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**EXPRESSION, PURIFICATION AND CHARACTERISATION OF  
ANTIMICROBIAL PEPTIDES OF HUMAN AND BOVINE  
LACTOFERRINS**

A thesis presented in partial fulfilment of the  
requirements for the degree of

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## Abstract

Lactoferrin (Lf), a basic, ~80 kDa iron-binding glycoprotein, is a member of the transferrin family. It is present in the milk and other secretory fluids of many, but not all, mammalian vertebrates. Several biological functions have been ascribed to this protein. These include bacteriostasis, modulation of the inflammatory response, iron nutrition, a role as an anti-oxidant and regulation of myelopoiesis.

Full-length human lactoferrin has been expressed in BHK cells, many strains of *Aspergillus* and with limited success in *Saccharomyces cerevisiae*. The main aim at the start of this project was to express full-length human lactoferrin (hLF) cDNA in the yeast *Kluyveromyces lactis* on whey-based media. Yeasts of the genus *Kluyveromyces* have been used for many years in the food industry and are classified as “Generally Regarded As Safe” (GRAS) organisms. *K. lactis* has impressive secretion capabilities and can grow on whey-based media (which is abundantly available in New Zealand). Attempts were made to sub-clone full-length hLF cDNA into the *K. lactis* vector, pEPS1 and the *S. cerevisiae* vector, pYEXS1 and to express the protein. The establishment of stable insert-carrying constructs of these yeast vectors in *E. coli* turned out to be an unattainable goal. Direct transformation of the ligation mix into *K. lactis* produced transformants, which secreted human lactoferrin protein products into the media as assessed by the lactoferrin ELISA assay. The secretion of hLF protein products by recombinant *K. lactis* continued for few generations, but gradually stopped. There are no known reports on the use of these vectors for the expression of any mammalian proteins in yeast.

Lactoferrin has antimicrobial activity against a broad range of Gram-negative and Gram-positive bacteria and against fungi. Originally, the antimicrobial effect of lactoferrin was attributed to its ability to tightly sequester two atoms of iron and hence inhibit microbial growth through nutritional deprivation of iron. Recently, an N-terminal peptide called lactoferricin, isolated from the acid-pepsin hydrolysate of lactoferrin was

shown to have greater antimicrobial activity than the intact protein.

Currently, the only way to obtain native lactoferricins is to isolate the peptides from the acid pepsin-hydrolysate of lactoferrin, which gives very low yields, or to synthesise them by protein chemical methods, which is very expensive on a large scale. So, heterologous expression of both human and bovine lactoferricins in *E. coli* was attempted in this study. Synthetic DNA fragments encoding both human and bovine lactoferricins and 3'-end variants of these fragments were sub-cloned into *E. coli* expression vectors, pPROEXHTa, pET-15b and pGEX-4T1. The constructs were designed to express lactoferricins either as poly-His- or as GST-fusion proteins. In all cases the fusion proteins were expressed as inclusion bodies. The inclusion bodies were urea solubilised and purified on appropriate affinity resins. However, none of the recombinant proteins remained soluble after the urea was removed and therefore could not be further characterised. A similar situation was encountered by other investigators who attempted to express cationic peptides in *E. coli*.

Both lactoferrin and lactoferricin have been shown to bind to the lipid A portion of the bacterial cell wall lipopolysaccharide (LPS), induce the release of LPS and kill the bacteria. In this work, five different *E. coli* strains were shown to have different susceptibility to native lactoferricin B. Transmission electron microscopy studies of the *E. coli* strains treated with lactoferricin B revealed considerable differences in their membrane ultrastructure. The most susceptible *E. coli* strain showed a direct outer membrane dislocation and effect on the cytoplasmic contents. A relatively resistant *E. coli* strain showed an initial formation of 'membrane blisters'. However, after a long exposure to lactoferricin B, a few cells of this strain showed an outer membrane dislocation and effect on the cytoplasmic contents. The formation of 'membrane blisters' might allow the relatively resistant strain of *E. coli* to reduce the lethal action of lactoferricin B.

## List of publications and presentations

This work has been published or presented in part in the following papers.

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# List of Contents

	Page
Abstract	i
List of publications and presentations	iii
List of Contents	iv
List of Tables	xii
List of Figures	xiii
Abbreviations	xv
Abbreviations of units	xviii

## CHAPTER 1

### Introduction And Literature Review

Group I	<i>The haem-containing proteins with catalytic or carrier functions</i>	2
Group II	<i>The iron storage proteins</i>	2
Group III	<i>Iron transport or iron-regulatory proteins</i>	3
<b>1.1</b>	<b>General properties of transferrin family of proteins</b>	3
<b>1.2</b>	<b>Location of lactoferrin in mammalian vertebrates</b>	6
<b>1.3</b>	<b>Biological functions of lactoferrin</b>	8
1.3.1	Lactoferrin in iron absorption and nutrition	8
1.3.2	Role of lactoferrin in growth promotion	11
1.3.3	Lactoferrin's role as a regulator of myelopoiesis	12
1.3.4	Lactoferrin in inflammation and immunomodulation	13
1.3.5	Lactoferrin as an anti-oxidant	17
1.3.6	Antimicrobial activity of lactoferrin	19
<b>1.4</b>	<b>Lactoferricin</b>	27
1.4.1.1	Identification, isolation and activity of the antimicrobial domain of lactoferrin	27

1.4.2	Structure vs biological activity relationships of the amino acid residues in lactoferricin	32
1.4.3	Antimicrobial activity of lactoferricin B	37
1.4.4	Affect of lactoferrin and lactoferricin on Gram-negative cell Membranes	39
1.4.5	Bovine Lactoferricin (LFcin-B)	40
1.4.6	Human Lactoferricin (LFcin-H)	41
<b>1.5</b>	<b>Expression of lactoferrin <i>in vivo</i></b>	41
<b>1.6</b>	<b>Heterologous expression of transferrin and lactoferrin and its variants</b>	42
<b>1.7</b>	<b>Structure of lactoferrin</b>	43
1.7.1	Human lactoferrin	44
1.7.2	Bovine lactoferrin	47
<b>1.8</b>	<b>Heterologous gene expression systems</b>	49
1.8.1	Yeast expression systems	49
1.8.2	<i>E. coli</i> expression vector systems	49
<b>1.9</b>	<b>Justification of this project</b>	50
	<i>Perspectives</i>	50
	<i>Aims</i>	51

## CHAPTER 2

### Materials And Methods

<b>2.1</b>	<b>Materials</b>	52
2.1.1	Enzymes and chemicals	52
2.1.2	Bacterial strains, yeast strains and plasmids used in this study	54
2.1.3	Genotypes of bacterial and yeast strains used in this study	55
2.1.4	Bacterial and yeast growth media	55
2.1.5	Commonly used solutions and buffers	56
2.1.6	Maintenance, storage and propagation of bacterial cultures	58
2.1.7	Growth and storage of M13 phage stocks	59

2.1.8	Growth and maintenance of yeasts <i>Saccharomyces cerevisiae</i> and <i>Kluyveromyces lactis</i>	59
<b>2.2</b>	<b>Methods</b>	59
2.2.1	General precautions in DNA handling	60
2.2.2	DNA and RNA quantitation	60
2.2.3	Protein quantitation	60
2.2.4	Preparation of M13 replicative form	60
2.2.5	Plasmid DNA and ds-M13 replicative form DNA preparations from <i>E. coli</i>	61
	<i>Plasmid DNA Minipreps</i>	61
	<i>Large-scale preparation of plasmid DNA</i>	62
2.2.6	Isolation of plasmids from <i>K. lactis</i>	62
2.2.7	Restriction digestion of DNA with endonucleases	62
2.2.8	Agarose gel electrophoresis of DNA	63
2.2.9	Size determination of DNA fragments	63
2.2.10	Precipitation of DNA and purification of DNA fragments from agarose gels	63
2.2.11	Modification of DNA fragments	64
2.2.12	Ligation of DNA fragments	64
2.2.13	Preparation of <i>E. coli</i> competent cells	64
2.2.14	Transformation of <i>E. coli</i> competent cells	65
2.2.15	Preparation of <i>K. lactis</i> competent cells	65
2.2.16	Electroporation of <i>K. lactis</i> competent cells	65
2.2.17	Transformation of <i>S. cerevisiae</i>	66
2.2.18	Sequence analysis of DNA	66
<b>2.3</b>	<b>Working with RNA</b>	66
2.3.1	Precautions to be taken in RNA work	66
2.3.2	RNA isolation from <i>E. coli</i>	67
2.3.3	RNA gel electrophoresis	67
2.3.4	Southern and northern blotting	67
<b>2.4</b>	<b>Expression and analysis of recombinant proteins</b>	68
2.4.1	Expression studies of recombinant proteins in <i>E. coli</i>	68
2.4.2	Disruption of <i>E. coli</i> cells	68
2.4.3	SDS-polyacrylamide gel electrophoresis using minigels	68

2.4.4	Fixing, staining and destaining of polyacrylamide gels	69
2.4.5	Electroblotting proteins from acrylamide gels	69
2.4.6	Western blotting	69
2.4.7	Enzyme-linked immunosorbant assay (ELISA) of lactoferrin	70
<b>2.5</b>	<b>Purification and characterisation of recombinant proteins</b>	<b>71</b>
2.5.1	Poly-His affinity tagged protein purification	71
2.5.1.1	Buffers for the purification of inclusion bodies on Ni-NTA column	71
2.5.2	GST-tagged protein purification	71
2.5.2.1	Buffers for the purification of GST-tagged proteins expressed as inclusion bodies	72
2.5.3	N-terminal protein sequencing of recombinant lactoferricins	72
<b>2.6</b>	<b>Antibacterial assays of native lactoferricin B (LFcin-B)</b>	<b>72</b>
2.6.1	Preparation of bacterial cultures for antibacterial assay	72
2.6.2	Determination of minimum inhibitory concentration (MIC) of LFcin-B	73
2.6.3	Assay of the bactericidal activity of LFcin-B	73
<b>2.7</b>	<b>Transmission Electron Microscopy (T.E.M.)</b>	<b>74</b>
2.7.1	Sample preparation	74
	<i>Primary fixation</i>	74
	<i>Post-fixation</i>	74
	<i>Embedding</i>	75
2.7.2	Cutting semi-thin and ultra-thin sections and their observation	75
	<i>Preparation of glass knife and observation of sections</i>	75

## CHAPTER 3

### Sub-Cloning And Expression Studies Of Full-Length And N-Terminal Lobe Of Human Lactoferrin cDNA In Yeast Vectors

<b>3.1</b>	<b>Introduction</b>	<b>76</b>
<b>3.2</b>	<b>Results</b>	<b>79</b>
3.2.1	Preparation of replicative form of M13 containing the N-lobe of hLF cDNA with a <i>SacI</i> restriction site	79

3.2.2	Sub-cloning of <i>SacI/SmaI</i> fragment from the replicative form of M13:Lfn (1-296) ( <i>SacI</i> ) into pGEM.hLF construct	79
3.2.3	Changing of <i>HindIII</i> at the 3'-end of hLF cDNA in pGEM.hLF (-1) ( <i>SacI</i> ) to <i>SacI</i>	81
3.2.4	Sub-cloning of ~2.1 kb hLF cDNA <i>SacI</i> fragment into pEPS1 and pYEXS1 expression vectors	83
	<i>Ligation of the fragment into vectors</i>	85
	<i>Size of the construct</i>	86
	<i>Nature of the hLF cDNA and the vectors</i>	87
3.2.5	Direct transformation of competent yeast cells with the ligation mix	87
3.2.6	Expression studies of full-length hLF or its fragments in recombinant <i>K. lactis</i>	87
3.2.7	Southern blot analysis of plasmid DNA isolated from recombinant <i>K. lactis</i>	88
3.2.8	Sub-cloning of hLF cDNA into pSPHO4, <i>K. lactis</i> vector with an inducible promoter	89
<b>3.3</b>	<b>Expression of the N-terminal lobe hLF cDNA in yeast-host vector systems</b>	89
3.3.1	Sub-cloning of the N-terminal lobe of hLF cDNA into <i>E. coli</i> vector pGEX4T-3	91
3.3.2	Conversion of <i>AatII</i> restriction site to either <i>SacI</i> or <i>BamHI</i> in pGEX4T-3/Lfn construct	91
3.3.3	Sub-cloning of <i>SacI</i> fragment of hLF cDNA N-lobe into yeast vectors	92
3.3.4	Sub-cloning of <i>BamHI</i> fragment of hLF cDNA N-lobe from pGEX4T-3/Lfn ( <i>BamHI</i> ) into pET-15b vector	93
3.3.5	Expression studies of pGEX4T-3/Lfn ( <i>SacI</i> ) and pET-15b/Lfn ( <i>BamHI</i> ) constructs in <i>E. coli</i>	93
<b>3.4</b>	<b>Discussion and conclusions</b>	94
(a)	Establishment of the constructs of yeast vectors with inserts of hLF cDNA and its N-terminal lobe	94
(b)	Direct transformation of yeast competent cells with the ligation mix by electroporation	94

## CHAPTER 4

### Expression Of Antimicrobial Peptides Of Human And Bovine Lactoferrins in *E. coli*

<b>4.1</b>	<b>Introduction</b>	96
<b>4.2</b>	<b>Results</b>	97
4.2.1	Design of overlapping synthetic oligonucleotides to construct full-length DNA for lactoferricin	97
4.2.2	Preparation of full-length lactoferricin H & B DNA from overlapping oligonucleotides	101
4.2.3	Cloning of the end-filled DNA fragments into <i>E. coli</i> expression vectors	102
4.2.4	DNA and amino acid sequences of the LFcIn-B constructs in pPROEXHTa <i>E. coli</i> expression vector	106
4.2.5	DNA and amino acid sequences of the LFcIn-H constructs in pPROEXHTa <i>E. coli</i> expression vector	107
4.2.6	Sub cloning of LFcIn-H & B fragments from ProA+LFCIn constructs into pGEX-4T1 and pET-15b <i>E. coli</i> expression vectors	109
<b>4.3</b>	<b>Analysis of all the constructs of LFcIn-H &amp; B</b>	112
4.3.1	Expression studies of the ProA+bp construct	112
4.3.1.1	Optimisation of expression of the ProA+bp construct	113
4.3.1.2	Isolation and solubilisation of inclusion bodies	113
4.3.1.3	Purification of solubilised inclusion bodies by column chromatography	114
4.3.1.4	Processing of the purified recombinant protein	115
	<i>Removal of urea, salt and imidazole by dialysis</i>	115
a.	Dialysis against Milli-Q water at pH 5.5 and pH 7.0	115
b.	Dialysis against 100 mM ammonium acetate	116
c.	Dialysis against 20 mM Tris-HCl buffer with and without 0.5 M NaCl	116
d.	Dialysis against 20 mM Tris-HCl buffer (pH 8.0) with a gradual decrease in urea concentration	116
e.	Dialysis against Milli-Q water and 20 mM Tris-HCl buffer (pH 8.0) both with 1 mM DTT (final concentration)	117
4.3.1.5	HPLC purification of recombinant LFcIn-B fusion protein	118
4.3.1.6	N-terminal sequencing of recombinant fusion protein expressed from the pPROEXHTa vector (ProA+bp construct)	119

4.3.2	Expression studies using the constructs Pro+hH, ProA+hH#, ProA+bp#, ProA+mbp and ProA+mhH	120
4.3.3	Expression studies using the constructs nT1+bp, nT1+hH, nT1+bp# and nT1+hH#	121
4.3.3.1	Purification of GST-fused proteins expressed by the nT1+bp# and nT1+hH# constructs	121
4.3.3.2	Thrombin cleavage of the GST-fusion protein	123
4.3.4	Expression studies using the constructs nET+bp, nET+hH, nET+bp# and nET+hH#	126
4.3.5	Plasmid stability in the induced cultures	126
4.3.6	Northern blot analysis of the LFcIn-H & B constructs	127
4.3.6.1	Analysis of LFcIn-H & B constructs with respective probes	127
4.3.6.2	Analysis of LFcIn-H & B constructs with GST-probe	130
4.3.6.3	Discussion of the northern blots	132
<b>4.4</b>	<b>Discussion and conclusions</b>	135
a.	Expression of synthetic gene sequences for LFcIn-H & B in <i>E. coli</i>	135
b.	Purification and characterisation of recombinant fusion proteins of lactoferricins	137

## CHAPTER 5

### Effects Of Native Lactoferricin B On Selected *E. coli* Strains

<b>5.1</b>	<b>Introduction</b>	139
<b>5.2</b>	<b>Results</b>	140
5.2.1	Calibration curves to assess the growth of <i>E. coli</i> strains used in this study	140
5.2.2	Estimation of native lactoferricin B concentration	142
<b>5.3</b>	<b>Antibacterial activity of lactoferricin B</b>	142
5.3.1	Determination of minimum inhibitory concentration (MIC) of lactoferricin B	143
5.3.2	Time-kill curves lactoferricin B	145
<b>5.4</b>	<b>Transmission electron microscopy (T.E.M.) studies of selected <i>E. coli</i> strains</b>	149

5.4.1	T.E.M. studies of lactoferricin B treated <i>E. coli</i> strain DH5 $\alpha$	150
5.4.2	T.E.M. studies of lactoferricin B treated <i>E. coli</i> strain 0111 (NCTC 8179)	152
5.4.3	Comparison of the T.E.M. pictures of <i>E. coli</i> DH5 $\alpha$ and 0111 at the same magnification	154
<b>5.5</b>	<b>Discussion and conclusions</b>	<b>157</b>

## CHAPTER 6

### General Discussion, Conclusions And Future Directions

<b>6.1</b>	<b>General discussion</b>	<b>159</b>
<b>6.2</b>	<b>Expression of full-length human lactoferrin (hLF) in <i>Kluyveromyces lactis</i></b>	<b>159</b>
6.2.1	Expression studies of the N-terminal lobe of hLF cDNA in <i>E. coli</i>	160
<b>6.3</b>	<b>Heterologous expression of human and bovine lactoferricins in <i>E. coli</i></b>	<b>161</b>
6.3.1	Northern blot analysis of LFCin-H & B constructs	162
6.3.2	Degradation of recombinant protein inside the <i>E. coli</i> cell by proteolysis	162
6.3.3	Production of inclusion bodies in <i>E. coli</i> : Problems and prospects	163
<b>6.4</b>	<b>Solubilisation and purification of recombinant proteins</b>	<b>164</b>
<b>6.5</b>	<b>Antibacterial assays of lactoferricin B</b>	<b>165</b>
<b>6.6</b>	<b>Transmission electron microscopy studies of lactoferricin B treated <i>E. coli</i> cells</b>	<b>166</b>
6.6.1	Interaction of lactoferricin B with biological membranes	166
<b>6.7</b>	<b>Conclusions</b>	<b>169</b>
<b>6.8</b>	<b>Future Directions</b>	<b>170</b>
6.8.1	Expression of human lactoferrin in <i>K. lactis</i> using a different set of proven expression vectors	171
6.8.2	Alternative expression systems for the expression of cationic peptides	171
6.8.3	Studies on the binding characteristics of lactoferricins and their variants to the LPS preparations of different <i>E. coli</i> strains	172

## List of Tables

Table 1.1	Iron compartments made by groups of iron binding (containing) proteins in normal humans	2
Table 1.2	Lactoferrin concentrations in various secretions of both human and bovine mammary glands	7
Table 1.3	Citrate to lactoferrin molar ratios in the secretions from the bovine mammary gland	22
Table 1.4	Effectiveness of antimicrobial activity of lactoferrin and its derivatives against <i>Escherichia coli</i> 0111	29
Table 1.5	Binding parameters of hLf, bLf, rhLf, EGS-loop rhLf and proteolytic fragments derived from hLf to <i>E. coli</i> 055B5 LPS	33
Table 1.6	Sequence alignment of antimicrobial peptides with lactoferrins including regions beyond the antimicrobial segments	34
Table 1.7	List of various microorganisms tested for antimicrobial activity with lactoferricin B	37
Table 1.8	Heterologous expression of transferrin and lactoferrin proteins and their variants	43
Table 2.1	Bacterial and yeast strains and plasmids used in this study	54
Table 2.2	Genotypes of bacterial and yeast strains used in this study	55
Table 4.1	Synthetic oligonucleotides for bovine lactoferricin (LFcin-B)	99
Table 4.2	Synthetic oligonucleotides for human lactoferricin (LFcin-H)	100
Table 4.3	Expression constructs of LFcin-B & H	111
Table 4.4	Overview of all lactoferricin constructs and their expression pattern in <i>E. coli</i> BL21(DE3)	134
Table 5.1	MIC values of lactoferricin B for the five selected <i>E. coli</i> strains grown on 1% peptone media at cell densities of $3 \times 10^6$ CFU/ml and $3 \times 10^7$ CFU/ml	143
Table 5.2	MBC values of lactoferricin B for the five <i>E. coli</i> strains at $3 \times 10^6$ CFU/ml and $3 \times 10^7$ CFU/ml cell densities	145

## List of Figures

Fig. 1.1	Schematic representation of the immunoregulatory activity of lactoferrin	15
Fig. 1.2	Comparison of N-terminus sequences of human, bovine and murine lactoferrin and human transferrin	30
Fig. 1.3	Primary loop structure of Lactoferricin B	40
Fig. 1.4	Primary loop structure of Lactoferricin H	41
Fig. 1.5	Tertiary structure of Human Lactoferrin	45
Fig. 1.6	Tertiary structure of Bovine Lactoferrin	48
Fig. 3.1	Partial restriction map of pGEM:hLF	78
Fig. 3.2	Partial restriction map of M13:hLF (1-296) ( <i>SacI</i> )	78
Fig. 3.3	Analysis of M13:Lfn ( <i>SacI</i> ) by <i>SacI</i> and <i>SmaI</i> digestion	79
Fig. 3.4	Schematic diagram of the preparation of pGEM:hLF (-1) ( <i>SacI</i> ) construct	80
Fig. 3.5	Restriction digestion analysis of pGEM:hLF	82
Fig. 3.6	Restriction digestion analysis of pGEM:hLF (2) ( <i>SacI</i> )	82
Fig. 3.7	Restriction digestion analysis of pGEM:hLF (-1) <i>SacI</i> and pGEM:hLF (2) ( <i>SacI</i> ) constructs	82
Fig. 3.8	Schematic diagram of the preparation of the constructs of hLF cDNA in yeast expression vectors	84
Fig. 3.9	Ligation test between the <i>SacI</i> digested and CIAP treated linear pEPS1 and ~2.1 kb hLF fragment with <i>SacI</i> ends	86
Fig. 3.10	Schematic representation of sub-cloning of N-terminal lobe of hLF cDNA into <i>E. coli</i> expression vector pGEX4T-3	90
Fig. 3.11	Restriction digestion analysis of pGEX4T-3/Lfn ( <i>SacI</i> ) and pGEX4T-3/Lfn ( <i>BamHI</i> )	92
Fig. 4.1	End-filled fragments of LFcIn-H & B DNAs	101
Fig. 4.2	Restriction enzyme digested and purified LFcIn-H & B DNAs	101
Fig. 4.3	Schematic diagram of sub-cloning strategy of lactoferricin DNA into <i>E. coli</i> expression vector pPROEXHTa	103
Fig. 4.4	Restriction digestion analysis of LFcIn-B & H constructs in the pPROEXHTa vector	105
Fig. 4.5	Schematic diagram of sub-cloning strategy for introduction of lactoferricin DNA into <i>E. coli</i> expression vectors pGEX-4T1 and pET-15b from pPROEXHTa/LFcIn-H & B constructs	110

Fig. 4.6	Purification of poly-His tagged recombinant LFcIn-B expressed in the pPROEXHTa on Ni-NTA column	115
Fig. 4.7	Analysis of the purified poly-His tagged recombinant LFcIn-B in the presence of DTT	117
Fig. 4.8	Purification of poly-His tagged recombinant LFcIn-B on a reverse-phase HPLC column	118
Fig. 4.9	SDS-PAGE analysis of the lyophilised peak fractions of the HPLC purified poly-His tagged recombinant LFcIn-B	119
Fig. 4.10	N-terminal sequence of the poly-His fusion protein obtained from construct ProA+bp	120
Fig. 4.11	Affinity purification of GST-fused LFcIn-B (with stop-codon at the 3'-end of insert) protein	123
Fig. 4.12	Affinity purification of GST-fused LFcIn-H (with stop-codon at the 3'-end of insert) protein	123
Fig. 4.13	Thrombin cleavage of recombinant LFcIn-H & B peptides from GST-fusions	124
Fig. 4.14	Northern blot analysis of total RNA from cells harbouring the LFcIn-H constructs	128
Fig. 4.15	Northern blot analysis of total RNA from cells harbouring the LFcIn-B constructs	129
Fig. 4.16	Northern blot analysis of total RNA from cells harbouring the LFcIn-H constructs with the GST probe	131
Fig. 4.17	Northern blot analysis of total RNA from cells harbouring the LFcIn-B constructs with the GST probe	132
Fig. 5.1	Calibration curves (drawn between OD <sub>600</sub> vs Log <sub>10</sub> CFU) for the five selected <i>E. coli</i> strains	141
Fig. 5.2	Antibacterial assay curves of <i>E. coli</i> strains grown on 1% peptone at initial cell densities of 3x10 <sup>6</sup> CFU/ml and 3x10 <sup>7</sup> CFU/ml to determine MIC values of lactoferricin B	144
Fig. 5.3	Time-kill curves of lactoferricin B for the five selected <i>E. coli</i> strains at 3x10 <sup>6</sup> CFU/ml cell density	147
Fig. 5.4	Time-kill curves of lactoferricin B for the five selected <i>E. coli</i> strains at 3x10 <sup>7</sup> CFU/ml cell density	148
Fig. 5.5	T.E.M. pictures <i>E. coli</i> DH5α cells treated for different time intervals with lactoferricin B	151
Fig. 5.6	Comparison of the changes in the membranes of the untreated and treated <i>E. coli</i> 0111 (NCTC 8179) cells at different time intervals (T.E.M. pictures)	153

Fig. 5.7	T.E.M. pictures of <i>E. coli</i> strains DH5 $\alpha$ and 0111 at the same magnification (15,300 x)	155
Fig. 6.1	Tentative molecular model depicting mode of action of dermaseptin with membranes	168
<b>Appendices</b>		190

## Abbreviations

amp or Ap	ampicillin
ARS	autonomous replicating sequence
ATP	adenosine triphosphate
A <sub>x</sub> or OD <sub>x</sub>	absorbance at x nm
BHK	baby hamster kidney cells
bLF	bovine lactoferrin
bp or bps	base pairs
BRL	Bethesda Research Laboratories
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
cpm	counts per minute
C-terminal	carboxy terminal
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double-stranded

DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
EDTA	ethylene diamine tetra acetic acid
EEO	electroendosmosis
ELISA	enzyme-linked immunosorbant assay
GM	granulocyte macrophage
GM-CSF	granulocyte macrophage colony stimulating factor
GRAS	generally regarded as safe
HEPES	N-2-hydroxy ethyl piperazine-N'-2-ethane sulfonic acid
hGH	human growth hormone
hLF	human lactoferrin
HPLC	high performance liquid chromatography
hTf	Human transferrin
HSA	human serum albumin
Ig	immunoglobulin
IPTG	isopropyl-D-thiogalactopyranoside
Kan	kanamycin
L or l	litre
LB	Luria-Bertani
Lf	Lactoferrin
LFcin-B	Bovine lactoferricin
LFcin-H	Human lactoferricin
Lfn	the amino terminal half of human lactoferrin
LMP	low melting point
MCS	multiple cloning site
MES	2 (N-morpholino) ethane sulphonic acid
MM	minimal medium
MOPS	morpholinopropane sulphonic acid
MW	molecular weight
MWCO	molecular weight cut off
N-terminal	amino terminal
NTA	nitrilotriacetic acid

OD	optical density
ORF	open reading frame
ori	origin of replication
PAGE	polyacrilamide gel electrophoresis
PB	phosphate buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
psi or lb/in <sup>2</sup>	pounds per square inch
RIA	radio immuno assay
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecylsulphate-polyacrilamide gel electrophoresis
ss	single-stranded
SSC	standard saline citrate
sTf	serum transferrin
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TCA	trichloroacetic acid
TE	Tris (10 mM) EDTA (1 mM), pH 8.0
TEMED	N,N,N',N'-tetra methyl ethylene diamine
Tris	Tris- (hydroxy methyl) amino methane
TTP	thymidine triphosphate
UV	ultraviolet
YEPD	rich glucose medium
YEPL	rich lactose medium
X-gal	5-bromo-4-chloro-3-indolyl -D-galactopyranoside

### Additional Abbreviations

APR	Acute Phase Reaction
EGTA	Ethylene Glycol-bis ( $\beta$ -aminoethyl Ether) N,N,N',N'-Tetraacetic Acid
GST	Glutathione S-Transferase
HCMV	Human cytomegalo virus
hrLF	human recombinant Lactoferrin
IL	Interleukin
KN	Korean Native
Lbp	Lactoferrin binding protein
LFcin	Lactoferricin
MIC	Minimum Inhibitory Concentration
NK	Natural Killer
OPD	O-Phenylene Diamine
PIPES	Piperazine N,N'-bis[2-ethanesulphonic acid]
PMN	Polymorphonuclear
PMNs	Polymorphonuclear leukocytes
PVDF	Polyvinylidene difluoride
RES	Reticuloendothelial system
RNAse	Ribonuclease
TB	Tris buffer
TNF	Tumour Necrosis Factor
YPD	Yeast extract Peptone Dextrose (rich glucose medium)

## Abbreviations of units

°C	degrees Celsius
g	gram(s)
Hr or hr	hour(s)
kb	kilobases
kDa	kilodaltons
L	litre(s)
M	molar
min	minute(s)
m	metre(s)
rpm	revolutions per minute
% (w/v)	percentage weight by volume
%(v/v)	percentage volume by volume
μf	microfarads
Ω	resistance in ohms
s	second(s)
V	volts