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**Production, Characterization and Utilization of the Bacteriocin
Produced by *Enterococcus faecalis* B9510**

**A thesis presented in partial fulfilment of the requirements for the degree of
Doctor of Philosophy in Biotechnology
at Massey University, Palmerston North, New Zealand**

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ABSTRACT

Bacteriocins are antimicrobial proteins and peptides produced by bacteria, antagonistic to other bacterial species but not affecting the producer species. The bacteriocins from Lactic Acid Bacteria (LAB) are particularly well-studied and exploited as safe and natural food preservatives. The current research aimed to identify and characterize a bacteriocin-like substance produced by *Enterococcus faecalis* B9510, a local isolate from silage. The production of this antimicrobial during the growth phase, destruction of antimicrobial activity by proteolytic enzymes and self-immunity of the producer strain indicated that the antimicrobial is a bacteriocin. The bacteriocin was heat-labile as the antimicrobial activity was destroyed by heating at 60 °C for one hour. This bacteriocin was also found to lyse sensitive cells.

To further characterize the bacteriocin to the protein level, the producer strain was grown in a completely defined medium, devoid of any proteins and peptides, to facilitate downstream processing. Purification was done by passing the culture supernatant through 10 kDa and 30 kDa ultrafiltration membranes. The 30 kDa ultrafiltration retentate showed antimicrobial activity and was then subjected to SDS-PAGE. The in-gel bacteriocin activity was then determined by incorporating dead cells of a sensitive strain *Lactococcus lactis* ssp. *cremoris* 2144 in a parallel SDS-PAGE gel, which was renatured after the electrophoresis. After renaturation a zone of clearance was observed around the active band, at approximately 35 kDa. The active band was excised and analyzed by mass spectrometry. The results revealed that the amino acid sequence matched a known bacteriocin enterolysin A. This was confirmed when the enterolysin A gene was amplified from the producer strain using PCR followed by DNA sequencing.

The earlier studies on enterolysin A primarily focused on the structural gene, and primary structure of enterolysin A. No information is available on the function of neighbouring genes of the enterolysin A structural gene. An attempt was made in the current study to elucidate the function of genes found in close proximity to the structural gene, with the aim to find the immunity gene.

Experiments were also conducted to find the mode of action of enterolysin A. Earlier studies have reported that enterolysin A is an endopeptidase which degrades the cell walls of sensitive Gram-positive bacteria. However, the cleavage site within the cell wall moiety has not been reported. The current study has revealed that enterolysin cleaves a peptide bond between D-glutamic acid and L-alanine in the stem peptide and N-

terminus of L-lysine and C-terminus of D-aspartic acid within the interpeptide bridge of peptidoglycan units of sensitive bacterial strains. Furthermore, transmission electron microscopy of enterolysin A treated cells gave new insight into the morphology of damaged cells.

The antimicrobial spectrum of enterolysin A already reported was also extended to other species in the current study. The results revealed that in addition to its activity against the bacterial species already reported, enterolysin A is also active against *Lactobacillus helveticus*, *Lactobacillus casei* and *Lactobacillus delbrueckii* ssp. *bulgaricus*.

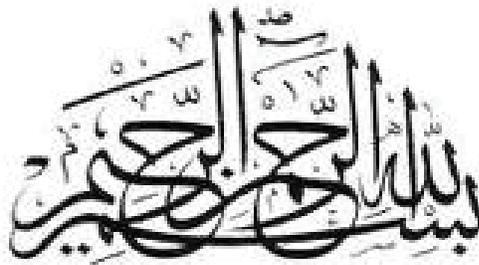
To conclude the project, enterolysin A was coated on polyethylene film. This film was found to effectively control the growth of *L. casei* and thus can be incorporated into antimicrobial packaging against spoilage microorganisms.

DEDICATED

TO

MY KIND AND LOVING MOTHER

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In the name of Allah, most benevolent, ever merciful

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FREQUENTLY USED ABBREVIATIONS

aa	Amino acids
ABC	ATP Binding Cassette
API	Analytical Profile Index
AS	Ammonium Sulphate precipitation
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
AU	Arbitrary Units
AWDA	Agar Well Diffusion Assay
bp	Base pairs
CAT	Catalytic Domain
CDM	Complete Defined Medium
CEX	Cation Exchange Chromatography
Cm	Chloramphenicol
Cm-r	Chloramphenicol resistance cassette
Da	Daltons
DNA	Deoxyribonucleic Acid
FPLC	Fast Protein Liquid Chromatography
FRC	Fonterra Research Centre, Palmerston North, New Zealand
GF	Gel Filtration Chromatography
GRAS	Generally Regarded As Safe
h	hour
HIC	Hydrophobic Interaction Chromatography
IAC	Immuno-Affinity Chromatography
IEX	Ion-Exchange Chromatography
IPTG	Isopropyl- β -D-galactopyranoside
kDa	kilodaltons
LAB	Lactic Acid Bacteria
LDPE	Low Density Polyethylene
MALDI, TOF	Matrix Assisted Laser Desorption Ionisation, Time of Flight
min	minutes

MIC	Minimum Inhibitory Concentration
ML, MU	Microbiology Laboratory, Massey University, Palmerston North
NAG	N-acetyl Glucosamine
NAM	N-acetyl Muramic Acid
NCBI	National Centre for Biotechnology Information
NCTC	National Collection of Type Cultures
NMWCO	Nominal Molecular Weight Cut-Off
OD	Optical Density
ORF/ <i>orf</i>	Open Reading Frame
PCR	Polymerase Chain Reaction
pH	Negative decadal logarithm of the [H ⁺] ion concentration
pI	Iso-electric point
PTS	Mannose Phospho-Transferase System
rcf	Relative Centrifugal Force
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
SD	Standard Deviation
SDM	Simplified Defined Medium
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
sec	Seconds
SRD	Substrate Recognition Domain
ssp.	Subspecies
TEM	Transmission Electron Microscopy
TEMED	Tetramethyl Ethylene Diamine
TLC	Thin Layer Chromatography
UF	Ultrafiltration
μ	Specific growth rate (h ⁻¹)

ABBREVIATIONS OF NUCLEIC ACIDS

A	Adenine
T	Thymine
G	Guanine
C	Cytosine
U	Uracil

ABBREVIATIONS OF AMINO ACIDS

Amino Acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

