The search for *Lactobacillus* proteins that bind to host targets

A thesis presented in partial fulfilment of the

requirements for the degree of

Master of Science

in

Microbiology

at Massey University, Turitea

New Zealand.

Zoe Amber Erridge

2014

Abstract

Interactions between microorganisms and host cells in the gastrointestinal tract are crucial to the host's health. Probiotic bacteria, such as the lactobacilli provide numerous benefits to human health thought to be mediated by bacterial proteins called effectors. *Lactobacillus rhamnosus* HN001 (*L. rhamnosus* HN001) is a cheese-fermenting isolate with probiotic characteristics and *Lactobacillus reuteri* 100-23 (*L. reuteri* 100-23) is a coloniser of the rodent forestomach. Whereas *L. rhamnosus* HN001 was shown to reduce eczema in children, *L. reuteri* 100-23 reduces inflammation in mice. The effector proteins for these strains are largely unknown. In this thesis, phage display technology was used to search for proteins that bind specific ligands. Shot-gun genomic phage display library of *L. rhamnosus* HN001 was affinity screened on fibronectin as bait, leading to enrichment of specific recombinant clones. Analysis of 10 candidate clones, however, determined that these are not genuine binders, but may have been selected due to a potential growth advantage during amplification steps of the library. The *L. reuteri* 100-23 genomic shot-gun phage display library was subjected to two affinity screens on two baits: fibronectin and murine stomach tissue. The aim of the screen on the murine stomach tissue was to identify keratin-binding proteins, as this strain naturally colonises the murine keratinous forestomach. Whereas no enrichment was detected in the screen on fibronectin as a bait, a strong enrichment of a phagemid displaying a short peptide, IGINS, derived from a cell-surface protease of *L. reuteri* 100-23 was identified. Identifying and characterising probiotic bacterial proteins that positively influence health will lead to a greater understanding of gastrointestinal tract interactions. Ultimately, this aids development of probiotic use as therapeutic agents.

Foreword and Acknowledgements

Through perseverance we move forward in life. Perseverance is achieved with ‘One More Try’. This mantra comes from two hardworking men and also an angel who is dearly loved by me. They never give up in the face of hardship. This thesis is not only the product of two years study and experimentation into gastrointestinal interactions, but a reflection on a lifelong journey and pursuit of learning.

My interest in science began at primary school. This involved learning about space, dinosaurs, geology and uncovering the remains of ancient civilisations. My interest in the genetic and chemical sciences began in high school. At University, by majoring in genetics and microbiology, I became highly interested in microorganisms and their significant influence. Therefore, this thesis is the product of all those years, hours and energy studying science and its role in our community.

There are some significant people who have had a positive impact in my life. In particular, I would like to thank the following people: Peter Flynn, for being the greatest maths teacher I have known; Vicki Bothwell, for being the greatest English teacher I have known; Jasna Rakonjac, my supervisor, for her expertise and guidance; Marie McGlynn, my mother, for raising me to do my best and follow my dreams; Catherine Dyhrberg, for her long lasting friendship and advice; Misha Collins, for being a role model for universal love and kindness; Luke Phoenix, for his support and inspiring me to be stronger in all walks of life.

I would also like to thank my lab mates at D.401, especially Wesley Wen and Julian Spagnuolo for their advice; Dragana Gagic, for constructing the vectors and phage display libraries; Linda Johnson, Richard Johnson, Gwenda Gopperth, Sharon Wright, Lindsay Wright and other members of my family.

In memory of my father who would be proud of what I have achieved.

Table of Contents

Abbreviations

Amp	Ampicillin
BLAST	Basic Local Alignment Search Tools
BSA	Bovine Serum Albumin
Cm	Chloramphenicol
DCs	Dendritic Cells
E. coli	*Escherichia coli*
ECM	Extracellular Matrix
GIT	Gastrointestinal Tract
IECs	Intestinal Epithelial Cells
IL_(number)	Interleukin (number) (i.e Interleukin 17)
Kn	Kanamycin
L. rhamnosus GG	*Lactobacillus rhamnosus* GG
L. rhamnosus HN001	*Lactobacillus rhamnosus* HN001
L. reuteri 100-23	*Lactobacillus reuteri* 100-23
M/PAMPs	Microbial/Pathogen Associated Molecular Patterns
NFkB	Nuclear Factor kappa B
NEC	Necrotizing enterocolitis
O.D.	Optical Density
PSA	Polysaccharide A
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffered Saline with Tween-20
PEG	Polyethylene Glycol
pmol	Pico mole
PP	Phagemid Particles
RF	Replicative Form
SDS	Sodium Dodecyl Sulfate
SOC	Super Optimal broth with Catabolite repression
ssDNA	single stranded Deoxyribose Nucleic Acid
TDPs	Transducing Phagemid Particles
2xYT	Yeast Extract Tryptone Broth

LIST OF FIGURES

LIST OF TABLES

1.0 Introduction

1.1 The gastrointestinal tract

The gastrointestinal tract (GIT) is the biggest mucosal surface in the body as it contains about 100 m^2 of intestinal epithelial cells (IECs). The GIT is colonised by a complex microbial community of up to 10^{14} organisms interacting symbiotically with epithelial and immune cells (Artis, 2008). Adult humans have a unique microbial community that is stable for months. On the other hand, infants have a less stable and variable composition of micro flora (Palmer *et al*., 2007). Numerous interactions are occurring between pathogens, commensals, probiotics and eukaryotic host cells within the GIT. The commensal microbial community regulate immune responses, strengthen the epithelial cell barrier, and defend the host from pathogenic microorganisms, as has been shown by comparisons of germ-free conventionally reared mice. The host provides nutrients (carbon and energy from the diet of the host) and shelter for the microbes (Velez *et al.*, 2010).

The host genome does not encode all the factors required for complete development and protection in the GIT. Therefore, the metagenome (the collective genomes from the community of microorganisms) is required (Mazmanian *et al.*, 2008). This symbiotic relationship is crucial to animal and human health. Unnecessary gut inflammation is prevented in a healthy host as the immune system is able to distinguish between beneficial and pathogenic bacteria. Homeostasis, the balance between microorganisms and the immune system, limits damage in the healthy host. This balance can be disturbed due to food allergies, pathogen invasion or inflammatory bowel disease and intestinal cancer (Macdonald and Monteleone, 2005). Homeostasis is a challenge in the GIT, as the population of microorganisms is dense.

Microbial antigens and other stimuli interact with the GIT and some can be absorbed and affect systemic organs. Gut-associated lymphoid tissues such as Peyer's patches make an active but reduced response to bacterial products that cross the intestinal barrier (Macdonald and Monteleone, 2005). Microbe antigens are also sampled in the lumen of the GIT, by "antigen-presenting" dendritic cells (DCs) which extend their long filopodia-like extensions (dendrites) between the IECs. It has been

shown that IECs themselves are not only barriers against microorganism invasion (through sealing the epithelia by tight junctions and the production of antimicrobial peptides) but also can distinguish between commensals and pathogens (Karlsson *et al.*, 2012).

IECs as well as DCs and other immune cells recognise pathogen/microbial associated molecular patterns (P/MAMPs) (also known as ligands) on microorganisms in the GIT through interaction with pattern-recognition receptors (PRRs) such as Toll-like receptors. This interaction induces several signalling pathways such as Nuclear Factor kappa B (NFkB) (Blander and Sander, 2012). Common MAMPs for the lactic acid bacteria are peptidoglycan, (Lipo)teichoic acids, and extracellular polysaccharides. This interaction induces signals that allow the host to recognise the microbe's presence. These signals sometimes result in internalization of the microbe (Corthesy *et al.*, 2007).

The extracellular matrix (ECM) is a "stable macromolecular structure underlying epithelial and endothelial cells and surrounding connective tissue cells" (Westerlund and Korhonen, 1993). Extracellular matrix molecules, such as fibronectin, laminin and collagen, can be receptors for many bacteria. A damaged mucosal layer exposes the ECM to the intestinal lumen, allowing colonisation by pathogens, which in turn results in infection (Styriak *et al.*, 2003). Probiotic bacteria could prevent gastrointestinal infections by competing with invading pathogens for binding to ECM. The basolateral side of IECs and in between the IECs are covered with ECM. The ECM is exposed to bacteria in the lumen when tissue trauma occurs. Often pathogens bind to ECM to colonize and infect (Lorca *et al.*, 2002). Thus, commensal and probiotic bacteria might competitively exclude pathogens by occupying these receptors.

However, the precise molecular mechanisms that regulate immune responses are largely unknown for commensal and probiotic bacteria. Thus, it is of interest to investigate bacterial structures/proteins (effectors) that interact with host cells and may be responsible for this immunomodulation. Some of these interactions out-compete pathogens or modulate immune responses in a species-/strain-dependent manner by manipulating/inhibiting downstream signalling pathways (van Hemert *et al.*, 2010, Bron *et al.*, 2012). For example**,** probiotic bacterium *Bacteroides thetaiotaomicron* modifies a protein in the MAMP receptor-induced NFkB pathways causing inhibition of the inflammatory signalling pathway (Areschoug *et al.*, 2004).

1.2 Probiotic bacteria

Probiotic comes from the Greek '*pro*' and '*biota*' which means 'for life'. Probiotic bacteria are living microorganisms that are beneficial to human health (and not for nutritional requirements) (Lin *et al.*, 2008). Most probiotic bacteria are lactic acid bacteria. There are many genera of lactic acid bacteria; the majority belong to the genera *Lactobacillus* and *Bifidobacterium*. Lactic acid-producing bacteria are gram-positive fermenters of carbohydrates that naturally inhabit plant environments, fresh meat and vegetables and, most importantly, oral, vaginal and GIT niches. These probiotic bacteria ferment, preserve, improve food, and have been implicated in a large range of health benefits (Holzapfel *et al.*, 2001). For example, *Lactobacillus rhamnosus* GG and *Lactobacillus casei* were shown to displace pathogenic *Escherichia coli* (*E. coli*) strains and *Salmonella* species from CaCo-2 cells (CaCo-2 cells are a continuous cell line of heterogeneous human epithelial colorectal adenocarcinoma cells*)* (Lee *et al.*, 2003).

Supplementation of live cultures (as well as using cell wall components) to animals and humans improves health (Twetman *et al.*, 2009). A successful probiotic has characteristics that enable it to persist or colonize the GIT by withstanding environmental conditions (e.g. low pH, high bile); clear or reduce pathogens and is proven safe for human consumption. Therefore, they are generally selected from human GIT flora or are already used in the food industry (Pirarat *et al.*, 2006). More beneficial characteristics include: an adhesive ability to epithelial cells and the ECM; modulation of immune responses, and/or exclusion/reduction of pathogen adhesion (Jankovic *et al.*, 2007). An adhesive ability to IECs is important as it suggests a longer persistence in the GIT.

Therefore, its use as a probiotic is enhanced due to a longer-term colonisation advantage over other strains that do not have an adhesive ability (Lee *et al.*, 2011). Often though, potential probiotic bacteria will not have all desirable characteristics. In the 1990's, over 2000 New Zealand strains of lactic acid bacteria were tested for probiotic fundamental characteristics. Four probiotic strains were found: *Lactobacillus rhamnosus* HN001, *Lactobacillus acidophilus* HN017, *Lactobacillus rhamnosus* HN067 and *Bifidobacterium lactis* HN019 (Prasad *et al.*, 1998).

1.3 *Lactobacillus*

The genus *Lactobacillus* is the focus for probiotic studies as there are about 148 recognised species that have beneficial effects on human health. These bacteria often have intestinal niche-specific adaptations. Some species are autochthonous – they colonise the GIT throughout the host's life – while some are allochthonous – they only persist for short periods of time in the GIT. Probiotic lactobacilli isolated to date typically are derived from fermentation products and are therefore allochtnonous (von Ossowski *et al.*, 2011).

Lactobacilli have been found to displace pathogenic bacteria. *Lactobacillus rhamnosus* GG was shown to be able to displace pathogenic *E. coli* and *Salmonella in vitro* (Lee *et al*., 2003). Lactobacilli can reduce *Listeria monocytogenes* and also prevent diarrhoea and alleviate the symptoms of lactose intolerance (Lee *et al*., 2011).

Pathogens can cause secretion of interleukins which cause inflammation. For example, *Helicobacter pylori (H. pylori)* injects cytotoxin-associated gene A (CagA) into the IEC which then secretes interleukin 8 (IL-8), causing inflammation. *Lactobacillus salivarius* UCC118 was found to inhibit the *H. pylori*-induced response in human stomach cancer cells (Ryan *et al.*, 2009).

Lactobacilli also have the potential to prevent inflammatory conditions such as necrotizing enterocolitis (NEC) (Lin *et al.*, 2009). NEC is responsible for deaths in neonates and very low birthweight infants. In these young children, commensal colonisation is delayed and inappropriate bacteria predominate (such as *Clostridium perfringens*) (Lin *et al*., 2008).

Lactobacilli also regulate adherence junction proteins by differentially expressing epithelial-cadherin and beta-catenin. This modulation of the epithelial barrier was shown by an increase in transepithelial electrical resistance. Four probiotic lactobacilli were used in this experiment to induce intestinal epithelial cells from a human colon carcinoma (T84 cell line). *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, and *Lactobacillus rhamnosus* affected the adherence junction differently, showing species specificity (Hummel *et al.*, 2012).

1.4 The search for lactobacilli effector molecules

Extracellular structures, and secreted proteins (also called "effector" molecules) may be responsible for immunomodulation and species-/strain-dependent interactions that determine the host immune response to a probiotic bacterium (van Hemert *et al*., 2010). Genomic work has bioinformatically identified thousands of candidate surface and secreted proteins (van Pijkeren *et al.*, 2006, Foligne *et al.*, 2007).

The surfaces of bacteria are the main interaction interfaces with the host, therefore, the majority of these effector molecules are likely to be found on the cell surface. *Lactobacillus plantarum* strain-specific cytokine responses were compared by comparative genome hybridisation profiles, 6 loci were identified to have immunomodulation roles in human cells. This approach also allowed these researchers to identify a mannose-specific adhesion and genes involved in DC responses (van Hemert *et al*., 2010).

Modifications to peptidoglycan have been found to modulate interactions of *Lactobacillus* with the host. For example, peptidoglycan N-acetylation and O-acetylation of glycan strands leads to resistance to lysozymes. Furthermore, extracellular polysaccharides are also found to be immunomodulatory by sheltering their MAMPs from detection by immune cell receptors (Kleerebezem *et al.*, 2010). For example, Polysaccharide A (PSA) in *Bacteroides fragilis* protected mice from experimentally induced colitis. PSA decreased pro-inflammatory interleukin 17 (IL-17) and tumour necrosis factor alpha (TNFa) while PSA increased regulatory interleukin 10 (IL-10) and this was required for anti-inflammation (Mazmanian *et al*., 2008). Teichoic and lipoteichoic acids, which are embedded in the proteoglycan layer, have also been shown to interact with the immune system.

Also, surface proteins, which are anchored in the membrane or are covalently attached to peptidoglycan are typically considered to be involved in adhesion (Kleerebezem *et al*., 2010). Surface-layer protein Slp of *Lactobacillus helveticus* R0052 binds to epithelial cells, preventing adherence of *E. coli* O157 (Twetman *et al*., 2009).

Another surface-layer protein, SlpA, of *Lactobacillus brevis* ATCC 8287 has high affinity to fibronectin and IECs; therefore, binding to fibronectin was found to be essential for adherence of this organism to IECs (Jakava-Viljanen and Palva, 2007). In

Lactobacillus acidophilus NCFM, fibronectin-binding protein FbpA is an important factor for the organism as it acts as a bridge between the bacterium and the ECM. Adhesion to Caco-2-cells was reduced by 76% in an FbpA inactivation mutant (Buck *et al.*, 2005). In another study, mutants of potential effector proteins were investigated. A significant decrease was seen in the mutants whose genes encoding fibronectin-binding and mucin-binding proteins were deleted. Therefore, multiple surface proteins can affect an organism's adherence (Buck *et al*., 2005).

Under selective pressures in the GIT, bacteria adapt by altering the molecular composition of their surface in order to avoid inducing inflammation and consequently inducing bacterial clearance through the PRR signalling on IECs or DCs (Kleerebezem *et al*., 2010). This includes modification by glycosylation, such as the *Lactobacillus rhamnosus* GG major secretion protein (Msp1) that was found to be O-glycosylated. This protein was implicated in protecting the bacterium from proteases rather than manipulating immune cell signalling (Lebeer *et al.*, 2012). Understanding protein glycosylation in probiotic bacteria could lead to isolation of an immunomodulatory glycan that could be used for human therapy.

It is highly plausible that there are many effector molecules to be discovered. Development of molecular and microbiological techniques to uncover more effectors is an important area of study. It is important to note that the specific effector molecules found should be treated case-by-case. This means that while one probiotic bacterium is found to impact a certain pathway (or have a certain cytokine profile, or a specific adhesion protein), another species or another strain of the same species could have a crucial difference in immune responses. Also, researchers have seen organ-specific effects in different strains (such as colon versus the stomach). (Castro *et al.*, 2003). Thus, lactobacilli have significant differences in regards to their mechanism of interaction and their location of colonisation. Therefore, probiotic interactions with host cells is a complex area of study. Each species and each strain requires heavy experimental investigation to predict and verify protein functions, and other critical factors that influence immune responses in order to fully understand the interaction with host human cells.

1.5 *Lactobacillus rhamnosus*

Lactobacillus rhamnosus GG (*L. rhamnosus* GG) is a well-known strain, it is important to discuss the studies that identified *L. rhamnosus* GG effector proteins. A *L. rhamnosus* GG adhesin named MabA, modulator of adhesion and biofilm (encoded by gene *lgg_01865*) was discovered that binds to the CaCo-2 intestinal cell line and fibronectin in the ECM. *lgg_01865* knockout mutants had decreased adhesion and biofilm formation. However, MabA does not seem to be important for the anti-inflammatory effect of *L. rhamnosus* GG and hence it was proposed that MabA stabilises late stages in adhesion to host cells after the pili has mediated the initial attachment. LGG_01866 is a transcriptional regulator of *L. rhamnosus* GG that controls expression of MabA under stress. Given that it is an environmentally-regulated transcriptional regulator, LGG_01866 could be pleiotropic and thus affect multiple surface proteins with roles in adhesion and immunomodulation partly by indirectly affecting cytokine production and signalling (Velez *et al*., 2010).

Another *L. rhamnosus* GG adhesin, LGG_02337, is a mucus adhesin protein that is located all along the surface of *L. rhamnosus* GG. However, it binds to mucus less significantly than the *L. rhamnosus* GG pili (von Ossowski *et al*., 2011). A novel pilus structure was discovered in *L. rhamnosus* GG that is encoded by the spaCBA gene cluster. SpaA forms the shaft; SpaB is found at the base and thought to be involved in pilus assembly termination; SpaC appears to be involved in the binding to mucosal cells as SpaC is uniformly distributed along the pili. *Lactobacillus rhamnosus* GG is currently the only probiotic found to have mucus-binding pili, which is likely to allow colonisation of the GIT and a competitive advantage over bacterial species that do not bind to GIT surfaces (Reunanen *et al.*, 2012). However, given that very similar pili in pathogenic bacteria are considered to be virulence factors, this poses a question over the overall benefit of the permanent colonisation to human health.

Lactobacillus rhamnosus HN001 (*L. rhamnosus* HN001) is one of the strains used in this thesis. *Lactobacillus rhamnosus* HN001 is a cheese-fermenting strain isolated in New Zealand that showed excellent characteristics to be a probiotic (Prasad *et al*., 1998). This strain is marketed under Fonterra as DR20^{TM} and is used in a wide

range of dairy products. Therefore, *L. rhamnosus* HN001 is safe and well-tolerated in humans and infants (Dekker *et al*., 2009).

Previously, *L. rhamnosus* HN001 was tested for binding to ECM components and *L. rhamnosus* HN001 bound to fibronectin (Authier, 2003). Attempts to identify a protein were performed. A fibronectin-binding-like protein was expressed by a GST fusion system but difficulties in expression and purification hindered characterisation (Authier, 2003). The draft genome of *L. rhamnosus* HN001 was submitted in April 2009 by the New Zealand Dairy Board at the University of Otago (Integrated Microbial Genomes website, retrieved from ://img.jgi.doe.gov/cgi-bin/w/main.cgi).

Lactobacillus rhamnosus HN001 has demonstrated many beneficial effects on humans. *Lactobacillus rhamnosus* HN001 halves the risk of developing eczema at 2 years of age (Chiang *et al.*, 2000). This probiotic bacteria has also been found to improve the immune capacity of the elderly and is associated with decreased *Clostridium difficile* (Lahtinen *et al.*, 2012). Treatment with *L. rhamnosus* HN001 does not change the major microbial groups or the faecal immune markers (Lahtinen *et al*., 2012).

Lactobacillus rhamnosus HN001 can protect the host against *Salmonella* infections (Gill *et al.*, 2001). Mucus may be crucial for the adhesion of *L. rhamnosus* HN001. Compared to CaCo-2, *L. rhamnosus* HN001 adhered 2-3 times more to the mucus-secreting cell line, HT29-MTX (Gopal *et al*. 2001). As well as this, *Lactobacillus rhamnosus* HN001 is an immunostimulant and could potentially be used in cancer immunotherapy (Cross *et al*., 2002). However, molecular mechanisms of all these effects remain ambiguous.

1.6 *Lactobacillus reuteri*

A trial using supplementation of *Lactobacillus reuteri* (*L. reuteri*) showed decreased IgE-associated eczema in human infants. The subjects were receiving the supplement through mothers from the 36th week of pregnancy, and then directly through to the first year of the baby's life. This shows that *L. reuteri* may enhance immune-regulation in infants (Forsberg *et al*., 2013). As well as this, *L. reuteri* strains were found to have a unique metabolism that has the potential to be exploited for bio-reducing harmful compounds in the GIT (van Niel *et al.*, 2012).

Some *L. reuteri* proteins have been discovered that are involved in the forestomach adherence. This includes the collagen-binding protein (CnBP) of *L. reuteri* NCIB 11951 (Aleljung *et al.*, 1994). Also, mutants of a high molecular mass surface protein (Lsp) and methionine sulfoxide reductase B (MsrB) (of which both have homologues in other lactobacilli) showed decreased adherence to the mouse forestomach (Walter *et al.*, 2005).

Lactobacillus reuteri strain 100-23 (*L. reuteri* 100-23) is one of the strains focused on in this study, along with *L. rhamnosus* HN001 (described above). *Lactobacillus reuteri* 100-23 is an autochthonous commensal. A single inoculum of this bacterium results in colonisation for life. The hosts are rodents, rhesus monkeys and swine. It forms a biofilm in the forestomach that sloughs off. Therefore, *L. reuteri* 100-23 is present continuously in the forestomach and the digestive tract (Tannock, 2004, Tannock *et al.*, 2012).

When *L. reuteri* 100-23 is introduced into *Lactobacillus*-free mice, increased populations of regulatory T cells (Treg) are detected, in parallel with a decrease in inflammation, by 21 days. The increased Treg cells are also associated with the development of immune tolerance to *L. reuteri* 100-23 (Livingston *et al.*, 2010).

The completed genome sequence of *L. reuteri* 100-23 was released in May 2008 by the Community Sequencing Program at the DOE Joint Genome Institute (Integrated Microbial Genomes website, retrieved from //img.jgi.doe.gov/cgi-bin/w/main.cgi)

1.7 Phage display technology

In this thesis, phage display technology is used with the intention of finding effector proteins in the two strains mentioned above. First, a simplified description of the phage biology is as follows. Filamentous phages (Ff) infect the host *E. coli* by attachment to the F-pilus and binding causes a structural rearrangement between N domains. The pilus retracts bringing the phage in closer contact to the cell. When the virion single stranded DNA (ssDNA) enters the *E. coli* cytoplasm, a negative strand is synthesised by the host cell machinery. The double stranded DNA of the phage genome is referred to as the replicative form (RF). Using the RF template, the positive stand is synthesised, followed by transcription and translation. Phage proteins pI/pXI and pIV form the assembly/exit complex in the host's envelope. The ssDNA is coated with pV

and as the ssDNA passes through the complex, pV is removed and pVIII forms the sheath around the DNA. The addition of pIII and pVI stops assembly and releases the phage (Cabilly, 1999, Rakonjac and Model, 1998). Therefore the progeny phages are secreted from host bacteria without cell lysis.

George Smith was the first to display a foreign protein on the surface of a bacteriophage, in 1985. In this original experiment, fragments of DNA from an organism were fused to viral capsid-encoding genes in a phage genome, and as a result foreign proteins are displayed on the surface of the virion (Smith, 1985). Subsequently, phagemid vectors (plasmids containing f1 origin of replication) were adapted for use in phage display. In this system, a phagemid expressing a fusion of a peptide or protein, or a library of peptides/proteins to a virion protein is introduced into an *E. coli* host, which is subsequently infected with a helper phage that contributes the virion proteins needed for replication of phagemid from the f1 origin of replication and for assembly of the phagemid-expressed virion protein fusion into the phagemid genome-containing particles (phagemid particles or PPs).

The usefulness of phage display comes from the physical link between displayed proteins and the DNA sequence encoding it (Mullen *et al.*, 2006). Recombinant proteins displayed on the surface of the virions are selected (panned) for by interaction of the protein with its ligand. Rare variants that have affinity for a target (bait) are identified by their coding sequences which are packaged inside the phage particles (Fig 1.).

The other benefits of this technology include massive genome coverage and the ability to affinity-screen routinely large number of clones (over 10^{12} per mL) against an immobilised target. Importantly, proteins with high specificity to a target can be retrieved by affinity selection. Also, phage display allows easy purification and functional characterisation of displayed proteins by purifying the PPs or phage virions using simple polyethylene-glycol (PEG) precipitation from the culture supernatant.

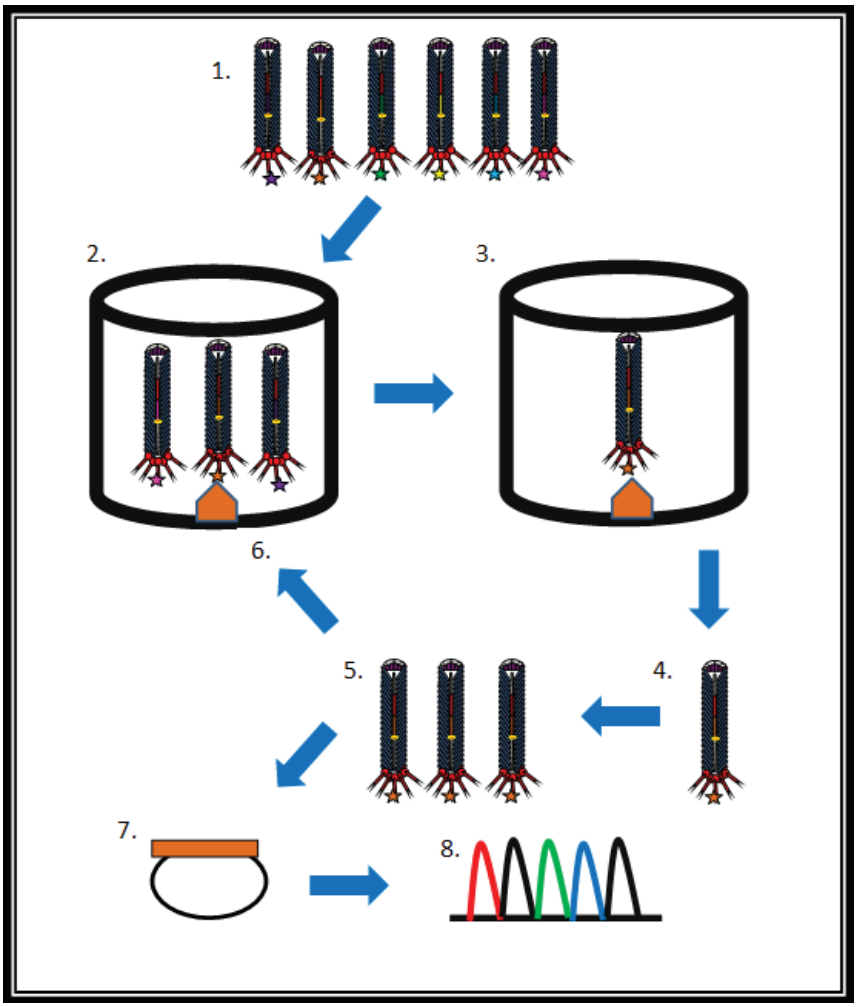

Figure 1. Library screening for fibronectin-binding proteins

1. Shot gun phage display library encodes many protein variants expressed on the surface of virions. 2. Library is incubated with the bait/ligand 3. Non-binders are washed away. 4. Phage particles (PPs) are eluted. 5. Followed by amplification in *E.coli* 6. Panning is repeated for a total of three or four rounds. 7. Plasmid DNA extracted from PPs 8. Enriched bands visible by agarose gel electrophoresis (represent enriched binders) and are excised from the gel and sequenced.

Jacobsson and Frykberg were the first to use phage display to identify proteins in *Staphylococcus aureus* that interact with ECM components (Jacobsson and Frykberg, 1995, Jacobsson and Frykberg, 1996). This method also successfully identified a fibronectin binding protein in *Staphylococcus epidermins* (Williams *et al.*, 2002). In an another study, affinity screening for bacterial proteins that bind patient serum immunoglobulins, revealed six immunodominant *Mycobacterium tuberculosis*

antigens absent from the vaccine strain (BCG). Three antigens were novel as they had not been discovered by other methods (Liu *et al.*, 2011). Phage display was used to identify ECM-interacting proteins (collagen and fibronectin) of *Lactobacillus casei* BL23; this work confirmed that surfaces of lactobacilli are decorated with interacting proteins that bind broad-spectrum targets (Munoz-Provencio and Monedero, 2011).

1.8 Project Aims

The molecular mechanisms underlying the immune responses caused by probiotic bacteria are largely unknown. Few bacterial effector proteins have been discovered and identified. Learning about these proteins will increase understanding of the beneficial effects associated with probiotic bacteria. This study focuses on the search for effector proteins in *Lactobacillus rhamnosus* HN001 and *Lactobacillus reuteri* 100-23. These bacteria are both highly beneficial to humans. By using phage display to search and functionally identify bacterial proteins that interact with fibronectin and the keratinous lining in mouse stomachs, progress can be made towards understanding the mechanisms of probiotic interactions with gastrointestinal cells.

2.0 Materials and Methods

2.1 Materials

The bacterial strains, plasmids and phage used in this study are described and referenced in Tables 1 and 2. Genotypes of the bacterial strains used are described in Table 1. The resistance markers for the plasmids are recorded in Table 2.

2.1.1 Bacterial strains, plasmids and phage

Table 1. Bacterial Strains used in this study

Strains	***Genotype***	***Reference***
E. coli K1819 (TG1)	K12, Δ*(lac-pro)*, *supE44*, *thi*, *hsdR5*, F' *traD36*, *proA*$^+$*B*$^+$, *lacI*^{q}, lacZDM14	(Baer *et al.*, 1984)
E. coli K1976	TG1 carries pJARA112	(Jankovic, 2009)
E. coli K1994	TG1 carries pNJB50 [pGZ *psp-pelB*-gIII (C154)]	(Bennett *et al.*, 2011a)

Table 2. Plasmids and phage used in this study

Plasmids	***Description/Reference***	***Resistance***
pJARA112	pBR322 *psp-gIII* (Rakonjac *et al.*, 1997)	Ampicillin
pNJB50	pGZ *psp-pelB*-gIII (C154) (Bennett *et al*., 2011a)	Chloramphenicol
pYW01	(Jankovic, 2009)	Chloramphenicol
pJARA144	pPSP2 *Δrom* (Beekwilder *et al.*, 1999)	Ampicillin
pDJ01	(Jankovic, 2009)	Chloramphenicol
pDJ04	(Jankovic, 2009)	Chloramphenicol
Phage		
VCSM13	Stratagene (USA)	Kanamycin
VCSMI13d3	VCSM13 *ΔgIII* (Rakonjac *et al.*, 1997)	Kanamycin

Phagemid vector, pYW01, (Fig. 2) was used in the construction of the phage display libraries used in this study. The display cassette in this vector (pelB signal sequence - c-myc-peptide tag - pIII) was expressed from a phage-shock-protein (*psp*) promoter. The *psp* promoter was only expressed after infection of helper phage and has been shown to minimise the toxicity of pIII fusions; a problem often encountered in phage display (Beekwilder *et al*., 1999).

Helper phage expresses proteins required for replication of phagemid from the f1 origin of replication and proteins of the virion, as well as the assembly machinery. Infection with the helper phage therefore results in assembly of phagemid ssDNA into the virions (called Phagemid Particles (PPs) that display phagemid-endoded fusion proteins.

Expression of the N-terminal domains of pIII in *E. coli* results in resistance to helper phage infection. This vector only expressed the C-terminal domain of pIII, hence the resistance to helper phage infection was avoided in those recombinant phagemids in the shot-gun genomic libraries that contained inserts including promoters upstream of displayed genes (Rakonjac *et al*., 2011).

The libraries were constructed by Dragana Gagic (former last name was Jankovic) (Jankovic, 2009, Jankovic *et al*., 2007). The library inserts ranged from 0.3 to 4 Kbp. The primary sizes of the *L. rhamnosus* HN001 and *L.reuteri* 100-23 libraries were 10^8 and $5x10^7$ recombinant clones, respectively. The size of the libraries maximises the chance to display the entire proteome. Using the following theoretical equation, $N = Log_{10} (1-P)/ Log_{10} [(1-I/G)]$, where N equals the number of clones in the libraries; P equals the probability of the library containing all parts of the genome; I equals the average cloned insert size (kb) (about 2 kb even though the inserts had a large range of size); and G equals the size of genome (kb), the P value was calculated. The P value then had to be multiplied by 18 to account for the fusion in frame with upstream signal sequence, downstream pIII C domain, and correct orientation. The coverage for both *L. rhamnosus* HN001 and *L. reuteri* 100-23 was calculated to be over 99.99%. However, in reality the library coverage is not 100% due to the potential toxicity of the inserts to *E. coli* (Jankovic, 2009).

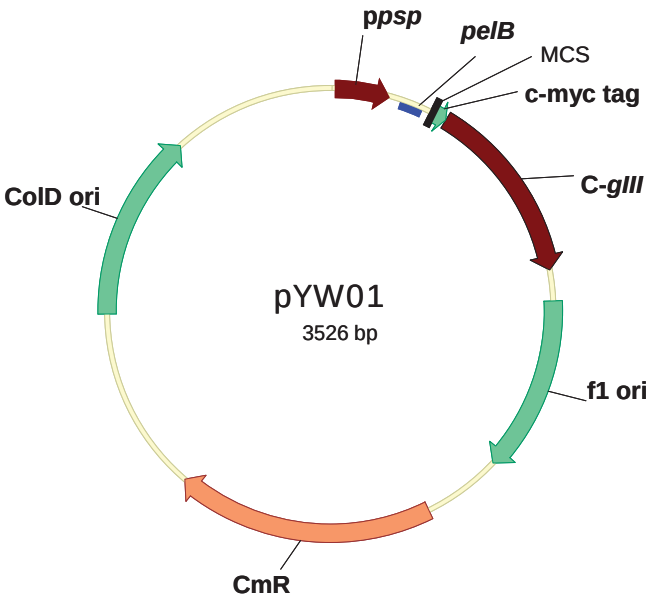

Figure 2. Phagemid vector, pYW01, used to construct the shot-gun phage display libraries

CmR, chloramphenicol resistance; **ColD ori**, the ColD plasmid origin of replication; **p*psp***, phage shock protein promoter; **pelB**, leader sequence; **MCS**, multiple cloning site; **c-myc**, a peptide tag followed by a single amber stop codon; **g*IIIC***, C-domain of *gIII*; **f1 ori**, the f1 phage origin of replication for generation of ssDNA for packaging into the phagemid particles (Jankovic, 2009).

2.1.2 Media, Buffers and other solutions

General chemicals, antibiotics and were purchased from Sigma-Aldrich (Australia) and Merck Limited (Australia). Super fibronectin (S5171) was also from Sigma-Aldrich but shipped from the United States. Invitrogen Life Technologies Incorporated (USA) manufactured the oligonucleotides. Restriction enzymes were purchased from either Invitrogen Life Technologies Incorporated (USA), Roche Molecular Biochemicals (Germany) or New England Biolabs Incorporated (USA). The bacteriological media, 2xYT, was purchased from Becton-Dickson and Company (USA). The Nunc-Immuno MaxiSorp™ plates came from Nunc™, Denmark.

Yeast Extract Tryptone broth (2xYT) and 2xYT agar

The 2xYT broth contained 16 g pancreatic digest of casein, 10 g of yeast extract and 5 g of NaCl per litre of MilliQ water. The 2xYT agar contained 31 g/L agar in 2xYT broth. After the 2xYT agar was autoclaved at 121˚C for 15 minutes, it was cooled to 50˚C and then the correct antibiotic was added. Ampicillin (Amp) plates contained 50 μg/ml. Chloramphenicol (Cm) plates contained 25 μg/ml. The 2xYT broth and agar plates were stored at 4˚C.

Soft agar

The 2xYT broth had 15.5 g/L agar added instead of usual 31 g/l agar to produce soft agar.

Super Optimal broth with Catabolite repression (SOC) medium

SOC medium was prepared by the addition of 20 g tryptone, 5 g yeast extract, 0.6 g NaCl and 0.2 g KCl per litre of MilliQ water. SOC medium was autoclaved and when the solution was cool, it was altered to contain 1 M $MgCl_2$ and 20% glucose.

10X Phosphate Buffered Saline (PBS), pH 7.4

The 10X PBS solution contained 0.01 M KH_2PO_4, 0.1 M Na_2HPO_4, 1.37 M NaCl and 0.027 M KCl. The solution was adjusted to pH 7.4.

1X PBS, pH 7.4

This 10X PBS solution was diluted to 1X PBS with MilliQ water.

1X Phosphate Buffered Saline with Tween-20 (PBST), pH 7.4

The 10X PBS solution was diluted to 1X PBS with MilliQ water and 0.1% Tween-20 was added.

30% Polyethylene glycol (PEG), 3M NaCl

It is important that the PEG is dissolved before the addition of NaCl. This solution is stored at 4˚C.

1 Kb+ ladder

This ladder was made by: 12.5 µl of 1 µg/µl stock plus 455 µl MilliQ water and 32 µl loading dye for a final concentration of 25 ng/µl.

6X DNA loading buffer

The 6X DNA loading buffer contained 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol.

50X TAE, pH 8

The 50X TAE solution contained 50 mM Tris-HCl, 0.11% (v/v) glacial acetic acid and 1 mM EDTA. The solution was adjusted to pH 8.

1X TAE, pH 8

The 50X TAE was diluted to 1X TAE with MilliQ water.

Sodium Dodecyl Sulfate (SDS)-containing buffer

The SDS-containing buffer was made with 1% SDS, 1 X TAE buffer, 5% glycerol and 0.25% bromophenol blue.

Agarose gels (0.7-1%)

Agarose gels, ranged from 0.7% to 1% agarose, were prepared by 0.7 to 1 g, respectively, of agarose to 100 ml 1X TAE buffer and heated until dissolved.

100mg Bovine Serum Albumin (BSA)/ml PBS

BSA was prepared to 100 mg/ml in 1x PBS. The solution was then diluted to 1% BSA in 1X PBS.

Super fibronectin

Super fibronectin (S5171) was supplied as 2 mg/ml solution. The super fibronectin was immobilised on flat bottom microtiter plates (Nunc-Immuno MaxySorp^{TM}, Denmark) at a final concentration of 5 µg/ml, 100 µl per well.

2.1.3 Animal organs used

Murine stomachs used for bait in the affinity-screening

The murine stomachs came from female BALB/c mice. BALB/c mice are albino House Mice (*Mus Musculus*). The animals were obtained from the Small Animal Production Unit (SAPU) at Massey University, and were housed at the same facility in standard cages with five animals per cage. The animals were provided with standard rodent chow and water. These mice were subjects of an unrelated experiment under appropriate ethics approval [MUAEC Protocol 11/85, updated 21-9-12]. At the end of that experiment (an immunisation trial), the animals were euthanized at 19-21 weeks of age under the SOP 09/03 Procedure for Performing Euthanasia in Mice, Rats, Hamsters and Guinea Pigs using CO_2. Organs were harvested post-mortem and the stomachs were stored at -80°C.

2.2 Methods

2.2.1 Bacterial growth conditions and storage

E. coli strain TG1 (Table 1) was used for the construction of and propagation of the helper phage stocks, phagemid vectors and phage display libraries. *E. coli* TG1 cells were incubated in 2xYT at 37ºC with aeration. When required, 25 µg/ml Cm or 100 µg/ml Amp were added to the media. Bacterial stocks were stored long-term at -80˚C in 3 volumes of overnight culture and 1 volume of glycerol and were stored short-term on solid media at 4˚C.

2.2.2 Competent cells

An *E. coli* colony was inoculated into 5 ml of 2xYT and incubated at 37˚C overnight. The overnight culture was used to inoculate 100 ml of 2xYT and incubated until it reached an optical density (O.D.) of 0.25-0.3 at 600 nm. All equipment and solutions had to be cold (4˚C). After centrifugation at 4000 rpm for 5 minutes, the medium was aspirated (discarded) and the pellet was resuspended in 10 ml of 0.1 M $CaCl_2$. Centrifugation and aspiration were repeated and the pellet was resuspended in 1 ml 0.1 M $CaCl_2$, 10% glycerol. The competent cells prepared in this way were dispensed into 1.5 ml tubes (about 50 µl per tube) for storage at -80˚C.

2.2.3 Transformation

For routine transformations, 0.5 µl of plasmid (containing about 20 ng of DNA) was added to 50 µl of competent cells and incubated on ice for 30 minutes. The cells were heat-shocked at 42˚C for 90 seconds (without shaking) and then transferred to ice for 2 minutes. SOC medium (800 µl) was then added and the cell suspension was incubated at 37˚C for 45 minutes with aeration by rotatory shaking. The transformed culture was spread on appropriate selection plate (100 µl undiluted and 100 µl of a 10-fold dilution).

2.2.4 Phage preparation and propagation

Phages are virions that contain helper phage genomes, while phagemid particles (PPs) are virions that contain phagemid genomes. To produce PPs, exponential cultures (10^8 cells/ml) of *E. coli* TG1 carrying either *L. rhamnosus* HN001 or *L. reuteri* 100-23 shot-gun phage display library were infected with the helper phage at a multiplicity of infection of 50 (50 phage to 1 bacterium) to release the virions. After 4 hours of shaking at 37 ºC, the cells were pelleted by centrifugation (9000 rpm for 20 minutes) and the supernatant collected. To precipitate and purify PPs in the supernatant, 30% PEG/ 3M NaCl was added to a final concentration of 5% PEG /0.5 M NaCl. The solution was incubated overnight at 4 ºC. After centrifugation (9000 rpm for 30 minutes), the supernatant was aspirated and the pellet was resuspended in 1 ml of 1X PBS. Phage and PPs were quantified (see **2.2.7 Titration of phage or phagemid particles** and **2.2.8 Densitometry**) and stored at 4°C for short term storage or stored at -80°C in 7% dimethyl sulfoxide for long term storage.

2.2.5 Amplification of phagemid particles

To amplify PPs after each round of library panning, 200 µl of eluted PPs were added into 100 ml of an exponential *E. coli* TG1 culture (to transfect their DNA into the cells by infection). After 30 minutes, appropriate antibiotic was added to the culture and incubation was continued overnight at 37 ºC. A 5 ml aliquot of the overnight culture was taken for later extraction of circular double-stranded DNA of the library phagemid pool, by the plasmid miniprep kit (Roche Applied Sciences, Germany). A 10 ml aliquot was added into 1 L of 2xYT medium. The rest of the procedure was followed as described in **2.2.4 Phage preparation and propagation** above (therefore the stages that

followed were: culture reached exponential stage, helper phage added, cells centrifuged, supernatant collected and PEG precipitated, cells centrifuged and the pellet resuspended).

Another amplification method involved using 2xYT agar plates. Transfected cells were pelleted by centrifugation; the pellet was resuspended in 10 ml of 2xYT and plated on 10 Cm plates (1 ml per plate) for amplification. The following day, the plates were scraped with 2 ml of 2xYT per plate. A 2 ml aliquot was taken to inoculate 200 ml of 2xYT containing appropriate antibiotic. Helper phage, VCSM13, was added when the culture reached exponential phase, to produce the PPs. After a 4 hour incubation at 37°C, a 5 ml aliquot was taken to prepare the circular double-stranded phagemid DNA for analysis. The rest of the culture was subsequently PEG-precipitated as described in **2.2.4 Phage preparation and propagation** and the PPs were titrated as described in **2.2.7 Titration of phage or phagemid particles**.

2.2.6 Helper phage stocks

To produce a working stock of helper phage, a single-plaque derived stock was added (at multiplicity of infection of 50 phage per cell) to 100 ml of an exponential culture of *E. coli* TG1. The culture was shaken at 37 ºC for 4 hours. Next, the cells were spun down by centrifugation; the supernatant collected; filtered (0.45 µm filter); heated at 65 ºC to eliminate bacteria, and titrated. The same protocol was used for obtaining infectious stock of VCSM13d3 phage, except that the host strain for this phage was *E. coli* TG1/pJARA112.

2.2.7 Titration of phage or phagemid particles

Titration was used to quantify phage or PPs. To titrate phage, serial dilutions of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, and 10^{-9} were dispensed as 10 µl drops onto an agar plate (along with a 10 µl drop of the undiluted phage). In total, three plates were counted to determine the titre accurately. The agar plate consisted of a 2xYT agar bottom layer (25 ml) and a top layer of soft agar (2.5 ml of 0.5% agar in 2xYT medium) mixed with 150 µl of an overnight *E. coli* TG1 culture. After incubation overnight at 37 ºC, plaques were counted in each dilution and calculated.

To titrate PPs, a similar procedure was carried out. However, the only difference was the plate used. To titrate PPs, the agar plate consisted of an 2xYT bottom layer

with the appropriate antibiotic (20 ml); a middle layer of 1.5% agar in 2xYT without antibiotic (buffer zone; 9 ml); and the top layer (2.5 ml) of soft agar as above.

2.2.8 Densitometry

Densitometry was also used for determining the amount of phage or PPs. The sample to be quantified was serial diluted to 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, and 10^{-9}. The same serial dilutions were used for a phage stock of a known concentration. The phage stock of known concentration was used as a standard. The samples and standards had SDS-containing buffer added (the buffer contains 1% SDS) and were heated at 70°C for 20 minutes to release the DNA from the virions. The samples and standards were subjected to agarose gel electrophoresis in 1X TAE buffer on a 0.8% agarose gel at 1.5 V/cm for 16 hours. The gel was photographed with a CCD camera (Biorad, USA) and analysed with Image gauge V4.0 to determine the amount of ssDNA present in the samples. Calculations were used to convert the ssDNA present into the amount of phage. This was based on the molecular weight of the ssDNA genome which was calculated from the length and base composition of the phage.

2.2.9 Pilot Study

A test panning experiment was performed for familiarity of the affinity screening process. The super fibronectin was immobilised on flat bottom microtiter plates (Nunc-Immuno MaxySorp^{TM}, Denmark) at a final concentration of 5 µg/ml, 100 µl per well. The plate was incubated overnight at 4 ºC with slow rotation. After overnight incubation at 4 ºC, the wells were washed with 1X PBST. The wells were then blocked with 1% BSA for 2 hours at room temperature. The test PPs had a translational fusion of *Streptococcus pyogenes* fibronectin-binding repeats (FnBR) to virion protein pIII (pDJ04) and thus would display FnBR on the surface of the phage and phagemid particles (Rakonjac *et al.*, 1995). The negative control contained an empty vector (pDJ01) and thus would not display any foreign proteins (Rakonjac *et al.*, 1995). PPs were added (10^{11} PPs in 100 µl of 1X PBS) and incubated at room temperature for 3 hours with slow rotation; followed by washing of the wells. Optimal PPs to use in affinity-screening was described in **2.1.1 Bacterial strains, plasmids and phage**. To elute bound PPs, 100 µl of elution buffer (100mM glycine-HCI, pH 2.2) was added to each well and incubated for 30 minutes at room temperature. Eluted PPs were

transferred to clean microfuge tubes and then neutralized by dispensing 6 µl of unbuffered 2 M Tris and titrated. DNA from the PPs was introduced into *E. coli* TG1 (as described in **2.2.5 Amplification of phagemid particles**). To produce PPs, transfected and amplified cultures were infected by VCSM13 helper phage**;** the resulting PPs were concentrated by PEG precipitation (see **2.2.4 Phage preparation and propagation**). Concentrated PPs were used in the subsequent round of affinity-panning on immobilised fibronectin**.** In total, three rounds of panning were carried out.

2.2.10 Affinity-screening of *L. rhamnosus* HN001 and *L. reuteri* 100-23 libraries with Super fibronectin

Affinity screening was performed on *L. rhamnosus* HN001 an *L. reuteri* 100-23 libraries with super fibronectin as bait. The procedure was identical to **2.2.9 Pilot Study**. Also, in total, three rounds of panning were carried out.

2.2.11 Blocking phage experiment

It was anticipated that phage would stick avidly to murine stomachs (that were to be used in one of the panning experiments). To lower the background binders, instead of just using BSA as a blocking agent, a blocking stock of non-infectious filamentous phage virions was created. The non-infectious phage in this blocking stock contained truncated pIII, from which the receptor-binding domains N1 and N2 were deleted. To obtain such stock, *E. coli* TG1 cells transformed with plasmid pNJB50 (which express the C-terminal domain of pIII) were infected with VCSM13d3 helper phage containing a complete deletion of *gIII* (Bennett *et al*., 2011). The VCSM13d3 stock used to infect pNJB50-containing cells was obtained as described in section **2.2.6 Helper phage stocks**. This produced non-infectious virions that were purified and concentrated by PEG precipitation as described in section **2.2.4 Phage preparation and propagation** and quantified by densitometry as described in section

To assess the amount of PPs bound to the murine stomach tissue (and therefore the background that interferes with library panning) in the presence or absence of the non-infectious blocking stock, a binding assay using PPs derived from cells containing empty vector pYW01 was performed. Standard 1.5 ml centrifuge tubes were coated overnight at 4 °C with either 1 ml of 1% BSA or the non-infectious blocking stock. The

following day, 10^{12} PPs in 1 ml of 1X PBS was added to the tube, then a single murine stomach (defrosted from -80 °C storage) was added.

After a 3 hour incubation at room temperature, on a roller, the stomach was washed five times in 1X PBS. The washed stomach was transferred into 100 ml of exponentially growing *E. coli* TG1 and incubated for 30 minutes at 37 ºC to elute the binders. A 1 ml aliquot was taken from the infected *E. coli* TG1 cells for titration, to quantify the number of TDPs that were bound to the stomach tissue.

2.2.12 Affinity screening of *L. reuteri* 100-23 using mouse stomachs as bait

As described in the results section **3.5 Screening *L. reuteri* 100-23 phage display library for binding to murine stomach tissue,** the blocking stock was unnecessary therefore to affinity screen *L. reuteri* 100-23 using mouse stomachs as bait, the standard 1.5 ml centrifuge tubes were coated overnight at 4 °C with 1 ml of 1% BSA. The affinity screening method was the same as above (**2.2.11 Blocking phage experiment**) except that four rounds of panning were used. Therefore, when the 1 ml aliquot was taken for titration, the remaining cells were used to produce and amplify PPs using a plate method (**2.2.5 Amplification of phagemid particles)** for the subsequent round of panning. The transfected cells were pelleted by centrifugation; the pellet was resuspended in 10 ml of 2xYT and plated on 10 Cm plates (1 ml per plate) for amplification. The following day, the plates were scraped with 2 ml of 2xYT per plate. A 2 ml aliquot was taken to inoculate 200 ml of 2xYT containing appropriate antibiotic. Helper phage, VCSM13, was added when the culture reached exponential phase, to produce the PPs. After a 4 hour incubation at 37°C, a 5 ml aliquot was taken to prepare the circular double-stranded phagemid DNA for analysis. The rest of the culture was subsequently PEG-precipitated and the PPs titrated as described in sections **2.2.4 Phage preparation and propagation** and **2.2.7 Titration of phage or phagemid particles** and used in a subsequent round of panning. Four rounds of panning were completed.

2.2.13 Analysis of selected inserts

After the last round of panning, the eluted PP pool was used to transfect recombinant phagemid DNA into the host *E. coli* TG1 (**2.4 Phage preparation and propagation**) and allowed to amplify by an overnight incubation in medium containing

the appropriate antibiotic. Circular double-stranded phagemid DNA was then extracted from transfected cells by a High Pure Plasmid Isolation Kit. The phagemid profile was analysed by agarose gel electrophoresis (**2.2.16 Agarose gel electrophoresis**)

To isolate enriched recombinant phagemids, predominant bands obtained after electrophoresis of the phagemids were excised from the gel. DNA was extracted from agarose gel slabs by the Agarose Gel DNA Extraction Kit (Roche Applied Science, Germany).

To clonally isolate individual recombinant phagemids, eluted plasmid bands were transformed into *E. coli* TG1 and plated on solid medium to obtain individual colonies. Alternatively, the TDPs from the final round of panning were incubated with *E. coli* TG1 to get single colonies on appropriate antibiotic selection plates. Colonies were individually picked and inoculated into separate tubes to set overnight cultures of recombinant phagemid clones. Double-stranded circular DNA of individual phagemids was extracted by High Pure Plasmid Isolation Kit for further analysis. Sizes of inserts were determined by restriction analysis by either *Nde* I, whose cut sites flank the inserts, or by *Eco* R1 and *Sph* I double digests (see **2.2.15 Restriction digestion of DNA**). To reveal the identity of inserts, they were analysed by sequencing (see **2.2.17 Sequencing**).

2.2.14 Quantification of DNA

DNA was quantified by NanoDrop® spectrophotometer and/or Qubit® fluorometer. DNA was stored at -20°C.

2.2.15 Restriction digestion of DNA

Restriction digests were performed by the following method:

2 µl of 10X Restriction Enzyme Buffer (buffer depends on enzyme)
1 unit of Restriction Endonuclease (RE) (Invitrogen, USA)
Required amount of DNA (100 ng per reaction)
Required volume of sterile water in a total volume of 20 epithelial-cadherin µl.

Each reaction was incubated for an hour at 37°C and DNA fragments were visualised by agarose gel electrophoresis.

2.2.16 Agarose gel electrophoresis

Various DNA fragments were visualised by agarose gel electrophoresis (containing molecular biology grade agarose at 0.7-1 %) in 1X TAE buffer. DNA loading dye was added to the samples before loading. The 1Kb+ ladder or supercoiled ladder were used as markers for size determination for linear and double-stranded circular plasmid DNA, respectively. The voltage and time of electrophoresis varied depending on the particular experiment, between 1.5 and 100 V/cm and 16 to 1 hour. Agarose gels were stained with 0.5 μg/ml solution of ethidium bromide in water for 15 minutes then destained in distilled water before being visualised on an UV transilluminator.

2.2.17 Sequencing

Sequencing was done using 150 ng of DNA in a 15 μl reaction containing 3.2 pico moles (pmol) of primer. The primers used are listed in Table 3. The reverse primer is located downstream of the multiple cloning site, within the pIII coding sequence. The forward primer is located upstream of the multiple cloning site.

Table 3. Oligonucleotides used for Sequencing

Name	***Sequence***	***Application***
pspF03	atgttgctgttgattcttca	Forward primer for sequencing
pspR03	tgcctttagcgtcagactgtagc	Reverse primer for sequencing

Sequencing was performed by Sanger DNA sequencing at the Massey University Genome Analysis Facility, Massey University, Palmerston North. The instrument used was a capillary ABI3730 Genetic Analyzer, from Applied Biosystems Incorporated. Sequence analysis programme Vector NTI (Life Technologies) and searches using NCBI BLAST algorithm (Altschul *et al*., 1990) were used to analyse the sequencing results.

2.2.18 Binding assays of purified clonal recombinant PPs

Recombinant plasmids that encoded potential binders to the bait were individually assayed (affinity-screened) for binding to the bait, to determine whether

they are true binders (see **2.2.12 Affinity screening of *L. reuteri* 100-23 using mouse stomachs as bait**). The colonies that were DNA sequenced were used to produce PPs (see **2.4 Phage preparation and propagation**). Control PPs came from colonies derived from *E. coli* TG1 containing empty vectors pYW01 (Cm^R) or pJARA144 (Amp^R). These PPs were used in simple or competitive binding assays. The following mixed combinations of PPs were used for competitive binding: recombinant phagemid isolated from the library and pJARA144; empty vector pYW01 and pJARA144. The eluted PPs were titrated on selective plates, depending on vector-encoded markers: Cm (recombinant phagemid and pYW01) or Amp (pJARA144).

3.0 Results

3.1 Pilot Study

A pilot fibronectin-binding assay testing of PPs displaying a fibronectin-binding-repeat (FnBR) domain from *Streptococcus pyogenes* surface protein called Serum Opacity Factor was performed, in order to establish the conditions for the library screening (Rakonjac *et al.*, 1995). The FnBR domain has a high affinity for fibronectin, therefore an increase in binding of the PPs displaying this domain was expected relative to the PPs derived from the empty vector. The pilot assay (see **2.2.9 Pilot Study)** showed that, as expected, the number of FnBR-displaying PPs eluted from immobilised fibronectin were higher than that of the empty vector-derived PPs. Therefore, the PP production and purification, as well as the panning procedure, were carried out correctly.

Table 4. Pilot fibronectin-binding assay

	Input[a]	**Output**[b]	**Ratio**[c]
FnBR-displaying PPs	7.2×10^{9}	8.0×10^{6}	1.1×10^{-3}
Empty vector-derived PPs	1.2×10^{9}	$<2.0 \times 10^{2}$	$<1.7 \times 10^{-7}$

[a] The input is the total amount of PPs that was mixed with the bait in each round of panning.

[b] The output is the total amount of PPs that was retrieved from the bait (eluted) after each round of panning.

[c] The ratio is obtained by dividing the output with the input.

3.2 Affinity screening of the libraries with Super fibronectin as bait

To identify putative fibronectin-binding proteins of *L. rhamnosus* HN001 and *L. reuteri* 100-23, a phage display approach was used. Three rounds of panning were completed for each of two libraries derived from these two species, as described in section **2.2.10 Affinity-screening of *L. rhamnosus* HN001 and *L. reuteri* 100-23 libraries with Super fibronectin.** Super fibronectin was used as the ligand. Titration of the eluted PPs (output) and input (the number of PPs mixed with the bait) in each round of panning was used to monitor enrichment for high-affinity binders (if any). High relative amount of eluted PPs were observed in the first round of panning, in comparison to the second and third round, which was unexpected. Normally a relative increase in the number of eluted PPs is observed. However, in some pannings the enrichment of low-affinity binders may not be reflected in an increase of the number of eluted particles, if the interaction between the displayed ligand and the bait is of low affinity (Jankovic, 2009).

Table 5. *L. reuteri* 100-23 and *L. rhamnosus* HN001 phage display library screening using Super fibronectin as a bait

Panning round	**Input**	**Output**	**Ratio**
1			
L. reuteri 100-23	$1.0x10^{10}$	$1.4x10^{8}$	$1.4x10^{-2}$
L. rhamnosus HN001	$1.0x10^{10}$	$5.4x10^{7}$	$5.4x10^{-3}$
2			
L. reuteri 100-23	$1.0x10^{12}$	$1.2x10^{5}$	$1.2x10^{-7}$
L. rhamnosus HN001	$1.4x10^{12}$	$2.3x10^{4}$	$1.6x10^{-8}$
3			
L. reuteri 100-23	$1.3x10^{12}$	$2.3x10^{4}$	$1.8x10^{-8}$
L. rhamnosus HN001	$1.5x10^{12}$	$2.1x10^{4}$	$1.4x10^{-8}$

[a] The input is the total amount of PPs that was mixed with the bait in each round of panning.

[b] The output is the total amount of PPs that was retrieved from the bait (eluted) after each round of panning.

[c] The ratio is obtained by dividing the output with the input.

3.3 Recombinant phagemids enriched for by affinity screening on Super fibronectin

After each round of panning, phagemid double-stranded closed circular DNA (or the Replicative Form, RF) was extracted from the culture at the step of amplification and subjected to agarose gel electrophoresis.

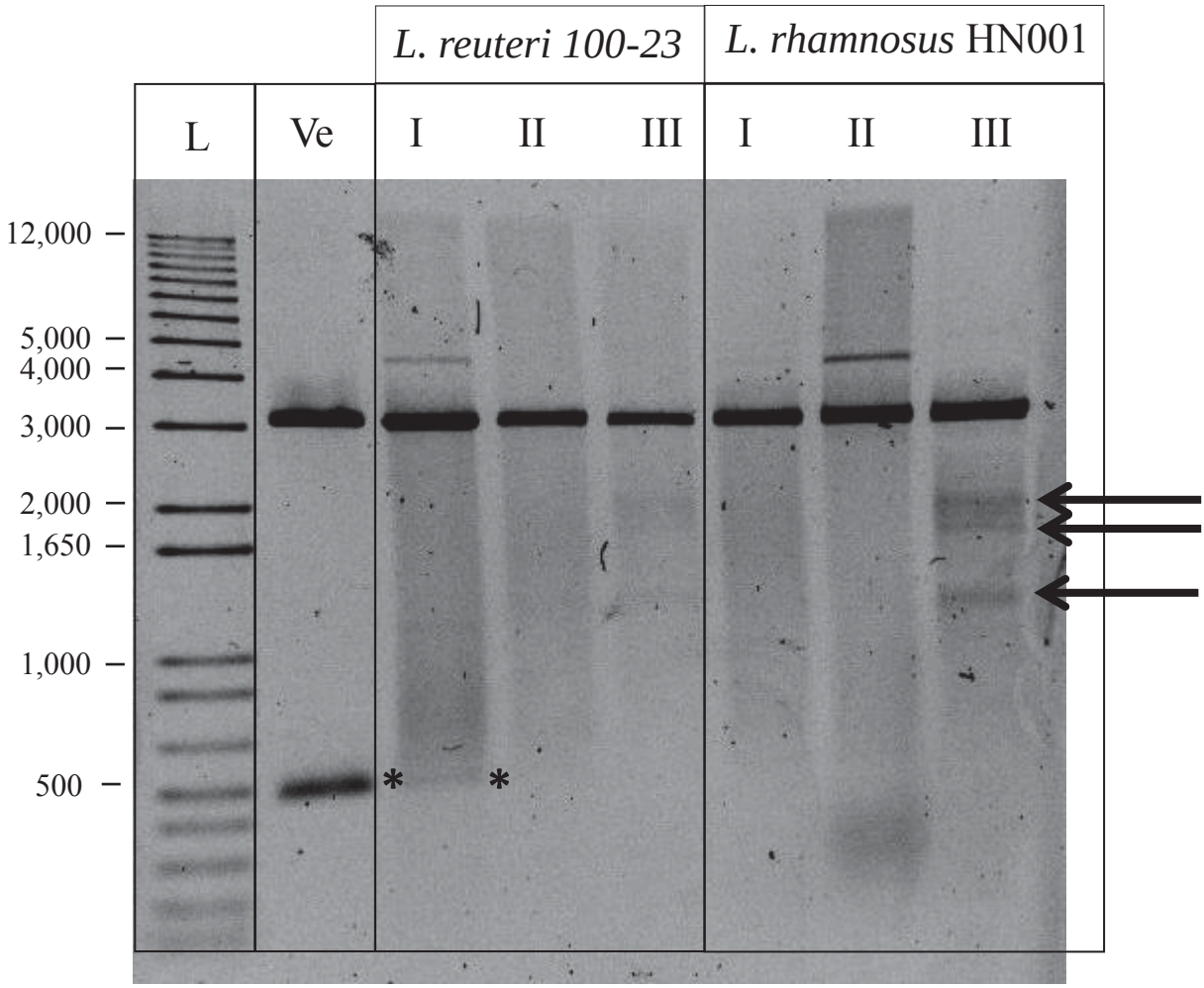

Figure 3. *Nde* I restriction digest of pooled phagemid DNA during affinity enrichment experiment. Lanes: **L**, 1 kb+ ladder; remaining lanes contain the *Nde* I-cleaved RF DNA of the following phagemid samples: **Ve**, pYW01 vector; ***L. reu* 100-23**, *Lactobacillus reuteri* 100-23 phagemid DNA from: **I**, the first round of panning; **II**, the second round of panning; **III**, the third round of panning; ***L. rh* HN001**, *Lactobacillus rhamnosus* HN001: **I**, the first round of panning; **II**, the second round of panning; **III**, the third round of panning. Arrows indicate enriched bands. Asterisks indicate the *Nde* I fragment obtained by cleaving the vector pYW01, also present in the library prior to the library panning

Due to the many different sizes of insert present in the libraries, RF DNA migrates as a smear on the gel, especially in the early rounds of panning when the diversity of the library is very high (Fig 3, lanes II). Selection of specific fibronectin-binding clones is expected to result in enrichment for one or several recombinant plasmids, resulting in the observed discrete recombinant phagemid bands instead of (or in addition to) the smear after agarose gel electrophoresis. By using a restriction enzyme, *Nde* I, which cleaves the vector sequence flanking the inserts, identification of the dominant insert bands was facilitated, as the supercoiled and relaxed circular forms of phagemid RF DNA were eliminated. Three discrete bands were visible in the third round of panning of *L. rhamnosus* HN001, but not for *L. reuteri* 100-23 library after agarose gel electrophoresis of *Nde* I-cleaved pooled RF of recombinant phagemid DNA (Fig. 3). To isolate the enriched recombinant phagemids of *L. rhamnosus* HN001, the uncut RF DNA was separated by agarose gel electrophoresis and the visible bands were excised. The DNA from excised agarose gel slabs was purified and transformed into *E. coli* TG1, to obtain individual recombinant clones.

3.4 Identification of inserts from the enriched recombinant phagemids

Transformation of DNA purified from the fastest-migrating of the three bands gave transformants. The DNA from the other two bands did not produce transformants. Phagemid DNA (RF) was isolated from ten transformant colonies which corresponded to individual recombinant phagemid clones. The DNA was digested with *Nde* I restriction nuclease to determine the size of the inserts (Fig.4). The insert size was not uniform, even though all transformants were derived from the same enriched phagemid band. This size range could be due to an overlap in migration between supercoiled and relaxed circular DNA forms of different recombinant plasmids in the preparative gels. Two clones (1 and 2) were empty (did not have an insert).

The inserts in clones 3, 4, 5, 6, 7, 8, 9 and 10 were analysed by DNA sequencing to identify the open reading frames in frame with the virion protein pIII encoded by the vector, a condition for display of peptides on the surface of the PPs. Sequence analysis programme Vector NTI (Life Technologies) and searches using NCBI BLAST algorithm (Altschul *et al.*, 1990) were used to analyse the sequencing results.

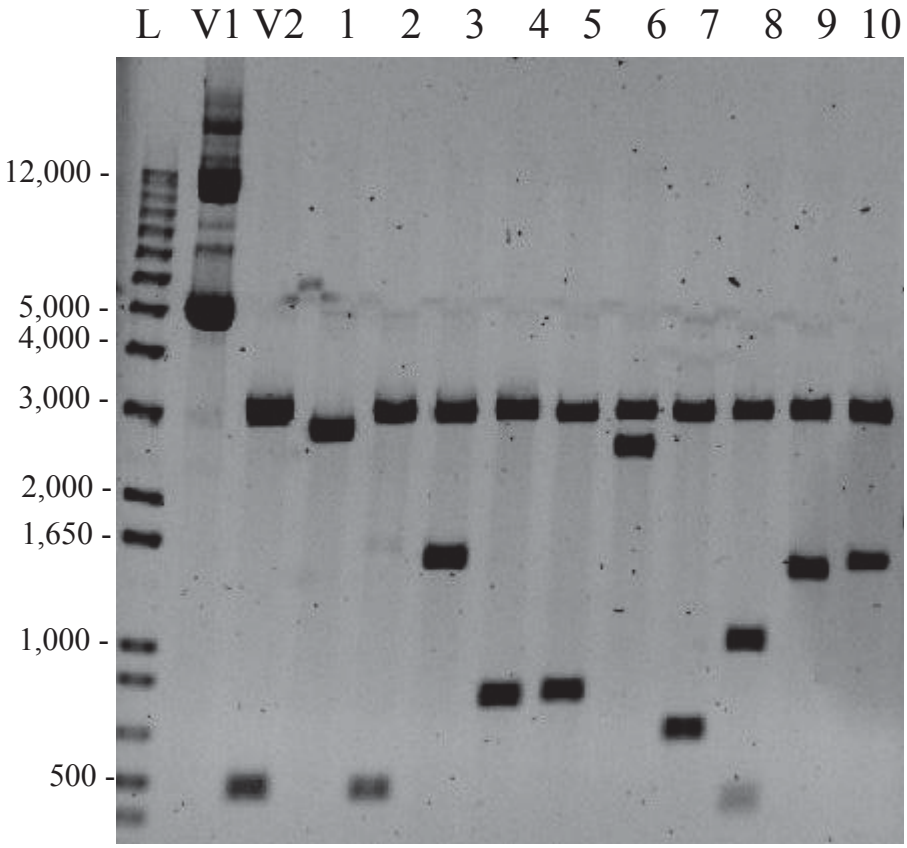

Figure 4. *Nde* I restriction analysis of individual recombinant clones isolated from enriched phagemid bands. Lanes: **L**, 1 kb+ ladder; **V1**, uncut pYW01 vector; **V2**, cut vector; **1 – 10**, *Nde* I digested clones 1 - 10.

Inserts in clones 3, 4, 5, 6, 8, and 9 encoded only very short peptides (equal to or less than 20 residues) in frame with the vector-encoded virion protein pIII, and also these peptides were not in frame with the upstream pelB signal sequence (they contained upstream untranslated genomic DNA). A signal sequence (typically 18-30 amino acid residues) is required for targeting of protein fusions to pIII to the inner membrane of *E. coli* and assembled into the virion, however it is cleaved by signal peptidase in the process of membrane targeting and before assembly into the PPs. Because of their short length, the peptides encoded by the clones 3, 4, 5, 6, 8 and 9, even if they encoded a signal sequence, would have been removed from the pIII fusion and no additional amino acids besides those encoded by the vector would have been displayed on the surface of the PPs. Inserts from clones 7 and 10, respectively, encode polypeptides of 102 and 72 amino acids in length, in frame with pIII. These two

peptides could potentially display peptides or functional domains that bind to fibronectin (Table 6).

Table 6. Amino acid sequence of recombinant clones

Recombinant clone	*Protein Sequence*
7	lnaeikrttyeipaadrccwsaaprcpagdghgpcmpridnvfgklly lfpkcahiaasrhlckgptfnhlikqtgmlhqsgiplrmdndrnhatlhhfkqh
10	lngldylhqhrnilrrqqlchcgslnrmikslgslilikqlkqqvr stvwasrkltttrelagnyfrrwrpl

A BLASTn search of clone 7 insert (searching the NCBI nucleotide database against the DNA sequence as query) showed that the sequence overlaps with an annotated gene in *L. rhamnosus* HN001 draft genome, encoding a predicted phosphotyrosine protein phosphatase. However, the peptide that is in frame with pIII is encoded by the antisense strand of that gene, hence the peptide that is displayed is functionally unrelated to the phosphotyrosine protein phosphatase. The BLASTn search using the clone 10 insert sequence matched a gene encoding an acetyltransferase of *L. rhamnosus GG*. However, the 72-residue long peptide (that was in frame in pIII) matched the translation of the -1 frame of the acetyltransferase gene, hence its amino acid sequence is completely different from that of the annotated *L. rhamnosus* GG protein. The BLASTp search using the amino acid sequences that are in frame with pIII (Table 6), did not match any predicted proteins in the NCBI database. Both clone 7 and 10 translational products have a predicted CTG translational start codon, which can serve as an alternative prokaryotic start codon (instead of ATG).

Analysis using algorithms for prediction of signal sequence (SignalP4.0) and transmembrane helices (TMHMM) (Center for Biological Sequence analysis, retrieved from www.cbs.dtu.dk/services/SignalP-4.0; www.cbs.dtu.dk/services/TMHMM) showed that the 102- and 72- residue peptides in frame with pIII do not contain a predicted signal sequence or transmembrane helices, which are required for display of a pIII fusion on surface of the PPs, hence they are unlikely to be displayed. Overall, this analysis has shown that none of the analysed recombinant phagemids carry inserts that could be displayed on the surface of the PPs and therefore are “background” clones.

Enrichment of "background" clones in the absence of real binders has been observed, and it is often due to the growth and PP assembly advantage over other clones in the amplification stages of the library screening. Alternatively, the true binders were missed in this analysis, and larger number of clones would have to be analysed in order to identify the true fibronectin binders.

3.5 Screening *L. reuteri* 100-23 phage display library for binding to murine stomach tissue

Lactobacillus reuteri 100-23 is a coloniser of keratinous surface in the murine forestomach. *Lactobacillus reuteri* proteins that mediate attachment of the cells to this surface have not been identified as yet. Phage display approach could potentially be used to identify binding protein(s). However, it has been noted that filamentous phage non-specifically bind to stomach tissue at a frequency of 1/1000 (D. Gagic, personal communication). These phage represent very high background that may prevent selection of true binders. It was therefore necessary to attempt to decrease this non-specific binding by using non-infectious filamentous phage particles to block the non-specific PP binding sites. A complete murine stomach, longitudinally cut to expose the keratinous surface, was used as a bait in this assay (and subsequent library panning), as it is difficult to dissect the keratinous tissue away from the rest of the stomach tissue due to a small size. The stomach tissue was salvaged after completion of an unrelated experiment that resulted in sacrifice of 30 BALB/c mice and was taken out of the -80 °C storage and immediately used in the assay (see **2.1.3 Animal organs used** for more details).

A trial assay was performed in which the unspecific binding of PPs derived from the empty vector (pYW01) to murine stomach tissue was monitored in the presence or absence of non-infectious filamentous phage (blocking stock) (see **2.2.11 Blocking phage experiment).** The titration of eluted PPs showed that there was a higher number of binders in the presence of the blocking stock than in its absence, an opposite of what was expected (with the blocking stock the output was $8x10^8$ PPs, and without the blocking stock the output was $4.4x10^7$ PPs). The filamentous phage has been reported to aggregate by adhering to each other along the axis of the filament at high concentrations. The already bound non-infectious phage have, instead of blocking the binding site, promoted binding of the PPs, possibly by forming the bridges between the

blocking phage and the non-binding PPs. Therefore, additional blocking was not going to be efficient and thus the standard blocking solution (1% BSA in PBS) was used in the subsequent library screening experiments.

Table 7. *L. reuteri* 100-23 phage display using keratinous mouse lining

Panning round	Input[a]	Output[b]	Ratio[c]
1	$1.0x10^{12}$	$6.0x10^{7}$	$6.0x10^{-5}$
2	$3.8x10^{10}$	$2.0x10^{6}$	$5.2x10^{-5}$
3	$1.0x10^{10}$	$4.0x10^{6}$	$4.0x10^{-4}$
4	$1.0x10^{11}$	$2.4x10^{7}$	$2.4x10^{-4}$

[a] The input is the total amount of PPs that was mixed with the bait in each round of panning.

[b] The output is the total amount of PPs that was retrieved from the bait (eluted) after each round of panning.

[c] The ratio is obtained by dividing the output with the input.

Four rounds of panning were completed for the *L. reuteri* 100-23 library as described in section **2.2.12 Affinity screening of *L. reuteri* 100-23 using mouse stomachs as bait** using the murine stomachs as bait. Table 7 shows the titration results in each round of panning and the corresponding ratios of eluted PPs (output) to the PPs mixed with the bait (input). The ratio of output to input increased with each round, indicating that clone(s) of increased affinity are becoming enriched.

3.6 Analysis of enriched recombinant phagemids

After each round of panning, the phagemid double-stranded closed circular DNA (or the Replicative Form, RF) was extracted from the culture at the step of amplification and subjected to agarose gel electrophoresis. Selection of specific recombinant library clones was expected to result in enrichment for one or several recombinant plasmids, resulting in discrete recombinant phagemid bands. By using a restriction enzyme, *Nde* I, which cleaves the vector sequence flanking the inserts, enriched bands were more visible, as the supercoiled and relaxed circular bands were eliminated (Fig. 5).

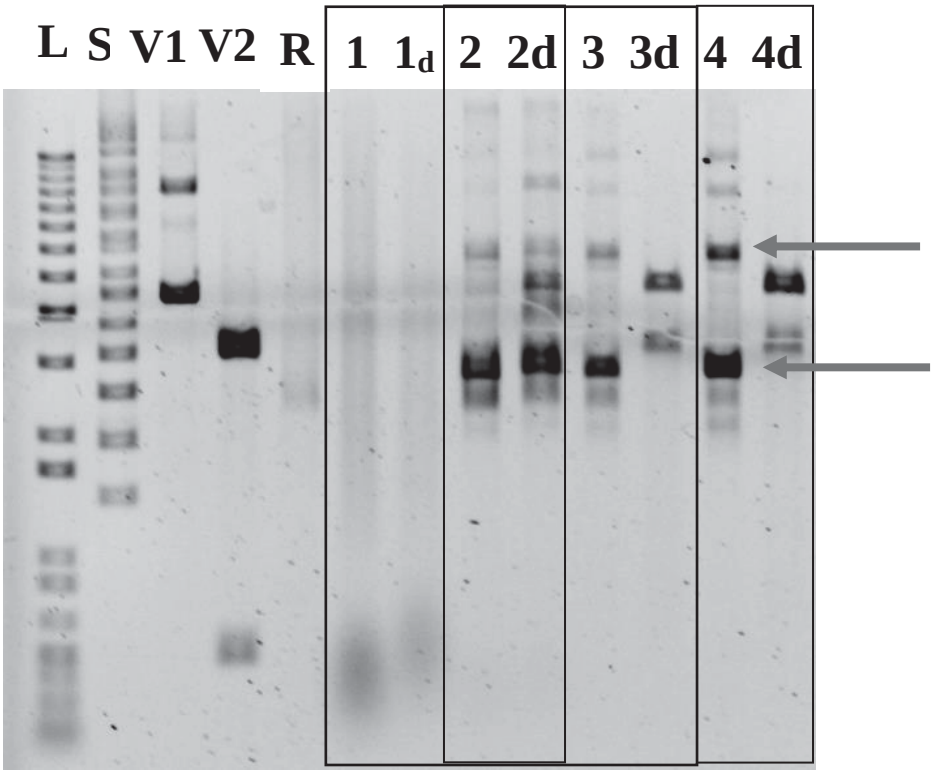

Figure 5. Monitoring enrichment for specific recombinant phagemids during the library panning on murine stomach tissue. Lanes: **L**, 1 kb+ ladder; **S**, supercoiled ladder; **V1**, pYW01 vector DNA, uncut; **V2**, pYW01 *Nde* I digest; **R**, *L. reuteri* 100-23 phage display library input, uncut, ***L. reu* 100-23**, *Lactobacillus reuteri* 100-23 phagemid DNA from: **1**, phagemid pool after the first round of panning, uncut; **1d,** *Nde* I-digest of 1; **2**, phagemid pool after the second round of panning, uncut; **2d**, *Nde* I digest of 2; **3**, phagemid pool after the third round of panning; **3d**, *Nde* I digest of 3; **4**, phagemid pool after the fourth round of panning; **4d**, *Nde* I digest of 4. Arrows indicate enriched bands. Please note that the DNA in lanes 1 and 1b was degraded by an unspecific nuclease; it was not possible to purify additional DNA.

The analysis showed high enrichment of a plasmid band after rounds 3 and 4 of panning (Fig. 5, lanes 3, 3d, 4 and 4d); however this phagemid lacked one of the two *Nde* I sites, resulting in a single band instead of two bands after agarose gel electrophoresis of *Nde* I-cut DNA (the two sites are located 81 nucleotides upstream,

and 391 nucleotides downstream of the cloning site). This in turn indicated that the dominant band could be a recombinant phagemid in which some of the pYW01 vector sequence flanking the insert was deleted, by recombination in the course of library panning and/or amplification.

To isolate the enriched recombinant phagemids of *L. reuteri* 100-23, the enriched phagemid band from the fourth round of panning (from the undigested lane on agarose gel; Fig. 5, lane 4) was excised. The DNA from excised from agarose gel slabs was purified and transformed into *E. coli* TG1 to obtain individual recombinant clones.

3.7 Analysis of dominant recombinant phagemid(s)

Transformation of DNA from the enriched band obtained in the fourth round of panning gave transformants. Phagemid DNA (RF) was isolated from nine individual colonies on the transformation plate (named 1, 2, 4, 5, 6, 7, 8, 9, and 10) which corresponded to individual recombinant phagemid clones. Purified plasmid DNA was double- and single-digested with *Sph* I and *Eco* RI, two enzymes that are adjacent to the *Sma* I site used for library construction in the multiple cloning site of the vector pYW01, to determine the size of the inserts and to analyse if and where the deletion of the vector sequence has occurred (Fig. 6).

The DNA extracted from colonies 4 and 6 was cleaved by both *Sph* I and *Eco* RI restriction enzymes, however the product was of identical size to that of the empty vector, pYW01, suggesting that these are not recombinant plasmids, but rather represent "background" of empty vector which is present in the library. The DNA extracted from colonies 1, 2, 7, 8, 9, and 10 contained an *Eco* RI site, which in the pYW01 vector is located downstream of the insert, but not an *Sph* I site which is located upstream of the insert. This was consistent with the result of the *Nde* I digest of the DNA pool from the 4^{th} round of panning, in which the dominant recombinant band was cleaved only once by *Nde* I. The cleavage product of *Eco* RI digests from these six clones was larger by about 700 nucleotides in comparison to the *Eco* RI-digested vector (Fig 6). All these findings point to the presence of an insert, and a deletion at the upstream joint of the insert and vector.

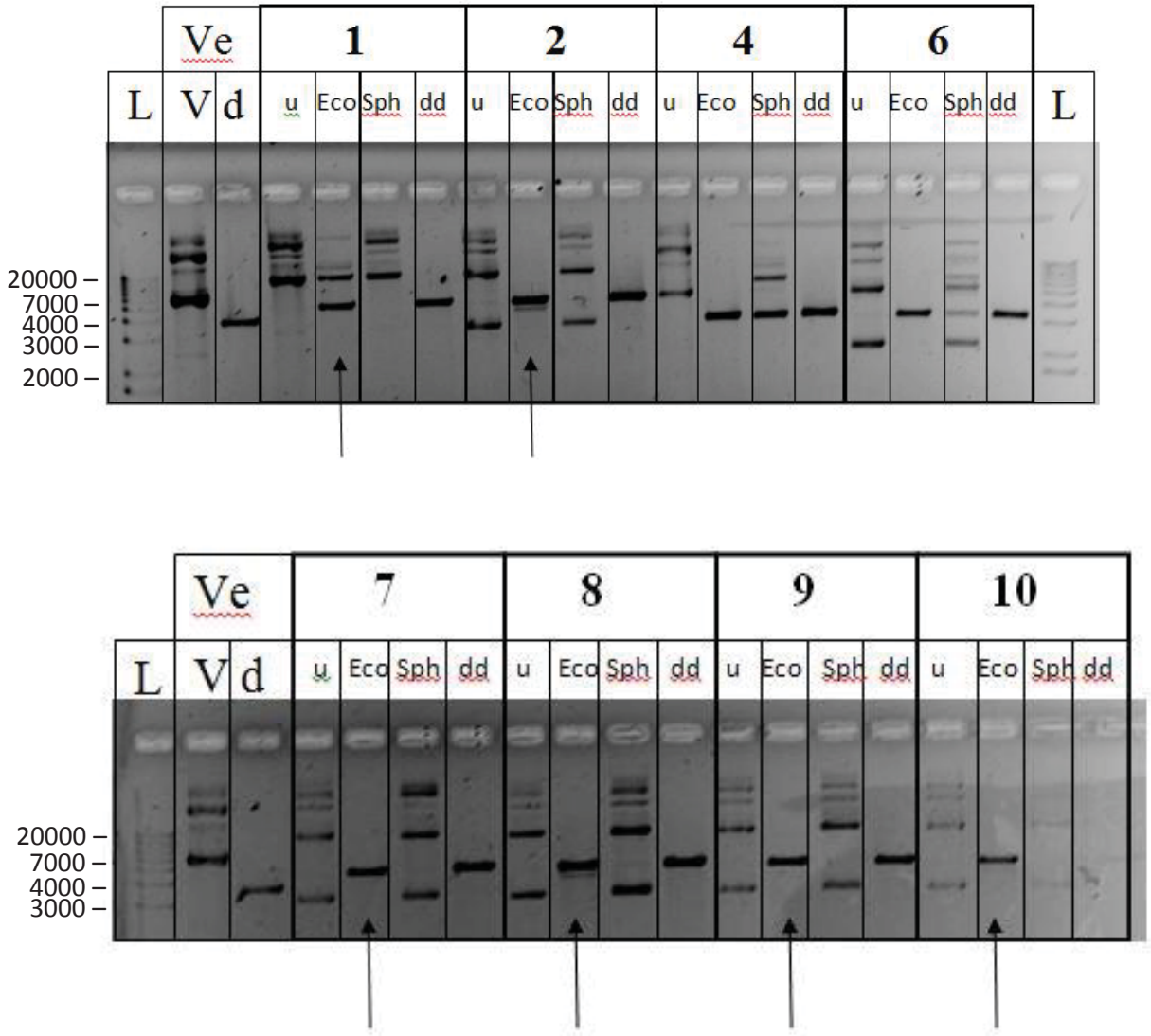

Figure 6. *Sph* I and *Eco* RI restriction analysis of individual recombinant clones isolated from an enriched phagemid band.

Lanes: Lanes: **L**, 1 kb+ ladder; **V**, uncut pYW01 vector; **d**, pYW01 *Eco* RI digest; **1-10,** *L. reuteri* recombinant phagemids, **u**, undigested; ***Eco***, *Eco* RI digest; ***Sph***, *Sph* I digest; **dd**, *Eco* RI and *Sph* I double digest. Inserts are present in clones 1, 2, 7, 8, 9, and 10. Arrows indicate the clones with *Eco* RI digested bands that are migrating ~700 nucleotides slower than the linearized pYW01 vector.

The inserts in clones 1, 2, 7, 8, 9 and 10 were analysed by DNA sequencing to identify the open reading frames that they carry. Sequence analysis programme Vector NTI (Life Technologies) and searches using NCBI BLAST algorithm (Altschul *et al.*,

1990) were used for analyse the sequencing results. The reverse primer resulted in successful sequencing. The forward primer did not result in successful sequencing, suggesting that its complementary sequence is missing from the recombinant phagemid. The sequences of clones 1, 2, 7, 8, 9 and 10 obtained using the reverse primer were identical, therefore they came from an identical clone.

The sequencing showed the presence of a short insert sequence (24 nucleotides). The insert was in frame with the upstream pelB signal sequence and downstream pIII, hence the translated peptide encoded by the insert was displayed on the surface of PPs. The BLASTn search showed that 17 nucleotides in the centre of this insert are identical to the coding sequence of a protease from *L. reuteri* strain TD1 genome sequence (locus accession number CP006603 and protein accession number AGR63532.1) and also from *L. rhamnosus*. The displayed peptide is located near the C-terminal end of the protease domain, towards a linker to the C-terminal PDZ domain. The translation of displayed peptide that matches AGR63532.1 is IGINS (Ile, Gly, Ile, Asn, Ser).

3.8 Competition assays of IGINS-displaying PPs binding to the mouse stomach tissue

Colony 7 produced the best sequence so it was chosen to test for binding to the bait in a competition or enrichment assay. In this assay, the clone 7-derived PPs are combined with an excess of PPs derived from a phagemid vector, pJARA144 (Amp^R), that has a different antibiotic resistance marker from clone 7 and pYW01 vector (Cm^R). The Amp^R and Cm^R PPs can be distinguished by titration using Cm- vs, Amp-containing plates, respectively (Table 8; see **2.2.18 Binding assays of purified clonal recombinant PPs** for details). Three independent assays were carried out. In all three assays, both the vector in which the library was constructed (pYW01) and clone 7, the dominant 4^{th} round library screening clone, were enriched over the competitor vector, pJARA144. Interestingly, the enrichment was very variable in three experiments, ranging from 19-fold to 290,000-fold. Furthermore, the enrichment of pYW01 vector, despite overall experiment-to-experiment variability, was consistently about 10- to 20-fold.

Table 8. Enrichment assays with clone 7

	Input[a]		In ratio[b]	Output[a]		Out ratio[b]	Enrichment[c]
Assay 1	Cm	Amp	Cm/Amp	Cm	Amp	Cm/Amp	Out R/In R[c]
pJARA144 + clone 7	$2.4x10^5$	$1.4x10^{11}$	$1.7x10^{-6}$	$4.0x10^4$	$5.6x10^5$	$7.1x10^{-2}$	$4.2x10^4$
pJARA144 + pYW01	$4.2x10^7$	$1x10^{12}$	$4.2x10^{-5}$	$8.0x10^6$	$6.6x10^5$	$1.2x10^1$	$2.9x10^5$
Assay 2							
pJARA144 + clone 7	$8.0x10^6$	$2x10^{11}$	$4.0x10^{-5}$	$1.5x10^5$	$2.0x10^8$	$7.5x10^{-4}$	$1.9x10^1$
pJARA144 + pYW01	$4x10^8$	$2x10^{12}$	$2.0x10^{-4}$	$1.0x10^5$	$7.4x10^5$	$1.4\ x10^{-1}$	$6.8x10^2$
Assay 3							
pJARA144 + clone 7	$1.1x10^8$	$1.8x10^{11}$	$6.1x10^{-4}$	$1.3x10^4$	$3.8x10^5$	$3.4x10^{-2}$	$5.6x10^1$
pJARA144 + pYW01	$1.2x10^8$	$6.0x10^{11}$	$2.0x10^{-4}$	$8.0x10^4$	$4.0x10^5$	$2.0x10^{-1}$	$1.0\ x10^3$

[a]The input and output titres, respectively, represent the total number of PPs mixed with the bait (input) and eluted from the bait (output).

[b]The "In ratio" and "Out ratio" represent, respectively, the ratios of Cm^R (clone 7 or pYW01) to Amp^R (pJARA44) PPs in the input and output

[c]The enrichment was calculated by dividing the Out ratio with In ratio (Out R/In R)

3.9 Identity of the clone 7

Further sequence analysis was carried out for clone 7. The 24 nucleotides between the pelB- and pIII-encoding sequences, that encode IGINS identical to a peptide from a *L. reuteri* and *L. rhamnosus* protease, and the upstream sequence that differed from pYW01 were found to be actually derived from vector p3CS (constructed in the laboratory; unpublished). The p3CS vector contains a *tac* promoter and a full-length $lacI^q$ gene. The pYW01 vector, in contrast, contains a *psp* promoter and no $lacI^q$ gene. This p3CS phagemid vector has a multiple cloning site including *Eco* RI at the C-terminus of pIII, and displays a c-myc tag at the C-terminus of pIII; the same tag is displayed at the N-terminus of pIII in vector pYW01. The size difference between pC3S and pYW01 sequences corresponds to the difference observed by agarose gel electrophoresis.

In summary, the *L. reuteri* 100-23 library was contaminated with an unrelated phagemid, which by coincidence displays a *L. reuteri*-derived peptide IGINS at the N-terminus of pIII. The enrichment assays with clonally purified p3CS-derived PPs showed that they were enriched over the competitor PPs derived from vector

pJARA144 (another phagemid phage display vector containing a different antibiotic marker), however the enrichment was not more pronounced than of that of the PPs derived from vector pYW01 used in the library construction. Given the specific enrichment of this plasmid in the library screening on the stomach tissue over other library recombinant phagemids, but not in the screening using fibronectin as bait, there is a possibility that the IGINS peptide binds to a ligand in the stomach tissue.

4.0 Discussion

Affinity screening of *L. rhamnosus* HN001 and *L. reuteri* 100-23 phage display libraries on fibronectin as bait was performed with the intention of identifying fibronectin-binding proteins in these two bacterial strains. The lack of enriched bands from the panning experiment in the case of *L. reuteri* 100-23 library may not necessarily mean that fibronectin-binding protein(s) do(es) not exist in this organism; it is possible that there is low-affinity to fibronectin-binding protein(s) that could not be enriched for in the three rounds of panning. Alternatively, the fibronectin-binding protein may not fold correctly when displayed on the surface of PPs. It is less likely that the sequence encoding the fibronectin-binding domain is missing from the library, as the library size is very large ($5x10^7$ primary clones).

Detection of discrete phagemid bands of *L. rhamnosus* HN001 are an indication of enrichment. However, the heterogeneity of insert sizes obtained from transformation with a single excised apparently enriched band indicate that the background population of non-binders is still highly represented. This insert heterogeneity is also indicated by sequence analysis of 8 clones, which all contained different (non-overlapping) inserts. Among the analysed recombinant clones, two encoded polypeptides, 102 and 72 residues in length, were in frame with pIII encoded by the vector, a condition for display on the surface of PPs. These polypeptides are large enough to represent functional protein domains (Table 6). However, BLAST searches showed that the 102-residue polypeptide was encoded by antisense strand of an otherwise annotated open reading frame, whereas the 72-residue polypeptide was encoded by -1 reading frame relative to an annotated reading frame in the genome of *L. rhamnosus*.

Even though the two polypeptides were not the annotated translational products of the corresponding coding sequence, it was still possible that they are genuine open reading frames that overlap with the annotated genes. Presence of a promoter and/or ribosome-binding-site motifs in this case would indicate a genuine open reading frame. These motifs were searched for in the DNA sequence upstream of the predicted translational start codons of clone 7 and 10 inserts, using prokaryotic promoter- and ribosome-binding-site-finding algorithms: Promoter 2.0 and Glimmer (Knudsen, 1999, Delcher *et al.*, 2007). No promoter sequences or ribosome-binding sites were found

immediately upstream of the translational sites. Although in the case of clone 10 the promoter was not relevant, given that it was encoded by the sense strand of a transcribed gene, the ribosome binding site would have been required for translation. The lack of ribosome binding sites preceding the putative start codons in both inserts suggests that they are unlikely to be translated, even if they were transcribed. Furthermore, given that the peptides in-frame with pIII were not fusions to the upstream vector-encoded pelB signal sequence, they would have to contain a signal sequence or an N-terminal transmembrane helix in order for the protein fusion with pIII to be targeted to the inner membrane of *E. coli*, another condition for display on the surface of the PPs. Analyses using the SignalP and TMHMM algorithms showed that these two peptides had no signal sequence or transmembrane helix, hence they could not have been displayed on the surface of the PPs, even if they were transcribed and translated.

Affinity screening of the *L. reuteri* 100-23 phage display library was next performed on the mouse stomach tissue as bait, to identify proteins that bind to the keratinous lining of the mouse forestomach. Since *L. reuteri* 100-23 colonises this area of the mouse stomach, it was likely that a protein/s that bind the stomach tissue exist in the *L. reuteri* 100-23 genome.

Detection of a discrete and very strong phagemid band in the second, third and fourth round of panning for *L. reuteri* 100-23 were an indication of enrichment. The purified dominant plasmid band was transformed into *E. coli* and the inserts from eight transformants were analysed by sequencing. Two out of eight sequenced phagemids were equivalent to the pYW01 vector; the remaining six phagemids were identical and they all corresponded to the dominant enriched band from the 4th round of panning based on their size by agarose gel electrophoresis. Sequence analysis showed that this recombinant phagemid contained a short insert; however it appeared that deletions occurred upstream and downstream of the insert, nevertheless preserving the reading frame with respect to the pelB signal sequence and pIII. Out of 24 nucleotides inserted between the signal sequence and pIII, 17 nucleotides were identical to the sequence of a DegP-family protease encoded by the *L. reuteri* and *L. rhamnosus* genomes. Detailed analysis, however, showed that the selected phagemid was equivalent to a phage display vector (p3CS, unpublished) that was used in the laboratory at the time when the library was being constructed. Also, the "insert" sequence corresponded to a synthetic linker in that vector. The PPs derived from this vector must have contaminated the library at a

very low frequency and were enriched during panning on the murine stomach. In contrast to the affinity-screen on the stomach tissue, this clone was not enriched in the screening on fibronectin, even though the library stock was the source of PPs in both screens. This indicates the specific enrichment of this plasmid on the stomach tissue as bait, hence it is possible that the displayed peptide binds the stomach tissue.

The assay using the clonally purified p3CS-derived PPs (Cm^R) spiked into an excess of PPs derived from an Amp^R phagemid and showed the enrichment of the former over the latter in three independent assays. However the PPs derived from the pYW01 vector, in which the library was constructed, was consistently enriched in the same three assays to an even higher extent. All three vectors displayed a c-myc peptide tag, which is included into the cloning and display cassette; hence binding of this tag to a target in the murine stomach tissue should not have resulted in an advantage to PPs derived from any of the three vectors. However, this was not the case, as the library affinity screen on the murine stomach tissue showed dominance of p3CS PPs over the empty vector pYW01 PPs observed as early as the second round of panning (Fig. 5), despite the much more prominent presence of empty vector pYW01 in the library prior to the panning (Fig. 3, the signature *Nde* I band is labelled with asterisks). Furthermore, p3CS was not enriched during screening on Super fibronectin, arguing against a simple growth advantage during amplification phase of screening. Further assays, examining binding of the PPs to the stomach tissue proteins resolved by SDS-PAGE and blotted onto a membrane, could resolve whether the p3CS PPs bind to a protein in the bait.

The IGINS peptide displayed on p3CS is located near the C-terminus of a *L. reuteri* DegP-like protease. This protease is predicted to be a surface protein, anchored via an N-terminal transmembrane helix and having a C-terminal domain exposed on the surface of *L. reuteri* cells, hence the IGINS peptide is predicted to be displayed on the surface of the bacterium and available for interactions with the bait. This protease is, however, not specific to the 100-23 strain of *L. reuteri*. Therefore, even if it does mediate binding to the stomach tissue, it is unlikely to be a keratin-specific binding protein characteristic of the murine-tropic strains.

5.0 Conclusion

By use of phage display technology, recombinant phagemids were enriched after affinity screening of *L. rhamnosus* HN001. Sequence analysis showed that these were unlikely to be expressed and displayed on the surface of PPs and therefore can be considered the "background" of non-binders. The *L. reuteri* 100-23 library did not show any enriched phagemid bands after three rounds of panning on super fibronectin. However, screening of the same library on the murine stomach tissue as bait resulted in one highly dominant band. The sequence analysis showed that this clone corresponded to an unpublished phage display vector from the laboratory, however it displayed, by coincidence, a peptide (IGINS) identical to a sequence near the C-terminus of a surface protease homologous to DegP. Further investigation into possibility of this peptide mediating binding to the stomach tissue will be undertaken.

Overall, this thesis has investigated a phage display approach in finding specific proteins of probiotic or commensal lactobacilli that interact with the host tissue. Serendipitously, one stomach-tissue-binding *L. reuteri* peptide may have been isolated in the course of this work. Confirmation of binding for this peptide could not be obtained in this thesis, but warrant further experiments to investigate this possibility.

6.0 Future Directions

6.1 Further characterisation of identified proteins

The peptide IGINS that potentially binds to the bait should be confirmed by additional experiments. Phage blots could be used to confirm binding of the p3CS-derived PPs to the proteins of stomach tissue proteins (Jankovic, 2009). Furthermore, the C-terminal of protease from which the IGINS peptide is derived should be expressed in a standard expression system or on the surface of the phage and examined for binding to the murine stomach tissue.

6.2 Further investigation into fibronectin binders

Affinity screening of the *L. reuteri* 100-23 on fibronectin could be continued through round 4 and 5 to decrease the background in the prospect that enriched binders would appear in later panning. It is not uncommon that five rounds of panning are required to select for sufficient enrichment of low-affinity binders (Jankovic, 2009). Conditions of binding and elution could also be manipulated to improve the enrichment (e.g. change in pH or ionic strength of binding or elution buffers). Furthermore, a larger number of recombinant clones from all enriched phagemid bands from the round 3 of panning could be analysed.

6.3 Identify adhesin(s) that bind(s) CaCo-2 cells

Lactobacillus rhamnosus HN001 was found to interact with intestinal CaCo-2 cells (Authier, 2003). It is expected that *L. rhamnosus* HN001 contains surface proteins that mediate this interaction. To identify these proteins, affinity enrichment of *L. rhamnosus* HN001 phage display library could be carried out, followed by sequencing as described in this thesis. In this case, CaCo-2 cells will be used as bait instead of immobilised fibronectin or the murine stomach tissue. Different proteins are present on the apical and basal side of the polarised epithelial cell layer. Therefore, panning should be implemented on both sides of the cell layer to compare the *L. rhamnosus* HN001 protein interactions.

6.4 Identify adhesin(s) that bind(s) signalling proteins of Toll and Nod pathways

Intracellular targets of bacterial manipulation are proteins of the two pathways that are induced by microbe-associated molecular patterns (MAMPs). These pathways are induced by binding to molecules that are specific to bacteria, in the case of Gram-

positive bacteria such as the lactobacilli. These specific molecules are: lipoteichoic acids, cell wall and extracellular polysaccharides, as well as formyl-methionine and bacteria-specific DNA modifications) (Blander and Sander, 2012). All these molecules induce innate immunity responses through two main pathways, Toll and Nod, resulting in induction of interleukin synthesis and secretion (i.e IL-8) (Athman and Philpott, 2004, Fritz and Girardin, 2005). A number of proteins along these pathways, most notably transcription factor NFkB and upstream kinase cascade could be used as bait (Vance *et al.*, 2009, Philpott and Girardin, 2004). If any binding proteins are found they will be purified, displayed and assayed for binding to individual components.

7.0 References

Aleljung, P., Shen, W., Rozalska, B., Hellman, U., Ljungh, A., and Wadstrom, T. (1994). Purification of collagen-binding proteins of *Lactobacillus reuteri* NCIB 11951. *Current Microbiology 28*: 231-236.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology 215*: 403-410.

Areschoug, T., Carlsson, F., Stalhammar-Carlemalm, M., and Lindahl, G. (2004). Host-pathogen interactions in *Streptococcus pyogenes* infections, with special reference to puerperal fever and a comment on vaccine development. *Vaccine 22 (1)*: S9-S14.

Artis, D. (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nature Reviews Immunol*ogy *8*: 411-420.

Athman, R., and Philpott, D. (2004). Innate immunity via Toll-like receptors and Nod proteins. *Current Opinion in Microbiology 7*: 25-32.

Authier, A. (2003). Characterisation of adhesion of a probiotic bacterium *Lactobacillus rhamnosus* HN001 to extracellular matrix proteins and the intestinal cell line CaCo-2 (Master's thesis, Massey University, Palmerston North, New Zealand).

Baer, R., Bankier, A. T., Biggin, M. D., Deininger,P. L., Farrell, P. J., Gibsonl T. J., Hatfull, G., Hudson, G. S., Satchwell, S. C., Seguin, C. and *et al*. (1984). DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature 310*: 207-211.

Beekwilder, J., Rakonjac, J., Jongsma, M., and Bosch, D. (1999). A phagemid vector using the *E. coli* phage shock promoter facilitates phage display of toxic proteins. *Gene 228*: 23-31.

Bennett, N. J., Gagic, D., Sutherland-Smith, A. J., and Rakonjac, J. (2011). Characterization of a dual-function domain that mediates membrane insertion and excision of Ff filamentous bacteriophage. *Journal of Molecular Biology 411*: 972-985.

Blander, J. M., and Sander, L. E. (2012). Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. *Nature Reviews Immunology* **12**: 215-225.

Bron, P. A., van Baarlen, P., and Kleerebezem, M. (2012). Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. *Nature Reviews Microbiology 10*: 66-78.

Buck, B. L., Altermann, E., Svingerud, T., and Klaenhammer, T. R. (2005). Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. *Applied and Environmental Microbiology 71*: 8344-8351.

Cabilly, S. (1999). The basic structure of filamentous phage and its use in the display of combinatorial peptide libraries. *Molecular Biotechnology 12*: 143-148.

Castro, A., Mandart, E., Lorca, T., and Galas, S. (2003). Involvement of Aurora A kinase during meiosis I-II transition in *Xenopus* oocytes. *Journal of Biological Chemistry 278*: 2236-2241.

Chiang, B. L., Sheih, Y. H., Wang, L. H., Liao, C. K., and Gill, H. S. (2000). Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (*Bifidobacterium lactis* HN019): optimization and definition of cellular immune responses. *European Journal of Clinical Nutrition 54*: 849-855.

Corthesy, B., Gaskins, H. R., and Mercenier, A. (2007). Cross-talk between probiotic bacteria and the host immune system. *Journal of Nutrition 137*: 781S-790S.

Cross, M. L., Mortensen, R. R., Kudsk, J., and Gill, H. S. (2002). Dietary intake of *Lactobacillus rhamnosus* HN001 enhances production of both Th1 and Th2 cytokines in antigen-primed mice. *Medical Microbiology and Immunology 191*: 49-53.

Dekker, J. W. Wickens, K., Black, P. N., Stanley, T. V., Mitchell, E. A., Fitzharris, P., Tannock, G. W., Purdie, G., and Crane, J. (2009). Safety aspects of probiotic bacterial strains *Lactobacillus rhamnosus* HN001 & *Bifidobacterium animalis* subsp. *lactis* HN019 in human infants aged 0–2 years. *International Dairy Journal 19*: 149-154.

Delcher, A. L., Bratke, K. A., Powers, E. C., and Salzberg, S. L. (2007). Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics 23*: 673-679.

Evrard, B., Coudeyras, S., Dosgilbert, A., Charbonnel, N., Alame, J., Tridon, A., and Forestier, C. (2011). Dose-dependent immunomodulation of human dendritic cells by the probiotic *Lactobacillus rhamnosus* Lcr35. *Public Library of Science (PLOS ONE) 6*: e18735.

Foligne, B., Nutten, S., Grangette, C., Dennin, V., Goudercourt, D., Poiret, S., Dewulf, J., Brassart, D., Mercenier, A., and Pot, B. (2007). Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. *World Journal of Gastroenterology 13*: 236-243.

Forsberg, A. Abrahamsson, T. R., Björkstén, B., and Jenmalm, M. C. (2013). Pre- and post-natal *Lactobacillus reuteri* supplementation decreases allergen responsiveness in infancy. *Clinical and Experimental Allergy 43*: 434-442.

Fritz, J. H., and Girardin, S. E. (2005). How Toll-like receptors and Nod-like receptors contribute to innate immunity in mammals. *Journal of Endotoxin Research 11*: 390-394.

Gill, H. S., Shu, Q., Lin, H., Rutherfurd, K. J., and Cross, M. L. (2001). Protection against translocating *Salmonella typhimurium* infection in mice by feeding the immuno-enhancing probiotic *Lactobacillus rhamnosus* strain HN001. *Medical Microbiology and Immunology 190*: 97-104.

Gopal, P. K., Prasad, J., Smart, J., and Gill, H. S. (2001). *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. *International Journal of Food Microbiology 67*: 207-216.

Holzapfel, W. H., Haberer P., Geisen R., Bjorkroth J., and Schillinger U. (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. *The American Journal of Clinical Nutrition 73*: 365S-373S.

Hummel, S., Veltman, K., Cichon, C., Sonnenborn, U., and Schmidt, M. A. (2012). Differential Targeting of the E-Cadherin/beta-Catenin Complex by Gram-Positive Probiotic Lactobacilli Improves Epithelial Barrier Function. *Applied Environmental Microbiology 78*: 1140-1147.

Jacobsson, K., and Frykberg, L. (1995). Cloning of ligand-binding domains of bacterial receptors by phage display. *Biotechniques 18*: 878-885.

Jacobsson, K., and Frykberg L. (1996). Phage display shot-gun cloning of ligand-binding domains of prokaryotic receptors approaches 100% correct clones. *Biotechniques 20*: 1070-1081.

Jakava-Viljanen, M., and Palva, A. (2007). Isolation of surface (S) layer protein carrying *Lactobacillus* species from porcine intestine and faeces and characterization of their adhesion properties to different host tissues. *Veterinary Microbiology 124*: 264-273.

Jankovic, D. (2009). Direct selection and phage display of *Lactobacillus rhamnosus* HN001 secretome (Doctoral dissertation, Massey University, Palmerston North, New Zealand).

Jankovic, D., Collett, M. A., Lubbersk M. W., and Rakonjac, J. (2007). Direct selection and phage display of a Gram-positive secretome. *Genome Biology 8*: R266.

Karlsson, M., Scherbak, N., Reid, G., and Jass, J. (2012). *Lactobacillus rhamnosus* GR-1 enhances NF-kappaB activation in *Escherichia coli*-stimulated urinary bladder cells through TLR4. *BMC Microbiology 12*: 15.

Kleerebezem, M., Hols, P., Bernard, E., Rolain, T., Zhou, M., Siezen, R. J., and Bron, P. A. (2010). The extracellular biology of the lactobacilli. *FEMS Microbiology Rev 34*: 199-230.

Knudsen, S. (1999). Promoter2.0: for the recognition of PolII promoter sequences. *Bioinformatics 15*: 356-361.

Lahtinen, S. J., Forssten, S., Aakko, J., Granlund, L., Rautonen, N., Salminen, S., Viitanen, M., and Ouwehand, A. C. (2012). Probiotic cheese containing *Lactobacillus rhamnosus* HN001 and *Lactobacillus acidophilus* NCFM((R)) modifies subpopulations of fecal lactobacilli and *Clostridium difficile* in the elderly. *Age (Dordrecht, Netherlands) 34:* 133-143.

Lebeer, S., Claes, I. J., Balog, C. I., Schoofs, G., Verhoeven, T. L., Nys, K., von Ossowski, I., de Vos, W. M., Tytgat, H. L., Agostinis, P., Palva, A., Van Damme, E. J., Deelder, A. M., De Keersmaecker, S. C., Wuhrer, M., and Vanderleyden, J. (2012) The major secreted protein Msp1/p75 is O-glycosylated in *Lactobacillus rhamnosus* GG. *Microbial Cell Factories 11*: 15.

Lee, J., Yun, H. S., Cho, K. W., Oh, S., Kim, S. H., Chun, T., Kim, B., and Whang, K. Y. (2011). Evaluation of probiotic characteristics of newly isolated *Lactobacillus* spp.: immune modulation and longevity. *International Journal of Food Microbiology 148*: 80-86.

Lee, Y. K., Puong, K. Y., Ouwehand, A. C., and Salminen, S. (2003). Displacement of bacterial pathogens from mucus and CaCo-2 cell surface by lactobacilli. *Journal of Medical Microbiology 52*: 925-930.

Lin, P. W., Myers, L. E., Ray, L., Song, S. C., Nasr, T. R., Berardinelli, A. J., Kundu, K., Murthy, N., Hansen, J. M., and Neish, A. S. (2009) *Lactobacillus rhamnosus* blocks inflammatory signaling *in vivo* via reactive oxygen species generation. *Free Radical Biology and Medicine 47*: 1205-1211.

Lin, P. W., Nasr, T. R., Berardinelli, A. J., Kumar, A., and Neish, A. S. (2008). The probiotic *Lactobacillus rhamnosus* GG may augment intestinal host defense by regulating apoptosis and promoting cytoprotective responses in the developing murine gut. *Pediatric Research 64*: 511-516.

Liu, S., Han, W., Sun, C., Lei, L., Feng, X., Yan, S., Diao, Y., Gao, Y., Zhao, H., Liu, Q., Yao, C., and Li, M. (2011). Subtractive screening with the *Mycobacterium tuberculosis* surface protein phage display library. *Tuberculosis (Edinburgh, Scotland) 91*: 579-586.

Livingston, M., Loach, D., Wilson, M., Tannock, G. W., and Baird, M. (2010). Gut commensal *Lactobacillus reuteri* 100-23 stimulates an immunoregulatory response. *Immunology and Cell Biology 88*: 99-102.

Lorca, G., Torino, M. I., Font de Valdez, G., and Ljungh, A. A. (2002). Lactobacilli express cell surface proteins which mediate binding of immobilized collagen and fibronectin. *FEMS Microbiology Letters 206*: 31-37.

Macdonald, T. T., and Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut. *Science 307*: 1920-1925.

Mazmanian, S. K., Round, J. L., and Kasper, D. L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature 453*: 620-625.

Mullen, L. M., Nair, S. P., Ward, J. M., Rycroft, A. N., and Henderson, B. (2006). Phage display in the study of infectious diseases. *Trends in Microbiology 14*: 141-147.

Munoz-Provencio, D., and Monedero, V. (2011). Shotgun phage display of *Lactobacillus casei* BL23 against collagen and fibronectin. *Journal of Microbiology and Biotechnology 21*: 197-203.

Palmer, C. Bik, E. M., DiGiulio, D. B., Relman, D. A., and O'Brown, P. (2007). Development of the human infant intestinal microbiota. *PLOS Biology 5*.

Philpott, D. J., and Girardin, S. E. (2004). The role of Toll-like receptors and Nod proteins in bacterial infection. *Molecular Immunology 41*: 1099-1108.

Pirarat, N., Kobayashi, T., Katagiri, T., Maita, M., and Endo, M. (2006). Protective effects and mechanisms of a probiotic bacterium *Lactobacillus rhamnosus* against experimental *Edwardsiella tarda* infection in tilapia (*Oreochromis niloticus*). *Veterinary Immunology and Immunopathology 113*: 339-347.

Prasad, J., Gill, H., Smart, J., and Gopal, P. (1998). Selection and characterisation of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics. 8: 993-1002. *International Dairy Journal* 8: 993-1002.

Rakonjac, J., Jovanovic, G., and Model, P. (1997). Filamentous phage infection-mediated gene expression: construction and propagation of the gIII deletion mutant helper phage R408d3. *Gene 198*: 99-103.

Rakonjac, J., and Model, P. (1998). The roles of pIII in filamentous phage assembly. *Journal of Molecular Biology 282*: 25-41.

Rakonjac, J. V., Robbins, J. C., and Fischetti, V. A. (1995). DNA sequence of the serum opacity factor of group A streptococci: identification of a fibronectin-binding repeat domain. *Infection and Immunity 63*: 622-631.

Reunanen, J., von Ossowski, I., Hendricks, A. P., Palva, A., and de Vos W. M. (2012). Characterization of the SpaCBA Pilus Fibers in the Probiotic *Lactobacillus rhamnosus* GG. *Applied and Environmental Microbiology 78 (7).*

Ryan, K. A., O'Hara, A. M., van Pijkeren, J. P., Douillard, F. P., and O'Toole, P. W. (2009). *Lactobacillus salivarius* modulates cytokine induction and virulence factor gene expression in *Helicobacter pylori*. *Journal of Medical Microbiology 58*: 996-1005.

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science 228*: 1315-1317.

Styriak, I., Nemcova R., Chang Y. H., and Ljungh A. (2003). Binding of extracellular matrix molecules by probiotic bacteria. *Letters in applied microbiology 37*: 329-333.

Tannock, G. W. (2004). A special fondness for lactobacilli. *Applied and Environmental Microbiology 70*: 3189-3194.

Tannock, G. W., Wilson, C. M., Loach, D., Cook, G. M., Eason, J., O'Toole, P. W., Holtrop, G., and Lawley, B. (2012). Resource partitioning in relation to cohabitation of *Lactobacillus* species in the mouse forestomach. *The ISME Journal 6*: 927-938.

Twetman, L., Larsen, U., Fiehn, N. E., Stecksen-Blicks, C., and Twetman, S. (2009). Coaggregation between probiotic bacteria and caries-associated strains: An *in vitro* study. *Acta Odontologica Scandinavica 67*: 284-288.

van Hemert, S., Meijerink, M., Molenaar, D., Bron, P. A., de Vos, P., Kleerebezem, M., Wells, J. M., and Marco, M. L. (2010). Identification of *Lactobacillus plantarum*

genes modulating the cytokine response of human peripheral blood mononuclear cells. *BMC Microbiology 10*: 293.

van Niel E. W. J., Larsson, C. U., Lohmeier-Vogel, E. M., and Rådström, P. (2012). The potential of biodetoxification activity as a probiotic property of *Lactobacillus reuteri. International Journal of Food Microbiology 152*: 206-210.

van Pijkeren, J. P., Canchaya, C., K. Ryan, A., Li, Y., M. Claesson, J., Sheil, B., Steidler, L., O'Mahony, L., Fitzgerald, G. F., van Sinderen, D., and O'Toole, P. W. (2006). Comparative and functional analysis of sortase-dependent proteins in the predicted secretome of *Lactobacillus salivarius* UCC118. *Applied and Environmental Microbiology 72*: 4143-4153.

Vance, R. E., Isberg, R. R., and Portnoy, D. A. (2009). Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. *Cell Host and Microbe 6*: 10-21.

Velez, M. P., Petrova, M. I., Lebeer, S., Verhoeven, T. L., Claes, I., Lambrichts, I., Tynkkynen, S., Vanderleyden, J., and De Keersmaecker, S. C. (2010). Characterization of MabA, a modulator of *Lactobacillus rhamnosus* GG adhesion and biofilm formation. *FEMS Immunology and Medical Microbiology 59*: 386-398.

von Ossowski, I., Satokari, R., Reunanen, J., Lebeer, S., De Keersmaecker, S. C., Vanderleyden, J., de Vos, W. M., and Palva, A. (2011). Functional characterization of a mucus-specific LPXTG surface adhesin from probiotic *Lactobacillus rhamnosus* GG. *Applied and Environmental Microbiology 77*: 4465-4472.

Walter, J., Chagnaud P., Tannock, G. W., Loach, D. M., Dal Bello, F., Jenkinson, H. F., Hammes, W. P., and Hertel, C. (2005). A high-molecular-mass surface protein (Lsp) and methionine sulfoxide reductase B (MsrB) contribute to the ecological performance of *Lactobacillus reuteri* in the murine gut. *Applied and Environmental Microbiology 71*: 979-986.

Westerlund, B., and Korhonen, T. K. (1993). Bacterial proteins binding to the mammalian extracellular matrix. *Molecular Microbiology 9*: 687-694.

Wickens, K., Black, P. N., Stanley, T. V., Mitchell, E., Fitzharris, P., Tannock, G. W., Purdie, G., and Crane J. (2008). A differential effect of 2 probiotics in the prevention of eczema and atopy: a double-blind, randomized, placebo-controlled trial. *Journal of Allergy and Clinical Immunology 122*: 788-794.

Williams, R. J., Henderson, B., Sharp, L. J., and Nair, S. P. (2002) Identification of a fibronectin-binding protein from *Staphylococcus epidermidis*. *Infection and Immunity 70*: 6805-6810.