Investigating probiotic biofilms: probing the link between biofilm formation and antimicrobial production

by

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ABSTRACT

INVESTIGATING PROBIOTIC BIOFILMS: PROBING THE LINK BETWEEN BIOFILM FORMATION AND ANTIMICROBIAL PRODUCTION

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Probiotics are live microorganisms known to confer a host benefit when ingested and represent a feasible option to alleviate gastrointestinal microbiota imbalances. Alleviation may occur via several mechanisms including bacteriocin production by probiotics to limit pathogen colonization. Unfortunately, probiotic efficacy may be restricted by their inability to incorporate into the naturally-occurring, static biofilms lining the human gut. This thesis examines the biofilm forming ability of fourteen commercial probiotic strains via a high-throughput crystal violet assay and scanning electron microscopy. All probiotics formed biofilms in vitro in at least one condition tested. More specifically, strong biofilm formation was observed by Pediococcus acidilactici under several conditions. Further investigation demonstrated that both anaerobiosis and bacteriocin production alter P. acidilactici biofilm formation. Moreover, it was shown that aeration, medium, and mode of growth modify bacteriocin production from P. acidilactici, highlighting the importance of environmental factors that influence both biofilm formation and bacteriocin production.
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Author’s Declaration of Work Completed

I declare that all work presented in this thesis is my own, with the following exceptions: Allison Williams assisted with development of large-scale biofilm screen.
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List of Abbreviations

CD, Crohn’s disease
CLSM, Confocal laser scanning microscopy
dH₂O deionized water
EM, Electron microscopy
FDA, Food and drug administration
GI, Gastrointestinal
GRAS, Generally regarded as safe
h, Hours
IBD, Irritable bowel disease
LAB, Lactic acid bacteria
Man-PTS, Mannose phosphotransferase system
MRS, de Man, Rogosa, and Sharpe
PBS, Phosphate buffered saline
RCM, Reinforced Clostridial medium
PC-CFS, Pediocin-containing cell free supernatant
SEM, Scanning electron microscopy
sIgA, Secretory immunoglobulin A
TJ, Tight junction
TSB, Tryptic soy broth
v/v, Volume per volume
UC, Ulcerative colitis
w/v, Weight per volume
Chapter 1: Introduction

The contribution of probiotics to human gastrointestinal tract health

The bacteria which colonize the human gastrointestinal (GI) tract provide important metabolic, trophic, and immune-modulatory functions and thus play an important role in the establishment of both health and disease (Guarner and Malagelada, 2003). Probiotics, microorganisms that when ingested in adequate amounts confer an advantage to the host, may contribute to improving GI health by temporarily alleviating ‘dysbiosis’, i.e. microbial imbalances in the natural gut microflora (Holzapfel et al., 1998). The benefits of probiotics are strain specific (Sultana et al., 2013); for example, various strains of Bifidobacterium longum demonstrate the ability to modify diverse components of the immune system in varying amounts relative to one another (Medina et al., 2007). The wide range of clinical benefits demonstrated by probiotics include; alleviation of flares of inflammatory bowel diseases such as Crohn’s disease (CD) (Fujimori et al., 2007) and ulcerative colitis (UC) (Bibiloni et al., 2005), decreased hypersensitivity reactions (Barletta et al., 2013), and treatment of various forms of diarrhea, with symptom mitigation being dose-dependent (Sultana et al., 2013; Gao et al., 2010; Pagnini et al., 2010).

Probiotic benefits are thought to be maximized in the colon, the most densely populated microbial ecosystem in the human body with microbial loads of approximately $10^{12}$ cells/g (Guarner and Malagelada, 2003). Several factors favour the selection of such a dense microbial ecosystem, including environmental factors such as slow transit time, high nutrient availability, favourable pH, and low levels of proteolytic enzymes (Yang et al., 2002). Because colonic diseases such as CD and UC respond in a clinically favourable way to probiotics, most likely through modulation of the gut microbiota, there is scope to develop probiotic strains for
therapeutic use that are optimized based on promotion of their beneficial effect while also ensuring optimization under colonic conditions.

Probiotic Selection

Specific criteria must be met in order for a bacterial strain to be termed a probiotic: typically, the strain should be isolated from the same species as the intended host to ensure compatibility for human consumption. Selected microorganisms must have a demonstrated beneficial effect, be non-pathogenic, and non-toxic, thus maximizing their safety for the immunocompromised (Anukam et al., 2008). The bacterium must also have the ability to survive in the GI tract and demonstrate stability during storage (Collado et al., 2009). These strict criteria allow probiotics to be classified as Generally Regarded as Safe (GRAS) by the Food and Drug Administration (FDA). The FDA considers probiotics as food additives, and GRAS status is required for a given probiotic to be legally recognized as such (FDA, 1997).

Probiotic selection is a multi-stage process beginning with the isolation and identification of a potential probiotic strain from a source such as; breast milk, fermented foods, or the gut microbiota of healthy individuals. Identification is followed by characterisation of the bacterium, including acid and bile resistance to assess GI tract survivability, in vitro adhesion to intestinal epithelial cells and/or mucus, and sensitivity to broad-spectrum antibiotics (Fontana et al., 2013). Favourable results during characterisation are followed by preclinical in vitro and in vivo (animal) studies to evaluate beneficial effects such as antimicrobial production or immunomodulation. Human clinical evaluations constitute the final stage before commercialization and can only proceed with the success of the former steps (Fontana et al., 2013).
**Mechanism of probiotic action**

Probiotics exert their beneficial effects through diverse and multifactorial mechanisms, including combinations of; increased barrier function, modulation of the immune system, competition, and antimicrobial production. Each of these will be reviewed as they are probiotic mechanisms relevant to the GI tract.

**Enhanced barrier function**

The mucus layer covering epithelial cells lines the GI tract and is the initial surface microbes confront in the colon (Kirjavainen et al., 1998). This layer is considered an important site for adhesion (Collado et al., 2007), colonization (Alemka et al., 2010), lubrication (Atuma et al., 2001), and protection of the host from harmful bacteria (Mack et al., 1999). The mucus layer is primarily composed of mucins; a family of heavily glycosylated proteins responsible for the gel-like mucus texture. Mucus integrity i.e. the ability of mucus to withstand and recover from insult (Slomiany et al., 1992), such as injury as a result of inflammation, is thought to be crucial for healing in inflammatory bowel disease (Shirazi et al., 2000). Probiotic-induced mucin increases have been observed in vivo: a 60% increase in mucin production was observed in rats fed $3 \times 10^9$ bacteria of probiotic supplement VSL#3, an extensively researched and commercially available multi-species probiotic supplement, for 7 days versus control animals (Caballero-Franco et al., 2007). Additionally, experiments analyzing mucin mRNA expression in probiotic-treated epithelial cells exhibited over a two-fold increase in mucin production as a result of Lactobacillus plantarum, but not Lactobacillus acidophilus (Caballero-Franco et al., 2007; Mack et al., 1999).
Cytoskeletal rearrangements of colonic epithelial cells may also enhance barrier function to regulate host defense mechanisms at the mucosal interface by preventing direct contact with commensals or potential pathogens. Pre-treatment of the colonic epithelial T84 cell line with *L. acidophilus* or *L. rhamnosus* resulted in attenuation of pathogen-induced decreases in tight junction (TJ) integrity, an important barrier in limiting paracellular permeability (Sherman *et al.*, 2009; Jepson *et al.*, 2000). Additionally, Sultana *et al.* (2007) used human keratinocytes to demonstrate that *B. longum* and various lactobacilli strains were able to sustain increases in transepithelial resistance values, the preferred method for assessing TJ integrity by ensuring the proper polarity is maintained across the epithelium, for up to 72 hours versus controls. The author suggests these effects may be due to increases in TJ protein expression levels and activation of signaling pathways involved in barrier formation.

**Immunomodulation**

Modulation of the immune system is one of the most extensively studied probiotic benefits and can occur via suppression and stimulation of the innate and adaptive immune system. The innate immune system is an immediate, non-specific collection of responses which do not result in immunologic memory, but represent the host’s first line of defence; such defences include the recruitment of various leukocytes, including mast cells associated with wound healing, and phagocytic cells, including macrophages that are recruited by mast cells. Probiotics are known to modulate and enhance multiple aspects of this branch of immunity. For example, treatment with the mixed species probiotic, VSL#3, was able to significantly decrease Crohn’s disease-like ileitis in an animal model by stimulating local tumor necrosis factor alpha production, a pro-inflammatory cytokine involved in stimulating protective local inflammation to
prevent the further establishment of chronic widespread damaging inflammation (Ji et al., 2013; Pagnini et al., 2010). Additionally, mouse models exemplify how probiotics are implicated in toll-like receptor signaling resulting in amelioration of experimental colitis and activation of innate cytokines (Kim et al., 2006; Rachmilewitz et al., 2004). Collectively, the above studies reveal the diverse ways that probiotics can alter the innate immune system.

In contrast, acquired immunity has both specificity and memory. Probiotic ingestion has been shown to significantly up regulate the secretion of immunoglobulin A (sIgA), the most abundant intestinal immunoglobulin. sIgA is critical in mucosal immunity against a variety of pathogens and their products, including Salmonella enterica (de LeBlanc et al., 2010), cholera toxin (Yasui et al., 1992), and the protozoan Giardia intestinalis (Goyal and Shukla, 2013). sIgA also mediates adhesion of protective commensal bacteria in the gut (Bollinger et al., 2003) and activates B cells, the principle function of which is the production of antibodies (Ibnou-Zekri et al., 2003). The production of T regulatory cells, components of the immune system involved in “self-checks” to prevent an excessive immune reaction, is stimulated by treatment with ITR5, a probiotic mixture, which in turn causes suppression of not only gut associated disease like IBD, but also dermatitis and rheumatoid arthritis (Kwon et al., 2010).

**Competition as a means of survival in the gut**

The ability of probiotics to outcompete other microorganisms, generally pathogens, is thought to occur in one of two ways; competing for nutrients and competing for adhesion sites (Woo and Ahn, 2013). Scavenging for binding sites may take the form of either i) competition, where probiotic and pathogenic organisms contend for the same sites/nutrients, ii) exclusion, where pathogen binding is inhibited by probiotics that are already present, or iii) displacement,
where the probiotic displaces an already present pathogen. *In vitro* studies using various lactobacilli and *Bifidobacteria* have demonstrated significant competitive effects against numerous pathogenic bacteria. Co-culture experiments with probiotic monospecies demonstrate significant decreases in *Listeria monocytogenes* adhesion via competition, exclusion, and displacement assays; in contrast, *Salmonella typhimurium* is only susceptible during exclusion assays (Woo and Ahn, 2013), highlighting the specificity of these interactions. Additionally, Collado *et al*. (2007) analysed the ability of single and mixed probiotic species to inhibit or displace pathogen adhesion to human intestinal mucus. The majority of probiotic-pathogen combinations resulted in an array of pathogen inhibition and displacement. Notably, certain combinations resulted in enhanced pathogen adhesion upon probiotic addition; specifically, the combination of probiotic *L. rhamnosus* LC-705 when challenging *Escherichia coli* K2 resulted in 36.4% greater inhibition than without probiotic addition. It has been suggested that drastic adhesion differences may be due to the number of binding sites or to the presence of a soluble factor in the medium, although the precise mechanism is unknown (Lee *et al*., 2002).

Less evidence is available supporting the ability of probiotics to out-compete other bacteria for nutrients. It has been observed that probiotic *E. coli* Nissle 1917 was able to out compete *S. typhimurium* colonization by scavenging iron more effectively (Deriu *et al*., 2013). It was shown that the mechanism behind enhanced scavenging was due to *E.coli* Nissle 1917’s ability to synthesize siderophores that are unaffected by host proteins that are known to restriction iron acquisition in *S. typhimurium*. 
Probiotic bacteriocins

Bacteria produce a wide range of antimicrobial compounds, and this review will focus on a subset of these, antimicrobial peptides termed bacteriocins, because of their increasing clinical relevance and potential use in food preservation (Kjos et al., 2011). Bacteriocins are placed into one of four categories (Fig. 1.1), and represent a main microbial defense mechanism against other bacterial species. Bacteriocins generally have a narrow effective range to ensure a survival advantage to the producing bacteria against closely-related species, though some bacteriocins may have a more extended target range that can include pathogens. Although bacteriocins have proven beneficial to humans via inhibition of pathogen colonization (Millette et al., 2008), their role in the natural environment remains unclear, but may include population control within microbial communities (James et al., 1991).

Interest in bacteriocins has grown due to the shortage of new antibiotics, increased microbial resistance to existing antibiotics, and growing evidence related to the deleterious effects of antibiotic-induced collateral damage to resident microflora caused by non-specific killing. As potential therapeutic agents, bacteriocins are attractive due to their potency (Fimland et al., 2005), low toxicity (to host cells), narrow spectrum of activity, amenability to bioengineering (Sleator and Hill, 2008), and minimal effect of the normal intestinal microflora (Le Blay et al., 2007). Nisin, the most-researched bacteriocin to date, is produced by the Gram positive bacterium Lactococcus lactis and was shown to be 8-16 times more active than vancomycin in a challenge study with Streptococcus pneumoniae in a mouse model (Goldstein et al., 1998). Additionally, nisin is effective at killing other Gram positive pathogens including Staphylococcus aureus (van Staden et al., 2012) and Clostridium difficile (Nerandzic and Donskey, 2013). Other categories of bacteriocins show excellent inhibition of Gram positive
bacteria as well as spores (Wolf and Gibbons, 1996), however, the protective outer membrane of
Gram negative bacteria may only be bacteriocin-susceptible if a concomitant sub-lethal injury is
introduced (e.g. heat shock) (Kalchayanand et al., 1992). Although not as well characterized,
bacteriocins produced by Gram negative bacteria, including colicins and microcins produced by
E. coli, are also being investigated for their potential to kill Gram negative pathogens (Murinda
et al., 1996).
Figure 1.1 An illustration of bacteriocin organization. Shown are the four categories that bacteriocins are allocated into, with subgroups of each category included.
*Pediococcus acidilactici as a probiotic*

*Pediococcus acidilactici* is a commercially available probiotic which is a homofermentative member of the lactic acid bacteria (LAB). *P. acidilactici* is an economically important bacterium in the food industry, where it is required for the fermentation of vegetables (Gardner *et al.*, 2001), meat (Albano *et al.*, 2009), and some dairy products (Caldwell *et al.*, 1996). The benefits of *P. acidilactici* as a probiotic have been observed across multiple species including; pigs, fish, mice, and humans.

Large-scale commercial animal farming has led to a growing interest in the use of probiotics, particularly *P. acidilactici*, as a prophylactic therapy for restoration of the normal microbiota and prevention of infectious disease. Oral *P. acidilactici* supplementation has demonstrated benefits in the aquaculture industry including; a 17% increase in animal survival (Ferguson *et al.*, 2010), 75-100% decrease in pathogenic organisms (Castex *et al.*, 2008), and a 10% decrease in the prevalence of spinal deformities, comparable to levels of protection from traditional antibiotic florfenicol (Aubin *et al.*, 2005). In hog production, both weaning and antibiotic treatment to prevent enteric infection act as stressors in piglets and is associated with the risk of GI disruptions and increased disease susceptibility (Lessard *et al.*, 2009). *P. acidilactici* oral supplementation in these animals has resulted in up to 5% increases in weight, improved measures of intestinal epithelial health including a 9% increase in villi height and 14% increase in crypt depth (Di Giancamillo *et al.*, 2008), reduced *E. coli* attachment to ileal mucosa by 11% (Daudelin *et al.*, 2011), and reduced coliform counts (Le Bon *et al.*, 2010). Human consumption of *P. acidilactici* may help to treat to peptic ulcers by reducing the colonizing numbers of a key causative agent, *Helicobacter pylori* in the stomach. Kaur *et al.* (2013) used a mouse model to demonstrate that ingestion of *P. acidilactici* BA28 significantly reduced levels of inflammation compared to control animals as a result of *H. pylori* infection. *P. acidilactici*
mm33 was also observed to significantly reduce intestinal colonization of vancomycin-resistant Enterococci in mice after 3 days, with undetectable levels after 6 days (Millette et al., 2008). Furthermore, when ingested orally (1X10⁹ CFU/kg of diet) *P. acidilactici* supplementation led to a decrease in *E. coli* translocated to mesenteric lymph nodes following *E. coli* challenge (Lessard et al., 2009).

The ability of *P. acidilactici* to act as a probiotic in addition to producing pediocin makes it an increasingly relevant and desired organism from a health and economic standpoint.

**Pediocin organization, gene products, and protein structure**

As described above, bacteriocins are placed into one of four categories. Pediocins, a bacteriocin produced by some pediococci, are found in class IIA (Fig. 1.1) and are best known for their anti-listerial activity, making them an attractive target for the meat industry. Pediocins are generally plasmid encoded on an approximately 9.5kb operon (Miller et al., 2005; Venema et al., 1995) that contains four genes; *pedA*, *pedB*, *pedC*, and *pedD*. *pedA* encodes the 62 amino acid unmodified preprediocin molecule (Henderson et al., 1992) while *pedB* encodes the 112 amino acid immunity protein to protect the pediocin producing bacterium against its own pediocin. *pedC* and *pedD* encode a 174 residue protein essential for pediocin secretion via ATP-binding cassette (ABC) transporters and 724 residue ABC homologue to transport pediocin across the membrane (Venema et al., 1995), respectively.

The final pediocin molecule is 44 residues in length, hydrophobic, and positively charged, becoming active after PedD cleaves the *pedA* leader sequence to produce the mature pediocin molecule (Chikindas et al., 2010). This mature pediocin molecule is divided into 3 parts, with the first 17 residues representing the highly conserved hydrophilic N-terminus (Haugen et al., 2011) containing the conserved YGNGV/L “pediocin box” motif proposed to
contribute to pediocin activity, as altering specific residues results in a dramatic loss of activity (Miller et al., 1998; Quadri et al., 1997). The N-terminus is followed by a flexible hinge separating the N and C termini, allowing the two domains to move relative to each other. This hinge grants the required flexibility to allow the less conserved, more hydrophobic C-terminus to penetrate into the hydrophobic portion of the target membrane to induce irreparable damage to the target cell (Haugen et al., 2011).

Generally, pediocins are bactericidal (Chikindas et al., 1993) with maximum binding at pH 6 and death of the target cell occurring in as little as 30 seconds (Bhunia et al., 1991). Pediocins mainly affect Gram positives, with Gram negatives vulnerable to pediocins only if a sub-lethal injury is inflicted; this lack of susceptibility is thought to be due to the outer membrane of Gram negative cells (Kalchayanand et al., 1992).

**Pediocin target cell recognition and mode of action**

Understanding pediocin target cell recognition allows for establishment of how sensitive bacteria may develop resistance, though this facet of bacteriocin research remains poorly understood. Initial investigations by Diep et al. (2007) and further work by Kjos et al. (2010) reveal that class IIa bacteriocins target the mannose phosphotransferase system (man-PTS), the major glucose transporter for many bacteria. Made up of four structural domains, IIA, IIB, IIC, and IID, hybrid constructs of man-PTS insensitive *L. lactis* and pediocin-sensitive *Listeria monocytogenes*, along with N-terminus chimeras, amino acid alignment, and site directed mutagenesis demonstrated that N-terminus residues of domain IIC are implicated in pediocin sensitivity.
Pediocins target the cytoplasmic membrane of Gram positive bacteria via initial interactions between the positively charged pediocin N-terminus and negatively charged bacterial phospholipid membrane. Insertion of the C-terminus into the membrane of sensitive cells cause hydrophilic pore formation exhibited via leucine counterflow (Chikindas et al., 1993) and results in the dissipation of the pH gradient and membrane potential as well as inhibition of amino acid transport in a dose-dependent manner (Christensen and Hutkins, 1992). Investigations using electrodes to monitor electrical potential changes via distribution of tetraphenylphosphonium, a lipophilic cation, demonstrate immediate dissipation of electrical potential in sensitive cells and the release of potassium ions after pediocin treatment (Bhunia et al., 1991; Chikindas et al., 1993). Furthermore, the uptake and dissipation of an alanine analogue and glutamate via pediocin addition demonstrated that pediocin forms pores in the cell membrane allowing for efflux of small solutes, with higher concentrations increasing the pediocin-induced pore exclusion limit (Chikindas et al., 1993).

**Pediocin production: optimizing yield and production under gastrointestinal conditions**

Traditionally, pediocin production in the laboratory focuses on maximizing yield, with output observed to be influenced by multiple environmental factors such as nutrient availability (Guerra and Pastrana, 2002; Biswas et al., 1991), temperature (Anastasiadou et al., 2008b; Biswas et al., 1991), aeration (Anastasiadou et al., 2008a, 2008b), and pH (Nel et al., 2001; Biswas et al., 1991), depending on the strain. Experimentally these types of studies are conducted either in relevant culture media or chemostats to understand how pediocin production is affected under physiologically relevant GI conditions. Studies of this nature also aim to
maximize pediocin yield to enhance pathogen exclusion (Garcia-Almendarez et al., 2008; Collado et al., 2007).

On route through the GI tract, probiotics must first survive transit through the harsh environments of the stomach and small intestine. Fernandez et al. (2013), and Kheadr et al. (2010) investigated the ability of P. acidilactici UL5 to survive and produce pediocin in a dynamic in vitro GI model. Although P. acidilactici demonstrated poor survival through the upper GI tract, as exhibited by a reduction of over 90% after 90 minutes of simulated digestion, the majority of P. acidilactici isolated from upper GI compartments were able to produce pediocin to the same level as unstressed cells. Fernandez et al., (2012) also assessed the ability of P. acidilactici UL5 to produce pediocin under simulated colonic conditions. Pediocin PA-1 production was significantly reduced under simulated colonic medium versus the control medium, MRS (8192 versus 128 arbitrary units (AU), despite similar growth kinetics). Again, this may be due to improper carbon/nitrogen ratios in the medium, making the broth not optimal for pediocin production (Guerra and Pastrana, 2002). Furthermore, mice administered P. acidilactici UL5 demonstrated the ability to produce pediocin PA-1 from fecal stool samples, though the bacterium was only able to persist for 1 day (Dabour et al., 2009).

**Planktonic versus biofilm lifestyle**

Two modes of growth, planktonic and biofilm, dominate the bacterial world. Planktonic cells represent individual, free-swimming entities, whereas biofilms are characterized as heterogeneous, complex, and structurally dynamic multispecies communities encased in a gel-like matrix. Biofilm-related research spans decades and reveals both ubiquity and importance in diverse environments (Stoodley et al., 2004; Zobell, 1943). Biofilm formation is observed in
natural environments, such as ponds and rocks, as well as artificial ones, including various plastics, glass and metals. As such, artificial surfaces pose potentially serious complications due to pathogenic biofilm formation in the food processing and nosocomial settings. Vast alterations in protein expression are noted between the two cell types depending on the organism, location within the biofilm, and stage of the biofilm development of the organism (Fig. 1.2) (Park et al., 2014; Sauer et al., 2002). Changes in protein expression includes the loss of motility appendages (Sauer et al., 2002), decreased metabolic activity (Bester et al., 2010), and increases in protein expression involved in multidrug efflux pumps leading to enhanced antibiotic resistance (Southey-Pillig et al., 2005). The general lifecycle of a biofilm is depicted in Figure 1.2; initially, planktonic cells adhere to a surface (Fig. 1.2A) where microcolony formation is initiated and the biofilm matrix, composed primarily of extracellular polysaccharides, begins to be produced acting as an adhesive to protect the young biofilm against physical forces (Fig. 1.2B). Continued bacterial growth and matrix expansion allows for further biofilm development (Fig. 1.2C) forming a fully mature community (Fig. 1.2D). The subsequent dispersal of a mature biofilm may be due to environmental or signalling within the biofilm itself, causing the release of planktonic cells back into the environment and the potential to colonize new locations (Fig. 1.2E). The specific details of biofilm development will be described below.
Figure 1.2 An overview of biofilm development. Biofilm formation is a sequential process initiated by free-floating planktonic cells (A) coming into contact with a surface. These cells begin to divide (B) leading to the development of microcolonies held together by the biofilm matrix (C). Microcolony growth and formation of microhabitats within the biofilm lead to biofilm maturation and the formation of macrocolonies (D). Macroculture formation is usually followed by detachment and dispersal of biofilm cells under unfavourable conditions to allow for continued proliferation in a more favourable environment (E).
Initial bacterial attachment is dependent on the colonizing microorganism (Borucki et al., 2003), surface (Terada et al., 2012), and surrounding environment (Guerra and Pastrana, 2002). Initial interactions of the bacteria with the substratum are reversible (Schembri et al., 2003) and eventually transition to an irreversible attachment. Both physical and cellular derived components contribute to bacterial surface adhesion. One example are the teichoic acids that are responsible for Gram positive cell wall rigidity and contribute to the bacterial cell wall net negative charge (Walter et al., 2007). Teichoic acid mutants lacking the D-alanine esters essential for the maintenance of cationic homeostasis (Fabretti et al., 2006) exhibit reductions in colonization and subsequent biofilm formation on abiotic surfaces. Resulting reductions in biofilm accumulation of S. aureus (Gross et al., 2001), L. monocytogenes (Abachin et al., 2002), and Staphylococcus epidermidis (Holland et al., 2011) is suspected to be a result of the mutant’s alteration in net charge of the mutants. Initial adhesion of bacteria to a surface may also be affected by surface conditioning, the treatment of a surface, generally with food-derived particles, to increase microbial interactions. In the mouth, host derived salivary pellicles coat oral surfaces to directly influence primary colonizers in as little as thirty minutes (Hood and Zottola, 1997). This concept has been extended to probiotics by using prebiotics or bacteria known to stimulate mucus production to improve colonization capacity (Lebeer et al., 2007).

The transition from irreversible attachment to mature biofilm occurs via an intermediate stage made up of microcolonies, bacteria aggregates of three to five cells high with approximately fifty cells per colony (Zhao et al., 2013). Although microscopic investigations have revealed this intermediate step, the mechanism(s) of how individual cells associate to form microcolonies is/are not well understood. Transcription factor sigma B has been implicated in microcolony formation, with confocal laser scanning microscopy (CLSM) demonstrating
increased microbial aggregation when sigma B is placed under the influence of an inducible promoter (Bateman et al., 2001).

Classically studied microorganisms such as *Pseudomonas aeruginosa*, *E. coli*, and *S. aureus* continue to serve as models to elucidate the multifaceted structure of biofilms. A mature biofilm contains numerous micro-niches as a result of nutrient availability, extracellular polymeric substances (Ryu et al., 2004), oxygen levels (de Beer et al., 1994), and pH fluctuations (Hunter and Beveridge, 2005). Water channels may flow through the biofilm to allow for nutrient influx and waste removal (Stoodley et al., 2002), with fluid filled voids located in the center of the biofilm ensuring water and nutrient flow into deeper regions of the biofilm. The heterogeneity and protective nature of a mature biofilm continually demonstrates an advantageous lifestyle, with longevity attributed to increased antibiotic resistance versus planktonic cells (Drenkard and Ausubel, 2002), heightened UV resistance (Elasri and Miller, 1999), and increased genetic exchange (Palmer et al., 2007). Relevant to this work, mathematical modelling predicts bacteriocin production may be advantageous in a spatially structured community, such as a biofilm (Durrett and Levin, 1997; Frank et al., 1994).

Biofilms sensing unfavourable changes in their environment, due to physical forces (Picioreanu et al., 2001), nutrient levels (Rice et al., 2005), enzyme production (Boles and Horswill, 2008), and excess metabolites (Barraud et al., 2006) undergo detachment and dispersal from a surface, releasing cells into the external environment to seek out a new environment with more favourable conditions to continue the biofilm lifestyle. Biofilm disassembly most likely includes steps involving matrix degradation and physiological changes to a more planktonic lifestyle, including regained sensitivity to antibiotics due to conversion to planktonic cells and reappearance of motility appendages.
Evidence of colonic biofilms

The large surface area required for nutrient uptake, high nutrient availability, and confirmed presence of indigenous microbial populations make the human GI tract an ideal environment for the development of microbial biofilms. Furthermore, biofilms are presumed to be unable to transverse the epithelial barrier, making them a less invasive type of bacterial growth (Thullner and Baveye, 2008; Palestrant et al., 2004). Though biofilms are found throughout the human GI tract (von Rosenvinge et al., 2013) only colonic biofilms are relevant to this review and will be discussed.

The visualization of biofilms continues to be dominated by CLSM, a technique which gives information about the organization and architecture of biofilm communities in a hydrated state (Pamp et al., 2009) with increased resolution over conventional light microscopy and elimination of out-of-focus areas (Lawrence et al., 1991). However, there are technical limitations of biofilm visualization using CLSM, including the difficulty of obtaining unaltered in vivo samples, ensuring minimal disruption during planktonic cell removal, and the potential for damage of the biofilm through chemical treatments during preparation; all of these factors restrict our ability to effectively visualize biofilm structures. Additionally, sampling is an issue: ethical constraints prevent the collection of colonic biopsy material from healthy individuals, and stool samples may not be representative of the biofilm-rich fraction within the colon.

Microscopy reveals colonic biofilms residing in the mucus layer covering epithelial cells (Palestrant et al., 2004; Banwell et al., 1985). These mucosal biofilms are hypothesized to contribute to microflora stability throughout life; with rapid colonic epithelial cell shedding indicating cell proliferation must occur at a rapid rate to ensure the indigenous microflora does
not get washed out because of the flow and shear force of digested material (Xu and Gordon, 2003).

A study by Banwell et al. (1985) employed scanning and transmission electron microscopy to demonstrate biofilm formation in the small and large bowel of rats fed phytohemagglutinin lectins. A more comprehensive study by Palestrant et al. (2004) utilized electron microscopy (EM), cryogenic sample preparation, and acridine orange to visualize human, baboon, and rat GI tissue. EM micrographs of all species depict classic biofilm characteristics; distinct layers throughout the biofilm (Macfarlane and Macfarlane, 2006), porous extracellular matrix, and parallel orientation of the microbes with respect to flow. Scanning electron microscopy (SEM), live/dead stains, florescence in situ hybridization, and CLSM revealed the distribution, height, and architecture of biofilms on food pellicles from fecal bacteria, displaying differences in complex carbohydrate fermentation in adherent (biofilm) versus non-adherent (planktonic) cells (Macfarlane and Macfarlane, 2006). Physiologically relevant in vitro studies using chemostats demonstrated how certain species appear to form biofilm microcolonies in mixed species biofilms (Macfarlane and Dillon, 2007; Macfarlane et al., 2005; Macfarlane and Macfarlane, 2006). This finding is not surprising due to the nature of the microhabitats throughout the colon; pH variability and nutrient variability. This view may change in the future in light of the realization that not all biofilms will form thick, dense aggregates seen in mentioned organisms; this continues to be a huge research focus.

**Extending probiotic benefits**

Evidence supporting the presence of colonic biofilms may be implicated in observed lifelong microflora stability (Sonnenburg et al., 2004), though probiotic research demonstrates
transiency (Velez et al., 2010) after ingestion, ensuring continued benefits only through daily supplementation. A growing interest lies in identification of probiotics that provide the greatest benefit, strategies to optimize these populations, and determination of their precise mechanism of action. The efficacy of these treatments is limited by the inability of most probiotics to be permanently established in the colon, an attribute that may be due to the inability to incorporate into the naturally occurring static gut biofilms. Certain *P. acidilactici* strains are commercially available as a probiotic, making it an exciting target for additional screening to potentially enhance already available *P. acidilactici* benefits to further improve human health.
Thesis overview and research objectives

Extensive progress has been made in understanding and characterizing the probiotic benefits and the mechanisms that result in digestive health benefits. Adhesion to mucus represents an important probiotic criterion (Anukam et al., 2007), with adherence associated with immune system stimulation (Beachy., 1981), amongst other benefits. Unfortunately, probiotic transiency due to lack of prolonged colonization of the GI tract continues to be a major hurdle despite the advances in probiotic research.

In attempt ing to address the aforementioned transiency issue, we hypothesize that incorporation of probiotics into the naturally occurring mucosal biofilms lining the gut, specifically the colon, would extend probiotic colonization and allow for prolonged relief of GI perturbations. The first step towards this long-term goal and the first objective of this work is to evaluate the environmental conditions favouring probiotic biofilm formation. By doing this a baseline of biofilm formation of each strain under multiple conditions can be established and subsequently altered for enhanced formation. Additionally, probiotic bacteria demonstrating biofilm formation are generally targeted for future studies, as probiotics with the ability to form biofilms are thought of as being able to prolong colonization (Terraf et al., 2012). In this thesis, I assessed the ability of several commercial probiotic strains to form biofilms using a semi-quantitative assay under 256 experimental conditions. From the results of this objective, I identified the probiotic strain, P. acidilactici, strain R1001 as the optimal biofilm-producer. Because of this, and because of the large number of conditions resulting in biofilm formation for this probiotic strain, P. acidilactici was chosen for further experimentation, with emphasis on pediocin production.

With this information the second objective was to characterize P. acidilactici strains 7/4A, ATCC 25740, R1001, mm33, mm33a, Rbl39, and Rbl40 biofilm formation using the same
crystal violet biofilm assay and SEM to characterize \textit{P. acidilactici} biofilm morphology. The third and final objective was to investigate how specific environmental factors including; anaerobiosis, media, and microbial lifestyle alter pediocin production.
Chapter 2: Materials and Methods

Bacterial strains and growth conditions

Probiotics used for the initial large-scale biofilm screen are listed in Table 2.1 and were provided by Dr. Thomas Tompkins (Instuit Rosell, QC). *P. acidilactici* strains for further biofilm work are summarized in Table 2.2; strain 7/4A was donated by Dr. Emma Allen-Vercoe (University of Guelph, Guelph, ON), pediocin producer mm33 and plasmid-cured mm33a were acquired from Monique Lacroix (Armand Frappier Instituit, Laval, QC), and pediocin producer Rbl39 and plasmid- cured Rbl40 were attained from Ismail Fliss (Université Laval, Laval, QC). *P. acidilactici* 25740 and *L. monocytogenes* 7644 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Probiotics were cultured in their preferred medium, de Man, Rogosa, and Sharpe (MRS), Oxoid, Nepean, ON; Reinforced Clostridial Medium (RCM), Oxoid, Nepean, ON; Tryptic Soy Broth (TSB), Becton Dickson, Mississauga, ON; Elliker broth (appendix) at 37°C under both anaerobic and aerobic environments. Plates and cultures utilized for anaerobic growth were first degassed in an anaerobic chamber (Ruskin Bug Box) at a rate of 5 mL/hr and subsequently streaked or inoculated to ensure complete anaerobiosis before further experimentation. *L. monocytogenes* 7644 was grown in nutrient broth (Oxoid, Nepean, ON) supplemented with 0.7% yeast extract (Becton Dickson, Mississauga, ON), is pediocin sensitive and was utilized for the pediocin efficacy studies. Bacterial glycerol stocks were made by adding 750 μl of overnight culture to 250 μl of sterile glycerol (80% v/v) and kept at –80°C.
**Initial probiotic biofilm screen**

Probiotics listed in Table 2.1 were subjected to 256 different conditions to assess biofilm formation (Fig. 2.1). Overnight liquid cultures of each strain were diluted in their respective medium to an OD$_{600\text{nm}}$ of 0.2; 200 µl was subcultured into 5 ml of sterile medium and grown to a cell density of $1 \times 10^8 \text{CFU/ml}$, as determined by optical density at 600nm, and 10 µl and was added to a 96-well plate filled with 200 µl of the desired medium for a final cell density of about $1 \times 10^6 \text{CFU/ml}$. Biofilms grown in the presence of air were sealed with parafilm and placed in a container with a small amount of water to prevent evaporation of liquid cultures; anaerobic cultures were grown in a Ruskinn anaerobic work station at 80% humidity. Microtitre plates were removed of excess air by degassing the plates overnight before use. All cultures were incubated at 37°C and biofilm formation was evaluated after incubation periods of 24, 48, 72, and 96 h. At each time point the planktonic and loosely-bound cells were removed by pipetting the media up and down three times. Plates were washed via pietting with 200 µl of phosphate buffered saline (PBS; appendix) three times and left to dry. 200 µl of 0.2% crystal violet (w/v) prepared in dH$_2$O was added to wells for 15 minutes and excess crystal violet removed by submersion of plates into a container filled with dH$_2$O. This was repeated 3 times, with the final wash completed in a separate container. Plates were dried and biofilm formation was rated based on crystal violet retention to polystyrene wells. The rating was based upon crystal violet saturation and coverage of biofilm formed at the bottom of microtitre plate (Table 2.3). Each condition was subjected to two biological replicates. Results from the probiotic biofilm screen were utilized to guide subsequent experimentation.
Table 2.1 Probiotic strains utilized in large scale probiotic biofilm screen. Unless stated in brackets growth is facultative.

<table>
<thead>
<tr>
<th>Strain Designation</th>
<th>Species</th>
<th>Preferred Medium</th>
<th>Preferred Aeration</th>
</tr>
</thead>
<tbody>
<tr>
<td>R0052</td>
<td><em>Lactobacillus helveticus</em></td>
<td>MRS</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>R0389</td>
<td><em>Lactobacillus helveticus</em></td>
<td>MRS</td>
<td>Aerobic</td>
</tr>
<tr>
<td>R0215</td>
<td><em>Lactobacillus paracasei</em></td>
<td>MRS</td>
<td>Aerobic</td>
</tr>
<tr>
<td>R1012</td>
<td><em>Lactobacillus plantarum</em></td>
<td>MRS</td>
<td>Aerobic</td>
</tr>
<tr>
<td>R0011</td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>MRS</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>R0071</td>
<td><em>Bifidobacterium bifidum</em></td>
<td>RCM</td>
<td>Anaerobic (strict)</td>
</tr>
<tr>
<td>R0033</td>
<td><em>Bifidobacterium infantis</em></td>
<td>RCM</td>
<td>Anaerobic (strict)</td>
</tr>
<tr>
<td>R0175</td>
<td><em>Bifidobacterium longum</em></td>
<td>RCM</td>
<td>Anaerobic (strict)</td>
</tr>
<tr>
<td>R0179</td>
<td><em>Bacillus subtilis</em></td>
<td>TSB</td>
<td>Aerobic</td>
</tr>
<tr>
<td>R0026</td>
<td><em>Enterococcus faecium</em></td>
<td>MRS</td>
<td>Aerobic</td>
</tr>
<tr>
<td>R1058</td>
<td><em>Lactococcus lactis</em></td>
<td>Elliker</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>R1001</td>
<td><em>Pediococcus acidilactici</em></td>
<td>MRS</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>R0049</td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>MRS</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>R0343</td>
<td><em>Lactobacillus rhamnosus GG</em></td>
<td>MRS</td>
<td>Anaerobic</td>
</tr>
</tbody>
</table>
Table 2.2 *P. acidilactici* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Year Isolated</th>
<th>Pediocin Production?</th>
<th>Primary usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/4A</td>
<td>Human feces</td>
<td>-</td>
<td>No</td>
<td>None</td>
</tr>
<tr>
<td>ATCC 25740</td>
<td>Plant</td>
<td>1969</td>
<td>No</td>
<td>Pediocin indicator</td>
</tr>
<tr>
<td>R1001</td>
<td>Cheddar cheese</td>
<td>1991</td>
<td>No</td>
<td>Commercially available probiotic</td>
</tr>
<tr>
<td>mm33</td>
<td>Human feces</td>
<td>2007</td>
<td>Yes (PA-1)</td>
<td>Prevent enteric pathogen colonization</td>
</tr>
<tr>
<td>mm33a</td>
<td>Human feces</td>
<td>2007</td>
<td>No</td>
<td>mm33 minus pediocin encoding plasmid</td>
</tr>
<tr>
<td>Rbl39</td>
<td>Cheese</td>
<td>1991</td>
<td>Yes (PA-1)</td>
<td>Pediocin production under GIT conditions</td>
</tr>
<tr>
<td>Rbl40</td>
<td>Cheese</td>
<td>1991</td>
<td>No</td>
<td>Rbl39 minus pediocin encoding plasmid</td>
</tr>
</tbody>
</table>
Figure 2.1 Outline of conditions tested in the large-scale probiotic biofilm screen. Each probiotic strain was tested against 256 conditions to ensure coverage of many major factors influencing biofilm formation; the presence of air, medium, nutrient level, time, and pH. All combinations of these conditions were created to maximize the likelihood of biofilm formation for each strain.
Table 2.3 Rating system employed in the initial large-scale probiotic biofilm screen. Rating biofilm formation was based on crystal violet saturation; deeper colouring represents increased stained biomass, and individual well coverage. A rating of three or greater based on the below criteria was considered satisfactory biofilm formation.

<table>
<thead>
<tr>
<th>Rating</th>
<th>Description</th>
<th>Visual Representation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Low colour saturation with biofilm around outer perimeter of well.</td>
<td><img src="471x575" alt="Visual Representation" /></td>
</tr>
<tr>
<td>2</td>
<td>Low colour saturation with biofilm covering bottom of well.</td>
<td><img src="471x575" alt="Visual Representation" /></td>
</tr>
<tr>
<td>3</td>
<td>High colour saturation with biofilm around outer perimeter of well.</td>
<td><img src="471x575" alt="Visual Representation" /></td>
</tr>
<tr>
<td>4</td>
<td>High colour saturation with biofilm covering bottom of well.</td>
<td><img src="471x575" alt="Visual Representation" /></td>
</tr>
</tbody>
</table>
**P. acidilactici biofilm assay**

Biofilm formation of *P. acidilactici* strains were completed in 1/20 RCM pH 6 and 100% RCM pH 6. The assay protocol was identical to that described for the initial screen above with an additional step, the addition of 200 µl 30% glacial acetic acid to each well to solubilize the crystal violet after drying. Glacial acetic acid was mixed up and down by pipetting 5 times, allowed to sit for 15 minutes, and mixed by pipette once more to ensure the crystal violet was resuspended. The plates were read at an absorbance of 600nm. 3 biological and 3 experimental replicates were completed per each strain with negative control wells containing 200 µl of sterile medium in triplicate.

**Scanning electron microscopy (SEM)**

Using a Precision Brand Punch and Die set pieces of polystyrene 6mm in size were cut from the lids of 96-well microtitre plates, sterilized in 70% ethanol for 15 minutes, and air dried. 20 µl of agar was added to the bottom of each well and the polystyrene disc placed on top to secure it in place. 200 µl of 1/20 RCM pH 6 medium was added to each well and 10 µl of a *P. acidilactici* culture (cell density 1x10⁸ CFU/ml), prepared as previously described, was added. The microtiter plates were incubated for 12, 24, and 48 h. After incubation excess medium was removed by pipette, the polystyrene discs were transferred to a 24-well plate and washed with SEM buffer (appendix) 3 times. 2% glutaraldehyde, prepared in the SEM buffer, was added to samples for 30 minutes. The glutaraldehyde was removed, and the samples were washed three times in SEM buffer. A 1% osmium tetroxide solution was prepared in SEM buffer and used to stain the samples for 30 minutes. Samples were washed once more in buffer and dehydrated in an increasing series of ethanol washes (50, 70, 80, 90 and 3 times in 100% ethanol) in 10 minute intervals.
increments. Samples were critical point dried and coated with gold to a thickness of 15 nm using an Emitech K550 sputter coater and viewed using a Hitachi S-570 electron microscope at 10 kv.

**Harvesting pediocin-containing cell free supernatants (PC-CFS)**

Sterile pieces of polystyrene were prepared as outlined above and were added to wells of a 96-well microtitre plate. 200 µl of RCM pH 6 was added per well followed by the addition of 10 µl of *P. acidilactici* liquid culture, prepared as outlined above. After 24 h of static incubation at 37°C in the presence of air and also under anaerobic conditions planktonic and loosely-bound cells were removed by pipetting the media up and down 3 times and saved for further treatment. Each polystyrene piece was washed 3 times in PBS and placed into fresh medium in a new 96-well plate and re-incubated for a further 24 h to allow for biofilm derived cells to propagate. Both planktonic and biofilm cells were normalized to an OD$_{600nm}$ of 1.0 and filtered through a 0.22 µm filter plate for 30 min at 1000 rpm at room temperature. To ensure any observed inhibition was not due to an acidic culture pH or the presence of hydrogen peroxide, the supernatant was adjusted to pH 7 using 5 M NaOH, treated with 1 mg/ml catalase, and incubated for 30 minutes at 25°C. For comparison the same was completed in MRS. Cell-free supernatant was collected from strains mm33, mm33a, Rbl39, and Rbl40. 2 biological and 3 experimental replicates were completed per strain.

**Inhibition testing**

The ability of the pediocins to inhibit the indicators *P. acidilactici* ATCC 25740 and *L. monocytogenes* ATCC 7644 was accomplished via a minimum inhibitory concentration test conducted in 96-well microtitre plates. A series of two-fold dilutions ranging from a 100%
concentration of isolated pediocin to 1.56% were made with each well containing 100 µl of a single concentration of pediocin. The indicator organisms were grown overnight in their respective medium, 200 µl of the overnight culture was subcultured into 5 ml fresh medium and grown to a cell density of 1x10^8 cells/ml, an OD_{600nm} of approximately 0.2. 100 µl of indicator was added to each well, plates were incubated statically in the presence of air and under an anaerobic environment at 37°C, identical to those described in the biofilm screen. The plates were read at 1,2,3,4,5,7, and 10 h at an absorbance of 600nm, to evaluate growth inhibition as compared to controls. 200 µl of sterile medium served as a negative control and 100 µl of sterile medium with 100 µl of indicator served as the growth control. Purified PA-1 pediocin (Sigma-Aldrich Co, Oakville, ON) at a concentration of 0.125 µg/ml acted as the positive control.
Figure 2.2 PC-CFS inhibition outline. PC-CFS were serially diluted two-fold and added to wells of a 96-well plate. The indicator organism was added and plates read at various time points. PC-CFS were isolated in duplicate and tested in triplicate. Percentages represent amount of initially isolated PC-CFS (100%) present in each dilution. Abbreviations: MC₁, medium control (medium minus inoculum) for first pediocin isolation 1; MC₂, medium control for second pediocin isolation 2; MCₚₐ, medium control for positive control (purified PA-1 at 0.125µg/ml); GC₁ growth control f (medium and inoculum only) for first biological replicate; GC₂, growth control for second biological replicate; GCₚₐ, growth control for positive control.
Statistical analysis

Statistical analyses were completed in Prism v5.0. (GraphPad Software, Inc., San Diego, CA). Biofilm comparisons were completed using unpaired Student’s t-tests with a modified t-test for data sets displaying significant variances (Welch’s correction) with the significance level set at α=0.05.
Chapter 3: Results

Probiotics form biofilms under various conditions

To effectively screen probiotic biofilm formation, a high-throughput assay was developed based on work by Stepanovic et al. (2000) using 96-well microtitre plates and crystal violet staining, the standard method for assessing biofilm formation. This screen provided an initial assessment of the conditions promoting probiotic biofilm formation. Due to the large number of conditions tested, conventional biofilm quantification via crystal violet solubilisation, spectrophotometry, and statistical analysis proved too laborious. Thus, to compare the biofilm forming ability of each probiotic within each condition, a rating system based on crystal violet saturation and surface coverage was developed for efficient and consistent analysis of biofilm formation (Table 2.3). All probiotics tested formed biofilms in at least one of the conditions tested, however, no patterns were observed when assessing for a common factor or condition that led to biofilm formation of all tested strains. Figure 3.1 summarizes the large-scale probiotic biofilm screen based on crystal violet staining; biofilm formation was assessed based on medium, pH, presence or absence of oxygen at initial incubation, and nutrient level. Each shade of blue represents the degree of biofilm formation observed, light blue representing the least biofilm formation detected and dark blue the greatest. The results are consistent with previous reports which demonstrated the ability of probiotics to form biofilms may fluctuate within a species (Terraf et al., 2012) and be altered when grown in similar conditions (Lebeer et al., 2007). Interestingly, L. rhamnosus GG, a popular strain in probiotic research due to its recognized adhesive ability to many biotic and abiotic surfaces (Alander et al., 1999) and ability to form biofilms (Lebeer et al., 2007), demonstrated biofilm formation under only 13/256 conditions in our assay. Though this was one of the strains resulting in the greatest number of
conditions resulting in biofilm formation, it was surprising since the literature describes its enhanced biofilm forming ability versus other probiotics. Strains such as *L. lactis* R1058 and *P. acidilactici* R1001 formed biofilms in 33 and 48 of the conditions tested, respectively (Fig. 3.1). *L. lactis* has previously demonstrated the ability to form biofilms (Luo *et al.*, 2005; Mercier *et al.*, 2002) whereas biofilm formation by the most prolific biofilm former in our assay, *P. acidilactici* R1001, has yet to be described. The ability of the species *P. acidilactici* to form biofilms does not agree with previous work described in that we were able to demonstrate biofilm formation versus other groups (Borges *et al.*, 2013; Merrifield *et al.*, 2011; Albano *et al.*, 2009), making it an interesting species for further studies. Additionally, some strains of *P. acidilactici* have the ability to produce pediocins that may enhance human health via the reduction of intestinal colonization by antibiotic resistant pathogens (Millette *et al.*, 2008).
<table>
<thead>
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<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<td>4</td>
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</tbody>
</table>

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A

---

PA 1001
BL 1012
EF 1025
BB 1071
LR 1011
LG 10343
LH 10052

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37
**Figure 3.1 Summary of the large-scale probiotic biofilm screen.** Heat maps demonstrating biofilm growth after (A) 24 h (B) 48 h (C) 72 h and (D) 96 h. Each coloured block corresponds to a rating based on crystal violet staining, with a rating of 1 representing the least biofilm formation (light blue) and 4 representing the greatest biofilm formation (dark blue). Each probiotic strain was tested against 256 conditions based on medium (column 1), pH (column 2), aeration (column 3), and nutrient level (column 4). Dendrogram generated by results similarity.

Refining biofilm formation with a focus on *P. acidilactici*

As biofilm formation by *P. acidilactici* has yet to be described, we wanted to ensure that *P. acidilactici* R1001 biofilm formation observed in the large-scale screen was not a random occurrence. Multiple *P. acidilactici* strains from diverse sources including; the gut, cheese, and plants (Table 2.2) were examined under conditions that promoted biofilm formation. *P. acidilactici* biofilm formation was assessed using two different media. The first was a low nutrient medium, a 1/20 dilution of RCM at pH 6 as this was the medium demonstrating the strongest biofilm formation of *P. acidilactici* R1001 in the large-scale screen. The second medium was undiluted RCM adjusted to pH 6 and was chosen as it was the medium from the high-throughput assay that most closely resembled those used in previous studies when studying pediocin production that also resulted in *P. acidilactici* R1001 biofilm formation (Papagianni and Anastasiadou, 2009). Biofilm formation was assessed at various time points to evaluate changes in biofilm development over time. Biofilms grown in low nutrient medium were analyzed at 12, 24, 48, 72, and 96 h to investigate biofilm formation shortly after initial inoculation and following periods of extended incubation. Biofilm formation in undiluted RCM pH 6 was evaluated at 24, 48, 72, and 96 h, with the 12 h interval excluded; the primary purpose of this medium was to investigate pediocin production, which is normally assessed after approximately 24 h (Albano *et al.*, 2009; Anastasiadou *et al.*, 2008a), in addition to biofilm formation.

**Anaerobiosis and ability to produce pediocin modifies *P. acidilactici* biofilm development**

Many factors have been identified to contribute to biofilm formation, such as anaerobiosis (Yoon *et al.*, 2002). Because *P. acidilactici* is a facultative anaerobe, we wanted to determine how the presence or absence of air with cultures would alter biofilm formation. Anaerobically grown biofilms required static incubation due to the inability of shaking.
equipment to enter the anaerobic chamber. To keep conditions the same biofilms grown in an aerobic environment were also placed under static conditions; over time this may have potentially altered the aerobic nature of the cultures due to the limited amount of oxygen diffusing into the culture.

Under nutrient limited conditions the effect fluctuating the amount of air on *P. acidilactici* biofilm formation was observed to be most predominant at the 12 h time point, as demonstrated by statistically significant increases in anaerobic biofilm formation in gut isolate *P. acidilactici* 7/4A and the pediocin producing strain isolated from human feces, *P. acidilactici* mm33 (Fig. 3.2A and 3.3A). Conversely, the second pediocin producing strain, *P. acidilactici* Rbl39, isolated from cheese, demonstrated an increase in biofilm formation in an aerobic environment at the 12 h time point (Fig. 3.3B). Though this effect was observed at the earliest time point, no trends were observed for future time points. Interestingly, when grown in a high nutrient medium, an aerobic or anaerobic environment had little impact on biofilm growth at all time points (Fig. 3.4 and 3.5).

Next, we investigated whether *P. acidilactici* biofilm formation was altered as a result of pediocin production, itself an energetically demanding process, by comparing the biofilm formation of pediocin producing strains, *P. acidilactici* mm33 and Rbl39, to their non-pediocin producing counterparts, *P. acidilactici* strains mm33a and Rbl40, respectively. The difference between the producing strains and their counterparts lies in the presence of a plasmid carrying the pediocin producing gene, immunity gene, and an ATP-binding cassette. Both pediocin producers demonstrated significant increases in biofilm development under both an aerobic and anaerobic environment at 12 h when grown under nutrient limited conditions (Fig. 3.3A and B).
though no differences in biofilm formation were observed in the high nutrient medium (Fig. 3.5A and B).
Figure 3.2 Effect of nutrient limiting conditions on *P. acidilactici* biofilm formation.

Quantification of biofilm formation by means of crystal violet staining and glacial acetic acid solubilisation at various time points for *P. acidilactici* strains. Panel A: 7/4A, Panel B: ATCC 25740, and Panel C: R1001. Biofilm production was assessed in an aerobic environment (at inoculation) (white bars) and anaerobically (hatched bars). Symbols: (*) p ≤ 0.05, (**) p ≤ 0.01, (*** p ≤ 0.001, (****) p ≤ 0.0001.
Figure 3.3 Effect of nutrient limiting conditions on *P. acidilactici* biofilm formation.

Quantification of biofilm formation by means of crystal violet staining and glacial acetic acid solubilisation at various time points for *P. acidilactici* strains. Panel A: mm33 and mm33a and Panel B: Rbl39 and Rbl40. Biofilm production was assessed in an aerobic environment (at inoculation) (white bars) and anaerobically (hatched bars). Maroon bars represent pediocin-cured strains mm33a and Rbl40 in graph A and B, respectively. Symbols: (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$, (****) $p \leq 0.0001$. 
Figure 3.4 Effect of excess nutrient conditions on *P. acidilactici* biofilm formation.

Quantification of biofilm formation by means of crystal violet staining and glacial acetic acid solubilisation at various time points for *P. acidilactici* strains. Panel A: 7/4A, Panel B: ATCC 25740, and (C) R1001. Biofilm production was assessed in an aerobic environment (at inoculation) (white bars) and anaerobically (hatched bars). Symbols: (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$, (****) $p \leq 0.0001$. 
Figure 3.5 Effect of excess nutrient conditions on *P. acidilactici* biofilm formation.

Quantification of biofilm formation by means of crystal violet staining and glacial acetic acid solubilisation at various time points for *P. acidilactici* strains. Panel A: mm33 and mm33a and Panel B: Rbl39 and Rbl40. Biofilm production was assessed in an aerobic environment (at inoculation) (white bars) and anaerobically (hatched bars). Maroon bars represent pediocin-cured strains mm33a and Rbl40 in panels D and E, respectively. Symbols: (*) p ≤ 0.05, (**) p ≤ 0.01, (***) p ≤ 0.001, (****) p ≤ 0.0001.
**Scanning electron microscopy validates *P. acidilactici* biofilm formation**

SEM was used to complement the high-throughput crystal violet assay performed on *P. acidilactici*. Complementation of the crystal violet assay was completed to ensure *P. acidilactici* biofilm formation was not a result of re-adhering planktonic cells; this would be visualized by a lack of cells on the sample surface due to initial wash steps, as SEM samples were treated in an identical manner to biofilm samples. Furthermore, the presence of microcolony and/or macrocolony formation would be indicative of biofilm formation, as planktonic cells do not form communities with complex architecture, but are generally monolayers and single cells. SEM was used to image all *P. acidilactici* strains, which were cultured on polystyrene cut-outs in 96-well microtiter plates using conditions identical to those used to assess biofilm formation. Micrographs were obtained at 12, 24, and 48 h in the same nutrient limited medium used for the biofilm assay to visualize the temporal development of biofilm formation (Fig. 3.6-3.10). To examine if pediocin production altered the observed biofilm structure micrographs of strains mm33a and Rbl40, the non-pediocin producing counterparts to mm33 and Rbl39, respectively, were obtained for cultures grown for 12 h. Only the 12 h time point was assessed for the non-pediocin producing counterparts as this was the only time point where both pediocin producers demonstrated statistical significance in biofilm formation versus their non-producing counterpart.

Table 3.1 summarizes the observations from the SEM micrographs of *P. acidilactici* biofilm formation. Biofilm formation is described in terms of microcolony or macrocolony formation, where a microcolony is defined as an aggregate of approximately fifty cells of three to five cells high (Zhao *et al.*, 2013), with a macrocolony being any larger observable colonies. This description relays information about the stage of biofilm development and the strains preferred colony size of the strains, as some probiotics are observed to disperse after
microcolony formation and do not continue to form macrocolonies (Macfarlane and Macfarlane, 2006). Biofilms are also defined in terms of their elevation off the surface, as a high elevation may lead to sloughing of biofilm due to shear forces (Chambless and Stewart, 2007). In this work estimations of elevation are described as either flat against surface or raised up, with biofilm cells elevated from the surface. Finally, biofilms will be described in terms of the space between individual microcolonies and/or macrocolonies (gaps). This descriptor is divided into; no gaps: cells are tightly associated with no or very few outlying cells; minimal gaps: small spacing between the majority of colonies where the empty space could be filled with a few cells and little exposed surface; and extensive gaps: large gaps between adjacent colonies with a majority of surface exposed. This information is pertinent as certain probiotics have an observed tendency to disperse and form small microcolonies when adhering to a surface (Macfarlane and Macfarlane, 2006), where in contrast other organisms are seen to form close associations between colonies (Jones and Versalovic, 2009). For clarification, the white arrow of Figure 3.6C is an example of a microcolony versus the white triangle, a representative example of a macrocolony. In the same frame the microcolony would be described as even with the surface and the macrocolony as raised off the surface. Figure 3.7D, E, and F are representative examples of extensive, minimal, and no gaps, respectively. SEM images demonstrated crystal violet staining was not based on re-adherent planktonic cells but biofilm formation, which was observed in all P. acidilactici strains due to the presence of cellular communities taking the form of microcolonies and/or macrocolonies.

Observed biofilm formation of certain strains demonstrated different morphologies when grown in an aerobic (at time of initial inoculation) or anaerobic environment (Table 3.1). For example, biofilm formation of P. acidilactici R1001 at the 12 h time point showed a tightly
associated, raised, single macrocolony when grown under aerobic conditions (Figure 3.8A), but low-lying microcolonies when grown anaerobically (Fig. 3.8C). Differences in observed biofilm structure are also noted when comparing pediocin producers to their non-producing counterparts. When biofilms were grown anaerobically, strain Rbl39 was observed to have small, low-lying microcolonies with extensive gapping (Fig 3.10E) versus strain Rbl40 that showed a tightly-associated, raised macrocolony under the same conditions (Figure 3.10B). Taken together, SEM was shown to complement and validate the crystal violet assay; demonstrating that retained crystal violet in the biofilm assay was due to biofilm formation, as planktonic cells would have been removed from the surface when washed. Additionally, variations in morphology are noted depending on the presence or absence of air and ability to produce pediocin.
Figure 3.6 Scanning electron microscopy of *P. acidilactici* 7/4A biofilms. Micrographs of biofilms grown in an aerobic environment at A: 12 h, B: 24 h, and C: 48 h as well as anaerobically at D: 12 h, E: 24 h, and F: 48 h.
Figure 3.7 Scanning electron microscopy of *P. acidilactici* ATCC 25740 biofilms.

Micrographs of biofilms grown in an aerobic environment at A: 12 h, B: 24 h, and C: 48 h as well as anaerobically at D: 12 h, E: 24 h, and F: 48 h.
Figure 3.8 Scanning electron microscopy of *P. acidilactici* R1001 biofilms. Micrographs of biofilms grown in an aerobic environment at A: 12 h, B: 24 h, and C: 48 h as well as anaerobically at D: 12 h, E: 24 h, and F: 48 h.
**Figure 3.9 Scanning electron microscopy of *P. acidilactici* mm33 and mm33a.** Micrographs of A: mm33 biofilms grown in an aerobic environment at 12 h, B: mm33a at 12 h, C: mm33 at 24 h, and D: mm33 at 48 h and anaerobically of E: mm33 at 12 h, F: mm33a at 12 h, G: mm33 at 24 h, and H: mm33 at 48 h.
Figure 3.10 Scanning electron microscopy of *P. acidilactici* Rbl39 and Rbl40 biofilms. Micrographs of A: Rbl39 biofilms grown in an aerobic environment at 12 h, B: Rbl40 at 12 h, C: Rbl39 at 24 h, and D: Rbl39 at 48 h and anaerobically of E: Rbl39 at 12 h, F: Rbl40 at 12 h, G: Rbl39 at 24 h, and H: Rbl39 at 48 h.
Table 3.1. Summary of *P. acidilactici* SEM biofilm observations based on colony development, elevation, and gaps (Flat, colony is flat against surface; Raised, colony is elevated from surface). Descriptors were developed for this work.

<table>
<thead>
<tr>
<th>Strain and Condition</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/4A Aerobic</td>
<td>-Microcolonies (flat) -Minimal gaps</td>
<td>-Single macrocolony (raised) -Raised off surface -No gaps</td>
<td>-Macrocolonies (raised) and microcolonies (raised) -Minimal gaps</td>
</tr>
<tr>
<td>7/4A Anaerobic</td>
<td>-Macrocolonies (flat) and microcolonies (flat) -Minimal gaps</td>
<td>-Single macrocolony (raised) and multiple microcolonies (flat) -No gaps</td>
<td>-Single macrocolony (raised) -No gaps</td>
</tr>
<tr>
<td>ATCC 25740 Aerobic</td>
<td>-Single macrocolony (raised) -No gaps</td>
<td>-Multiple microcolonies (flat) -Minimal and extensive gaps</td>
<td>-Single macrocolony (raised) and multiple microcolonies (flat) -Minimal gaps</td>
</tr>
<tr>
<td>ATCC 25740 Anaerobic</td>
<td>-Multiple microcolonies (flat) -Extensive gaps</td>
<td>-Multiple microcolonies (flat) -Minimal gaps</td>
<td>-Single macrocolony (flat) -No gaps</td>
</tr>
<tr>
<td>R1001 Aerobic</td>
<td>-Single macrocolony (raised) and multiple microcolonies (flat) -Raised off surface -Extensive gaps</td>
<td>-Single macrocolony (raised) and multiple microcolonies (raised or flat) -Extensive gaps</td>
<td>-Multiple microcolonies (flat) -Extensive gaps</td>
</tr>
<tr>
<td>R1001 Anaerobic</td>
<td>-Macrocolonies (flat) and multiple microcolonies (flat) -Minimal gaps</td>
<td>-Single macrocolony (raised) and multiple microcolonies (flat) -Extensive gapping</td>
<td>-Single macrocolony (raised) and multiple microcolonies (flat and raised) -Minimal gapping</td>
</tr>
<tr>
<td>mm33 Aerobic</td>
<td>-Multiple microcolonies (flat) -Minimal gapping</td>
<td>-Multiple microcolonies (flat and raised) -Extensive gapping</td>
<td>-Single macrocolony (raised) -No gapping</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>------------------------------</td>
<td>-----------------------------</td>
<td></td>
</tr>
</tbody>
</table>
| mm33a Aerobic  | - Multiple microcolonies (flat)  
- Extensive gapping | Not investigated            |
|                |                              | Not investigated            |
| mm33 Anaerobic| - Multiple microcolonies (flat)  
- Extensive gapping | - Multiple macrocolonies (raised) and microcolonies (flat)  
- Minimal gapping |
|                | - Single macrocolony (raised)  
- Extensive gapping | - Extensive gapping         |
| mm33a Anaerobic| - Multiple macrocolonies (raised) and microcolonies (flat)  
- Minimal gapping | Not investigated            |
|                |                              | Not investigated            |
| Rbl39 Aerobic  | - Single macrocolony (raised)  
- No gaps         | - Multiple microcolony (flat)  
- Extensive gaps |
|                |                              | - Multiple microcolonies (flat)  
- Minimal and extensive gaps |
| Rbl40 Aerobic  | - Single macrocolony (raised)  
- No gaps         | Not investigated            |
|                |                              | Not investigated            |
| Rbl39 Anaerobic| - Microcolonies (flat)  
- Extensive gapping | - Microcolonies (flat)  
- Extensive gapping |
|                |                              | - Single macrocolony (raised)  
- Extensive gaps |
| Rbl40 Anaerobic| - Single macrocolony (raised)  
- Extensive gapping | Not investigated            |
|                |                              | Not investigated            |
Anaerobiosis, medium, and mode of growth alter *P. acidilactici* derived pediocin inhibition

To investigate and compare the ability of *P. acidilactici* strains mm33 and Rbl39 to produce pediocin under various growth conditions, we employed a standard pediocin inhibition assay based on a series of two-fold dilutions (Papagianni *et al*., 2006). Evaluating pediocin output was determined by testing PC-CFS against different indicator organisms (bacteria that are susceptible to pediocin) resulting from either bacteriostatic or bactericidal effects. The ability of pediocin within the PC-CFS to inhibit growth via bacteriostatic action of the chosen indicator organism can therefore be monitored via spectrophotometry. For this study, the highly sensitive indicator strain *P. acidilactici* ATCC 25740 (Papagianni *et al*., 2006), and clinically relevant indicator *L. monocytogenes* ATCC 7644 (Mattila *et al*., 2003) were used to test the bacteriostatic activity of pediocin within the PC-CFS. Due to time and equipment constraints, we used PC-CFS, unpurified pediocin, to examine its bacteriostatic effects and is the preferred method for analyzing the effects of pediocins (Papagianni *et al*., 2006). Other factors which might be perceived and contribute to inhibitor killing, such as a low pH and catalase, are accounted for during PC-CFS processing to ensure any bacteriostatic activity was due to the pediocins present in the PC-CFS.

Because the presence of varying levels of culture aeration is known to increase bacteriocin production (Anastasiadou *et al*., 2008a; Amiali *et al*., 1998), PC-CFS of *P. acidilactici* mm33 and Rbl39 as well as their pediocin-lacking counterparts, were collected from cultures grown in the presence and absence of air; though in this work cultures were not aerated but initially inoculated in either an aerobic or anaerobic environment. The activity of the collected PC-CFS concentrations were evaluated via two-fold dilutions in either fresh MRS or RCM with concentrations ranging, from 100% to 1.56%, where the 100% concentration
represented the initial PC-CFS isolated from various *P acidilactici* cultures. In the literature (Rasch and Knochel, 1998), bacteriocin values are generally presented as international or arbitrary units (IU/ AU) that cannot be compared between studies. Thus, for these experiments purified pediocin PA-1 at a concentration of 0.125 µg/ml was used as a positive control and reference point to compare the activity of pediocin in CFS during the experiments. An equal volume of indicator microorganism was added and spectrophotometry measurements were taken over a period of 10 h to determine how inhibition of growth varied over time. The greater the amount of pediocin present in the PC-CFS, the greater the inhibition, which will be reflected in a delay in the observed growth of the indicator microorganism versus the growth control, as detected by optical density. We investigated three different variables and their influence on pediocin production; the effect of air within cultures, medium, and mode of growth.

**The effect of an anaerobic environment on PC-CFS inhibition**

PC-CFS were isolated in both an initially aerobic and anaerobic environment to assess if variation in indicator growth inhibition was a result of the amount of air exposed to cultures. PC-CFS were further isolated planktonically in both MRS and RCM with and without the presence of air. Additionally, PC-CFS were isolated from biofilm derived cells grown in RCM. When comparing the effect of a more aerobic environment to an anaerobic environment both pediocin producing strains, mm33 and Rbl39, displayed greater growth inhibition against indicator *P. acidilactici* ATCC 25740 under an aerobic environment when planktonic cells were isolated from MRS (Fig. 3.11A and B; Fig. 3.12A and B); this is evidenced by microbial growth similar to that of the growth control at PC-CFS concentrations of 12.5-1.56% when grown in an anaerobic environment. The opposite effect was observed when assessing the effect of an
anaerobic environment was tested against indicator *L. monocytogenes* ATCC 7644; anaerobic PC-CFS demonstrated enhanced inhibition as observed by prolonged complete inhibition of indicator growth at 100% and 50% pediocin concentration (Fig. 3.13A and B; Fig. 3.14A and B).

When assessing the effect of an initially aerobic environment in which PC-CFS was isolated from planktonically grown cultures in RCM, both pediocin producing strains exhibited increased growth inhibition against indicator *L. monocytogenes* ATCC 7644 when PC-CFS was isolated in an aerobic environment (Fig. 3.13C and D; Fig. 3.14C and D); This can be observed at PC-CFS concentrations of 50% or less as anaerobically isolated cultures display growth similar to that of the growth control. Conversely, under these conditions, differences observed in activity against *P. acidilactici* ATCC 25740 were negligible (Fig. 3.11C and D; Fig. 3.12C and D).

Finally, we examined the effect varying the level of air had when PC-CFS were isolated from biofilm-derived cells in RCM; greater growth inhibition occurred during growth in an initially aerobic environment of both mm33 and Rbl39 when tested against *L. monocytogenes* (Fig. 3.13E and F; Fig. 3.14E and F). This can be observed by comparing mm33 and Rbl39 isolated pediocin to the known value of the positive control; isolation in an aerobic environment of most tested values fall below the dotted line, indicating a higher pediocin concentration resulting in increased microbial growth inhibition. When biofilm derived cells from RCM where tested against *P. acidilactici* ATCC 25740 differences were minor (Fig. 3.11E and F; Fig. 3.12E and F).
The effect of medium on PC-CFS inhibition

Comparisons of PC-CFS output based on culture growth medium, MRS or RCM, allowed for analysis of how variations in medium components may alter pediocin production, presenting itself as variations in indicator growth inhibition. Both pediocin producing strains demonstrated increased inhibition in MRS against indicator *P. acidilactici* ATCC 25740 when PC-CFS were isolated in an aerobic environment; this is exhibited by complete inhibition at 100%, 50%, and 25% concentrations of PC-CFS (Fig. 3.11A and C; Fig. 3.12A and C). Interestingly, the effect of medium on PC-CFS was reversed when isolated in MRS and RCM anaerobically, with an increased inhibition occurring when PC-CFS was isolated in RCM, as observed at concentrations of 6.25%, 3.125%, and 1.56% (Fig. 3.11B and D; Fig. 3.12B and D).

When inhibition based on growth medium was assessed using the indicator strain *L. monocytogenes* ATCC 7644 enhanced inhibition occurred in mm33 and Rbl39 when grown in RCM (Fig. 3.13A and C; Fig. 3.14A and C). Increased inhibition in RCM is evidenced by increased observed inhibition at 100% PC-CFS concentration in RCM. Anaerobically, mm33 and Rbl39 PC-CFS displayed increased inhibition in MRS, with large differences compared to the growth control after three to four hours, and inhibition from RCM pediocin showing growth similar to the positive control at lower concentrations in mm33 (25% and lower) (Fig. 3.13B and D; Fig. 3.14B and D).

The effect of the mode of growth on PC-CFS inhibition

The minimal data available pertaining to probiotic biofilms has restricted investigations related to bacteriocin production from these biofilms. In this section, comparisons were made between PC-CFS from planktonic cells grown in RCM and RCM-derived biofilm cells. This
medium was chosen for two reasons: it allowed for the use of a medium besides MRS (the medium most commonly used to isolate CFS); and growth in undiluted RCM resulted in biofilm formation after 24 h. Additionally, biofilm formation of *P. acidilactici* was not observed in undiluted MRS after 24 h (using a diluted medium would lead to insufficient growth and most likely undetectable levels of pediocin in CFS). When tested against indicator *P. acidilactici* ATCC 25740, planktonic and biofilm derived PC-CFS from mm33 and Rbl39 resulted in planktonic cells exhibiting increased inhibition. This distinction was noticeable after 7 h at pediocin concentrations of 100%-12.5% (Fig. 3.11C and E; Fig. 3.12C and E). Variation in the inhibition between the two modes of growth was indistinguishable in both strains when isolated anaerobically (Fig. 3.11D and F; 3.12D and F). When tested against indicator *L. monocytogenes* ATCC 7644, both pediocin producers demonstrated greater inhibition planktonically, as seen in the large differences in inhibition compared to the growth control in strain mm33 (Fig. 3.13C and E; Fig. 3.14C and E). When grown anaerobically mm33 and Rbl39 displayed increased inhibition when pediocins were isolated from biofilms, with the corresponding planktonic pediocins displaying growth rivalling the growth control at lower pediocin percentages (25-6.25%) (Fig. 3.13D and F; Fig. 3.14D and F).
Figure 3.11 Inhibition testing of PC-CFS isolated from *P. acidialcetici* mm33 and tested against indicator strain *P. acidialcetici* ATCC 25740. Coloured lines represent various percentages of initially isolated pediocin tested; 100% (red), 50% (yellow), 25% (green), 12.5% (blue), 6.25% (purple), 3.125% (pink), and 1.56% (grey). The growth control is depicted by a solid black line while the dashed black line represents the positive pediocin control, purified PA-1 pediocin at 0.125ug/mL. Graphs are separated by the presence or absence of air (vertically) as well as culture growth medium (horizontally- MRS or RCM) and mode of growth (horizontally- Planktonic of biofilm). Each point represents the mean of n=6 experiments (2 biological and 3 experimental replicates) +/- standard error of the mean.
Figure 3.12 Inhibition testing of PC-CFS isolated from *P. acidilactici* Rbl39 and tested against indicator strain *P. acidilactici* ATCC 25740. Coloured lines represent various percentages of initially isolated pediocin tested; 100% (red), 50% (yellow), 25% (green), 12.5% (blue), 6.25% (purple), 3.125% (pink), and 1.56% (grey). The growth control is depicted by a solid black line while the dashed black line represents the positive pediocin control, purified PA-1 pediocin at 0.125μg/mL. Graphs are separated by the presence or absence of air (vertically) as well as culture growth medium (horizontally- MRS or RCM) and mode of growth (horizontally- Planktonic of biofilm). Each point represents the mean of n=6 experiments (2 biological and 3 experimental replicates) +/- standard error of the mean.
Figure 3.13 Inhibition testing of PC-CFS isolated from *P. acidialctici* mm33 and tested against indicator strain *L. monocytogenes* ATCC 7644. Coloured lines represent various percentages of initially isolated pediocin tested: 100% (red), 50% (yellow), 25% (green), 12.5% (blue), 6.25% (purple), 3.125% (pink), and 1.56% (grey). The growth control is represented by the solid black line with the dashed black line being the positive pediocin control, inhibition of purified PA-1 pediocin at 0.125ug/mL. Graphs are separated by the presence or absence of air (vertically) as well as culture growth medium (horizontally- MRS or RCM) and mode of growth (horizontally- Planktonic of biofilm). Each point represents the mean of n=6 experiments experiments (2 biological and 3 experimental replicates) +/- standard error of the mean.
Figure 3.14 Inhibition testing of PC-CFS isolated from *P. acidialctici* Rbl39 and tested against indicator strain *L. monocytogenes* ATCC 7644. Coloured lines represent each different percentage of pediocin tested after dilution; 100% (red), 50% (yellow), 25% (green), 12.5% (blue), 6.25% (purple), 3.125% (pink), and 1.56% (grey). The growth control is represented by the solid black line with the dashed black line being the positive pediocin control, inhibition of purified PA-1 pediocin at 0.125ug/mL. Graphs are separated by the presence or absence of air (vertically) as well as culture growth medium (horizontally- MRS or RCM) and mode of growth (horizontally- Planktonic of biofilm). Each point represents the mean of n=6 experiments (2 biological and 3 experimental replicates) +/- standard error of the mean.
Chapter 4: Discussion

From a research standpoint, microbial adherence to the GI tract mucosa is considered an important criterion during probiotic selection (Collado et al., 2007; Juntunen et al., 2001; Kirjavainen et al., 1998). As such, the ability of probiotics to form biofilms may result in improved mucosal adhesion and may be considered a beneficial property in promoting colonization, potentially leading to increased retention to the host mucosa (Terraf et al., 2012). Accordingly, the initial goal of this research was to characterize the biofilm forming ability of various probiotic species over a wide range of conditions. Using a defined set of commercially available probiotics (Table 2.1) it was observed that each probiotic, including different strains of the same species, formed biofilms under diverse conditions (Figure 3.1). Furthermore, this work expands on these initial results and highlights the ability of *P. acidilactici* to form biofilms as well as assesses the variation in pediocin production that occurs under different growth conditions.

To enhance our understanding of how environmental factors may modify probiotic biofilm formation we tested each probiotic to determine how medium, nutrient level, pH, time, and an anaerobic environment may influence biofilm formation (Figure 3.1). To our knowledge, this work represents the first large-scale screen assessing the ability of probiotic strains to form biofilms. One of the caveats to bear in mind when investigating probiotic biofilm formation is the potentially detrimental effects biofilms may have on large-scale manufacturing equipment (Morikawa, 2006). Additionally, nutrient rich media generally used for robust growth in laboratories may not be economically feasible for large scale probiotic growth.

Biofilm forming capacity was assessed using crystal violet, the standard stain for evaluating biofilm formation (Stepanovic et al., 2000). As such, information on the viability of the cells
within the biofilms will not be determined, as crystal violet staining only conveys information for the total biofilms biomass (i.e. the cells and extracellular materials) (Peeters et al., 2008). A viability stain was not chosen for this work as crystal violet biofilm staining represents the standard method for assessing biofilm formation and we were interested in total biomass accumulated during the testing as a first step to assessing biofilm development.

This work demonstrated that all probiotics were able to form biofilms in at least one of the conditions tested, with certain strains exhibiting biofilm formation in a greater number of conditions. Previous work has demonstrated the ability of the probiotics to form biofilms, typically when grown in either MRS or TSB, in an aerobic environment, at a neutral pH, as these are generally the only conditions assessed for biofilm formation (van der Veen and Abee, 2011; Blair et al., 2008; Chavez de Paz, 2007; Heikens et al., 2007; Macfarlane and Macfarlane, 2006; Luo et al., 2005). Interestingly, Lactobacillus rhamnosus GG, an extensively studied probiotic strain (Ouwehand et al., 1999) because of its enhanced adhesive ability compared to other probiotics demonstrated biofilm formation in limited conditions tested versus what was expected based on previous literature (Fig 3.1). In vitro testing of the biofilm forming ability of L. rhamnosus GG exhibited how certain conditions, including media MRS and TSB, an acidic pH, and presence of mucin resulted in biofilm formation (Lebeer et al., 2007). Our results demonstrated L. rhamnosus GG biofilm formation rarely occurred in MRS and generally under neutral or basic pH values (Fig 3.1). P. acidilactici R1001, an understudied probiotic species that demonstrated the ability to form biofilms under numerous conditions; observed variation in probiotic biofilms exhibited in this work when comparing to other published probiotic biofilm reports may be due to the extensive number of conditions tested in this study, which have not been previously examined in such detail.
Additionally, it has been observed that slight alterations in environmental conditions may lead to modified probiotic biofilm formation (Lebeer et al., 2007); for example, variations in the availability of fermentable carbon and resulting high carbon: nitrogen ratio (Huang et al., 1994) is observed to alter biofilm formation, with lower ratios resulting in continual biofilm formation where an apparent steady state is not reached throughout the course of the experiment. This is in line with the work presented here as MRS is known to have a larger carbon: nitrogen ratio (1.9:1) versus RCM (1.4:1) and Elliker (1.4:1), with elliker being the media resulting in the greatest number of conditions that promotes biofilm formation.

The probiotic biofilm screen demonstrated the diverse conditions in which probiotic bacteria form biofilms. This may be beneficial, as many lactobacilli, one of the few indigenous genera of the GI tract (Martin et al., 2008), are observed to survive in environments with distinct nutrient compositions (Varma et al, 2010; Valeur et al, 2004; van Houte and Green, 1974). *P. acidilactici* was chosen for further study because of inconsistencies observed in the literature and increasing interest in this species for use as a probiotic because of its ability to produce pediocin. With this said, we decided to complete a more in depth analysis of the biofilm forming ability of *P. acidilactici*.

To better evaluate the ability of *P. acidilactici* to form biofilms, the high-throughput 96-well crystal violet screening assay was employed to examine multiple *P. acidilactici* strains. For these assays, quantitative analysis of *P. acidilactici* biofilms was achieved by solubilisation of the crystal violet and measurement via spectrophotometry (Fig. 3.2-3.5). The results demonstrated that every *P. acidilactici* strain tested was able to form biofilms in low and high nutrient RCM at a pH of 6. Of interest, the growth of biofilms under an initially aerobic environment versus a strictly anaerobic one was observed to play a role in biofilm formation; the effect of varying the
amount of air on biofilms grown in the low nutrient medium led to significant differences in all tested strains of *P. acidilactici* except R1001 (Fig 3.2 and 3.3). Interestingly, when grown in the low nutrient medium biofilm formation of strain 7/4A (Fig 3.2A), a human gut isolate, demonstrated significant increases under anaerobic conditions, whereas strains ATCC 25740 (Fig 3.2B) and Rbl39 (Fig 3.3A), a plant and cheese isolate, respectively, exhibited increased formation when grown in the presence of air. This information is in line with the presence or absence of air the strain was originally isolated from, as the gut is predominantly anaerobic and surfaces of plants and cheeses are aerobic. For example, work by Yoon *et al.*, (2002) demonstrated that biofilm formation by *Pseudomonas aeruginosa* isolated from mucus within the lungs of a cystic fibrosis patient (an anaerobic environment) resulted in more robust biofilm formation under anaerobic conditions.

This work also assessed the possibility of pediocin production impacting biofilm formation. To do this we compared the biofilms formed by pediocin producers, mm33 and Rbl39, to their non-pediocin producing counterparts, mm33a and Rbl39, respectively. Our results demonstrated that biofilms of both pediocin-producing strains showed significant increases in biofilm formation at the 12 h time point (Table 3.1). Interestingly, at all other time points where significant differences in biofilm formation in both the low and high nutrient media were demonstrated, the non-pediocin producing counterpart exhibited increased biofilm formation (Table 3.1). These results may suggest a role for pediocin production in early biofilm formation.
Though no relationship is known to exist between biofilms and bacteriocins it is hypothesized that the release of cellular chromosomal DNA, which occurs concomitantly with bacteriocin expression (van der Ploeg., 2005), may serve as a constituent of biofilm development, as this extracellular DNA is known to be an integral part of the biofilm matrix (Whitchurch et al., 2002). In contrast to this, the energetically demanding process of bacteriocin production (Riley and Wertz, 2002) may hinder biofilm formation at later time points. The energy saved by eliminating bacteriocin synthesis may be redirected to biofilm maintenance by increasing cell density, as bacteriocin production can alter rates of cell division (De Vuyst et al., 1996), or producing key cellular components that contribute to biofilm formation. By doing so this may contribute to enhancing the overall cellular fitness of cells within the biofilm.

To investigate the ability of specific environmental variables to influence pediocin output we isolated PC-CFS under various conditions and subjected PC-CFS to microorganisms known to be pediocin-susceptible. When PC-CFS were isolated based on the presence or absence of air within microbial cultures no trends were observed when comparing growth inhibition between the two indicators P. acidilactici ATCC 25740 (Fig 3.11 and 3.12) and L. monocytogenes ATCC 7644 (Fig 3.13 and 3.14). A study by Annuk et al., (2003) also demonstrated that different indicators show varying degrees of inhibition, as evidenced when various pathogenic indicators were tested against the antagonistic activity of lactobacilli under microaerobic or anaerobic aeration. This kind of comparison stresses the importance of aeration on pediocin output and chosen indicator.

Presently, little information is available comparing pediocin output based on air availability within cultures. Work by Anastasiadou et al., (2008, 2008a) provides the only two reports
establishing anaerobiosis alters pediocin production. This work showed that maximum pediocin production of *P. acidilactici* NRRL B5627 occurs at 60% air saturation. Similarly, Amiali *et al*, (1998) also found that a 60% air saturation level led to an eight-fold increase in nisin production in supplemented whey permeate medium versus 0, 30, or 90% air saturation after 24 h. In contrast, other studies have found anaerobic conditions result in optimized nisin output (De Vuyst and Vandamme, 1994), making it clear the effect of aeration depends on the bacteriocin.

The influence of medium on pediocin production was also evaluated, though no general trends were observed. Few reports have investigated the influence of media components on pediocin production. Moreover, comparisons involving RCM-isolated bacteriocins have not yet been made, because RCM is generally used to culture *Clostridium* spp. and not *Pediococcus* spp., even though RCM can also support robust growth of certain probiotics, such as the Bifidobacteria. A study by Biswas *et al*, (1991) investigated the ability of medium components to alter pediocin output and found supplemented Tryptone Glucose Extract broth enhanced *P. acidilactici* pediocin AcH production by 15% versus MRS. Interestingly, the composition of this medium is much less nutrient dense versus MRS that contains 7.7% and 38% yeast extract and glucose, respectively; whereas Trypticase Glucose Extract contains only 1% of both the aforementioned nutrients. Medium components such as peptone, meat extract, yeast extract and nitrogen (peptone, beef extract, yeast extract) source are known to alter bacteriocin output depending on component concentration (Todorov and Dicks, 2004; Guerra and Pastrana, 2002). The above media constituents are present in nearly identical concentrations in MRS and RCM suggesting differences in output are attributed to other components. Investigating the effect of medium components on bacteriocin production outside the *Pediococcus* genus may also give inferences about how pediocin production may be modified. Alteration of bacteriocin output may
be due to the presence of MgSO₄, glucose, or K₂HPO₄ in MRS, as they have been observed to modify bacteriocin secretion from certain lactobacilli, depending on the concentration (Toldov and Dicks, 2004). Although the work of Toldov and Dicks (2004) is evidence of how medium components may modify bacteriocin production, the effect of each medium component on bacteriocin output may be strain specific; for example, Kelly et al., (1996) observed an increase in bacteriocin production of *Lactobacillus brevis* after the addition of 16% sodium acetate to an MRS based broth whereas Ogunbanwo et al., (2003) observed no such effect when sodium acetate was added in lower amounts (0-0.3%).

Currently, to our knowledge there are no reports investigating biofilm derived bacteriocin production. This gap in bacteriocin research is interesting to note due to the ubiquity of biofilms in numerous environments, current popularity of bacteriocin research, and greater numbers of bacteriocin-producing microbes being isolated and characterized. Furthermore, *Lactococcus lactis*, producer of the most researched and commercially utilized bacteriocin, nisin, demonstrates the capacity to form biofilm (Garcia-Almendarez et al, 2008; Mercier et al, 2002; Leriche et al, 1999). Interestingly, biofilm-derived cells displayed increased indicator inhibition under anaerobic conditions (Fig. 3.13D and F; Fig. 3.14D and F), a positive result due to the anaerobic nature of the GI tract, and more specifically, the colon. Due to the lack of information on this particular topic interpreting and understanding these results prove difficult. Mathematical modelling of bacteriocin evolution reveals bacteriocin production may be advantageous in structured communities such as those found in biofilms, suggesting bacteriocin production may have evolved specifically for the biofilm environment (Watnick and Kolter, 2000). In a spatially structured environment bacteriocin-producing bacteria may be able to successfully invade and out-compete susceptible bacteria even at low densities. Outcompeting microbes susceptible to
bacteriocin occurs at a specific bacteriocin concentration to ensure susceptible organism mortality and producer proliferation (Durrett and Levin, 1997). Because of the costly nature of bacteriocin production for a cell, bacteriocin proliferation was shown to be maximized when the concentration of producer species is 50%, relative to the susceptible species (Inglis et al., 2009). Depending on the critical bacteriocin threshold and initial inoculum, coexistence of producer and susceptible species (Bucci et al., 2011) or increased mortality of the susceptible organism may occur. Bacteriocin secretion may also be advantageous in environments with high nutrient availability, (Nadell et al., 2010) as bacteriocin production is shown to be proportional to growth rate (Bucci et al., 2011). This situation may serve to be advantageous to the bacteriocin producing strain in the colon due to the high nutrient availability.

In conclusion, the ability of probiotics to form biofilms was observed to occur under specific conditions and appears to be attainable for numerous probiotic species, with factors such as aeration; pH, medium, and nutrient levels contributing to increased biofilm formation. In addition, P. acidilactici biofilm formation was shown to be influenced by aeration and possibly the ability to produce pediocin. Finally, PC-CFS isolated from P. acidilactici was observed to be altered depending on culture conditions. Speculation about why certain factors result in altered biofilm formation and indicator inhibition of isolated PC-CFS have been presented, though this work represents the first step towards a deeper understanding of probiotic biofilms as a whole and pediocin production from P. acidilactici. To grasp a greater understanding of how and why specific environmental factors alter biofilm formation and pediocin production, continued investigation which builds on these initial results is required.
Future work

This thesis offers new information pertaining to the ability of probiotics to form biofilm under multiple, laboratory-based conditions, with a specific focus on *P. acidilactici* biofilm formation and pediocin output. Future work could include assessing the biofilm forming capacity of *P. acidilactici* under gut-simulated conditions, with a focus on the colon, to grasp how biofilm development is modified in the GI environment. Additionally, *in vitro* co-culturing of *P. acidilactici* with other probiotics may serve to enhance biofilm formation, as most biofilms in the natural environment are composed of multiple species acting synergistically (Burmolle *et al.*, 2006). Furthermore, a multispecies probiotic may enhance probiotic efficacy as evidenced by Collado *et al.* (2007) who observed that combinations of probiotic species display an increased ability to displace various pathogens compared to each probiotic tested individually. Differences in the biofilm forming capacity of *P. acidilactici* in individual species or in combination could be investigated using CSLM, the primary method for assessing biofilm formation due to the hydrated nature of the samples, giving information on height and other aspects of biofilm morphology. Additionally, two-dimensional gel electrophoresis coupled with mass spectrometry could be employed to determine any uniquely expressed proteins of probiotic planktonic and biofilm lifestyles. This would aid in understanding distinct quantitative changes while giving excellent coverage of the proteome (Park *et al.*, 2014) and subsequent implications on the mode of growth established by probiotics.

Experiments examining pediocin production in this work give insight into the ability of various parameters to alter pediocin production. Pediocin output under simulated gastrointestinal conditions has been experimentally determined under planktonic conditions (Kheadr *et al.*, 2010; Fernandez *et al.*, 2012; Fernandez *et al.*, 2013). Experiments investigating the ability of *P.
*acidilactici* biofilms to produce pediocin under simulated colonic conditions would allow for comparisons to planktonic cultures and as well would give initial information of pediocin output if *P. acidilactici* was incorporated into mucosal biofilms. Additionally, because the pediocin producing strains used in this work are not classified as probiotics, by isolating the pediocin-containing plasmid and transforming it into a known *P. acidilactici* probiotic, such as strain R1001, enhancement of an already known probiotic species may occur in the form of pathogen inhibition. This type of experiment would also be used to analyze how indigenously non-pediocin producing strains of *P. acidilactici* could produce pediocin and to what level.

Further investigations may provide answers to how and why probiotics form biofilms as well as how this phenomenon can be exploited. To make significant steps in moving forward, research should focus on increasing the physiological relevance by moving away from laboratory conditions and towards simulated colonic media or cell culture techniques to assess how biofilm formation and pediocin output are modified. For example, biofilm formation could be investigated anaerobically using Macfarlane’s medium to simulate colonic conditions, using Caco-2 cells to assess biofilm formation or by fluorescently labelling *P. acidilactici* strains to determine their ability to colonize gut microbial community simulators such as chemostats.

This thesis examined the conditions leading to probiotic biofilm formation. It was observed that all tested probiotics were able to form biofilms under at least one condition. Due to the numerous conditions leading to biofilm formation, lack of previous biofilm characterization, and current interest in pediocins, *P. acidilactici* was chosen for further experimentation. It was demonstrated that both anaerobiosis and ability to produce pediocin altered the biofilm forming ability of *P. acidilactici*. Lastly, it was observed variations in culture conditions including; medium, the absence of air, and mode of growth lead to variation in pediocin production. This
work will act as an initial step towards understanding the factors underlying biofilm formation and pediocin production, and to extend this knowledge to more colonic related environments. Future work will hopefully lead to the development of probiotics with the ability to prolong colonic retention to extend probiotic benefits.


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Appendix

Solution Preparation

SEM buffer

SEM buffer was prepared by mixing sodium phosphate dibasic (Na$_2$HPO$_4$), at a concentration of 0.07 M with potassium phosphate (KH$_2$PO$_4$), at concentration 0.07 M, in a 50/50 ratio.

PBS

The phosphate buffered saline (PBS) buffer used for biofilm assays was 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 1.8 mM KH$_2$PO$_4$, adjusted to pH 7.4 with HCl.

Medium Components

Elliker (31 g/l)

20 g Tryptone
5 g Yeast extract
4 g Sodium chloride
1.5 g Sodium acetate
0.5 g Ascorbic acid