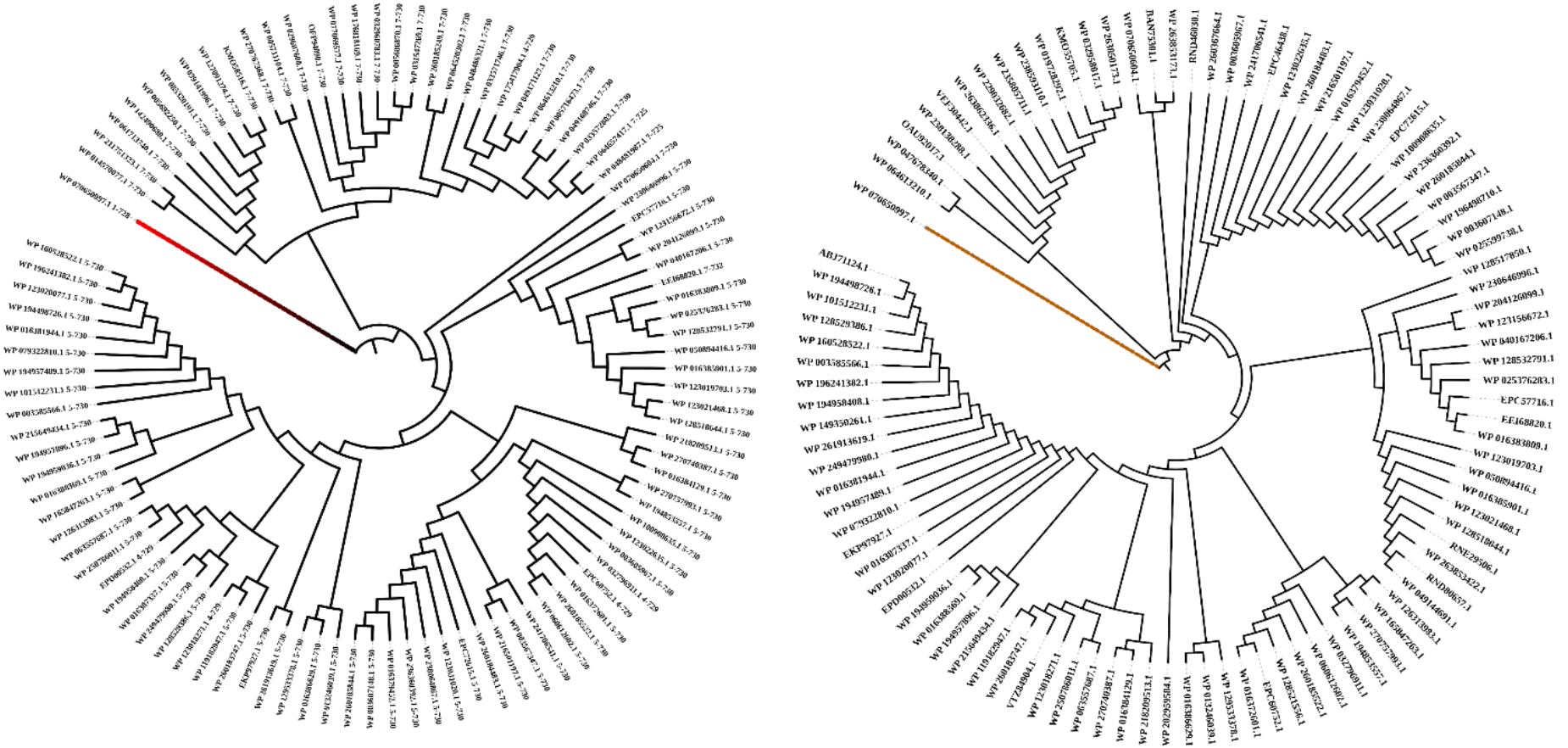


Figure 4.1: Cladogram of the first 100 more similar sequences to trcsx (red) and trcsy (yellow) ABC transpoters including the transmembrane domain. Legend can be found in Table 1-S and Table 2-S in the supplementary material.

The intermembrane predicting model tools' command line also supported these first results confirming the trcsx protein architecture. Differences include the six-transmembrane helical domain (6-TMD) of the peptidase-containing ATP-binding cassette transporters (PCATs) and the common C39 domain also found in trcsy. The outcome of the protein prediction structural analysis is summarised in **Figure 4.2**.

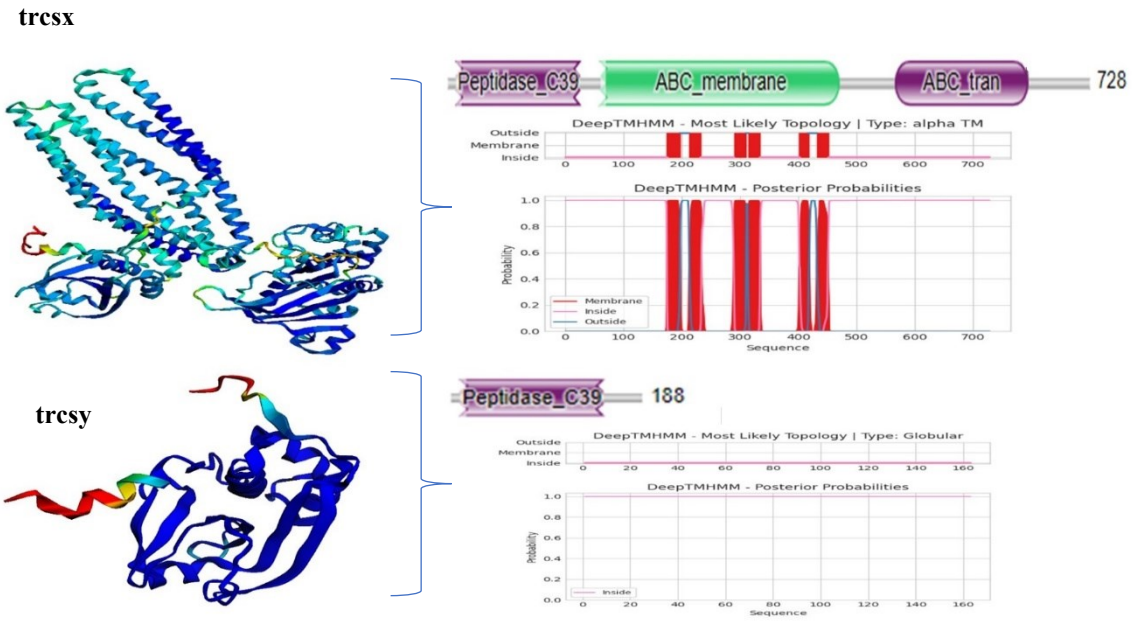


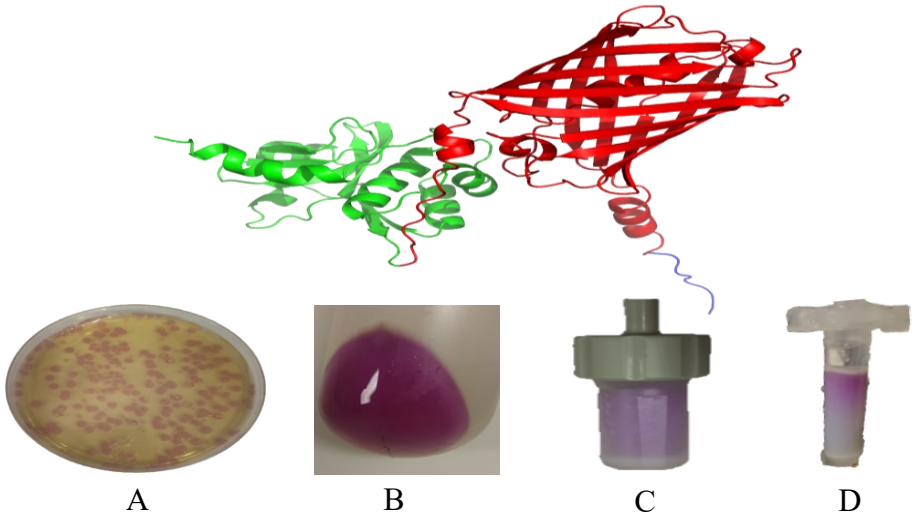
Figure 4.2: ABC transpoters 3D prediction model propose in according with confidence color plDDT from MembranFold. Legend: pLDDT > 90 (blue) high accuracy, pLDDT 70-90 (green - turquoise) generally good backbone prediction, pLDDT 50-70 (yellow) low confidence, pLDDT < 50 (red) not interpretable. Transmembrane region and protein motif sequence also reported.

Cloning, induction, and purification of bacteriocin

The PCR results confirmed the presence in the genome and length of *casx, casy, trcsx* and *trcsy* genes. Sequencing results validated that the correct genes were successfully inserted in the appointed MCS in all plasmids previously described for cloning and expression of proteins through control of expression by the T7 phage promoter inducible by IPTG. Heterologous expression and purification of 6xHis-tag-GFP-Precaseicin FS-X, 6xHis-tag-GFP-Precaseicin FS-Y and 6xHis-tag-GFP (used as control) were achieved following the method described. Initial attempts to express *trcs*x and *trcs*y genes cloned in pRSF-C39X, and pRSF-C39Y plasmids under a constitutive promoter in *E. coli* were unsuccessful. Based on this observation, a pRSF-C39MOTIF plasmid producer C39 MOTIF-Mcherry-8xHis-tag peptide was designed to evaluate the steps of expression and purification. Intentionally, only the homolog peptidase C39 motif was expressed, in order to then test the efficiency in cleaving Caseicin FS-X and Caseicin FS-Y. Adding mCherry genes led to visually localising the protein during heterologous expression and purification. Once the chimeric plasmid was constructed, the host cell of *E coli BL21* showed the ability to produce C39 MOTIF-Mcherry-8xHis-tag peptide after induction.

On the other hand, thanks to the presence of the red fluorescent protein, issues during extraction and elution steps from His-tag and ion exchange purification were detected. The result suggested the interaction of the protein C39 MOTIF with the cell membrane due to *E. coli* causing the loss of the majority expressed protein of interest. Secondly, nonachievement in elution and recovery of captured His-tagged protein from an HisTrap HP and DEAE Sepharose Fast Flow was also observed. **Figure 4.3** summarises the steps of production for the C39 MOTIF-Mcherry-8xHis-tag peptide.

C39 MOTIF-Mcherry-8xHis-tag peptide

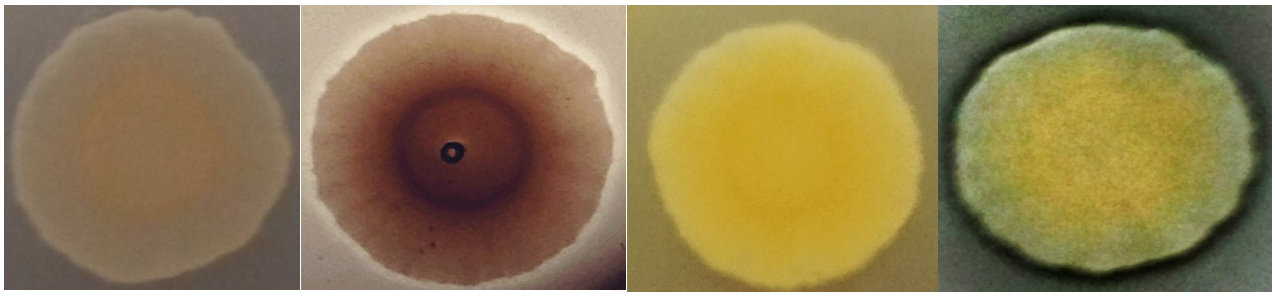


A B C D

Figure 4.3: Summary of the heterologous expression and purification step in this study causing the loss of peptide C39 MOTIF-Mcherry-8xHis-tag peptide. Legend: A-transformation of plasmid pRSF-C39MOTIF in *E. coli* BL21 host strain, B- *E coli* BL21 host cell lysate after centrifugation, C- HisTrap HP column after elution with SB500 buffer, D- DEAE Sepharose Fast Flow column after elution with 1M Tris 1M NaCl pH 7.5.

Overall, the protease properties theoretically involved in the maturation of Caseicin FS bacteriocin using this expression system and purification methods couldn't be properly evaluated. However, a partial interaction between the C39 MOTIF-Mcherry-8xHis-tag peptide and the cell membrane was observed. This phenomenon could be explained by the membrane protein to which the C39 motif belongs. Based on this evidence, a different expression system using a co-expression approach involving bacteriocin and ABC transporter genes into the same plasmid under the T7 phage promoter inducible by IPTG was chosen.

To this purpose, plasmids pACYC_Precaseicin_X, pACYC_Precaseicin_Y, pACYC_Precaseicin_X_ABC6, pACYC_Precaseicin_Y_ABC3, pACYC_GFP_Precaseicin_X and pACYC_GFP_Precaseicin_Y were design. Among these plasmids, only the pACYC _GFP_Precaseicin_X_ABC6 and pACYC_Precaseicin_X_ABC6 were able of bacteriocin secretion detected with an overlay layer of BHI soft agar inoculated with *L. monocytogenes* EDG-e in top of the host plasmid colony as indicated in **Figure 4.4**.



pACYC_Precaseicin_X pACYC_Precaseicin_X_ABC6 pACYC_Precaseicin_Y_ABC3 pACYC _GFP_Precaseicin_X_ABC6

Figure 4.4: *E. coli* BL21 colony expressing leaderless Caseicin bacteriocin alone was used as a control. Co-expression of leaderless Caseicin bacteriocin with ABC transporter trcsx or trcsy was tested to the ability to secrete the related mature peptide. Transformant cell co-expressing ABC transporter trcsx overlayed *with L. monocytogenes* EDG-e showed bacteriocin secretion.

Consequently, the transformants expressing pACYC_GFP_Precaseicin_X_ABC6 and pACYC_Precaseicin_Y_ABC3 plasmid were subjected to protein purification to recover the overexpressed GFP- Precaseicin_X in order to evaluate lost in size due the liberation of mature Caseicin FS-X. The three-step purification flow using Amberlite XAD-16, SP Sepharose and C18 SPE allowed the mature Caseicin FS-X recovery from cell-free supernatant of *E. coli* after induction of expression of pACYC _GFP_Precaseicin_X_ABC6 and pACYC_Precaseicin_X_ABC6 plasmid. Mature purified Caseicin FS-X antimicrobial activity was detected using a well-agar diffusion assay and overlayed Tricine-SDS-PAGE. **Figure 4.5** summarises the antimicrobial activity of the recovered Caseicin FS-X cell free supernatant cleaved from trcsx protease and overlaid SDS-PAGE gel.

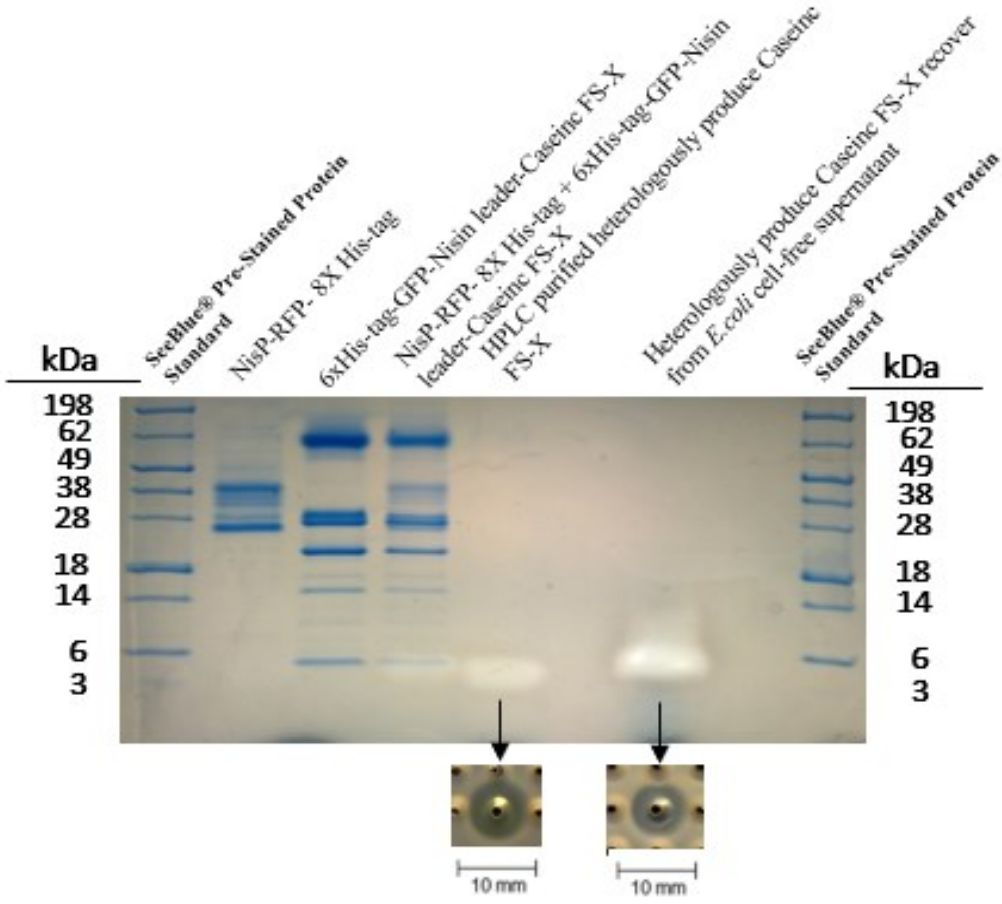


Figure 4.5: Desteined SDS-PAGE gel over an SDS-PAGE gel overlaid with BHI soft agar inoculated with *L. monocytogenes* EDG-e as a target strain.

Co-expression of heterologous ABC transporter *trcs*x in combination with GFP_Precaseicin_X reveals a reduction in protein production efficiency compared to ABC transporter *trcs*y. However, there was no evidence of variation in bacteriocin production between the pACYC_GFP_Precaseicin_X_ABC6 and pACYC_PreCaseicin_X_ABC6 plasmid.

In support of the evidence related to Caseicin FS-X maturation and secretion by pACYC_GFP_Precaseicin_X_ABC6 in *E coli* BL21, the purified GPF differences were finally highlighted. This suggests that the proteolytic domain C39 recognises the unique leader peptide of Caseicin FS-X. However, according to our results, only ABC transporter trcsx can complete an efficient bacteriocin secretion. Considering the homology and differences between trcsx, trcsy and Precaseicin

X/Y, the only explanation is that ABC transporter trcsy is incapable of completing the final step of maturation and secretion due to the lack of a complete specific export apparatus. **Figure 4.6** summarises the differences found under the co-expression plasmid system related to GFP-Precaseicin production, size and protein folding.

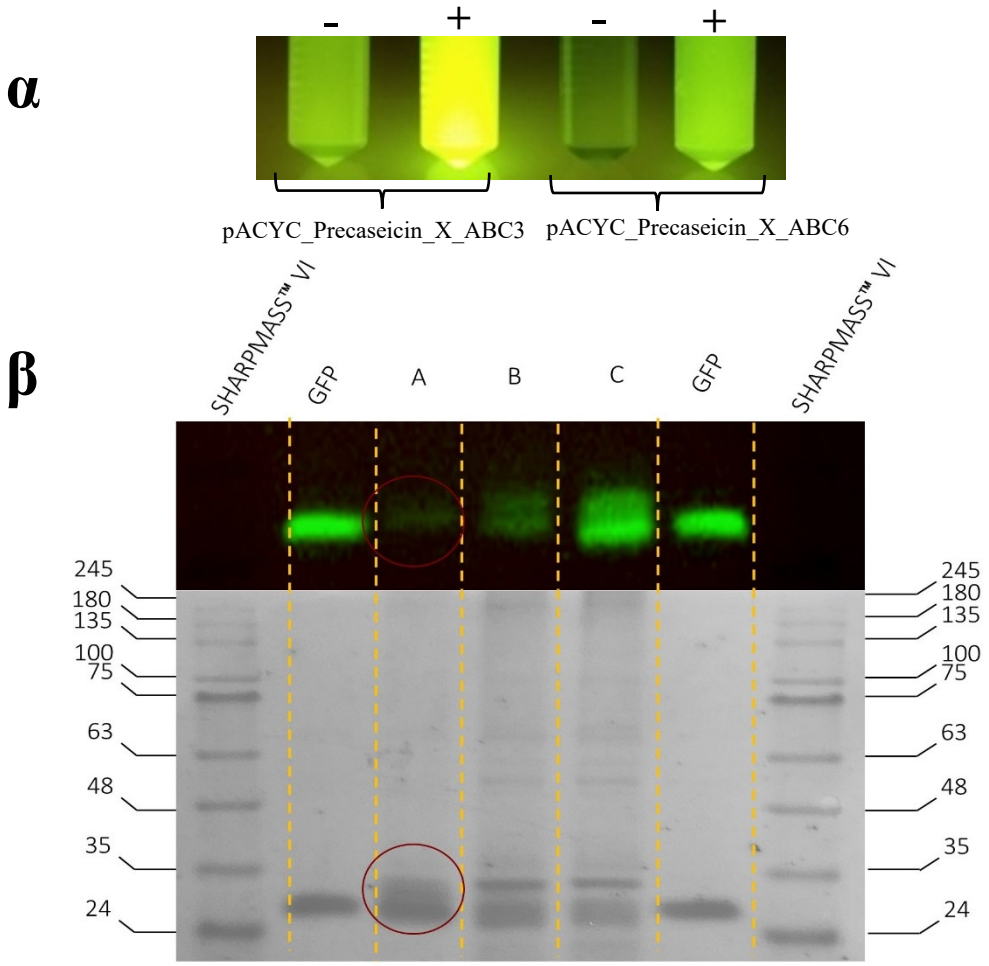


Figure 4.6: α- Differences in induced (+) and not induced (-) heterologous co-expression of 6xHis-tag-GFP-Precaseicin FS-X protein in with ABC transporter trcsx or trcsy β.- Tricine–SDS-PAGE 10%. Legend: A His-tag–GFP-Precasinc FS-X co-expressed with *trcs*x ABC transpoter, B- His-tag–GFP-Precasinc FS-X co-expressed with *trcs*y ABC transpoter, C- His-tag–GFP-Precasinc FS-X, GFP- His-tag–GFP.

DISCUSSION

Most of the ABC transporters are membrane translocators with a generally single substrate target, but many are also promiscuous. Their function governate the trafficking of a range of simple ions, an extensive collection of small molecules of sugars, lipids, amino acids, oligosaccharides, vitamins, xenobiotics, toxic metabolites, hydrophobic drugs, and small peptides or proteins (Holland, 2019). Also, bacteriocins are secreted by an ABC-dependent transporter where the C-terminus is removed by proteolytic cleavage between the two Gly-Gly residues. The authors Ishii et al., 2010 reported that the leader sequences ending with Gly-Ala; Gly-Ser are variant of the typical double Gly motif and then subjected to secretion by ABC transporters (Bobeica et al., 2019).
Cleavage is then achieved by the cytoplasmic C39 peptidase domain at the N-terminus of the transporter corresponding with the active catalytic centre, which is also essential for functional secretion via Type I secretion systems (T1SS) (Wu, Hsieh & Tai, 2012; Kanonenberg, Schwarz & Schmitt, 2013).

The precise role of the leader peptide in the export process remains unclear. However, the alignment between bacteriocin leader peptides with Gly-Gly motif showed a consensus sequence.
-LSX2ELX2IXGG- where X can be any amino acid, which was proposed as a recognition site by the export machinery (Håvarstein, Holo & Nes, 1994). In Chapter 2 we discuss the homologies and the differences in all variants of Caseicin operons. Including the presence of the YGNGV motif, which is highly conservative in the pediocin-like bacteriocin group and variation of the doubles Gly motif formed by five residues of this amino acid, which is the central topic in this chapter, due to unsure cleavage site in the 5xGly motif and the deep differences in the structure found in the related ABC transporters involved in these novel bacteriocins maturation, where only the catalytic component is conservative.

The leader peptide of Caseicin FS-X (casx) and Caseicin FS-Y (casy) is 24 aa long with substitution in position 7 by having either Valine (V) or Alanine (A) and in position 17 by having either Isoleucine (I) or Valine (V). In both leader from position 9 to 21, the -LSNAEISKITGG- and -LSNAEISKVTGG- sequences, which share the consensus cleavage site LSX2ELX2IXGG mention above, were localised. This suggested that the actual leader peptide is composed of 21 aa and Caseicin FS bacteriocin actually have an extra tree Gly in the mature peptide.
This has led to the exploration of alternative heterologous expressions necessary to elucidate the functionality of the secretion machinery required for recognising and processing these pre-peptides. Both the alignment and the three-dimensional models indicate the uniqueness in sequence of the peptidase C39 family. It has high identities only to trcsx and trcsy ABC transporter, suggesting the direct relation with the precaseicin peptide.

Previous studies demonstrated that the N-terminal peptidase C39 domain can be expressed as individual active domain (Furgerson Ihnken, Chatterjee & van der Donk, 2008). However, transport of intracellularly produced peptides in bacteria utilize the energy of ATP binding and hydrolysis to translocate substrates across the cell membrane (Beis, 2015). It was shown that overexpression of integral membrane proteins, is highly influenced by the choice of the T7 expression strain, which may have detrimental effects by their intrinsic function (Gubellini et al., 2011). The success of integral membrane protein overexpression in simple model organisms is influenced by many factors including the recombinant gene sequences, translation efficiency, incomplete processing of signal sequences, the metabolic burden on the host strain which require a case-by-case protocol adjustment (Francis & Page, 2010).

In recent years, substantial progress was accomplished in producing both prokaryotic and eukaryotic membrane proteins in engineered bacterial hosts, especially *E. coli* but also *Lactococcus lactis* and *Bacillus subtilis* (Schlegel et al., 2014). Furthermore, studies reported with success the overexpression of bacteriocin membrane using *E. coli* BL21(DE3) strain together with T7 promoter-based plasmids also in combination with fluorescent protein fusion strategy (Wu & Tai, 2004; Bobeica et al., 2019; Mathieu et al., 2019).

As observed, the results with and without the application of fusion strategy simply fail in obtaining a functional trcsx and trcsy in a feasible amount to validate their functions.
Considering this severe impediment, insight in the scientific approach using a co-expression system, which revealed that only the structural bacteriocin gene and the transporter encoded gene, are required for expression of fully functional bacteriocins. These results are in accordance with Mesa-Pereira et al. (2017) who analogously produced pediocin PA-1 and bactofencin A in *E. coli* using transporter and bacteriocin genes (Mesa-Pereira et al., 2017). In addition, co-expression in *E. coli* provides a useful alternative to the use a multiple plasmid expression system (Tolia & Joshua-Tor, 2006).

In general, overexpressed recombinant proteins in *E. coli* can be either in the periplasmic space or in the cytoplasm in inclusion bodies requiring appropriate purification steps to isolate the peptide of interest (Terpe, 2006). This is the case of bacteriocins, which have a toxic component and are mostly found in the insoluble fraction when expressed in *E. coli* host (Moon, Pyun & Kim, 2006). In contrast, the use of modified pACYC Duet-1 shuttle vector derived from *E coli* BL21(DE3) host allowed the expression of active bacteriocins directly into the medium. The presence of GFP as a fusion partner provided the advantage of clear v of the target proteins and confirmed the loss in size after the cleavage of Caseicin FS-X when co-expressed with the ABC transporter trcsx revealing also that there are no impediment in cleavage. The stabilizing effects provided by GFP for class IIa bacteriocins, without disrupting its auto fluorescent property, is well known (Guinane et al., 2015). However, in our study, the same result in terms of antimicrobial activities with and without the GFP as a fusion partner to the prebacteriocins was obtained.

CONCLUSION

The results presented here prove the inability of the naturally truncated ABC transporter *trcs*y to process and secrete precaseicin bacteriocins. In contrast, the role of ABC transporter *trcs*x posses a fully functioning bacteriocin processing machinery. This system could be useful for expressing new bacteriocins described in silico, opening the door to the heterologous expression and harnessing the potential of new bacteriocins in the future. In addition, identifying and overcoming the pitfalls of membrane protein expression remain a challenge. A novel approach in terms of bacteriocin production is one of the tasks required for implementing their application in food bioperservation, but more investigation of the mode regulation is required.

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CHAPTER 5

Preliminary inhibition of *Listeria monocytogenes* performance by addition of Caseicin FS-X and Caseicin FS-Y in food

ABSTRACT

Pediocin-like is a bacteriocin group produced by lactic acid bacteria that has broad-spectrum activity against *Listeria monocytogenes*, a foodborne pathogen of particular concern in the food industry. This study aimed to evaluate Caseicin FS, the newly identified bacteriocin belonging to class IIa with promising antilisterial properties, in vitro. High scaling the heterologous protein production, first the variation in susceptibility of pathogen strains was investigated. Among the different tested *L. monocytogenes* strains, the more resistant to Caseicin FS in vitro was used as target pathogen in food trials. In particular, the effectiveness of the bacteriocin was tested against the pathogen on purposed inoculated in stracchino cheese and sliced ham and conserved under refrigerated conditions of storage simulating the conditions during the shelf life of these kind of products. This study is a first evaluation of biocontrol in food by applying bacteriocin Caseicin FS as an antimicrobial agent to confirm the potential inhibition of the pathogen *L. monocytogenes* which is responsible for listeriosis, a foodborne disease with related socioeconomic impacts, especially in ready-to-eat (RTE) products.

KEYWORDS: Antilisterial, bio preservation, bacteriocin, food safety

INTRODUCTION

Foodborne pathogens (e.g. viruses, bacteria, parasites) are biological agents that can cause a foodborne illness event. Bacteria are the majors cause of foodborne diseases and exist in a variety of shapes, which include *Bacillus cereus, Campylobacter jejuni, Clostridium botulinum, Clostridium perfringens, Cronobacter sakazakii, Escherichia coli, Listeria monocytogenes, Salmonella spp., Shigella spp., Staphylococcus aureus, Vibrio spp. and Yersinia enterocolitica* (Bintsis, 2017). Among these bacteria, *Listeria monocytogenes* is one of the leading causes of death for foodborne pathogens, especially in pregnant women, newborns, the elderly, and immuno-compromised individuals (Martins & Leal Germano, 2011). *Listeria monocytogenes* is a Gram-positive, mobile and ubiquitous bacterium. It can multiply at low temperatures (1-4 °C), low pH values and high salt concentrations compared to other bacteria. Because of these characteristics, this bacterium can proliferate in many different foods, especially those with a long shelf-life. Vegetables, fish, meat, fruit and cheese are food with a high *Listeria monocytogenes* risk (McAuliffe & N. Jordan, 2012). For this reason, numerous of outbreaks of listeriosis occur every year. The genus *Listeria* is currently comprised of 17 species, including 9 *Listeria* species newly described since 2009, among which *L. monocytogenes* and *L. ivanovii* are the pathogenic to humans causing listeriosis (Guillet et al., 2010; Buchanan et al., 2017).

Listeriosis, is a foodborne illness that is dangerous to humans and animals. Although with the introduction of HACCP, GMP and GHP regulations, cases of listeriosis are fewer compared to other foodborne illnesses, the lethality of this disease is 20-40% higher than those caused by other more common bacteria, such as *Salmonella* spp. and *Campylobacter* spp. (Iacumin, Manzano & Comi, 2016; Chlebicz & Śliżewska, 2018). The growth/no growth boundary for *L. monocytogenes* is defined by a number of physico-chemical characteristics such as pH, water activity, lactic acid concentration and temperature (Gonzales-Barron et al., 2020).

The increasing concern of *L. monocytogenes* infection among foods, is related especially with regard to ready-to-eat refrigerated products that do not require cooking or reheating before the final consumption (Martins & Leal Germano, 2011). One of the most recent and important, in terms of people affected, outbreaks events occurred between January 2017 and July 2018 in South Africa. There were 1060 registered cases. The World Health Organisation (WHO) described it as the largest outbreak of listeriosis ever recorded (Smith et al., 2019). In EU, based on the quantitative risk the biological hazards of *L. monocytogenes* is in increasing trend in various RTE food categories (heat-treated meat; smoked and graved fish; and soft and semi-soft cheese) (Ricci et al., 2018). Regulatory limits of *L. monocytogenes* in RTE foods vary from country to country ranging from zero tolerance (absence/25 g) for all RTE foods to 100 CFU/g for food that do not support growth (Buchanan et al., 2017; Archer, 2018). Lactic acid bacteria (LAB) are a group of beneficial Gram-positive bacteria, which have generally recognised as safe (GRAS) status representing the most studied protective cultures for RTE meat and fresh products for the ability to produce antimicrobial compounds including diacetyl, acetoin, hydrogen peroxide, carbon dioxide, reuterin, reutericyclin, antifungal peptides and bacteriocins.

In this regard, the pediocin-like bacteriocins are the more studied for their strong antilisterial proprieties and are one possible approach to control *L. monocytogenes* in RTE food by using the producing cultures or the bacteriocins in purified or crude preparations (Dong et al., 2021).

Antimicrobial agents, such as nisin and pediocin, are reported as a decontamination agent in dairy and meat products, which are prone to contamination by pathogenic microorganisms (Volpane et al., 2021; Woraprayote et al., 2016). Although anti-listeria efficiency of these bacteriocins is significantly different depending on the targeted indicator strains, the sample preparation method and assay conditions can influence the result in term of antimicrobial activities. Furthermore, pediocin is likely to have higher

activity and acts more specifically against *L. monocytogenes* than nisin without disturbing other bacteria including beneficials microbes (Cintas et al., 1998).
In this study the direct application of bacteriocins Caseicin FS as preservative agent, aimed to gain prior information related to the applicability of these novel antimicrobial peptides in food matrix. Preliminary in vivo evaluation was performed in stracchino cheese and dry cured ham (DCH) under refrigerated storage condition.

MATERIAL AND METHODS

Bacterial cultures

Lactiplantibacillus plantarum 423 bacteriocin producer were cultured at 30 °C in MRS broth (Oxoid, Milano, Italia). Target strains of *L. monocytogenes* ATCC 19111, *L. monocytogenes* ATCC 13932, *L. innocua* ATCC 33030, *L. ivanovi* ATCC 19119, *L. grayi* ATCC 25401 and *Enterococcus fecalis* 19433 were cultured at 37 °C in Brain Heart Infusion (Oxoid, Milano, Italia). Sampling for the enumeration of *Listeria* were perform in the chromogenic media ALOA® (Biolife, Milano, Italia) and enrichment was performed in Fraser broth (Oxoid, Milano, Italia). Decimal dilutions were then prepared from the sample diluted in sterile fisiologic water composed by 9 g/L NaCl (Sigma, Milano, Italia) and 1 g/L Bacteriological Peptone (Oxoid, Milano, Italia). Identification of *L. monocytogenes* was investigated using the *L. monocytogenes* identification method ISO 11290-2:2017. Colonies were counted, and the results were calculated as the means and standard deviation of three determinations per each replicate.

Bacteriocin production and minimal inhibitory concentration (MIC)

Bacteriocins Caseicin FS-X and Caseicin FS-Y were produced heterologously produce in according to the methods discussed in Chapter 2. Protein expression of 8×His-Tagged NisP_Mcherry GPF-Nisleader-Caseicin FS-X and GPF-Nisleader-Caseicin FS-Y proteins was split in 3 batch of 2 L of terrific broth. Protein purification followed in combination of his-tag and ion exchange chromatography for each protein including all 3 batches which were then unified per heterologously produced protein and filter sterilized with Minisart® NML Plus Syringe Filter 0.2 µm (Sartorius, Italy). Purified proteins concentration was determined using BCA Protein Assay Kit (Merck-Millipore, Germany). The reaction inducing the liberation of the bacteriocin were obtain due the mix of GPF-Nisleader-Caseicin FS X-Y peptide and 8×His-Tagged NisP_Mcherry in ratio 7/3 followed by and incubation at 16 °C overnight.

After cleavage liberation of Caseicin FS-X/Y antimicrobial activity were tested with agar well diffusion assay as described by Balouiri et al. (2016) using Brain Heart Infusion (BHI) soft agar 0.8% (w/v) agar (Oxoid, Milano). Briefly, overnight cultures of *L. monocytogenes* ATCC 19111, *L. monocytogenes* ATCC 13932, *L. innocua* ATCC 33030, *L. ivanovi* ATCC 19119, *L. grayi* ATCC 25401 and *Enterococcus fecalis* 19433 were inoculated independently at approximately 10^7 CFU/mL into 45 ml of soft BHI agar (0.7% agar). Wells were made using a sterilised 96-well PCR plate placed into melted inoculated media.
Lactiplantibacillus plantarum 423 cell free supernatant pH adjusted of an overnight culture was used as a positive control. Cleaved Caseicin FS-X/Y Caseicin FS-X and Caseicin FS-Y were diluted in sterile 50 mM Tris, 200 mM NaCl, pH 7.5 elution buffer per 8 time in ratio 1/1 (v/v) and spotted in independent well to determine the MIC. Elution buffer (50 mM Tris, 200 mM NaCl, pH 7.5), Uncleaved Caseicin FS and heterologous NisP protease were also spotted as a negative control.

The inhibition spectrum was evaluated after an overnight incubation at 37 °C for each strain tested. Clear inhibition zone ≥ 2 were referred to an arbitrary unit calculated using the following formula reported by (Ansari et al., 2018):

$$(Au\ ml^{-1}) = \frac{Reciprocal\ of\ the\ Highest\ Dilution}{Amount\ of\ Bacteriocin\ Used} \times 1000$$

In vitro evaluation of inhibitory potency of Caseicin FS-X (time killing test)

Listeria monocytogenes ATCC 19111, *L. monocytogenes* ATCC 13932, *L. innocua* ATCC 33030, *L. grayi* ATCC 25401 were mixed at equal concentration and used to inoculate 9.9 ml of sterile Brain Heart Infusion (Oxoid, Milano, Italia) at a final bacterial load of 100 CFU//ml. Then, 0.1 ml of Caseicin FS-X, which is 1/40 dilution of the purified protein, were added. The negative control was elution buffer (50 mM Tris, 200 mM NaCl, pH 7.5) and uncleaved Caseicin FS. Treated and untreated samples for each strain and sampling point were incubated at 37 °C for 8 h. Sampling was carried out in triplicate from an independent repetition of analysis condition at set intervals of 2 h to assess the growth of *L. monocytogenes*.

Evaluation of *L. monocytogenes* inhibition on ham and stracchino cheese with Caseicin bacteriocin

Dry Cured Ham (DCH) was kindly provided by the company Principe di San Daniele SpA (Italy). Stracchino cheese was purchased in an Italian supermarket. Both food matrix was portioned in an aseptic environment in 10 g (± 0.1 g) and then each sample was positioned in a sterile Whirl-Pak®bag (Sigma, Milano) under aseptic conditions. This evaluation compared the evolution of *L. monocytogenes* with and without the addition of Caseicin FS-X and Caseicin FS-Y bacteriocin*s.* Samples of stracchino cheese and raw ham were inoculated with the less sensitive to Caseicin strains of *L. monocytogenes* resulted *from the* MIC and time killing test at 100 and 1000 CFU/g as a final bacterial concentration, respectively. In order to evaluate the effect of mature bacteriocin Caseicin, 0.1 mL of the purified peptide was also added with a final concentration of 40 *(Au* ml^{-1}*)/g*. The inoculated and inoculated with the addition of bacteriocins samples were then vacuum packed (VM-16, Orved, Italy). The chosen inoculum concentration was different for the two foods matrix tested based on their compositional characteristics and, above all, on the consideration regarding the supportability or non-supportability to the growth of *L. monocytogenes*.

Samples and inoculum preparation

The strains *L. monocytogenes* ATCC 19111, *L. monocytogenes* ATCC 13932, *L. innocua* ATCC 33030, *L. grayi* ATCC 25401 regularly kept in the Department of Agri-food, Environmental and Animal Sciences of the University of Udine were chosen as a target strains. Inoculum was prepared by adding 0.1 mL of culture stock (stored at −80°C, for a maximum period of 90 d) to 50 mL sterile tubes containing 10 mL of BHI broth and incubated at 37 °C for 16 h. After its growth, each strain were harvested independently by centrifugation at 8000 × g for 10 min and then the spent broth was discarded. The obtained bacterial pellet was resuspended in physiologic water until OD_{600} was 0.1 detected by the Spectrophotometer Bio-Rad SmartSpec 3000 (Bio-rad, Italy). Dilutions were performed in physiological water and 0.1 mL of each dilution was plated in ALOA® (Biolife, Milano, Italia) in order to evaluate the exact count corresponding to OD_{600} of 0.1. The plates were incubated at 37 °C for 48 h.

Microbiological sampling

All Stracchino cheese samples were monitored during refrigerated storage (4 °C for 10 days) and thermal abuse (4 °C for 2 days and then 8 °C for the remaining 8 days). During this period, samplings were carried out at set intervals to assess the growth of *Listeria monocytogenes* ATCC 19111.

L. monocytogenes ATCC 19111 was monitored at 0, 1, 2, 6, 8 and 10 days after inoculation. Analyses were performed in triplicate.

Analogously, the inoculated and non-inoculated samples of Dry Cured Ham (DCH) were stored at 4°C for 20 days and under thermal abuse, 2 days at 4 °C and 18 days at 8 °C. The sampling points were at 0, 1, 2, 6, 13, and 20 days of storage.

pH and Aw measurements

The pH potentiometric measurements were carried out with a pin electrode pH-meter (Basic20 pH, Crison, Bacelona, Spain) that was inserted directly into the sample. The water activity (Aw) was determined with a water activity meter (AquaLab 4TE, Decagon Devices, USA). For both the analysis three independent measurements per each replicate were performed at the sampling point T_0 of both food matrix.

RESULTS

Antimicrobial activity detection

Purified expression batch of GPF-Nisleader-Caseicin FS-X, GPF-Nisleader-Caseicin FS-Y and 8×His-Tagged NisP_Mcherry produced 456,6 µg/mL 423,85 µg/mL 641,85 µg/mL.

The antimicrobial activity of heterologously produced Caseicin FS-X and Caseicin FS-Y bacteriocins showed a clear inhibition zone in agar when compared with negative control (uncleaved peptide) using *L. monocytogenes* ATCC 19111, *L. monocytogenes* ATCC 13932, *L. innocua* ATCC 33030, *L. ivanovi* ATCC 19119, *L. grayi* ATCC 25401 and *Enterococcus fecalis* ATCC 19433 as indicator strains (**Figure 5.1**).

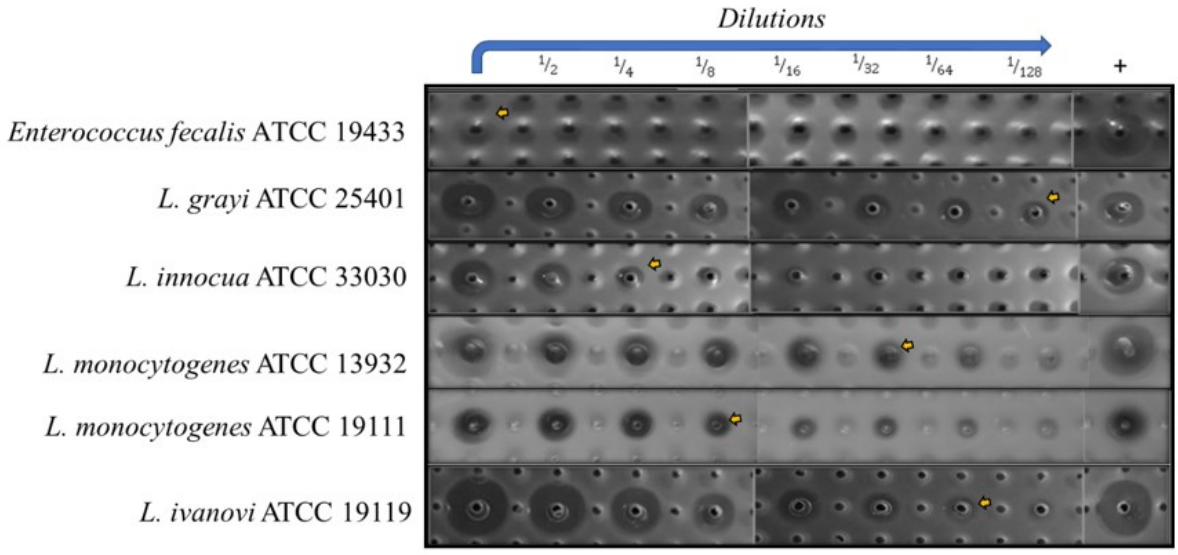

Figure 5.1: Dilution series of Caseicin FS-X tested in different target bacteria. For each dilution 50 µL were spotted in the wells including the positive control. The purified protein used as negative control 8×His-Tagged NisP_Mcherry GPF-Nisleader-Caseicin FS-X, GPF-Nisleader-Caseicin FS-Y and the elution buffer had no antimicrobial proprieties.

Clear inhibition zone ≥ 2 mm was considered as the minimal inhibition concentration (yellow arrow). Between the cleaved mature Caseicin FS-X and Caseicin FS-Y from these results, no differences in inhibition were found. Accordingly with the MIC, these peptides showed difference in effectiveness related to the strains tested as reported in **Table 5.1**.

Table 5.1: Resulted minimal inhibitory concentration (MIC) and relative conversion in arbitrary units of different target bacteria tested.

Strains	**Last dilution with antimicrobial activity detected**	$(\boldsymbol{Au\ ml^{-1}})$
Enterococcus fecalis ATCC 19433	-	-
L. grayi ATCC 25401	1/128	15,6
L. innocua ATCC 33030	1/4	500
L. monocytogenes ATCC 13932	1/32	62,5
L. monocytogenes ATCC 19111	1/8	250
L. ivanovi ATCC 19119	1/64	32,25

In vitro antimicrobial activity against *Listeria* spp.

To confirm the capability of Caseicin FS-X to explicate the bactericidal action in a closer to a real environment against *L. grayi* ATCC 25401, *L. innocua* ATCC 33030, *L. monocytogenes* ATCC 13932, *L. monocytogenes* ATCC 19111 were tested. For all strains a reduction of bacterial concentration was found in the treatment condition as shown in **Figure 5.2**. Application of Bacteriocin Caseicin FS-X at final concentration of 40 **(*Au ml*$^{-1}$*)/ml** exceptionally reduce >7-log after 8 h of incubations compared to the control under the same conditions. Only strain *L. monocytogenes* ATCC 19111 was countable after two hours of incubation. After enrichment step all strains show the presence of the pathogen inoculated.

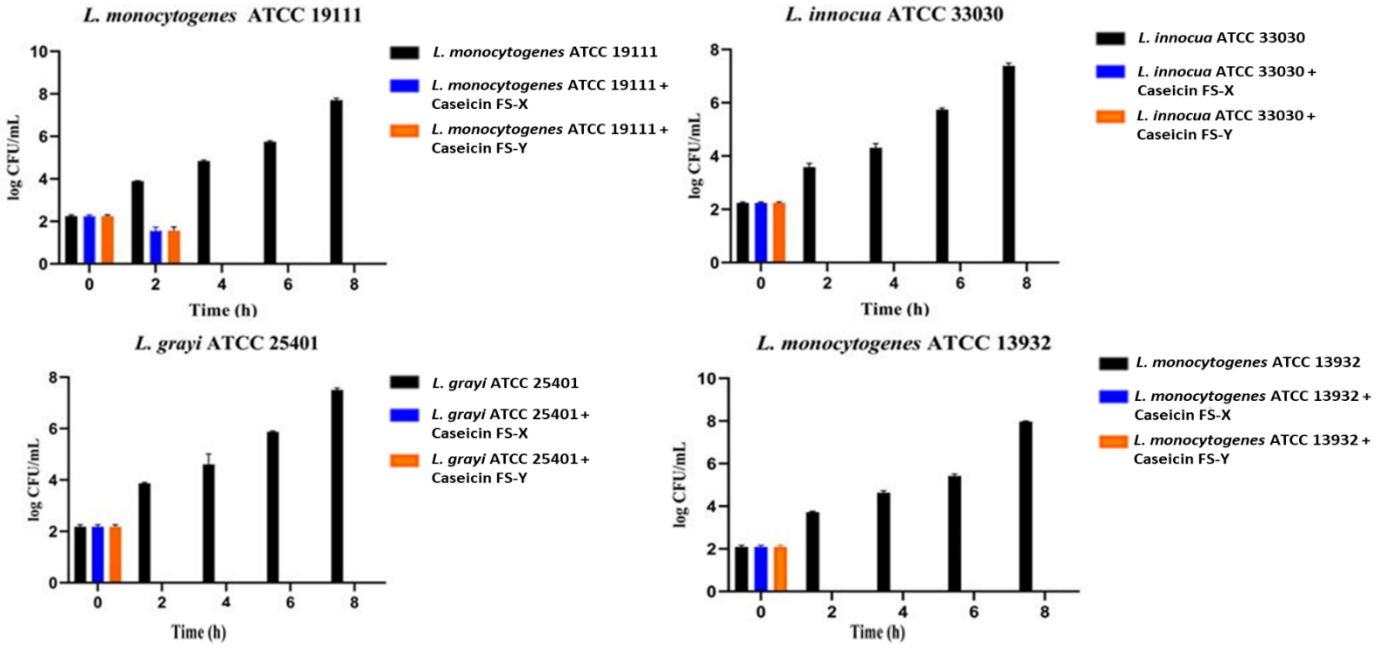

Figure 5.2: Growth evolution of *Listeria spp.* strains in BHI broth with or without bioprotective Caseicin FS-X at 37°C.

These results were in accordance with the Au ml-1, except for *L. innocua* ATCC 33030, which was >7-log after 8 h and was observed, these results followed the Au ml-1, but it is the stain with the high MIC (500 *Au* ml^{-1}). In this regard, *L. monocytogenes* ATCC 19111 was chosen as a model strain in the following analysis for consistently lower susceptibility to Casein FS.

Application of Caseicin bacteriocin in ham and stracchino cheese

Results showed that *L. monocytogenes* in both products was under the concentration detectable with the method after 24 h of conservation for the samples treated with Caseicin FS-X. In addition, *L. monocytogenes* was also undetected after the first and second enrichment steps. The results of the microbial counts are described in **Figure 5.3** and represent the average plate count value of the performed analysis.

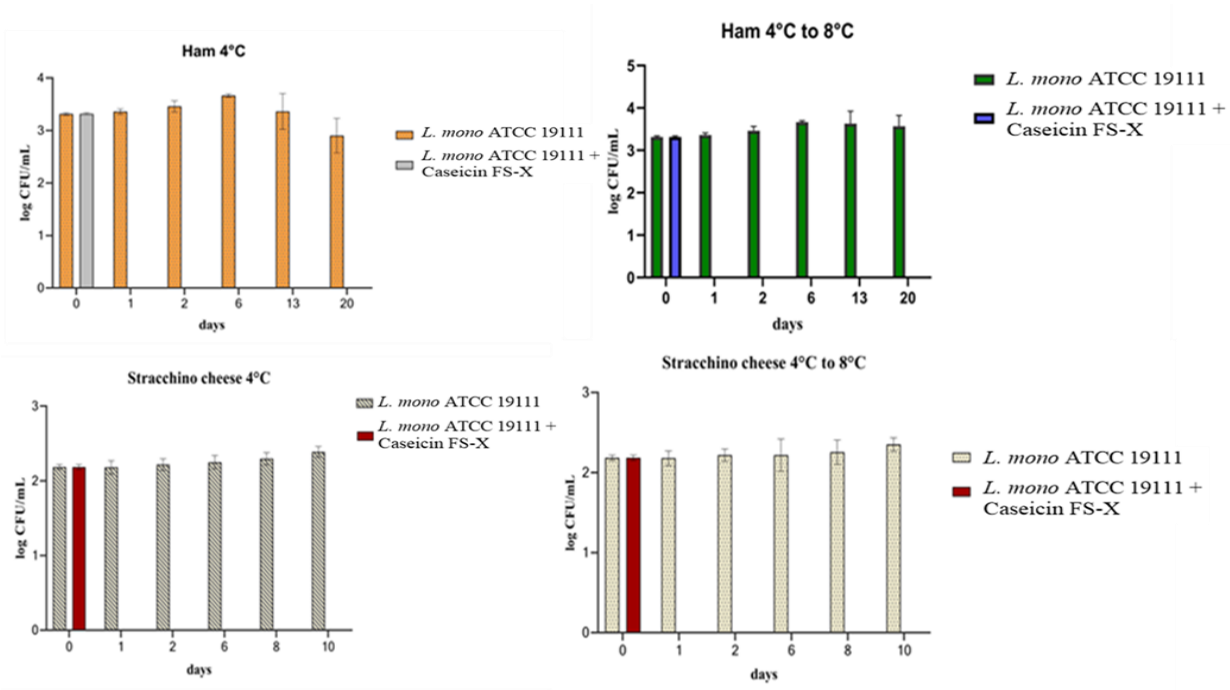

Figure 5.3: Evolution of the growth of *L.* monocytogenes ATCC 19111 treated and non-treated with Caseicin FS-X.

DISCUSSION

The application of bacteriocins as part of hurdle technology results in microbial inactivation. Pediocin-like antimicrobial activity against a wide spectrum of Gram-positive bacteria, many of them responsible for food spoilage or foodborne diseases.

A specific mode of action of pediocin-like is the specific binding to the mannose phosphotransferase transmembrane protein complex (Man-PTS) involved in the transport and metabolism of carbohydrates in bacteria (Zhu et al., 2022). Despite similarities of pediocins group in their primary structures, aminoacid sequence with hydrophilic, cationic proprieties, activity and target cell specificity differences has been reported (Eijsink et al., 1998). In this regard, a connection between the amount of disulfide bridge is considered the variable related to the target cell specificity and temperature dependency of the activity of pediocin-like bacteriocins (Fimland et al., 2000). However, the efficient application of different class IIa bacteriocin is unlinked to their disulfide bond when applied as preservatives.

In the present study, the antibacterial efficacy of the heterologously expressed Caseicin FS bacteriocin was evaluated *in vitro,* in stracchino cheese, and in ham as a food matrix in order to gain data on the potential applicability as an antimicrobial agent.

In Chapter 2, the MIC of Caseicin FS was set at µg/ml to explicate effective antimicrobial action with some variability in potency. Similar pattern of antimicrobial activity of others bacteriocins is in accordance with this result, where the potency of their action is dependent on the strain of bacteria targeted (Hugas et al., 2002).

The results obtained in this prior evaluation in vitro of heterologously expressed Caseicin FS-X/Y confirm that variability related to the sensitivity of each strain tested. However, we observed a higher inhibition halo of the bacteriocin plantaricin 423 used as a positive control for *L. grayi* ATCC 25401, *L. monocytogenes* ATCC 13932 and *L. ivanovi* ATCC 1919. Analogously, performance of the time-kill test confirmed the bactericidal effect of Caseicin FS-X against all strains tested. Only strain *L. monocytogenes* ATCC 19111 at concentration of 100 CFU/ml when treated with Caseicin FS-X, shows after two h of treatment with 1 log CFU/ml of bacterial concentration. Therefore, based on the degree of resistance to bacteriocins show that *Listeria monocytogenes* ATCC 19111 it was the least sensitive to antimicrobial action of Caseicin FS. More investigation involving different media, time and temperature of incubation, bacterial load and Caseicin FS concentration is required for a fully characterisation of the best condition for Caseicin FS action explicability. Overall inoculum size and growth rate are considered the most important variable in antimicrobial susceptibility testing (Pfaller, Sheehan & Rex, 2004).

The success of bacteriocins in various food systems and the challenges strategies employed to put them to work efficiently in various food systems have been discussed in several studies (Johnson et al, 2018). However, several factors can result limiting the effectiveness of bacteriocins in food preservation. In this regard the efficacy of bacteriocin application in food systems of bacteriocins can be influenced by pH, temperature, water activity, salt, the presence of other food components. Furthermore, the manufactural process involved the food production can influence their stability reducing or eliminating their antimicrobial function. For this reason, bacteriocin applicability requires the evaluation for each condition and technique integrated with the production process related to the final product.

Regulation (EC) No. 2073/2005 establishes the criteria within which a food product is considered to be microbiologically safe, including growth limits for *Listeria monocytogenes* which are set to a tolerance limit of 100 CFU/g for ready-to-eat (RTE) foods that do not support the growth of *Listeria monocytogenes*. Conversely, RTE foods that support the growth of *Listeria monocytogenes* have a zero-tolerance policy, meaning the absence of the bacteria in 25 g of the product.

Stracchino cheese due the value of pH and Aw ($pH > 4,4$; $a_W > 0,92$) support the proliferation of *L. monocytogenes.* In contrast, the processed ham values of pH and Aw are low ($pH < 4,4$; $a_W < 0,92$) are not supporting the grow of *L. monocytogenes*. Increased risk for the presence of *L. monocytogenes* in processed ham is the slicing process which cause (Vorst, Todd & Ryser, 2006; Zhang et al., 2018; Verma et al., 2022).

According to research conducted by Mataragas and Drosinos (2007), our findings indicate that *L. monocytogenes* did not grow at temperatures below 4°C. Therefore, a slight increase of 0.5-log in the number of CFU/g was observed during the shelf-life of untreated sliced ham samples. The growth of *L. monocytogenes* in untreated Stracchino cheese sample was also limited when conditioned at 4°C. This trend was observed in similar stracchino cheese varieties with an average pH of 6.50 and an average Aw of 0.95, such as salted cottage cheese. These types of cheese remained stable throughout the entire shelf-life when conditioned at 4°C, as reported by Giannou et al. (2009), Bellio et al. (2016), Coroneo et al. (2016), and Engstrom et al. (2020).

In both food matrix, the application of Caseicin FS induced the reduction of *L. monocytogene*s ATCC 19111. Evidence suggests that the storage environmental conditions in food preservation plays a key

role in controlling the growth of *Listeria monocytogenes* which is psychotropic pathogens. With this in mind, the use of Caseicin FS in eliminating target bacteria can be considered as a synergic effect with refrigeration temperature conditions applied in this study. the use of Caseicin FS action in eliminating target bacteria can be considered as a synergic effect with refrigeration temperature conditions applied in this study.
Concentration of the antimicrobial Caseicin FS, temperatures, other type of food belonging the RTE categories as well as the comparison with others bacteriocin against different pathogen strains will attribute a more detailed picture of the promising applicability of Caseicin FS.

There already exist many control measures within the food industry to prevent or minimise bacterial contamination, including good manufacturing practices. Furthermore, due the high risk of foodborne illness due to post-lethality contamination of RTE product effective methods of sanitation is required (McLauchlin et al., 2004). Bacteriocin application through producer strain have been used in combination with high pressure for improved control of *L. monocytogenes* on RTE product (Oliveira et al., 2015). However, even the application of hurdle technologies may not reduce cell counts of *L. monocytogenes* by more than 5 log, or prevent regrowth during storage (Teixeira et al., 2018). Aside from nisin and pediocin, the legal approval of other bacteriocins as safe food additives or bio-preservative agents, as well as their large-scale production, is a real challenge in their future application (Verma et al., 2022). In this regard, Caseicin is a new discovered bacteriocin, and the production requires protein expression techniques. There is not yet available a defined comparison methodology between expression and purification techniques allowing the direct comparison on potency of different peptides. However, from the result gained in this study it is reasonable to affirm that Caseicin FS X and Caseicin FS Y are potent as well as others pediocin-like bacteriocins. This study involved the first step of scaling-up production in order to proceed with the characterization of this antimicrobial peptide, which in accordance with our results and prediction is showing a great potential in food applications. Although results are promising, this study remains a proof-of-concept, and the use of Caseicin bacteriocin as food preservatives should be further investigated.

CONCLUSION

Isolation of new bacteriocins for food and medical applications is an increasing trend. However, the usage of bacteriocins or bacteriocin-producing starter cultures for the preservation of food products request the development of well-defined protocols including them as food antimicrobial agents to provide a complete picture of the effective action against foodborne pathogens in the food systems.
This preliminary study within Caseicin FS bacteriocin as preservative agent in stracchino cheese and raw ham resulted in a reduction of *L. monocytogenes* that opens promising further application as a versatile preservative that can be applied with different strategies in various food products.
Extensive research is essential to assess the safety and efficacy of Caseicin FS bacteriocin in food products. Nonetheless, it was demonstrated the effectiveness of Caseicin FS bacteriocin as a potent anti-listeria agent when directly applied stracchino cheese and raw ham makes it a thereby promising antimicrobial peptide.

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CHAPTER 6

Thermophilin 13: In Silico Analysis Provides New Insight in Genes Involved in Bacteriocin Production

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This chapter is formatted according to the Journal "Microorganims MDPI" publish the 28 February 2023

Citation: Salini, F.; Iacumin, L.; Comi, G.; Dicks, L.M.T. Thermophilin 13: In Silico Analysis Provides New Insight in Genes Involved in Bacteriocin Production. *Microorganisms* 2023, *11*, 611. https://doi.org/10.3390/microorganisms11030611

ABSTRACT

Bacteriocins are a large family of ribosomally synthesised proteinaceous toxins that are produced by bacteria and archaea and have antimicrobial activity against closely related species to the producer strain. Antimicrobial proteinaceous compounds are associated with a wide range of applications, including as a pathogen inhibitor in food and medical use. Among the several lactic acid bacteria (LAB) commonly used in fresh and fermented food preservation, Streptococcus thermophilus is well known for its importance as a starter culture for yoghurt and cheese. Previous studies described the bacteriocin thermophilin 13 exclusively in S. thermophilus SFi13 and the genes encoding its production as an operon consisting of two genes (thmA and thmB). However, the majority of bacteriocins possess a complex production system, which involves several genes encoding dedicated proteins with relatively specific functions. Up to now, far too little attention has been paid to the genes involved in the synthesis, regulation and expression of thermophilin 13. The aim of the present study, using in silico gene mining, was to investigate the presence of a regulation system involved in thermophilin 13 production. Results revealed the dedicated putative bacteriocin gene cluster (PBGC), which shows high similarity with the class IIb bacteriocins genes. This newly revealed PBGC, which was also found within various strains of Streptococcus thermophilus, provides a new perspective and insights into understanding the mechanisms implicated in the production of thermophilin 13.

Keywords: thermophilin; Streptococcus thermophilus; gene organization; class IIb bacteriocin

INTRODUCTION

Streptococcus thermophilus is a nonpathogenic lactic acid bacterium commonly isolated from bovine mammary tissue and raw milk, producing lactic acid, exopolysaccharides (EPS) and several organoleptic compounds from the fermentation of lactose and galactose, and also is a well-known starter culture used in the production of yoghurt and cheese [1]. The species has GRAS (Generally Regarded As Safe) status from the FDA (Food and Drug Administration) and QPS (Qualified Presumption of Safety) status from the EFSA (European Food Safety Authority). Several strains of S. thermophilus produce bacteriocins, which are small, ribosomally synthesized peptides with narrow or broad spectrum antimicrobial activity [2]. Examples include thermophilin A (strain ST134) [3], thermophilin T (strain ACA-DC 0040) [4], thermophilin 110 (strain 580) [5] and thermophilin 1277 (strain SBT1277) [6]. Two other unnamed bacteriocins were reported in *S. thermophilus* strain 81 and *S. thermophilus* strain 580, but little is known about their peptide sequence and genes encoding them [7,8].

Furthermore, apart from the mentioned examples, *S. thermophilus* SFi13, an isolate belonging to the Nestle' strain collection, produces thermophilin 13. The inhibitory spectrum of thermophilin 13 includes Clostridium botulinum, *Listeria monocytogenes, Lactococcus lactis, Bacillus cereus, Bacillus subtilis* and *S. thermophilus* [9].

Expression of bacteriocin genes is usually subject to external induction factors (IFs) regulation. The gene encoding the pre-peptide is normally located in the same operon as genes encoding the immunity protein, ABC transporter and accessory protein [10]. The accessory protein may also be involved in rendering immunity to the bacteriocin-producing cell [11]. The cleavage site that characterises peptides ThmA and ThmB is preceded by a double-glycine motif found in pre-peptides of class IIb bacteriocins [12,13]. However, thermophilin 13 has been described as an atypical bacteriocin in the sense that the activity of the antibacterial peptide ThmA is enhanced by the peptide ThmB, encoded by genes thmA and thmB, respectively, on a 960-bp operon (U93029.1) [9]. Thermophilin 13, in agreement with the classification proposed by Zouhir et al. (2010), shares common characteristics with class IIe bacteriocins by having a WX9GX3G motif ($1.02 \times 10^{-7} <$ p-value $< 7.01 \times 10^{-6}$) in the enhancer peptide ThmB. However, the YGNGV-C motif is missing in both the peptides ThmA and ThmB. The YGNGV-C motif is typical of the anti-Listeria-active peptides [13]. Marciset et al. (1997) described thermophilin 13 as an ionophoric poration complex formed by the interaction between ThmA and ThmB [9]. Their results did not provide any information about the involvement of genes other than thmA and thmB forming the operon, which regulates the production of thermophilin 13. Among bacteriocin-producer strains, lactic acid bacteria play a key role in fresh and fermented food preservation. The present study is focused on *Streptococcus thermophilus* SFi13 strain, which is the only producer reported in the scientific literature of the bacteriocin thermophilin 13. Based on current knowledge, only two genes, *thmA* and *thmB*, are involved in the thermophilin 13 productions, and to our knowledge, no further studies have been pursued to investigate this bacteriocin's mode of action and gene organisation. Therefore, our study aimed to investigate the identity and organisation of all the genes encoding proteins involved in thermophilin 13 regulation, synthesis, transport and immunity, using in silico DNA comparisons.

MATERIAL AND METHODS

Genome sequences

The thermophilin 13 operon sequence (U93029.1) amounting to 960-bp and listed in the National Center for Biotechnology (NCBI, Bethesda, MD, USA; https://www.ncbi.nlm.nih.gov/; accessed on 2 October 2022) nucleotide database was used to conduct a similarity search using the NCBI Basic Local Alignment Search Tool (BLAST) [14]. Similarities to the thermophilin 13 operon were determined using NCBI Sequence Viewer [15]. The complete genome sequences of all bacterial strains showing the presence of 960-bp with an identity of 100% with the thermophilin 13 operon (U93029.1) sequence were downloaded from the NCBI database.

Identification and analysis of the thermophilin 13 biosynthetic gene cluster (BGC)

To identify potential bacteriocins, biosynthetic gene clusters (BGC) of all genome sequences were analysed using the command-line antiSMASH version 5.0 [16] and BAGEL4 [17]. The ClusterFinder algorithm with additive cluster discovery was used. ClusterFinder source code is available from the GitHub repository (https://github.com/petercim/ClusterFinder; accessed on 2 October 2022).

Amino acid sequences with predicted ORFs (open reading frames) were compared against the non-redundant protein database using Blastp version 2.9.0+ (protein–protein BLAST) [18]. Using the Jukes–Cantor model, a Nearest-Neighbor-Interchange (NNI) tree with 1000 Bootstraps was constructed, including all bacteriocin gene sequences provided by antiSMASH and BAGEL4 hosted on the NCBI website. Analyses were conducted using the MEGA 11 software (Version 11.0.11) platform [19].

Putative bacteriocin genes within the respective genomes were annotated using the CLC Main Workbench—QIAGEN Bioinformatics software (CLC bio, Aarhus, Denmark). Sequence alignment was performed using Muscle WS [20] and displayed by Tree Of Life (iTOL) v4 online tool [21]. Comparative analyses of genomic datasets were performed using Operon-mapper [22] and loci of selected bacteriocin genes were visualised using cblaster (github.com/gamcil/clustermap.js; accessed on 16 November 2022) [23], Clinker & Clustermap.js (github.com/gamcil/clinker; accessed on 16 November 2022) [24] and protein 3D prediction was obtained with ColabFold open-source software available at https://github.com/sokrypton/ColabFold; accessed on 15 February 2023 [25].

RESULTS

Currently, no complete genome sequence of *S. thermophilus* SFi13 is available on the NCBI database. In a study by Comelli et al. (2002) and in the deposited patent USOO7491386B2, the authors described and evaluated bacterial strains with potential properties as oral probiotics, useful for the prevention of dental caries. According to the Nestlé Culture Collection (NCC), they also affirmed that strain *S. thermophilus* SFi13 was reclassified as *S. thermophilus* NCC 2008 [26,27].

All prior research on this bacteriocin only refers to the partial sequence with Accession Number U93029.1 (NCBI). Despite the reclassification of strain *S. thermophilus* SFi13 to *S. thermophilus* NCC 2008, the genome sequence is also unavailable on the NCBI database. The DNA sequences of operon U93029.1 contained genes thmA and thmB, putative promoter elements, ribosome binding sites, and a rho-independent terminator structure, as reported by Marciset et al. (1997) [9]. A similarity search using BLAST identified *S. thermophilus* B59671 (CP022547.1), *S. thermophilus* KLDS 3.1003 (CP016877.1), *S. thermophilus* STH_CIRM_1049 (LR822034.1), *S. thermophilus* STH_CIRM_1048 (LR822033.1), *S. thermophilus* CS9 (CP030927.1), *S. thermophilus* DMST-H2 (CP063275.1), TK-P3A (CP045596.1), ATCC 19258 (CP038020.1), *S. thermophilus* LMD-9 (CP086001.1), *S. thermophilus*

NCTC12958 (LS483339), and *Streptococcus macedonicus* 19AS (PEBN00000000.1) shares identical DNA sequences to the thermophilin 13 operon (U93029.1) of *S. thermophilus* SFi13. All these strains have 100% similarity and 100% identity with the operon U93029.1. Even though some sequences had 79.22–84% of identity with operon U93029.1, their query cover ranged from 8–14%, and for this reason, they were automatically excluded from this study. General information and identification code of these 11 strains are listed in **Table 6.1**.

Table 6.1: List of strains and associated GenBank accessions code for genomes in which a 960-bp sequence with 100% identity to U93029.1 was found using BLASTn.

Strains	GenBank Code	Isolation/Source	Reference
Streptococcus thermophilus B59671	CP022547.1	Milk	[28]
Streptococcus thermophilus KLDS 3.1003	CP016877.1	Lactic starter (for yoghurt production)	[29]
Streptococcus thermophilus LMD-9	CP086001.1	Lactic starter (for yoghurt and mozzarella production)	[30]
Streptococcus macedonicus 19AS	PEBN00000000.1 ***	Cheese	[31]
Streptococcus thermophilus STH_CIRM_1049	LR822034.1	Lactic starter (for yoghurt production)	[32,33]**
Streptococcus thermophilus STH_CIRM_1048	LR822033.1	Lactic starter (for yoghurt production)	[32,33]**
Streptococcus thermophilus CS9	CP030927.1	Fermented milk	[34]
Streptococcus thermophilus DMST-H2	CP063275.1	Probiotic products	[35]
Streptococcus thermophilus TK-P3A	CP045596.1	Pasteurised milk	[36]
Streptococcus thermophilus ATCC 19258 *	CP038020.1	Milk	[37]
Streptococcus thermophilus NCTC 12958 *	LS483339.1	Milk	[38]

* Genome sequences of *S. thermophilus* NCTC 12958 and *S. thermophilus* ATCC 19258 are identical. In this study, both genomes were analysed independently. ** The identification name of the strains of *Streptococcus thermophilus* STH_CIRM_1048 and *Streptococcus thermophilus* STH_CIRM_1049 is related to the accession codes for genomes valid for the NCBI database; the same strains are reported as *Streptococcus thermophilus* CIRM-BIA1048 *Streptococcus thermophilus* CIRM-BIA1049 in citation [32,33]. *** GenBank code PEBN01000000.1 and PEBN01000052.1 both refer to *Streptococcus macedonicus* 19AS strain in NCBI database.

Data obtained using BAGEL4 and antiSMASH version 5.0 confirmed the distribution of thermophilin 13 BGC (biosynthetic gene cluster) in all 11 strains of *S. thermophilus*. All strains showed the same area of interest (AOI), with some variation in nucleotide sequences. Strains *Streptococcus thermophilus* B59671 (CP022547.1) and *Streptococcus macedonicus* 19AS (PEBN00000000.1) were the most diverse based on AOI. **Figure 6.1** shows a cladogram tree that is derived from the multiple sequence alignments of AOIs identified from the in silico study.

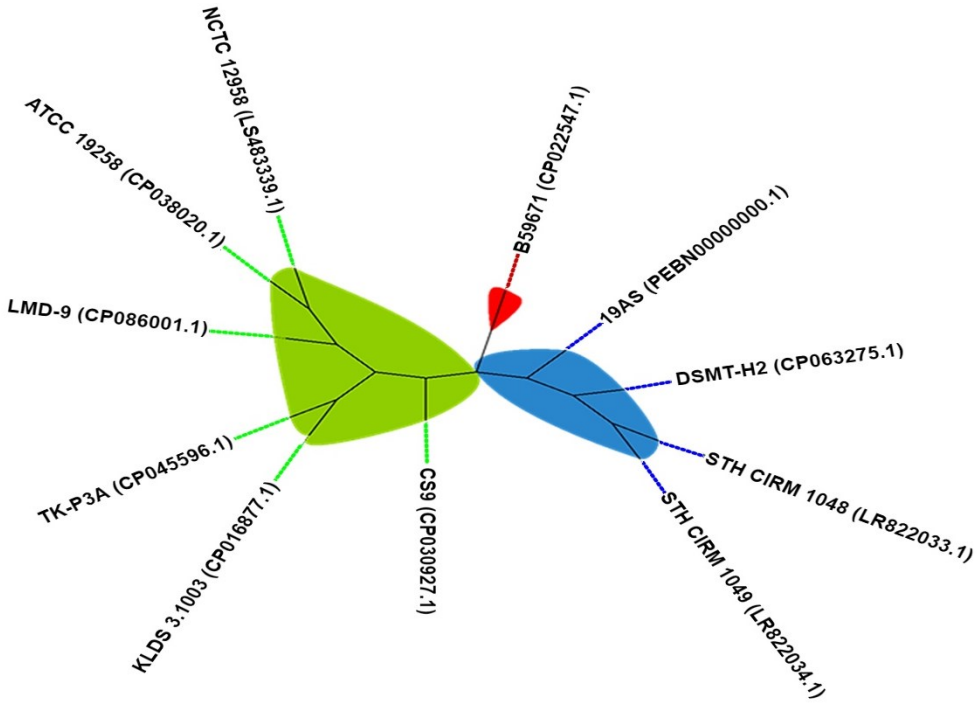

Figure 6.1: Cladogram tree showing the phylogenetic relatedness amongst gene loci, including thermophilin 13 (U93029.1). The tree was composed using the maximum likelihood method but visualised by removing branch length information. The three nodes are shown in different colours.

Known bacteriocin loci were detected, e.g., the lantibiotic salivaricin 9 operon in the genome of *S. thermophilus* NCTC 12958 and *Streptococcus thermophilus* ATCC 19258 and thermophilin 110 operon in *S. thermophilus* B59671 [5]. The entire locus of Salivaricin 9 was fully characterized from *S. salivarius* strain JIM8780, and it was shown to consist of eight genes, having the following putative functions: sivK, sensor kinase; sivR, response regulator; sivA, Sal9 precursor peptide; sivM, lantibiotic modification enzyme; sivT, ABC transporter involved in the export of Sal9 and concomitant cleavage of its leader peptide; and sivFEG, encoding lantibiotic self-immunity [39]. The broad-spectrum bacteriocin thermophilin 110 is encoded within the blp gene cluster. Furthermore, thermophilin 110 was reported to inhibit the growth of *Listeria monocytogenes*, *Streptococcus mutans*, *Streptococcus pyogenes* and *Propionibacterium acnes*.

Manual curation and annotation were performed to compare the differences between the ORFs predicted by the bacteriocin mining tools. Comparisons of AOIs indicated that the gene loci in the thermophilin 13 operon are organised into eight genes/ORFs encoding proteins related to bacteriocin production, plus the two thermophilin 13 structural genes. These were consistent for all strains and include a response regulator (RR), sensor histidine protein kinase (HPK), quorum-sensing system pheromone BlpC, ABC-transporter, bacteriocin accessory protein, thiol–disulfide oxidoreductases, CAAX protease and genes *thmA* and *thmB*. Despite the similarity in translation, three operon patterns were observed using the Operon-mapper web server. These variations, including all strains analysed in the present study, are schematically visualised in **Figure 6.2**.

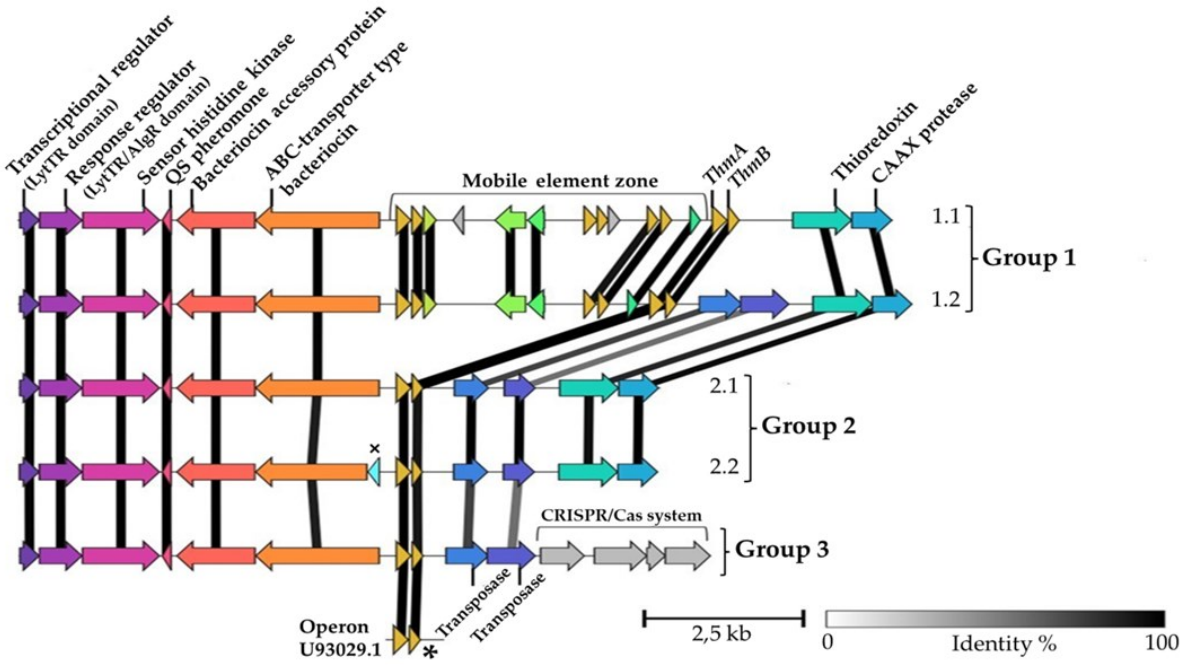

Figure 6.2: Comparison of the BGCs of thermophilin 13. Predicted ORFs are represented as arrows. Three main gene arrangements were detected. Subgroup 1.1 contains strain *S. macedonicus* 19AS and subgroup 1.2 strains *S. thermophilus* STH_CIRM_1049, *S. thermophilus* STH_CIRM_1048, *S. thermophilus* DMST-H2. Group 2 is divided into two subgroups, with strains *S. thermophilus* KLDS 3.1003, *S. thermophilus* TK-P3A, *S. thermophilus* CS9 in subgroup 2.1 and strains *S. thermophilus* LMD-9, *S. thermophilus* ATCC 19258 and *S. thermophilus* NCTC 12958 in subgroup 2.2. Group 3 is represented by strain *S. thermophilus* CS9. The symbol * indicates the third ORF (ORFC), encoded by the U93029.1 operon and symbol × indicate the C39 peptidase-like domains found only in subgroup 2.2. Colours are based on ORFs similarity found, including the mobile element zone, which were identified as small ORFs with apparent unrelated functions in the bacteriocins productions due to the prediction as hypothetical proteins.

A similar regulation and secretion system was observed for thermophilin 13 in groups 1, 2 and 3 (**Figure 6.2**). However, an additional nucleotide sequence (mobile element zone) was detected in group 1 (**Figure 6.2**). No variations in gene transcription up-stream and downstream of this area were observed. In this regard, the insertion element, which is present in all sequenced BGCs of cluster 1, requires further investigation to assess possible interference with thermophilin 13 production due to the presence of transposases. A third ORF (ORFC), encoded by the U93029.1 operon, was re-ported by Marciset et al. (1997) [9] and was found in all BGCs groups shown in **Figure 6.2**. Structure models of the poration complex formed by Thermophilin 13 were de-scribed as the ThmA enhancing ThmB peptide with maximal explication in antimicrobial activity in equimolar concentration. However, the peptide ThmA alone resulted in antibacterial activity against *S. thermophilus, Clostridium botulinum*, *Listeria. monocytogenes* and *Bacillus cereus*.

In this regard, the presence of GxxxG-motifs or GxxxG-like motifs AxxxA and SxxxS motif, instead of the GxxxG-motif and a high helical content were related to the two-peptide bacteriocins into form membrane-penetrating helix–helix structures, ex-plaining the increased helical content forming a dimer complex, in which an incremented antimicrobial action is attributable [40]. This dual peptide interaction was de-scribed in several class IIb bacteriocins including thermophilin 13 as is reported by the authors Oppegård et al. (2008) and Nissen-Meyer et al. (2010) [41,42]. However, this aspect requires further investigation due to multiple GxxxG motifs, located in positions ^{21}GxxxG^{25}, ^{32}GxxxG^{36}, ^{40}GxxxG^{44},

^{54}GxxxG^{58} for ThmA and ^{5}GxxxG^{9}, ^{14}GxxxG^{18}, ^{15}GxxxG^{19}, ^{19}GxxxG^{23}, ^{24}GxxxG^{28} for ThmB peptides, respectively, as is showed in **Figure 6.3**.

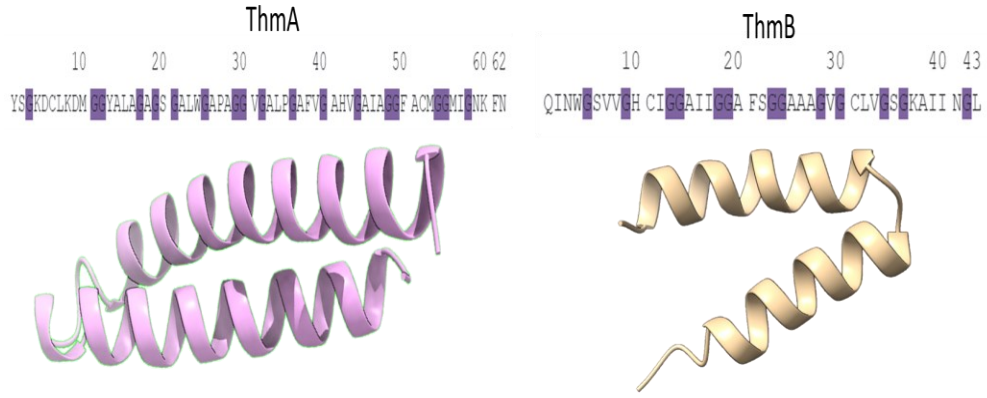

Figure 6.3: Amino acid sequences of the unmodified two-peptide subunit of thermophilin 13. The glycine residues in both peptides are matched in purple.

DISCUSSION

Most bacteriocin operons include genes involved in the post-transcriptional modification and/or secretion of these peptides [12]. Based on that, the present study examined the thermophilin 13 operon (U93029.1) described by Marciset et al. (1997) [9], which appears lacking in bacteriocin-regulating genes involved in bacteriocin synthesis.

In silico analysis is an excellent predictor of "bacteriocin-associated driver genes" within genomes genes adding information on the mechanism related to the specific bacteriocin production. Starting from genomic or amino acid sequences, the main advantages of these methods are a significant reduction in time in comparison to the traditional screening method and, subsequently, the costs embroiled to the use of laboratory materials. Antimicrobial genome-mining tools have been closing the gap between a large number of predicted biosynthetic gene clusters (BGC) encoding bacteriocins, including ribosomally synthesized, post-translationally modified peptides (RiPPs) and also polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) [43]. However, the presence of bacteriocin genes in a strain is always directly related to an effective translation into biological antimicrobial activity [44].

This comprehensive in silico study reveals a complete thermophilin 13 gene cluster containing genes encoding a response regulator (RR), sensor histidine protein kinase (HPK), quorum-sensing system pheromone BlpC, ABC-transporter, bacteriocin accessory protein, thiol–disulfide oxidoreductases, CAAX protease and genes *thmA* and *thmB*.

Furthermore, we confirm the presence of ORFC in all strains; however, no correspondence related to this peptide has been associated with bacteriocin production in the databases.

Similarly to other bacteriocin gene clusters, response regulators grouped as LytR/AlgR family (RR) were predicted [45]. These regulators explicate their function in binding to promoters that initiate the transcription after phosphorylation of Asp residues promoting bacteriocin production and autoactivating their respective operons [46]. LytR Regulatory Systems represents the most abundant type of transcriptional regulator in the prokaryotic kingdom involved as either activators or repressors of single or operonic genes; of genes, including those involved in virulence, metabolism, quorum sensing motility and bacteriocins [45,47]. Furthermore, histidine kinases and response regulators mediate the actual response regarding bacteriocin production by a two-component signal-transducing system [48–51].

Peptide MTKHRTSLTAFTELSPSELHRISGGDWWDWMKYFPSKQAIDSNKHKLG is present in all groups. By identifying the potential role in the bacteriocins biosynthetic gene cluster of this peptide, the prediction showed affinity to the quorum-sensing pheromone BlpC (PF03047 HMM), which is also appointed as ComC/BlpC family leader-containing pheromone/bacteriocin. Interestingly, these peptides are different but are reported in several quorum-sensing regulated bacteriocins in *S. thermophilus*, stimulating the production of BLP (bacteriocin-like peptides) as a signal peptide for the activation of bacteriocin synthesis through a three-component regulatory system consisting of a peptide pheromone, a membrane-associated histidine protein kinase, and response regulators [52,53]. Plantaricins A, E/F and J/K by *L. plantarum* of C11, sakacin A of *L. sakei* Lb706EF and sakacin P of *L. sakei* LTH673102 are the best examples of bacteriocin of class II regulated by the three-component regulatory system, including inducing peptide (an indicator of the cell density), which is sensed by the corresponding (HPK), resulting in the activation of the RR [54].

A dedicated bacteriocin ABC-transporter, including a peptidase C39 motif, predicted to be a bacteriocin/lantibiotic transporter based on conserved domains (COG227400), and a bacteriocin accessory protein generally associated with transport, was observed [55,56]. ABC-transporter proteins related to the class II bacteriocin maturation and secretion carry a proteolytic peptidase C39 domain in their N-termini. The proteolytic peptidase C39 cleaves a double glycine (GG) motif-containing signal peptide from substrates before secretion, modulated in association with an ATP-binding cassette component located in the same protein [57.58]. Differences in ABC transporter sequences in Group 2 were detected in the C39 motif. Interestingly, an independent protein containing C39 peptidase domains, in terms of amino acid sequences, is present in subgroup 2.2. This protein conformation is termed C39 peptidase-like domains (CLD); additionally, their role is not yet completely understood, and they appear degenerated with nonproteolytic activity [59,60]. Most endopeptidases of family C39 are the less conservative component in the entire bifunctional transporter protein with a dedicated catalytic function for the secretion of the antimicrobial peptide of interest [61].

Thiol–disulfide oxidoreductases (TDORs) in Gram-positive bacteria play an essential role in forming disulfide bonds, allowing correct folding in class II bacteriocins through the R−S−S−R′ bond of the CXXC catalytic site resulting in disulfide-bonded cysteines. [62,63]. In this regard, only thmA has two cysteine residues in positions 6 and 53 of the aminoacidic backbones. The aminoacid methionine and single cysteines are also vulnerable to oxidation, but it has never been reported the disulfide bridge formation with this conformation in bacteriocins. However, in this protein, the LPxTG motif membrane-anchored transpeptidase, which cleaves proteins between the threonine (Thr) and the glycine (Gly), is conserved.

Interestingly, ThmA and ThmB peptides lack Thr residues; this is in accordance with Marciset et al. (1997), who observed that the oxidation of methionines to methoxides in position (Met^{10}, Met^{54} and/or $Met^{57)}$ of ThmA seems the only possible explanation of the proposed poration complexes $(AB)_n$ (i.e., Thermophilin 13) [9].

CAAX metalloproprotease (bacteriocin-processing enzymes) detected in bacteriocin loci, including the Abi genes downstream of the bacteriocin structural genes, is likely involved in self-immunity. The role of these conserved motifs in the immunity function conferred a high degree of cross-resistance against each other's bacteriocins, suggesting the recognition of a common receptor. An example of this mechanism was found in *Latilactobacillus sakei* 23K [64]. Furthermore, the bacteriocin-like gene sak23Kalphabeta showed antimicrobial activity when expressed in a heterologous host, and the associated Abi gene sak23Ki conferred immunity against the related bacteriocin [65,66]. Genes encoding the production, secretion, regulation, and immunity of thermophilin 13 are similar to gene sequences reported for class II bacteriocins from *S. thermophilus* strains LMG18311, CNRZ1066, and

LMD-9 [67]. However, in *S. thermophilus* B59671, belonging to Group 3, TDOR and CAAX protease are replaced with a CRISPR/Cas system. Prior studies have noted the importance of quorum sensing induction peptides encoded by the different blp gene clusters found in *S. thermophilus* strains ST109, LMD-9, ST106, LMG18311, CNRZ1066, ND03, JIM8232, MN-ZLW002 and B59671 due to their homology to a bacteriocin-like peptide (blp) gene cluster in *S. pneumoniae* [28,51,68-69]. In relation to this aspect, the strains *S. thermophilus* B59671, ST106, ST109 and LMD-9 have been shown to produce a broad spectrum of bacteriocins encoded within a bacteriocin-like peptide (blp) gene cluster. However, the thermophilin 13 operon is also present in LMD-9 and B59671 strains but must not be confused with the bacteriocin-like peptide (blp) in *S. pneumoniae* gene clusters mentioned above. In this regard, strains LMD-9 and B59671 could be multiple-bacteriocins producer strains and should be highlighted for the necessary evaluation of the role of environmental factors and medium composition on bacteriocin production.

Bacteriocin production is an energy-utilising process involving a cascade of genetic mechanisms that varies greatly in how bacteriocin loci are organised. Among bacteriocin production mechanisms, in many strains, quorum-sensing (QS) circuits modulate various physiological responses, including the production of antimicrobial compounds [70,71]. However, in silico screens can be limited by their dependence on similarity to those previously described by Walsh et al. (2015) [72]. Further work is required to confirm that operon variation between strains influences the production of thermophilin 13. In summary, these results highlight that the production of peptides ThmA and ThmB is strongly related to its PBGC, which is not limited to only *thmA* and *thmB* genes. Therefore, it can be assumed that the QS system regulates the expression of thermophilin 13 bacteriocins in several *S. thermophilus* strains. It has to be considered that since 1997 no other investigations have been made on this bacteriocin. However, the evidence gathered in this study provides further insights into the mechanism of production and regulation of thermophilin 13; this has been observed and described to the scientific community after twenty-five years. All these reported strains are used in industrial applications, and their technological properties have already been proven, opening a new panorama of research that need further investigation. In light of the urgent need for new weapons to counteract pathogens without the use of antibiotics, the identification of the most suitable thermophilin 13 producer strain in terms of bacteriocin production and its applicability in food manufacturing is relevant.

CONCLUSION

The significance of antimicrobial peptides (AMPs) is growing for applicability in various fields, including as a bioprotector agent. There are still many challenges regarding bacteriocins looking for an answer, such as structural multiplicity, different modes of action, different classes, and the high cost of production. Furthermore, also for bacteriocins already applied as preservative agents, the major issues are connected to finding strategies for optimizing their maximum rate of production and developing more effective purification steps from the bacterial supernatant, which are currently long and complicated.

A large number of genomes available in public repositories offer novel approaches valuable in identifying novel bacteriocin genes and gene clusters [54]. Screening of putative bacteriocin gene clusters provides a deeper understanding of how these peptides are regulated. Genome mining indicates that operon thermophilin 13 is present in several strains grouped in three different clusters on the basis of the different genes organization of the eight genes involved in these bacteriocins' biosynthesis. As Marciset et al. (1997) suggested in their conclusion, thermophilin 13 showed peculiar and different characteristics in its mode of action that can share functional properties of lantibiotics. Our results also

indicated that the thermophilin 13 two-component peptide system belongs to class IIb with its own related genes cluster, composed of a response regulator (RR), sensor histidine protein kinase (HPK), quorum-sensing system pheromone BlpC, ABC-transporter, bacteriocin accessory protein, thiol–disulfide oxidoreductases, CAAX protease and genes *thmA* and *thmB*. However, the predictions obtained from the present research and the others in silico studies in general, must not be accepted as conclusive evidence for bacteriocin production, and we do not claim that all strains included in this study can produce thermophilin 13 in vitro and/or in vivo. Nevertheless, the information obtained in this study shed some light on the possible quorum sensing involvement in the mechanisms of regulation and secretion of thermophilin 13, which has already been reported in *Streptococcus thermophilus* strains having bacteriocin-like peptide (blp) gene cluster. This is a solid starting point for further investigation into a topic that has not been explored since 1997, which provides opportunities to expand the knowledge of this antimicrobial peptide in order to target effective applications for food safety.

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CHAPTER 7

General discussion and conclusion

GENERAL DISCUSSION

The history of bacteriocins begins at the same timeline as the discovery of the antibiotic penicillin. In 1925, the Belgian scientist André Gratia reported the first known bacteriocin named colicin (Waters & Crosa, 1991). Alexander Fleming's described the antibiotic penicillin in 1928 (Tan & Tatsumura, 2015). Since then, antibiotics have changed the medical approach to treat infectious diseases (Adedeji, 2016). In parallel, bacteriocins were associated as natural antimicrobials for food preservation (Contessa et al., 2021).

In both medical and food industry ùù, the topic of antibiotic resistance and the need for greater microbiological safety and stability of perishable food products are currently one of the most important challenges .(Andersson & Hughes, 2010; Silver, 2011; Peelman et al., 2013).

Bacteria can become resistant through genetic mutations, and concerning spread of antibiotic resistant genes, acquisition has been reported between species and across genera, including horizontal gene transfer of resistance elements (Mathers et al., 2011). The intensive abuse of antibiotics in medicine and agriculture/animal farming further exacerbates this problem, which has been prioritized with the need for identifying antibiotic alternatives, such as bacteriocins (Borzenkov, Surovtsev & Dyatlov, 2014). Discovery and development of new antibiotics have decreased over the past few decades, and investigation into novel antimicrobials effective against drug-resistant pathogens is therefore essential (Meade, Slattery & Garvey, 2020; Imade et al., 2021). It is considered that most bacteria can produce at least one antimicrobial peptide for self-preservation and competitive advantages in their ecological niche (Hanen et al., 2014). Bacteriocins have long been isolated from bacteria originating from diverse environmental backgrounds, including soil, marine life, food products and human pathogens (Dischinger, Basi Chipalu & Bierbaum, 2014; Volpane et al., 2021). More than 3000 antimicrobial peptides have already been identified due to advances in genome sequencing and mining, providing an ever-increasing number of peptides to be investigated (Wang, Li & Wang, 2016; Hao et al., 2018).

Lactic acid bacteria (LAB) are an integral part of the animal and human intestinal microbiota, of their diet, being the principal actors of food fermentation, or consumed as probiotic. They are utilized in the food industry as natural biopreservatives due to their antimicrobial properties generally related to the production of antimicrobial compounds, among which bacteriocins are one important example (Ren et al., 2022).

In this study, the strains *Lacticaseibacillus casei* UD2202 and *Lacticaseibacillus casei* UD1001 were chosen after bioinformatic screening of their whole genome. Results showed that these strains were the main candidates to be bacteriocins producing bacteria with novel antimicrobial properties sharing high homology in their genetic sequences. The identified potential antimicrobials belong to the class IIa bacteriocins and were named Caseicin FS-X and Caseicin FX-Y.

Homology based analysis also identified two other bacteriocin gene clusters that were classified as variants of the novel genes identified in *Lacticaseibacillus casei* UD2202 and *Lacticaseibacillus casei* UD1001. All four operons, CAS-X, CAS-Y, CAS-J and CAS-Z were novel with an undescribed double protease system. However, immunity protein and ABC transporter are typical from the class IIa bacteriocins involved in self-protection and secretion of these peptides.

The initial aims of this study were to isolate and characterize Caseicin FS-X and Caseicin FX-Y properties. However, our results indicated that *Lacticaseibacillus casei* UD2202 and *Lacticaseibacillus casei* UD1001 could not synthesize these antimicrobials which were in contrast to the in silico results.

In this regard, different antimicrobials were reported to be regulated by different environmental factors. Examples are the lantibiotics streptin and holoduracin, which are activated only in solid media (Wescombe & Tagg, 2003; McClerren et al., 2006). Several attempts with inconsistent results were directed to identify the trigger component responsible for activating the Caseicin production. Moreover, due to the complexity and variability of the several mechanisms of regulation governing the production of bacteriocins. Therefore, it could not be excluded that these strains are bacteriocin producers in a natural environment.

In order to confirm the effective antimicrobial properties, protein expression systems were designed with the application of commercial and self-produced proteases such as WELQut and NisP proteins to isolate the recombinant protein of interest, respectively. Heterologous expression and purification of mature Caseicin FS-X and Caseicin FX-Y confirmed the antilisterial properties. The discrepancy in the ABC transporter nucleotide sequence found in these variants of the operons suggested a possible complication during the maturation of Caseicin FS peptides. Only upstream of the CAS-X operon, a contingent insertion in nucleotide sequence located within unclassified transposon systems, was identified. It is known that bacteriocins operon clusters reside either in the genome, plasmids or other mobile genetic elements (Kumariya et al., 2019). However, based on the literature, it can be supposed that possible alterations in the genes expressed near the transposase could lead to the inactivation of the CAS-X operon (Vandecraen et al., 2017). More evidence is required to rely upon the mobile element with the Caseicin FS-X production.
For this reason, all genes regulating bacteriocins present in operons CAS-X and CAS-Y were evaluated in a modified NICE® system that has been used to express genes of various backgrounds. Production of bacteriocin was detected only in CAS-X operon, which confirmed the complete functionality in producing bacteriocin. On the contrary, CAS-Y did not produce bacteriocins, which focused the investigation on finding the reason causing the missed production between these variants, which is related mainly to the ABC transporter and the presence of an accessory protein found only in CAS-X.
In general, the accessory protein in the regulation of class IIa bacteriocins is rarely found, and its function is associated with the correct folding of disulfide bonds when more than two cysteine residues are present in the aminoacid backbone (Oppegård et al., 2015). However, the bacteriocin Caseicin FS possesses only two cysteines, which could result in only one possible disulfide bond conformation.

On the contrary, the ABC transporters have been described as essential components required for the correct maturation and secretions of class IIa bacteriocins using the type 1 secretion systems (Mesa-Pereira et al., 2017; Beis & Rebuffat, 2019). Several reports using heterologous overexpression in *Escherichia coli* found a direct relationship between the bacteriocins' evolution and their mature form, explicating antimicrobial action using ABC transporters (Franke et al., 1999; Xie et al., 2002).
A similar expression systems approach was proposed in combination with the native protease in the ABC transporter in a separate plasmid to evaluate the proteolytic action in the maturation of Caseicin FS. In this case, heterologously produced ABC transporter reveals complications in the purification. This complication was mainly associated with the membrane protein misfolding, which forms aggregates, rather than the insoluble fraction (inclusion bodies) of the *E. coli* host, as described by Korepanova et al., (2009).
To avoid this problem, successful co-expression of both genes encoding bacteriocin and ABC transporter confirmed that only the ABC transporter found in CAS-X operon can complete the maturation of Caseicin FS-X.

The *in vitro* characterization of Caseicin FS-X and Caseicin FS-Y, as well as the identification and annotation of its operons, including variants, contributing to the available information of the class IIa bacteriocins research.
However, from the results obtained within these chapters, it is evident that there are several areas where future research is needed:

1. All four Caseicin operons should be functionally characterized to confirm the putative annotations of genes with unknown functions, such as the two hypothetical proteins and the second proteases with the role still undescribed in relation to bacteriocin production.
2. Three approaches leaded to the comprehension of the mode of regulation of Caseicin FS operon and resulted in the production of this antimicrobial peptide. Further optimization of an expression system dedicated to the large-scale production is still needed to produce higher yields of this peptide.
3. More research in needed to characterize Caseicin's structure, chemical and physical properties in order to further narrow the best condition of applicability.

In vitro activity of Caseicin FS is typical of class IIa, especially against *L. monocytogenes*, which suggests possible application as natural antimicrobials for food preservation. A preliminary trial in food explores the efficacy of Caseicin treatment in stracchino cheese and sliced ham. In both food matrices, Caseicin was effective in reducing viable bacterial cells of *L. monocytogenes* intentionally inoculated. Bacteriocin addition showed promising results, reaching tolerance zero after 24 h of inoculum under the condition of refrigeration. However, these results request a deeper investigation within the evaluation of different temperatures, pH, strains target, peptide concentrations and combination with various technologies such as the polymeric nanofibers for active food packaging (Min et al., 2022).
Lastly, a comprehensive *in silico* analysis elucidating the mechanism of regulation of thermophilin 13, still considered an atypical bacteriocin with only two genes, *thmA* and t*hmB* (total length 960-bp) was described shedding some light on the possible quorum sensing involvement in the mechanisms of regulation and secretion of thermophilin 13.

FINAL CONCLUSION

This work explored the structural and functional characteristics of novel gene organizations identified in four different variants codifying an undescribed bacteriocin named Caseicin FS. These entire operons were annotated and contained genes related to the class IIa bacteriocins biosynthesis. Hence bactericidal properties are undetected from the wild strains characterisation of these novel peptides different expression system were designed to simplify the production and evaluation of Caseicin FS functionality. Furthermore, we reported three main plasmid-based systems dedicated to the comprehension of the mechanics of the production of this peptide. The achievement was the production of Caseicin FS, which is confirmed as a novel bacteriocin belonging to class IIa with proven antilisterial properties *in vitro* and *in vivo*. Additionally, these results provide valuable information on evaluating these operons, which seemed to be related to the evolutionary constraint component which could modify the genetics architecture of Caseicin FS. In conclusion, the developments of genome mining techniques still face several challenges in identifying target molecules and providing effective predictions of their mode of regulation. However, this work, combining in silico approach and overexpression of recombinant proteins, perfectly matched the expectations in discovering new bacteriocins and understanding their functionality for further application for a sustainable future. In addition, future studies should investigate the up-scaled production of Caseicin FS in bioreactors for improved yields applying expression systems suitable for large scale production.

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SUPPLEMENTARY MATERIAL

CHAPTER 4

Table S. 1: First 100 hits from BLASTp similarity search of trcsx ABC transporter.

Description	*Scientific Name*	*Query Cover*	*Per. Ident*	*Accession*
peptide cleavage/export ABC transporter	*Lacticaseibacillus zeae*	100%	100.00%	WP_070650997.1
peptide ABC transporter ATP-binding protein	*Lactobacillus sp. HMSC075D02*	99%	84.53%	OFP94090.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.53%	WP_005692250.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.39%	WP_049168746.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.39%	WP_077069577.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.25%	WP_005716473.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.39%	WP_005686870.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.39%	WP_270767368.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.39%	WP_064520302.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.39%	WP_142490688.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.25%	WP_031547269.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.12%	WP_033572803.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.39%	WP_085320191.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.39%	WP_039141996.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.39%	WP_061713740.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.25%	WP_176818169.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.12%	WP_064613210.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.39%	WP_127091274.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.25%	WP_260185249.1
peptide ABC transporter ATP-binding protein	*Lacticaseibacillus rhamnosus*	99%	84.25%	KMO58516.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.12%	WP_049171127.1

peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.25%	WP_211751323.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.12%	WP_033571746.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.25%	WP_032960783.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	98%	84.66%	WP_175417904.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.12%	WP_005711104.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	84.12%	WP_014570077.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	83.98%	WP_048486321.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	83.98%	WP_029607600.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_194498726.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	83.29%	WP_048481987.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	99%	83.29%	WP_064657417.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus zeae*	99%	82.60%	WP_070650604.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.33%	WP_218209513.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus*	99%	83.20%	WP_003585566.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_196241382.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_194958408.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_016388369.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_261913619.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_260183747.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_016381944.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.20%	WP_016383809.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_160528522.1

peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_013246039.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_270740387.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_079322810.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_270757993.1
ABC-type bacteriocin transporter	*Lacticaseibacillus paracasei*	99%	83.20%	EEI68820.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_238064867.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.20%	WP_040167206.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_194957489.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_101512231.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_126313983.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_100908635.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_194853557.1
ABC transporter	*Lacticaseibacillus paracasei*	99%	83.20%	EPC57716.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_063557687.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_194959036.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_128529386.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_194957896.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_119182947.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_165847263.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_123020077.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.78%	WP_016386629.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_123031020.1

ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	EPC72615.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_025376283.1
ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	EPD00532.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_016385901.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_050894416.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_123156672.1
ATP-binding component	*Lacticaseibacillus paracasei*	99%	82.92%	EKP97927.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_128532791.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_236360392.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_016379452.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.78%	WP_123018271.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_123019703.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.78%	WP_250786011.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_016384129.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.64%	WP_129533378.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_123022635.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.78%	WP_241706541.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_204126099.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	83.06%	WP_230646996.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.78%	WP_003605967.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.78%	WP_249479980.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_123021468.1

peptide cleavage/export ABC transporter	*Lacticaseibacillus*	99%	82.78%	WP_016372601.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus*	99%	82.78%	WP_003567347.1
ABC transporter	*Lacticaseibacillus paracasei*	99%	82.78%	EPC60752.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.64%	WP_016387337.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.78%	WP_032796911.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.78%	WP_215649434.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_128518644.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.92%	WP_260184483.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.78%	WP_216501197.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.78%	WP_003607148.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.64%	WP_260185522.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	99%	82.78%	WP_260185844.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	*99%*	*82.64%*	*WP_060612602.1*

Table S. 2: First 100 hits from BLASTp similarity search of trcsy ABC transporter.

Description	***Scientific Name***	***Query Cover***	***Per. Ident***	***Accession***
peptide cleavage/export ABC transporter	*Lacticaseibacillus zeae*	100.00%	100.00%	WP_070650997.1
bacteriocin-processing peptidase, Cysteine peptidase, MEROPS family C39	*Lacticaseibacillus paracasei*	98%	77.64%	ABJ71124.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus paracasei*	98%	77.64%	WP_049144691.1
Lactococcin-G-processing and transport ATP-binding protein LagD	*Lacticaseibacillus paracasei*	98%	77.64%	RND80657.1
Lactococcin-G-processing and transport ATP-binding protein LagD	*Lacticaseibacillus paracasei*	98%	77.02%	RND46030.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus paracasei*	98%	77.64%	WP_263853422.1

Lactococcin-G-processing and transport ATP-binding protein LagD	*Lacticaseibacillus paracasei*	98%	77.64%	RNE29506.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus paracasei*	98%	77.02%	WP_025599738.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus rhamnosus*	97%	77.99%	WP_263850173.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus rhamnosus*	97%	77.99%	WP_032958017.1
peptide ABC transporter ATP-binding protein	*Lacticaseibacillus rhamnosus*	97%	77.99%	KMO55705.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus paracasei*	98%	77.02%	WP_260367664.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus rhamnosus*	97%	77.99%	WP_019728292.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus rhamnosus*	97%	77.99%	WP_238593110.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus rhamnosus*	97%	77.99%	WP_235805711.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus rhamnosus*	97%	77.99%	WP_229032682.1
peptide ABC transporter ATP-binding protein	*Lacticaseibacillus rhamnosus*	97%	77.36%	OAU92017.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus casei*	97%	75.47%	WP_263853173.1
truncated bacteriocin ABC transporter ATP-binding and permease components	*Lacticaseibacillus casei*	97%	75.47%	BAN75301.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus rhamnosus*	97%	77.99%	WP_263862336.1
putative ABC transporter, ATP-binding protein ComA	*Lacticaseibacillus rhamnosus*	97%	77.99%	VEF30442.1
cysteine peptidase family C39 domain-containing protein	*Lacticaseibacillus rhamnosus*	97%	77.99%	WP_238138288.1
Lactococcin-G-processing and transport ATP-binding protein LagD	*Lacticaseibacillus paracasei*	98%	77.02%	VTZ84904.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	78.26%	WP_218209513.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_194498726.1
ATP-binding component of an ABC superfamily peptide transporter	*Lacticaseibacillus paracasei*	98%	77.64%	EKP97927.1

ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	EPD00532.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_101512231.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_128529386.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_160528522.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_270740387.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_250786011.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus*	98%	77.64%	WP_003585566.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_196241382.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_063557687.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_016384129.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_194958408.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_149350261.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_261913619.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_249479980.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_016387337.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_202959584.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_016386629.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_013246039.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_123020077.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_129533378.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_123018271.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_119182947.1

peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_260183747.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_194959036.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_016388369.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_194957896.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_215649434.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_016381944.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_123156672.1
ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	EPC57716.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_204126099.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_128518644.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_016383809.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_025376283.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_194853557.1
ABC-type bacteriocin transporter	*Lacticaseibacillus paracasei*	98%	77.64%	EEI68820.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_128532791.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_123021468.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_241706541.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_003607148.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_270757993.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_016385901.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_050894416.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_196498710.1

peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_123019703.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus*	98%	77.02%	WP_003567347.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_260185844.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_236360392.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_100908635.1
ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	EPC72615.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	97%	77.99%	WP_047678340.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_238064867.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_123031020.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_016379452.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_165847263.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_216501197.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_126313983.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_260184483.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_123022635.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_194957489.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_032796911.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_128521556.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus*	98%	77.02%	WP_016372601.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.64%	WP_040167206.1
ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	EPC60752.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_260185522.1

peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_060612602.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_079322810.1
ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	EPC46438.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_128517850.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	76.40%	WP_003605967.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus zeae*	97%	76.73%	WP_070650604.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus paracasei*	98%	77.02%	WP_230646996.1
peptide cleavage/export ABC transporter	*Lacticaseibacillus rhamnosus*	97%	77.99%	WP_064613210.1