University of Alberta

Selection of Lactic Acid Bacteria and Their Metabolites for Preventing and Treating Infections

by

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Abstract

Increasing prevalence of multidrug-resistance bacteria from extensive antibiotic usage and shortages of new classes of antibiotics have created urgent needs for alternative prevention and treatment options for infections. Based on the hypothesis that lactic acid bacteria (LAB) and their metabolites can prevent and treat infections, this thesis aims to demonstrate this by developing probiotic applications to target the problem of metritis in the dairy industry, and evaluate LAB synthesized glycans for their potential in preventing and treating post-weaning diarrhoea (PWD) in swine production.

Using culture-dependent analysis, PCR clone library construction, and quantitative PCR (qPCR), main bacterial species that constituted the microbiota of both healthy and infected bovine reproductive tracts were isolated and identified. Using selected LAB from healthy cows, freeze dried probiotics were prepared and used as intravaginal administrations for treating infected cows. Animal trial results obtained by Dr. Ametaj's research group have indicated improvements in the reproductive performance of the treated cows.

In terms of evaluating LAB derived glycans for preventing and treating PWD in swine, the porcine erythrocyte haemagglutination model was used to test for anti-adhesion properties in LAB exopolysaccharides (EPS). Glycans including reuteran, glucan and levan that were produced by *Lactobacillus reuteri* were found to exhibit anti-adhesion properties. However, animal studies will need to be conducted to confirm potential *in vivo* effects. In order to produce novel and

potentially anti-adhesive glycans that can prevent a wider range of pathogenic bacteria from adhering to their target hosts, alpha-galactosidases (α-Gal) from *Lb. reuteri,* which are capable of producing alpha-galacto-oligosaccharides (α-GOS) were cloned and characterized. Using different acceptor sugars, the production of a variety of α-GOS was detected by high performance liquid chromatography (HPLC) and electrospray ionization tandem mass spectrometry (ESI-MS/MS). Both *in vitro* and *in vivo* anti-adhesion studies will be required to further studies the potential anti-adhesive properties of novel enzyme-synthesized α-GOS.

Results obtained from this thesis research will facilitate the development of versatile novel probiotics and therapeutic glycans for applications in food and feed products in order to enhance the health of farm animals and humans alike.

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III. List of Abbreviations

α-Gal	Alpha-galactosidase
β-Gal	Beta-galactosidase
α-GOS	Alpha-galactooligosaccharides
β-GOS	Beta-galactooligosaccharides
AMPs	Antimicrobial peptides
ATP	Adenosine triphosphate
CCE	Crude cell extracts
CFU	Colony-forming unit
Da	Dalton
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DP	Degree of polymerization
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *Escherichia coli*
EPEC	*Enteropathogenic E. coli*
EPS	Exopolysaccharids
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
ETEC	*Enterotoxigenic E. coli*
g	Gram
GI	Gastrointestinal
GOS	Galacto-oligosaccharides
GRAS	Generally recognized as safe

HBD	Human beta defensin
HeOS	Heterooligosaccharides
HePS	Heteropolysaccharides
HMO	Human milk oligosacchardies
HPLC	High performance liquid chromatography
HoPS	Homopolysaccharides
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kb	Kilobase
kDa	Kilodalton
L	Litre
LAB	Lactic acid bacteria
LB	Luria-Bertani broth
MRS	deMan-Rogosa-Sharpe medium
min	Minute
mL	Mililitre
µL	Microlitre
µM	Micromoles per litre
mM	Milimoles per litre
mMRS	modified deMan-Rogosa-Sharpe medium
OS	Oligosaccharides
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDW	Post-weaning diarrhoea

qPCR	quantitative real time PCR
RAPD	Random amplification of polymorphic DNA
RCM	Reinforced clostridial medium
rDNA	ribosomal DNA
RDP-II	Ribosomal Database Project II
RFO	Raffinose family oligosaccharides
RI	Refractive index
RNA	Ribonucleic acid
rRNA	ribosomal RNA
SLT	Shiga-like toxin
STEC	Shiga-like toxin producing *E. coli*
TBE	Tris-Borate-EDTA
TGF	Transforming growth factor
TNBS	Trinitrobenzenesulphonic acid
VRE	Vancomycin-resistant enterococci

1. Introduction

1.1. Lactic Acid Bacteria

Lactic acid bacteria (LAB) are a group of diverse Gram-positive bacteria that are generally acid tolerant, non-spore forming, fastidious rods or cocci that grow under microaerophilic to strictly anaerobic conditions, and produce lactic acid as the major end product of carbohydrate fermentation (Carr et al., 2002; Stiles & Holzapfel, 1997). Since the original term of LAB historically lacked phylogenetic significance, the definition and taxonomic classifications of LAB have been riddled with controversies. LAB can be phylogenetically classified as members in the order of *Lactobacillales*, which is under the phylum of Firmicutes. According to Ludwig, Schleifer, and Whitman (2009) in Bergey's Manual Systematic Bacteriology, there are currently six families (*Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, *Streptococcaceae*) within the *Lactobacillales* order, which contain a total of 36 genera. As detailed in **Table 1-1**, habitat, pathogenicity and relevance to the food processing among these genera can vary enormously. A majority of *Lactobacillales* members are commensals that are capable of causing a variety of opportunistic infections. More specifically, the *Aerococcaceae* and *Carnobacteriaceae* families largely contain pathogens with little relevance to food; whereas *Lactobacillaceae, Enterococcaceae, Leuconostocaceae* and *Streptococcaceae* commonly occur in different foods. Although the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* were historically considered as the core group of LAB, principal LAB genera that are commonly

used for food fermentations, have currently expanded to include *Enterococcus*, *Carnobacterium*, *Lactococcus*, *Leuconostoc,* *Oenococcus*, and *Weissella* (Axelsson, 2004). The term "LAB" used in subsequence will exclusively refer to these relevant genera, and discussions on probiotics will be focused on probiotic LAB throughout this thesis.

Table 1-1. Lab families and genera with regards to habitat, pathogenicity and relevance to foods

Families with Genera Examples	Sources	Association with Diseases in Humans or Animals	Relevance to Foods	Ref.
Aerococcaceae				
Abiotrophia	Isolated from oral cavity	endocarditis, bacteraemia	n.a.	1–3
Aerococcus	Habitat: concomitant flora	urinary tract infections, bacteraemia	n.a.	4–6
Dolosicoccus	Isolated from human blood; habitat unknown	pneumonia	n.a.	7
Eremococcus	Isolated from horse reproductive tract; habitat unknown	n.d.	n.a.	8
Facklamia	Isolated from human clinical samples *e.g.* blood, spinal fluid, urine, abscesses; nasal cavity of juvenile elephant seal; habitat unknown	pneumonia, bacteraemia	n.a.	9–11
Globicatella	Isolated from human clinical specimens *e.g.* blood, urine and spinal fluid; lungs and joint-lesions of swine and ruminants; habitat unknown	bacteraemia, meningitis, meningo-encephalitis; purulent infections in domestic animals	n.a.	12–16
Ignavigranum	Isolated from human clinical specimens *e.g.* wound infection and ear abscess; habitat unknown	wound infection, mastoiditis	n.a.	17
Carnobacteriaceae				
Alkalibacterium	Isolated from decaying marine algae & seagrass, raw fish, salted fish and 'ka-pi' (salted & fermented shrimp paste) from temperate areas; also isolated from artificial alkaline environment *e.g.* edible-olive wash waters, *Polygonum* indigo fermentation liquor	n.d.	F/-/-	18–22
Allofustis	Isolated from porcine semen specimens	n.d.	n.a.	23
Alloiococcus)	Isolated from middle ear fluid of children with otitis media	acute otitis media & otitis media with effusion	n.a.	24,25
Atopobacter	Isolated from carcasses of common seals	n.d.	n.a.	26
Atopococcus	Isolated from moist powdered tobacco	n.d.	n.a.	27
Atopostipes	Isolated from an underground swine manure storage pit	n.d.	n.a.	28

Note: n.a., not applicable; n.d., not determined; bold: Family; non-bold: Genus; F: Food/feed fermentation; S: Food Spoilage, P: Probiotics

Table 1-1. Continued: Lab families and genera with regards to habitat, pathogenicity and relevance to foods

Families with Genera Examples	Sources	Association with Diseases in Humans or Animals	Relevance to Foods	Ref.
Carnobacterium	Habitat: temperate and polar aquatic and terrestrial environments; fresh water habitats; present in food including fish, meat and dairy products; isolated from marine sponges, and live fish often as fish pathogens	infections in fish	F/-/P	29–33
Desemzia	Isolated from the ovaries of the lyreman cicada (*Tibicen bacterium*)	n.d.	n.a.	34,35
Dolosigranulum	Isolated from human clinical specimens *i.e.* two sets of blood cultures	synovitis	n.a.	36,37
Granulicatella	Habitat: normal microbiota of human oral, urogenital & intestinal tracts	bacteraemia, infective endocarditis, eye ulcers	n.a.	1,2,38,39
Isobaculum	Isolated from small intestine of a badger carcass	n.d.	n.a.	40
Marinilactibacillus	Isolated from core samples of deep sub-seafloor sediments; coastal sub-seafloor sediments; living or decomposing sponges, algae, shellfish, crabs, and fish in temperate and subtropical areas	n.d.	n.a.	41–43
Trichococcus	Isolated from guano of Magellanic penguins (*Spheniscus magellanicus*) in Chilean Patagonia; gas-condensate-contaminated soil in Colorado	n.d.	n.a.	44–46
Enterococcaceae				
Enterococcus	Habitat: human intestinal tract, especially as members of neonatal intestinal microbiota & large bowel microbiota; natural microbiota gastro-intestinal microbiota of mammals and birds; microbiota of artisanal cheeses produced in southern Europe; Isolated from soil, plants, and water possibly due to contamination	nosocomial infections *e.g.* bacteraemia, endocarditis, urinary tract infections often followed by intra-abdominal and pelvic infections	F/S/P	47–53
Melissococcus	Isolated from diseased honey bee larvae with clinical signs	European foulbrood (an infectious disease of honeybee larvae)	n.a.	54,55
Tetragenococcus	Isolated from fermented squid liver sauce; a Japanese local traditional fermented fish sauce (Shottsuru); Japanese soy mash; Indonesian soy mash (kecap)	n.d.	F/-/-	56–58

Note: n.a., not applicable; n.d., not determined; bold: Family; non-bold: Genus; F: Food/feed fermentation; S: Food Spoilage, P: Probiotics

Table 1-1. Continued: Lab families and genera with regards to habitat, pathogenicity and relevance to foods

Families with Genera Examples	Sources	Association with Diseases in Humans or Animals	Relevance to Foods	Ref.
Vagococcus	Isolated from human clinical samples *i.e.* blood, peritoneal fluid & wounds, a root-filled tooth with periradicular lesions; domestic animals *e.g.* chickens (chicken faeces), pigs (swine clinical samples & swine manure storage pit), cattle (in ground beef), horses & cats; seal & harbour porpoise carcasses, common otter, fish with peritonitis (in Atlantic salmon, rainbow trout and brown trout); well & river water	vagococcosis in fish; potential association with opportunistic infections in humans and animals	n.a.	59–70
Lactobacillaceae				
Lactobacillus	Habitat: oral cavities, gastrointestinal and reproductive tracts of humans and animals; isolated from fermented foods which include dairy, meat, fish/seafood, plants & sourdough based foods; also isolated as spoilage bacteria beer & marinated herrings	bacteraemia & sepsis; endocarditis; urinary tract infections	F/S/P	71–86
Pediococcus	Habitat: microbial flora of forage crops & influences the fermentation characteristics of silage; commonly isolated from vegetable, dairy & meat fermentations; often isolated as beer-spoilage bacteria; also isolated from human respiratory tract; human/animal intestinal & reproductive tract; human clinical specimens *e.g.* saliva, urine, stool, wounds, abscesses, peritoneal fluid, and blood	endocarditis pneumonitis, bacteremia, septicemia, hepatic abscesses; gaffkemia in lobsters	F/S/P	85,87–104
Leuconostocaceae				
Leuconostoc	Habitat: green vegetation and roots; Isolated from vegetables, silage & fermented vegetable products, sourdough, dairy products, meat, cattle, fish, insects; human sources from faeces, vaginal samples & breast milk samples; cause severe quality problems in sugar beet processing and storage; clinical specimens of internal fluids (*e.g.* blood, urine & cerebrospinal fluid)	pneumonia, bacteremia, nosocomial urinary tract infections	F/S/-	85,105–117
Oenococcus	Habitat: restricted to wine and related ecological niches	n.d.	F/S/P	118–120

Note: n.a., not applicable; n.d., not determined; bold: Family; non-bold: Genus; F: Food/feed fermentation; S: Food Spoilage, P: Probiotics

Table 1-1. Continued: Lab families and genera with regards to habitat, pathogenicity and relevance to foods

Families with Genera Examples	Sources	Association with Diseases in Humans or Animals	Relevance to Foods	Ref.
Weissella	Habitat: soil; Isolated from fresh vegetables, sugar cane, carrot juice, fermented products including silage, sourdough, fish, meat; beaked whale (*Mesoplodon bidens*), raw milk & sewage; human & animal clinical samples *e.g.* canary liver, canine otitis sample & human faeces	septicemia in mona monkey (*Cercopithecus mona*); endocarditis in human	F/S/-	121–128
Streptococcaceae				
Streptococcus	Habitat: bacterial flora of fermented dairy products including yogurt, cheese & natural starters; commensal microbiota of the respiratory tract of humans & animals and can colonize tooth enamel; dental plaque of humans & monkeys (*Macaca fascicularis*); human infant faeces; bovine mouth, throat and faeces	pharyngitis, pneumonia, dental caries, bacteremia, toxic shock syndrome, rheumatic fever, Scarlet fever, acute glomerulonephritis, necrotizing myositis & fasciitis	F/S/P	129–146
Lactococcus	Isolated from green plant sources (*e.g.* broccoli, corn, potatoes, cucumbers, sweet peas, beans, cantaloupes); fresh & fermented dairy products (*e.g.* colostrum, raw milk, cream, cottage cheese, yogurt & aged cheeses); body & tail of cows & goats; regarded as important for starter cultures	infective endocarditis, liver abscess, and osteomyelitis in humans; mastitis in cows; infections in fish and fresh-water prawns	F/S/P	147–155
Lactovum	Isolated from acidic forest floor solution (with *in situ* pH of 4.5)	n.d.	n.a.	156

Note: n.a., not applicable; n.d., not determined; bold: Family; non-bold: Genus; F: Food/feed fermentation; S: Food Spoilage, P: Probiotics

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1.2. LAB as Probiotics and Their Applications in Humans and Animals

Since the probiotic concept was first introduced by Metchnikoff (1907), numerous LAB species within genera such as *Lactobacillus*, *Pediococcus* and *Enterococcus* have been recognized as probiotics, which are defined as "live micro-organisms that confer health benefits on their hosts when adequately administered" (FAO/WHO, 2001; Fuller, 1989; Klein et al., 1998; Osmanagaoglu et al., 2011; Sanders, 2008; Temmerman et al., 2003). Probiotic LAB have generally been viewed as useful and inexpensive prophylactic and treatment alternatives for human gastrointestinal infections by restoring disturbed intestinal microbiota with little side effects (Hatakka & Saxelin, 2008). While much of research and clinical applications in LAB probiotics have been primarily targeted toward infectious diarrhoea such as those caused by *Clostridium difficile*, the efficacy of these probiotics in preventing and treating infectious diseases at proximal mucosal surfaces of the gastrointestinal (GI) including the oral cavity and the stomach, as well as at distal mucosal surfaces of such as the upper respiratory tract, the lungs, and the urogenital tracts have also been investigated and reported (Dendukuri et al., 2005; de Vrese & Schrezenmeir, 2002). Despite the need for continuing effort in elucidating the underlying mechanisms of probiotics, convincing evidence of probiotic LAB in enhancing both local systemic immune responses of the host was observed (Sheih et al., 2001; Yasui et al., 1999). Hence it is important to note that through stimulating host immune systems or translocation, orally administered probiotics can either directly or

indirectly exert health effects elsewhere in the body, and that the administration of probiotics is not exclusive to the oral route (Morelli et al., 2004). In addition to infectious diarrhoea, urogenital infections, promising results in the uses of probiotic LAB to ameliorate human diseases associated with the immune system, which include inflammatory bowel disease, and food allergy have been demonstrated in both human clinical trials and animal studies (Gorbach, 1990; Jonkers & Stockbrügger, 2003; Majamaa & Isolauri, 1997; Malin et al., 1996; McGroarty, 1993).

Applications of probiotics in improving resistance against infectious bacterial diseases are shared among those for human use and animal use. However, probiotic use for farm animals places more emphases on the enhancement of animal growth performance (Collado, 2009). In dairy cattle and pigs, probiotic LAB have been reported to increase resistance to infections as well as to improve feed conversion ratios among the animals (Anadón et al., 2006; Dawson et al., 1990; Di Giancamillo et al., 2008). There have also been encouraging results of using LAB fecal isolates to reduce *Escherichia coli* O157:H7 shedding in cattle as well as potential uses of LAB metabolites for neutralizing enterotoxin from swine specific pathogenic *E. coli* (Brashears, Jaroni, & Trimble, 2003; Mitchell & Kenworthy, 1976).

Overall, probiotic LAB can elicit numerous health-promoting effects in their mammalian hosts, which include immune modulation, pathogen exclusion through interfering with pathogen adhesion, and pathogen growth inhibition from the production of bactericidal compounds (Hatakka & Saxelin, 2008; Lee, 2009).

All of the above mentioned health promoting aspects of probiotic LAB will be discussed in more details in the ensuing sections of this introductory chapter. Potential probiotic applications in preventing and treating metritis in dairy cattle and post-weaning diarrhoea in pigs will be further discussed in subsequent chapters.

1.3. Immunomodulation Effects of Probiotic LAB

Probiotic LAB modulate both of the mammalian innate and adaptive immune responses, which help to increase infection and disease resistance in their hosts (Walker, 2008). More specifically, the innate immunity can be enhanced by stimulating the secretion of anti-adhesive and antimicrobial compounds including anti-adhesive glycoproteins, namely mucins and antimicrobial peptides (AMPs) such as defensins, as well as increasing activities in key cells of the innate immune system such as macrophages and natural killer cells (Kato et al., 1983, 1984; Ogawa et al., 2006; Perdigón et al., 1986, 1988; Schiffrin et al., 1997). Mucins are primarily produced by goblet cells in the gut (Deplancke & Gaskins, 2001). Defensins can be produced by a wide range of cells including neutrophils, and epithelial cells of the skin, airways, pancreas, kidneys, intestinal tract and urogenital tracts (Ganz & Lehrer, 1994; Jones & Bevins, 1992; McCray & Bentley, 1997; Zhao et al., 1996). Defensins have been found to induce the recruitment of dendritic cells and T cells to the site of infection via chemotaxis (Yang, 1999). Through dendritic cells, the link between the innate and adaptive immune systems can be effectively established to enhance the host's defense against microbial invasions (Steinman & Hemmi, 2006). Dendritic cells confer

such protection by secreting protective proteinaceous interleukin-12 (IL-12) and type I interferons, as well as coordinating the differentiation, activation and proliferation of naïve CD4+ T cells into Th1, Th2 or Th3 cells (Delcenserie et al., 2008). Interestingly, probiotics appear to be able to directly modulate the immune response in dendritic cells. Specific gene loci that corresponded to such immunomodulation properties have been identified in a probiotic *Lactobacillus plantarum* strain using comparative genome hybridization (Meijerink et al., 2010). The binding of probiotics to dendritic cell via specific intercellular adhesion molecules is essential for inducing the differentiation of T cells into regulatory T cells, which resulted in increased production and release of the anti-inflammatory cytokine IL-10 (Smits et al., 2005).

Additional ways for probiotic LAB to stimulate the adaptive immune system include the activation of mucosal B cells, and the increase in the production of immunoglobulin A (IgA) (Corthesy et al., 2007; Kaila et al., 1995; Link-Amster et al., 1994; Majamaa & Isolauri, 1997; de Moreno de LeBlanc et al., 2008). IgA coats the surface of pathogenic microorganisms which prevents them from binding to the surface of the GI tract and facilitates their elimination by phagocytes. Furthermore, modulation of cytokine production by specific probiotic LAB strains systemically or locally in the host gut can elicit different inflammatory responses (Isolauri, 2001; Maassen et al., 2000). For example, using a murine colitis model, the administration of a probiotic mixture of *Lactobacillus* spp., *Bifidobacteria* spp., and *Streptococcus salivarius* (VSL#3) during remission periods was able to induce immunoregulatory responses in T-

cells in expressing transforming growth factor (TGF)-β, and thereby reduce the severity of intestinal inflammation (Di Giacinto et al., 2005). Moreover, strain specific properties such as the anti-oxidative properties in *Lactobacillus fermentum* through glutathione production, has been shown to modulate the production of mediators involved in intestinal inflammatory response such as TNFα and nitrogen oxides and expedite the recovery of inflamed tissue in the trinitrobenzenesulphonic acid (TNBS) model of rat colitis *(*Peran et al., 2006).

As the discussion on immune modulatory effects of probiotics is vastly complex and far beyond the scope of this thesis, further information on the influence of probiotics in the modulation of host immune responses especially with regards to luminal microecology and mucosal barrier functions can be obtained in reviews by Madsen (2006) and Sherman et al. (2009). Nevertheless, the roles that AMPs play in conferring host immunity in regards with defensins and bacteriocins will be discussed in more details.

1.4. Host Derived AMPs: Defensins

1.4.1. Antimicrobial Peptides

AMPs are ubiquitously found in living organisms ranging from single-celled microorganisms to plants, invertebrates including insects, and vertebrates such as fish, amphibians, birds and mammals (Martin, Ganz, & Lehrer, 1995; Wang et al., 2011; Wang & Wang, 2004). They are small peptides (10 to 100 amino acids) with molecular weights of less than 10 kDa, which confer broad-spectrum antimicrobial defense against pathogenic bacteria, fungi, protozoa and

viruses (Hancock, 1997; Kulkarni et al., 2009; Sang & Blecha, 2008). Since their discovery, research in AMPs have gained immense momentum, primarily in the pursuit of developing alternative antimicrobial therapy for treating bacterial infections, as multiple-drug resistant bacteria become increasingly prevalent in both clinical and veterinary settings (Fedders, Podschun, & Leippe, 2010; Giuliani, Pirri, & Nicoletto, 2007; Hancock & Sahl, 2006; Park, Park, & Hahm, 2011; Wang et al., 2011; Witte, 1998).

1.4.1.1. Mammalian Defensins

As part of an important group of AMPs found in higher vertebrates, defensins have been identified in animals including humans, non-human primates, cows, sheep, goats, rats and mice (Bals, Goldman, & Wilson, 1998; Boniotto et al., 2003; Del Pero et al., 2002; Ganz, Selsted, & Lehrer, 1990; Ganz & Lehrer, 1998; Huttner et al., 1998; Lehrer & Ganz, 1999; Meyerholz et al., 2004; Pardi et al., 1988; Roosen et al., 2004; Selsted & Harwig, 1989; Selsted et al., 1993; Yount et al., 1995; Zhao et al., 1999). Mammalian defensins (2.0 kDa to 6.0 kDa) are grouped into three subfamilies as α-, β- and θ-defensins, based on their structural differences (Ganz et al., 1985; Lehrer & Ganz, 2002; Tang, 1999).

1.4.1.1.1. Alpha-Defensins

Alpha-defensins are largely characterized by the presence of three intramolecular disulfide bonds that are formed from specific pairing of cysteine residues within a six-cysteine motif (Selsted et al., 1985). Of six human α-defensins (HNP-1-6) identified thus far, four α-defensins (HNP-1-4) were found to be expressed in granulocytes and particular lymphocytes, and the other two

(HD-5-6) were predominantly produced by secretory Paneth cells that reside within small intestinal crypts (Lehrer & Ganz, 2002; Ouellette & Bevins, 2001). Remarkably, HNP-1-3 collectively encompass 30% to 50% of the total protein content within the azurophil granules inside polymorphonuclear neutrophils, while HNP-4 defensins were found to be roughly 100 fold lower in abundance (Ganz et al., 1990; Selsted et al., 1985; Wilde et al., 1989). Furthermore, gene expression, immunolocalization, and secretion of HD-5 were detected in the human female reproductive tract epithelia, and evidence suggested that HD-5 is potentially an essential constituent of the innate female urogenital immune system and that hormonal and proinflammatory factors were most likely to have contributed in regulating the HD-5 gene expression (Quayle et al., 1998).

1.4.1.1.2. Beta-Defensins

Beta-defensins differ from α-defensins by precursor structures, the arrangement of three intramolecular disulfide bonds [(Cys1–Cys5, Cys2–Cys4, Cys3–Cys6) versus (Cys1–Cys6, Cys2–Cys4, Cys3–Cys5)], and gene expression patterns (Lehrer & Ganz, 1999). Unlike the presence of α-defensins which is limited to humans, monkeys and several rodent species, β-defensins are ubiquitously found in all mammalian species that have been studied to date (Yang et al., 2002). In domestic cattle, β-defensins are produced by epithelial cells present in a wide range of tissues, which include the epidermis, the tongue, and the respiratory and GI tracts (Selsted et al., 1993; Zhao et al., 1999). There are also at least thirteen β-defensins that were isolated from bovine neutrophils (Selsted et al., 1993; Zimmermann et al., 1995). After the initial discovery several

bovine peptides, the first human β-defensins (HBD) were isolated from hemofiltrate collected from patients diagnosed with end-stage renal diseases (Bensch et al., 1995). Since then, at least four HBDs (HBD1-4) have been detected and as extensively reviewed by Schneider et al. (2005), HBD-1 was found in the epithelia of the trachea, bronchi, small airways and mammary glands, as well as expressed in keratinocytes from normal skin, sweat glands, inflammatory skin lesions, and epithelial cells of the thymus, pancreas, kidneys, small intestines, prostate, testis, vagina, ectocervix, endocervix, uterus, fallopian tubes, and the placenta. Similarly, HBD-2 was expressed in the gingival mucosa, keratinocytes, and the tracheal epithelium; whereas HBD-3 have been isolated from keratinocytes from human lesional psoriatic scales, and found to be expressed in epithelia of the respiratory, gastrointestinal, and genitourinary tracts as well as non-epithelial tissues such as leukocytes, heart and skeletal muscles. Lastly, HBD-4 was found to be highly expressed in the gastric antrum and the testis, but lower levels of expression was also observed inside neutrophils and in the epithelia of thyroid glands, lungs, uterus, and kidneys.

1.4.1.1.3. Theta-Defensins

Theta defensins are cyclic octadecapeptides that are formed by the ligation of two truncated α-defensins (Selsted, 2004; Tang, 1999; Trabi, Schirra, & Craik, 2001). These defensins were first discovered in leukocytes and bone marrow of Rhesus macaques, but humans only possess pseudogenes of θ-defensins and express ancestral θ-defensins known as retrocyclins (Nguyen, Cole, & Lehrer, 2003).

1.4.1.2. General Mode of Action of Defensins

As extensively reviewed by Yang et al. (2002), defensins are not only key regulators of the innate immune system, but they also intricately affect the adaptive immune system of their host in order to provide protection against infections caused by a wide spectrum of bacteria, fungi and viruses (Bastian & Schäfer, 2001; Ganz & Lehrer, 1998; Lehrer & Ganz, 1999; Yasin et al., 2004). Although the mode of action of defensins is poorly understood, strong evidence indicated the disruption of the integrity and function of microbial phospholipid membrane through attachment, insertion, and channel formation was likely to cause death or inactivation of targeted microorganisms (Bauer et al., 2001; Ganz, 2003; Hoover et al., 2000; Hristova, 1997). In the case of inactivating viruses, it has been found that defensins can directly bind to them via specific viral receptors and prevent their entry into the host cell (Cole et al., 2002; Nguyen et al., 2003; Yasin et al., 2004).

1.5. Bacterial AMPs: Bacteriocins

1.5.1. Bacteriocins

Similar in composition as defensins, bacteriocins are bacterial AMPs that act as effective defence mechanisms for enhancing the survival of a particular strain of bacteria against other competing strains in the environment (Jenssen, Hamill, & Hancock, 2006; Nissen-Meyer & Nes, 1997; Riley, 1998). These inherent antimicrobial properties in bacteria are highly desirable in probiotic LAB strains as they offer promising alternative opportunities for preventing and treating intestinal and non-intestinal infectious diseases in mammalian hosts

(Hatakka & Saxelin, 2008). In general, bacteria produce bacteriocins that are effective against other closely related species. Examples include thuricin that is produced by *Bacillus thuringiensis* and colicins that are produced by *Escherichia coli* (Braun et al., 1994; Favret & Yousten, 1989; Héchard & Sahl, 2002). In comparison to AMPs produced by eukaryotic organisms, which are active at micromolar concentrations, bacteriocins are overall more potent, with effective concentrations at pico- to nanomolar ranges (Fimland et al., 2005). Interestingly, LAB synthesized bacteriocins were found to exhibit strong synergistic antimicrobial effect with the eukaryotic AMP pleurocidin from fish (Luders et al., 2003). Presently, bacteriocins produced by food-grade and probiotic LAB are particularly valued commercially for their antimicrobial applications in the preservation of food and feed against foodborne pathogens and spoilage bacteria (Gálvez et al., 2007; Nes et al., 2007). In addition, a broad-spectrum LAB bacteriocin named lacticin 3147, which was produced by food-grade *Lactococcus lactis*, have also demonstrated efficacy in preventing mastitis in dairy cattle (Ryan et al., 1999). The following sections aim to discuss the classification scheme of bacteriocins before the modes of action of specific bacteriocins including nisins and pediocins are discussed in more depth.

1.5.2. Classification of Bacteriocins

Bacteriocins are peptides that are ribosomally synthesized in bacteria and archaea, which can differ widely in terms of their spectrums of activity, modes of action, and structures of amino acids (Cotter, Hill, & Ross, 2005; Jack, Tagg, & Ray, 1995; Tagg, Dajani, & Wannamaker, 1976). Consequently, classification

and naming of bacteriocins has been extensively disputed due to groupings that were largely inconsistent and incoherent. The classification scheme devised by Cotter et al. (2005) has remained to be the most accepted, despite recent efforts by Zouhir et al. (2010) to reclassify bacteriocins according to their amino acid sequences, and the attempts made by Nes et al. (2007) to exclude class III bacteriocins (**Table 1-2** as indicated by [*]).

Table 1-2. Universal bacteriocin classification scheme (Cotter, Hill, & Ross, 2005)

Class I Lantibiotics	**Class II Unmodified peptides**	**Class III* Large Proteins**
Ia Linear	IIa Pediocin-like	IIIa Bacteriolytic
Ib Globular	IIb Multi-component	IIIb Non-lytic
Ic Multi-component	IIc Cyclic peptides	
	IId Miscellaneous	

* indicates possible exclusion from this classification system

1.5.2.1. Class I Bacteriocins

Class I bacteriocins, or more commonly known as lantibiotics, are peptides characterized with post-translationally modified thioether amino acids dehydroalanine, dehydrobutyrine, lanthionine and β-methyl-lanthionine, which confer structural conformation and stability (Chatterjee et al., 2005; Kellner et al., 1988; Klaenhammer, 1993; Skaugen et al., 1994). These bacteriocins can be further divided into three subclasses based primarily on secondary structural features and mode of action as they cannot be homogenously grouped by size, structure or mode of action alone (Guder et al., 2000; Willey & van der Donk, 2007). As traditionally classified by Jung (1991), Class Ia peptides are linear, cationic, and can inhibit bacteria by forming pores in the bacterial membrane; whereas Class Ib lantibiotics are more rigid and globular in structure. Class Ic

lantibiotics have more than one peptide in their structures, wherein a globular α-peptide can complex with an elongated β-peptide to collectively inhibit cell wall synthesis and form pores in sensitive bacteria (Dischinger et al., 2009; Morgan et al., 2005; Wiedemann et al., 2006).

1.5.2.2. Class II Bacteriocins

Unlike Class I bacteriocins, Class II bacteriocins are small, heat stable peptides that are not translationally modified with unusual amino acids (Nes et al., 1996). These bacteriocins can also be grouped into three subclasses. Class IIa is comprised of *Listeria* inhibitory and pediocin-like bacteriocins, which contain conserved amino acid sequences of YGNGVXCXXXXCXV and disulfide bridges that are formed by two cysteine residues in the peptide N-terminals (Eijsink et al., 1998). Class IIb multi-component bacteriocins include bacteriocins that often require two peptides to have antibacterial activities (Anderssen et al., 1998; Nissen-Meyer et al., 1992). Class IIc bacteriocins are cyclic bacteriocins that are formed by the covalent linkage of the N- and C- termini (Kawai et al., 2004; Maqueda et al., 2008). Class IId bacteriocins contain miscellaneous peptides that are linear, non-pediocin like, sometimes lacking a leader peptide or can be activated by reduced cysteine residues (Diep & Nes, 2002; Franz et al., 1999; Venema et al., 1993).

1.5.2.3 Class III Bacteriocins

Class III bacteriocins are heat-labile proteins with high molecular mass of greater than 30 kDa and inactivation conditions of between 60 to 100 ºC for 10 to 15 minutes (Jörger et al., 1986). Though highly debated and controversial, Class

IIIa bacteriocins are considered to be bacteriolyic and Class IIIb bacteriocins have non-lytic bactericidal activities (Nilsen et al., 2003; Tagg, 1984; Thompson et al., 1996; Vaughan et al., 1992). Examples of each class of bacteriocins are shown in **Table 1-3**.

1.5.3. General Characteristics of Class I and Class II Bacteriocins and Their Mode of Action

Class I and II bacteriocins are usually positively charged, amphiphilic and membrane-active peptides with low molecular weights of between 2 and 10 kDa; although neutral and anionic peptides also exist (Lee & Kim, 2011; Nes et al., 2007). Both classes of bacteriocins that are produced by LAB are especially suited for applications in food safety and preservation technology due to biosafety and their ability to resist extreme pH, temperature and salinity, albeit they are inactivated by proteolytic enzymes of pancreatic and gastric origins such as trypsin, chemotrypsin and pepsin (Alvarez-Cisneros et al., 2011). Since nisin and pediocin are key respective representatives of Class I and Class II bacteriocins and they share some common inhibition mechanisms, their modes of actions will hence be discussed in more depth.

In terms of mode of action of bacteriocins, they differ greatly in their chemical structures and their consequential effects on essential cellular processes in transcription, translation, replication, and cell-wall formation (Oscáriz & Pisabarro, 2001). Nevertheless, the most commonly found mechanistic action involves the generation of membrane pores or channels, which destroy sensitive cells by depleting their proton motive force and thus their energy potential.

Table 1-3. Examples of Class I, Class II and Class III bacteriocins produced by LAB

Bacteriocin	Producer strains	Inhibitory Spectra	References
Class Ia			
Nisin A/Nisin Z	*Lactococcus lactis*	*Bifidobacterium* spp.; *Clostridium* spp.; *Enterococcus* spp.; *Lactobacillus* spp.	Le Blay et al., 2007
Class Ib			
Lacticin 481	*Lactococcus lactis*	Wide range of LAB, *Staphylococcus carnosus*; *Clostridium tyrobutyricum*	Dufour et al., 2007; Piard et al., 1992
Thermophilin 1277	*Streptococcus thermophilus*	*Lactobacillus* spp.; *Clostridium* spp.; *Microbacterium* spp.; *Bacillus cereus*; *Enterococcus fecalis*	Kabuki et al., 2009; Kabuki et al., 2007
Class Ic			
Lacticin 3147	*Lactococcus lactis* DPC3147	*Acetobacter* spp.; *Bacillus* spp.; *Clostridium* spp.; *Enterococcus* spp.; *Lactobacillus* spp.; *Pediococcus* spp.; *Listeria monocytogenes*; *Streptococcus thermophilus*	Ryan et al., 1996
Lactocin S	*Lactobacillus sakei* L45	*Lactobacillus* spp.; *Pediococcus* spp.; *Leuconostoc mesenteroides*	Mørtvedt-Abildgaa et al., 1995
Class IIa			
Leucocin A	*Leuconostoc gelidum* UAL 187; *L. mesenteroides* TA33a	*Carnobacterium* spp.; *Enterococcus* spp.; *Lactobacillus* spp.; *Leuconostoc* spp.; *Listeria* spp.; *Pediococcus* spp.; *Weissella* spp.	Corbier, et al., 2001; Hastings et al., 1991; Papathanasopoulos et al., 1997; Papathanasopoulos et al., 1998

Table 1-3. Continued: Examples of Class I, Class II and Class III bacteriocins produced by LAB

Bacteriocin	Producer strains	Inhibitory Spectra	References
Pediocin PA-1/ AcH	*Pediococcus acidophilus* PAC 1.0	*Lactobacillus* spp.; *Listeria* spp.; *Staphylococcus* spp.; *Enterococcus faecalis*; *Clostridium sporogenes; Ped. pentosaceus*,	Cintas et al., 1998; Rodríguez, Martínez, & Kok, 2002
Piscicocin CS526	*Carnobacterium maltaromaticum* CS526	*L. monocytogenes*	Yamazaki et al., 2005
Plantaricin C	*Lactobacillus plantarum* LL441	*Enterococcus* spp.; *Clostridium* spp.; *Lactobacillus* spp.; *Pediococcus* spp.; *Leuconostoc cremoris*; *Strep. thermophiles*, *Staphylococcus camosus*; *Bacillus subtilis*	González et al., 1994
Sakacin A	*Lactobacillus sakei* Lb706	*Carnobacterium* spp.; *Enterococcus* spp. *Lactobacillus* spp.; *Listeria* spp.; *Brochothrix thermosphacta*	Holck et al., 1992; Schillinger & Lücke, 1989
Class IIb			
Acidocin J1132	*Lactobacillus acidophilus* JCM 1132	*Lactobacillus* spp.	Tahara et al., 1996
Lactacin-F	*Lactobacillus johnsonii* VPI11088	*Lactobacillus* spp.; *Enterococcus fecalis*	Allison et al., 1994; Muriana & Klaenhammer, 1987
Plantaricin NC8	*Lactobacillus plantarum* NC8	*Lactobacillus* spp.; *Pediococcus* spp. *Carnobacterium maltaromaticum*	Diep et al., 1995; Maldonado et al., 2003
Class IIc			
Carnocyclin A	*Carnobacterium maltaromaticum* UAL307	*Carnobacterium* spp.; *Brochothrix* spp. *Enterococcus* spp.; *Lactococcus* spp. *Lactobacillus sakei; Leuconostoc mesenteroides; Pediococcus acidilactici; Listeria monocytogenes; Staphylococcus aureus*	Martin-Visscher et al., 2008, 2009

Table 1-3. Continued: Examples of Class I, Class II and Class III bacteriocins produced by LAB

Bacteriocin	Producer strains	Inhibitory Spectra	References
Gassericin A	*Lactobacillus gasseri* LA39	*Bacillus* spp.; *Enterococcus* spp.; *Lactococcus* spp.; *Staphylococcus* spp. *Leuconostoc mesenteroides*	Arakawa et al., 2009; Kawai et al., 1998
Leucocyclicin Q	*Leuconostoc mesenteroides* TK41401	*Bacillus* spp.; *Enterococcus* spp.; *Lactococcus* spp.; *Pediococcus* spp.; *Streptococcus* spp.; *Weissella* spp.; *Lactobacillus sakei;Leuconostoc mesenteroides*; *Listeria innocua*	Masuda et al., 2011
Lactocyclicin Q	*Lactococcus* sp. *strain* QU 12	*Bacillus* spp.; *Enterococcus* spp.; *Lactococcus* spp.; *Lactobacillus* spp.; *Listeria* spp.; *Pediococcus* spp.; *Streptococcus* spp.; *Micrococcus luteus; Leuconostoc mesenteroides*; *Weissella cibaria*	Sawa et al., 2009
Reutericin 6	*Lactobacillus reuteri* LA 6	*Lactobacillus acidophilus* JCM 2125; *Lactobacillus delbrueckii* JCM 1002; *Lactobacillus delbrueckii* JCM 1148/JCM 1248	Kabuki et al., 1997; Kawai et al., 2004; Toba et al., 1991
Class IId			
Enterocin 96	*Enterococcus faecalis* WHE 96	*Bacillus* spp.; *Enterococcus* spp.; *Lactobacillus* spp.; *Lactococcus lactis; L. monocytogenes*	Izquierdo et a., 2009
Garvieacin Q	*Lactococcus garvieae* BCC 43578	*L. monocytogenes* ATCC 19115; other *Lb. garvieae*	Tosukhowong et al., 2012
Class IIIa			
Enterolysin A	*Enterococcus faecalis* LMG 2333	*Bacillus* spp.; *Enterococcus* spp.; *Lactococcus* spp.; *Pediococcus* spp.; *Lactobacillus brevis; Lactobacillus curvatus; Listeria innocua; Staphylococcus carnosus; Propionibacterium jensenii*	Nilsen, Nes, & Holo, 2003
Class IIIb			
Helveticin J	*Lactobacillus helveticus* 481	*Lactobacillus* spp.	Jörger & Klänhammer, 1986

1.5.3.1. Mode of Action of Nisin

Nisin was first recognized as a food preservative by FAO/WHO in 1969 (FAO/WHO, 1969). Since then, it remains to be the only bacteriocins approved as food additive and the most widely applied bacteriocin to date. As a result, the mode of action of nisin is also the best elucidated among all other bacteriocins. There are two natural variants of nisin, which only differ by one amino acid residue at position 27 i.e. a histidine in nisin A in place of an asparagine in nisin Z (Buchman, Banerjee, & Hansen, 1988; Mulders et al., 1991). Since the two nisin variants have no apparent differences in their activities, their modes of action will not be separately discussed.

The primary target for nisin in bacterial cells is the cytoplasmic membrane, where it initiates electrostatic association with negatively charged phospholipids in the membrane bilayer via its lysine rich C-terminus (Abee, Krockel, & Hill, 1995; Breukink et al., 1997; Driessen et al., 1995). Using lipid II first as a docking molecule, nisin achieves membrane pore formation with lipid II being incorporated into the pore (Breukink et al., 1999). Since lipid II is also an integral component for peptidoglycan biosynthesis, sequestering lipid II during pore formation allows nisin to be more active against Gram-positive bacteria with peptidoglycan-rich cell-walls than Gram-negative bacteria (Breukink & de Kruijff, 2006; Wiedemann et al., 2001). Although the exact mechanisms for pore formation is ambiguous and still under elucidation, processes of insertion and aggregation are likely to occur in the cytoplasmic membrane due to the amphiphilic properties of nisin (Lins et al., 1999; Moll et al., 1996; Yeaman &

Yount, 2003; van den Hooven et al., 1996). Furthermore, insertion of nisin into the membrane is likely to reduce membrane fluidity by immobilizing membrane phospholipids and thus confer synergistic effect in killing susceptible microorganisms with high hydrostatic pressure treatments (ter Steeg, Hellemons, & Kok, 1999).

Nisin forms pores in susceptible bacteria such as *Listeria monocytogenes* with diameters between 2 to 2.5 nm which are stable for seconds (Wiedemann, Benz, & Sahl, 2004). This results in the immediate loss of small and essential cytoplasmic compounds including ions, cellular adenosine triphosphate (ATP), and amino acids such as glutamic acid and lysine (Abee et al., 1994). Effluxes of monovalent and divalent cations such as K+ and Mg2+ lead to rapid depolarization of the cytoplasmic membrane and depletion of its proton-motive force (Bruno et al., 1992). These disruptions are often accompanied and escalated by further inhibition of respiratory activities, cessation of biosynthesis, cell lysis, which eventually cause cell death (Bierbaum & Sahl, 1987; Gao et al., 1991; Reisinger et al., 1980; Sahl et al., 1987). The antimicrobial effect of nisin also extends to inhibiting the outgrowth of spores and vegetative cells, in which membrane sulphydryl groups are inactivated especially in the case of *Bacillus cereus* (Delves-Broughton, 1990; Henning et al., 1986; Morris et al., 1984; Ramseier, 1960; Thorpe, 1960).

1.5.3.2. Mode of Action of Pediocin PA-1/AcH

Pediocin PA-1/AcH is often considered to be the best known pediocin thus far (Papagianni & Anastasiadou, 2009). It is another broad spectrum bacteriocin

with antimicrobial activity against Gram-positive bacteria, but with particularly strong bactericidal activity against *L. monocytogenes* (Rodríguez et al., 2002). Remarkably, resistance development toward Class IIa bacteriocins in mutant *L. monocytogenes* has been found to be associated with the mechanism of preventing the synthesis of mannose-specific phosphotranferase and the upregulation of phospho-β-glucosidase and β-glucoside-specific phosophotranferase (Gravesen et al., 2002).

Despite being compositionally and structurally different from nisin, the cytoplasmic membrane is also the main target for pediocin PA-1/AcH (Papagianni, 2003). Similar to nisin and analogous to other class IIa bacteriocins, pediocin PA-1/AcH first destabilizes the cytoplasmic membrane through binding and then forms pores by inserting its hydrophobic C-terminal regions into the membrane to induce permeabilization (Drider et al., 2006). While pediocin PA-1/AcH cannot be adsorbed pass the outer membrane and affect the inner cytoplasmic membrane of Gram-negative bacteria under normal conditions, many sublethally injured Gram-negative organisms such as *Salmonella enterica* serotype Typhimurium and *E. coli* become susceptible after the integrity of their outer membrane is compromised by freezing, sublethal heating, hydrostatic-pressure treatment, exposure to lactic acid or EDTA (Kalchayanand, Hanlin, & Ray, 1992). Therefore, it is necessary for pediocin PA-1/AcH to pass through the damaged outer membranes of Gram-negative cells in order to access the inner membranes and inflict lethal effects. Unlike the binding mechanism of nisin, pediocin PA-1/AcH was believed to rely on receptor mediated binding (Bhunia et al., 1991). As

pediocin PA-1/AcH impairs the functions of the cytoplasmic membrane, it is likely that the loss of membrane potential from depletion of intacellular ATP, amino acids and ions, influx of lactose from the medium, inhibition of glucose transport, and potential cell lysis occurring in cells with an autolysis system would ensue to cause cell death (Chen & Montville, 1995; Chikindas et al., 1993; Christensen & Hutkins, 1992; Pucci et al., 1988; Waite et al., 1998). In regards to bacterial spore inhibition, pediocin PA-1/AcH seems to be less effective than nisin as it cannot directly kill spores, but it can inhibit the growth of *Bacillus* spp. and *Clostridium* spp. once spore germination was induced (Kalchayanand et al., 1998). Nevertheless, potent inactivation synergism was observed when spores were jointly treated with a mixture of pediocin PA-1/AcH and nisin under hydrostatic pressure (Kalchayanand et al., 2003, 2004).

1.5.4. Bacteriocin Production in LAB as a Probiotic Trait

Bacteriocin production has been traditionally viewed as an important probiotic trait, and there are LAB studies that have unequivocally demonstrated this positive influence on the host health despite their overwhelming scarcity (Dobson et al., 2012). One study that clearly demonstrated and confirmed that bacteriocin production offered primary protection against two strains of *L. monocytogenes* in mice was conducted by Corr et al. (2007). They have rendered probiotic protection against hepatic and splenic *Listeria* infections in ineffective using both a stable mutant of *Lactobacillus salivarius* UCC118 that was incapable of producing the Abp118 bacteriocin, and infections from a *L. monocytogenes* strain that expressed a cognate Abp118 immunity protein AbpIM.

Although protection against *Salmonella enterica* serotype Typhimurium was also observed, the antagonist effect could not be solely attributed to bacteriocin production as the mutant strain was able to confer comparable protection. Using the same bacteriocin producing wild-type and a similar mutant strain of *Lb. salivarius* UCC118 that lacked bacteriocin-production, Riboulet-Bisson et al. (2012) reported that bacteriocin Abp118 production can partially impact the faecal microbiota composition of both mice and pigs, even though the bacteriocin was previously found to be ineffective against Gram-negative bacteria *in vitro*. Furthermore, only the administration of Abp118 active wild-type *Lb. salivarius* UCC118 was found to have an effect on members of the Firmicutes, which suggested microbiota modulation effects associated with bacteriocin production. Overall, the administrations of *Lb. salivarius* UCC118, which were independent of bacteriocin production, were not found to induce major changes to the murine microbiota. However, bacteriocin producing wild-type *Lb. salivarius* UCC118 was found to significantly decrease populations of *Spirochaetes* present in the swine microbiota. Direct *in vivo* evidence of bacteriocin-producing LAB in preventing pathogen colonization was provided by Millette et al. (2008) using a mouse model. Strong inhibitory activity against a clinical isolate of vancomycin-resistant enterococci (VRE) was revealed through comparing control treatments that were consisted of phosphate-buffered saline with intragastric administrations of human fecal isolates of *Lactococcus lactis* MM19 and *Pediococcus acidilactici* MM33, which respectively produced nisin Z and pediocin PA-1/AcH, and a non-pediocin producing mutant strain of *Ped. acidilactici* MM33A. While

Ped. acidilactici MM33 reduced the *Enterobacteriaceae* populations, *Lc. lactis* MM19 and *Ped. acidilactici* MM33A were observed to increase the total fecal LAB and anaerobe populations. Treatment with the two bacteriocin-producing LAB strains resulted in a reduction of VRE fecal population to levels below the detection limit six days after infection, and treatments with pediocin-negative mutant strains yielded statistically similar results as the control treatments.

In addition to the roles that LAB bacteriocins play as antimicrobials and their ability to modulate different microbiota through their contribution to competitive inhibition, bacteriocins also participate in extracellular communication as signaling peptides. As reviewed in detail by Kleerebezem (2004), nisin serves as a prime example of such quorum sensing effects as it can induce its own biosynthesis depending on surrounding cell-densities. Other examples include plataricin A, which has been found in sourdough fermentation, and salivaricin UCC118, which was previously described as being associated with the GI microbiota; as well as plantaricin NC8 and lactacin B from food environments that are capable of participating in inter-species communication (Flynn et al., 2002; Gobbetti et al., 2007; Maldonado, Ruiz-Barba, & Jimenez-Diaz, 2003; Tabasco et al., 2009). As with other health enhancing properties of LAB probiotics, greater understanding of the bacteriocin producing aspect will likely lead to improved future human and veterinary applications.

1.5.5. Other Antimicrobial Metabolites Produced by Probiotic LAB

Aside from bacteriocin production, probiotic LAB are also able to produce a number of other antimicrobial metabolites including organic acids, hydrogen

peroxide, nitric oxide and species specific inhibitory compounds such as reutericyclin and reuterin produced by strains of *Lb. reuteri* (Axelsson et al., 1989; Dahiya & Speck, 1968; Eschenbach et al., 1989; Gänzle, 2004; Karahan et al., 2005). Organic acids such as lactic acid have been shown to inhibit gastric pathogens such as *Helicobacter pylori*, and have been exploited as preservatives in large scale food and beverage production (Midolo et al., 1995; Ross, Morgan, & Hill, 2002). With regards to the production of hydrogen peroxide in LAB, the compound is not only effective in inhibiting urogenital pathogens, but it is also important for sustaining the long-term colonization of probiotic LAB in vagina (Reid, 2001; Tomás et al., 2003; Vallor et al., 2001). Acidification from the generation of high levels of nitric oxide in fermentation or GI environments may also contribute to the superior bactericidal properties of probiotic LAB (Jones et al., 2010; Sobko et al., 2006; Xu & Verstraete, 2001). Reutericyclin is a tetramic acid and was considered to be the first low-molecular-weight antibiotic that was purified from cultures of the sourdough isolate *Lb. reuteri* LTH2584 (Gänzle et al., 2000; Höltzel et al., 2000). It was found exhibit a broad inhibitory spectrum, especially toward *Lactobacillus* spp., *Bacillus subtilis*, *Bacillus cereus*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Listeria innocua*. It also inhibited the growth of lipopolysaccharide mutant strains of *E. coli*, but it was not able to inhibit the growth of wild-type Gram-negative bacteria. It is important to make clear distinctions between reutericyclin and reuterin. The terms “reuterin” refer to β-hydroxypropionaldehyde, which is an entirely different compound that is also produced by *Lb. reuteri* (Talarico & Dobrogosz, 1989). It confers a broad

antimicrobial activity spectrum against food-borne pathogens and spoilage organisms, but higher antimicrobial activities were observed for Gram-negative than for Gram-positive bacteria (Axelsson et al., 1989). Furthermore, neither reutericyclin nor reuterin are bacteriocins, unlike reutericin 6, which is a cyclic bacteriocin that was isolated from *Lb. reuteri* LA 6 (Toba et al., 1991).

It is evident that certain probiotic LAB strains such as *Lb. reuteri* are capable of producing wide varieties of antimicrobial compounds to exert health benefits on their host. Studies of using LAB produced glycans such as exopolysaccharides (EPS) and oligosaccharides to prevent pathogens from adhering to their host are emerging. As another facet of my doctoral research, the following section of the introduction will further discuss these glycans with regards to bacterial adhesion.

1.6. Complex Cellular Functions of Glycans

Macromolecules in the form of carbohydrates (or glycans) dominate the surface of mammalian cells as glycolipids and glycoproteins. These cell-surface glycans are biologically important for the development, growth and survival of the organism as they orchestrate crucial cellular processes such as cell adhesion, proliferation, recognition, migration and differentiation (Bertozzi & Kiessling, 2001; Bucior et al., 2004; Lowe, 2003; Podbilewicz, 2004). Equally important for microbial physiology and cell communication are glycans that play integral roles in providing structural integrity, and mediating bacterial-host interactions, which underpin bacterial pathogenesis (Comstock, 2009; Comstock & Kasper, 2006; Hooper & Gordon, 2001; van Heijenoort, 2001). The inherent chemical properties

of the monosaccharide units, in which glycans are composed of, allow vast repertoires of structurally and functionally different glycans to be generated in order to achieve highly specific control over all of these enormously complex biological processes. More specifically, each monosaccharide contains either five or six carbons, and there can be up to eleven different ways of linking two discrete six-carbon monosaccharides together (Weiss & Iyer, 2007). Therefore, different branching structures in glycans with the same monosaccharide composition but different linkage types readily confer unique biological functions. Aside from glycan conformation, positional isomerism, hydrogen bonding, and density contribute to the modulation of glycan functions as well (Kulkarni, Weiss, & Iyer, 2010). Moreover, the abilities of monosaccharides to conjugate with protein and lipid molecules further increase the numbers and the structural complexities of the available glycan types (Lis & Sharon, 1993; Parry et al., 2007).

1.6.1. Bacterial Adhesins and Targeted Glycans

The ability of bacteria to adhere to abiotic and biotic surfaces in the environment is vital for their proliferation and survival (Dunne, 2002). Furthermore, adherence to target skin or mucosal surfaces of animals is not only important for the successful establishment of a normal microbiota, but also crucial as a first step in initiating infections (Chiller, Selkin, & Murakawa, 2001; Boyle & Finlay, 2003). Bacteria achieve efficient host adhesion through the expression of different types of protein complexes, which are collectively known as “adhesins”. As illustrated in **Figure 1-1**, adhesins can be classified as pili/fimbriae, or afimbrial adhesins which can be bacterial integral cell wall proteins or bacterial

cell surface glycoproteins (Corrigan, Miajlovic, & Foster, 2009; Hoiczyk et al., 2000; Hultgren et al., 1993; Koretke et al., 2006; Niemann, Schubert, & Heinz, 2004; Varki et al., 2009). Pili and fimbriae are complex polymeric organelle structures that are assembled from specific repeating protein subunits (Huang et al., 2001; Ohlsen et al., 2009). They protrude from the bacterial cell surface and have carbohydrate recognition domains located at the tip of the structure (Klemm & Schembri, 2000; Lindhorst, et al., 2010; Peterson & Quie, 1981).

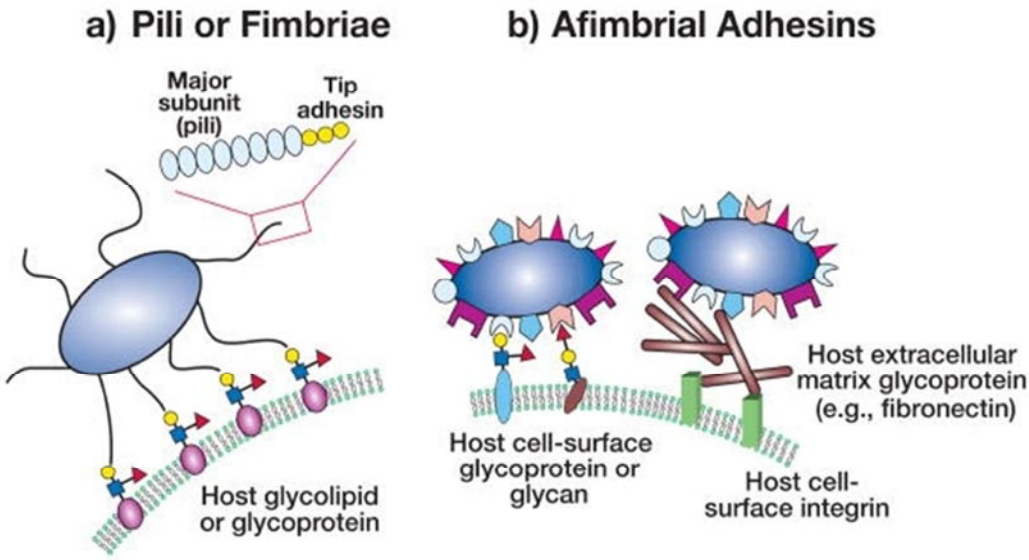

Figure 1-1. Illustrations of bacteria-host cell surface adherence mechanisms. a) Pili or fimbriae are organelle structures that protrude the bacterial cell surface. They are composed of repeating structural subunits of protein with a different protein tip that for recognizing specific glycan motifs as part of bacteria-host receptors. b) Afimbrial adhesins can be integral cell bacterial wall proteins or bacterial cell surface glycoproteins (Varki et al., 2009).

While some afimbrial adhesins attach to monomeric or oligomeric glycoproteins, glycosphingolipids, or glycosaminoglycans that are directly present on the host cell surface as receptors, others bind to extra cellular matrix glycoproteins such as fibronectin, collagen, laminin or mucin, which permit adherence to host mucosal surfaces (Eberhard et al., 1998; Erdem et al., 2007; Fink, Green, & St. Geme III, 2002; Kukkonen et al., 1993; Patti et al., 1994; Rostand & Esko, 1997).

Most bacteria possess multiple types of adhesins with different specificities for carbohydrate motifs on host receptors. Such variations in adhesin specificity confer the ecological niche that bacteria occupy which in turn also defines specific host tissues that are susceptible to colonization by relevant bacteria (Beachey, 1981; Ofek, Hasty, & Doyle, 2003; Tiralongo & Moran, 2010). For example, different from *Escherichia coli* with the type I fimbriae, P-fimbriated uropathogenic *E. coli* specifically bind to the disaccharide Galα1-4Gal (galabiose) either at internal sites or at the non-reducing end of globo series of oligosaccharides that are abundant in the human urinary tract (Strömberg et al., 1990; Sung et al., 2001). In fact, galabiose was found to be the minimal receptor structure required for adhesins of uropathogenic *E. coli* to achieve adhesion (Svenson et al. 1983). In addition to tissue tropism, bacterial adhesins can also confer species specificity. The K88 fimbriae of *E. coli* is one such example as infections caused by this microorganism only affect pigs (Cassels & Wolf, 1995; Searle et al., 2010). Additional examples of common pathogens with their respective host receptor glycan motifs used for adhesion are listed in **Table 1-4**.

Table 1-4. Examples of adhesin directed bacterial pathogen adherence to glycan receptors in host animal tissues

Microorganism	Adhesin	Receptor Glycan Structure	Target Tissue	Reference
Actinomyces naeslundii	Type II fimbriae	• Galβ1-3GalNAcβ1-3Galα1-4Gal-	Oral cavity	Strömberg & Borén, 1992
Campylobacter jejuni	n.a.	• Fucα1-2Galβ1-4GlcNAcβ- (H-2 antigen)	Intestinal tract	Ruiz-Palacios et al., 2003
Enteropathogenic E. coli (EPEC)	Bundle-Forming Pili (BFP)	• GalNAcβ1-4Gal-Galβ1-4GlcNAc-	Intestinal tract	van Maele, Heerze, & Armstrong, 1999
Enterotoxigenic E. coli (ETEC)	F17 fimbriae	• GlcNAc	Intestinal tract	Bertin et al., 1996
ETEC	F41 fimbriae	• GlcNAc • GalNAc	Intestinal tract	Lindahl & Wadström, 1986
ETEC	K88 fimbriae	• GalNAcβ1-4Galβ1-4Glc- • Galβ1-3GalNAcβ1-4Galβ1-4Glc-	Porcine Intestinal tract	Grange, 2002
ETEC	K99 fimbriae	Neu5Gcα2-3Galβ1-4Glc-	Intestinal tract	Sharon & Ofek, 2000
E. coli	Type I fimbriae	• Man • Manα1-3Man α1-6Man-	Urinary tract	Ofek, Mirelman, & Sharon, 1977; Sharon, 1987

Note: n.a., not available; Gal, Galactose; GalNAc, N-Acetylgalactosamine; Glc, Glucose; GlcNAc, N-acetyl-D-glucosamine; Fuc, Fucose; Man, Mannose; Neu5Gc, N-glycolylneuraminic acid

Table 1-4. Continued: Examples of adhesin directed bacterial pathogen adherence to glycan receptors in host animal tissues

Microorganism	Adhesin	Receptor Glycan Structure	Target Tissue	Reference
E. coli	P fimbriae	• Galα1-4Galβ1-4Glclβ1- • GalNAclβ1-3Galα1-4Gallβ1-4Glclβ1-	Urinary tract	Kuehn et al., 1992**;** Haslam et al., 1994
Salmonella enterica Enteritidis	n.a.	• (Neu5Acα2-6)Galβ1-4Glc • NAcβ1-2Man-	Intestinal tract	Sugita-Konishi et al., 2002
Streptococcus pneumoniae	n.a.	• GlcNAcβ1-3Gal- • GlcNAcβ1-4Gal- • GalNAcβ1-3Galα1-4Galβ1-4Glc-	Respiratory tract	Andersson et al., 1981; Andersson, Leffler et al., 1983; Cundell & Tuomanen, 1994; Krivan, 1988

Note: n.a., not available; Gal, Galactose; GalNAc, N-Acetylgalactosamine; Glc, Glucose; GlcNAc, N-acetyl-D-glucosamine; Fuc, Fucose; Man, Mannose; Neu5Gc, N-glycolylneuraminic acid

1.6.2. Bacterial Toxins and Targeted Glycans

Many pathogenic bacteria, especially those that cause infectious diarrhoea, secrete proteinaceous toxins that are also capable of binding host receptor glycans (**Table 1-5**). Particular examples include toxin A from *Clostridium difficile* and Shiga-like toxins in *E. coli*. Toxin A binds to terminal oligosaccharide sequences of Galα1-3Galβ1-4GlcNAc in animal host cell glycolipids (Clark et al., 1987). Likewise, Shiga-like toxins, which are protein cytotoxins that are functionally and structurally similar to the Shiga toxin produced by *Shigella dysenteriae*, bind to the same oligosaccharide sequence of Galα1-4Galβ1-4Glcβ in host cells (Lindberg et al., 1987; Samuel et al., 1990). All of the listed examples of glycan-binding toxins are two-component AB toxins, which are comprised of active A subunits that disrupt normal functions of host cells and B subunits for binding (Gerhard et al., 2008; Just et al., 1995; Pruitt et al., 2009). Furthermore, with the exception of Toxin A, the rest of the toxins are even more structurally similar as they all belong to the AB_5 toxin family, which is characterized by a hetero-hexameric assembly that is formed from a single toxic A subunit and a pentamer of five B-subunits (Fraser et al., 1994; Sixma et al., 1992; Stein et al., 1992; Zhang et al., 1995). Despite the lack of additional supporting data, it is highly conceivable that similar binding preferences of these toxins for specific glycan structures may be conferred by high glycan structural homologies. Examples of host glycan adherence to bacterial toxins are shown in **Table 1-5**.

Table 1-5. Host glycan adherence to bacterial toxins

Microorganism	Toxin	Key Receptor Glycan Structure	Target Tissue	Reference
Clostridium difficile	Toxin A	Galα1-3Galβ1-4GlcNAcβ- Galβ1-4Fucα1-3GlcNAcβ1- Fucα1-2Galβ1-4Fucα1-3GlcNAcβ1-	Large Intestine	Clark, Krivan, Wilkins, & Smith, 1987**;** Tucker & Wilkins, 1991
Escherichia coli (EPEC & EHEC)	Shiga-like toxins (Type I & II)	Galα1-4Galβ1-4Glcβ-	Small Intestine	Samuel et al., 1990
ETEC	Heat-labile toxin	Galβ1-3GalNAc β1-4(NeuAc α 2-3)Galβ-	Small Intestine	Fukuta et al., 1988
Shigella dysenteriae	Shiga toxin	Galα1-4Galβ1-4Glcβ-	Small intestine	Jacewicz et al., 1986; Lindberg et al., 1987; Mobassaleh et al., 1988
Vibrio cholera	Cholera toxin	Galβ1-3GalNAc β1-4(NeuAcα2-3)Galβ-	Small intestine	Merritt et al., 1994

Note: Gal, Galactose; GalNAc, N-Acetylgalactosamine; Glc, Glucose; GlcNAc, N-acetyl-D-glucosamine; Fuc, Fucose; Neu5Ac, N-acetylneuraminic acid (sialic acid)

1.7. Potential Receptor Carbohydrate Based Anti-adhesive Therapeutics

As studies revealed more glycan structures that pathogens and their toxins use for establishing adherence, research in the development of anti-adhesive therapy pertaining to the use of specific glycans as host receptor analogs also became more abundant (Ofek, Hasty, & Sharon, 2003; Zopf & Roth, 1996). In many cases, adhesion studies were conducted to support the determination of the receptor carbohydrate structures using carbohydrates from a variety of sources including plant biomass, chemical synthesis, milk *et cetera* (**Table 1-6**). Mannose was an early example of such anti-adhesive carbohydrates. Using *in vitro* adhesion assays with human epithelial cells, mannose was first discovered as a receptor carbohydrate for *E. coli* with Type I fimbriae (Ofek, Mirelman, & Sharon, 1977). Hooper and Gordon (2001) later found mannose to be effective in preventing ETEC colonization with an infant mice and infant mice intestinal segments. In addition, methyl α-D-mannopyranoside (the methylated form of mannose), which is also a competitive inhibitor of mannose-sensitive *E. coli* adhesins, was able to reduce the colonization of uropathogenic *E. coli* in the urinary track of mice, and prevent urinary infections cause by *Klebsiella pneumonia* in rats (Aronson et al., 1979; Fader & Davis, 1980).

Table 1-6. Studies of anti-adhesive carbohydrates and their targeted microorganisms

Carbohydrate Structure	Target Microorganism	Adhesion Test Model	References
D-Mannose	*E. coli* (Type I fimbriae); ETEC	*in vitro:* adhesion assay with human epithelial cells; *in vitro:* infant mice intestinal segment *in vivo:* infant mouse model	Ofek, Mirelman, & Sharon, 1977
Methyl α-D-mannopyranoside	uropathogenic *E. coli; Klebsiella pneumonia*	*in vivo:*mouse and rat models	Aronson et al., 1979; Fader & Davis, 1980
Galabiose (Galα1-4Gal)	uropathogenic *E. coli*	*in vitro*: haemagglutination assays with human/sheep erythrocytes; cell adhesion assays with T24 cell lines	Salminen et al., 2007
Globotriose (Galα1-4Galβ1-4Glc)	uropathogenic *E. coli*	*in vitro:* slide agglutination assay & flow cytometry assay with human erythrocytes *in vivo:* BALB/c mouse model	Leach et al., 2005
Globotriose–chitosan	uropathogenic *E. coli*	*in vitro:* Vero cell cytotoxicity assay *in vivo:* mouse model	Li et al., 2012
Globotetrose (GalNAcβ1-3Galα1-4Galβ1-4Glc)	uropathogenic *E. coli*	*in vitro:* mouse epithelial cells, haemagglutination human and guinea pig erythrocytes *in vivo:* BALB/c mouse model	Edén et al., 1982
Alpha1,2-linked fucosylated OS	*Campylobacter jejuni*	*in vitro:* HEp-2 cell line assay *in vivo:* mouse model *ex vivo:* human illeum mucosa Clinical: 2 year study with 93 mother-infant pairs	Ruiz-Palacios et al., 2003; Morrow et al., 2004

Note: Gal, Galactose; GalNAc, N-Acetylgalactosamine; Glc, Glucose; GlcNAc, N-acetyl-D-glucosamine; Neu5Ac, N-acetylneuraminic acid (sialic acid); OS, oligosaccharides; GOS, galactooligosaccharides; chito-oligosaccharides, COS; PD, polydextrose; EPS, exopolysaccharides

Table 1-6. Continued: Studies of anti-adhesive carbohydrates and their targeted microorganisms

Carbohydrate Structure	Target Microorganism	Adhesion Test Model	References
Lacto-N-neotetraose	*Haemophilus influenzae, Streptococcus pneumonia*	*in vitro:* human pharyngeal and buccal epithelial cell adhesion assays	Andersson et al., 1986
3'-sialyllactose (Neu5Acα2-3Gal β1-4Glc)	*Helicobacter pylori*	*in vitro:* human duodenum-derived cell line HuTu-80	Simon et al., 1997
Neutral OS	*Listeria monocytogenes* EPEC serotype O119 *Salmonella fyris Vibrio cholerae*	*in vitro:* human colon carcinoma cell line Caco-2	Coppa et al., 2003; Coppa et al., 2006
Sulphated EPS	*H. pylori*	*in vitro:* HeLa S3 cell line	Guzman-Murillo & Ascencio, 2000
Sulphated EPS	*H. pylori*	*in vitro:* Kato III and HeLa S3; human gastric cell lines	Ascencio et al., 2004
GOS alone or in combination with PD	*Cronobacter sakazakii*	*in vitro:* HEp-2 and Caco-2 cell lines	Quintero et al., 2011
GOS	EPEC	*in vitro:* HEp-2 and Caco-2 cell lines	Shoaf et al., 2006
Chitosan-OS	EPEC	*in vitro:* HT29 cell line	Rhoades et al., 2006
Beta-GOS	*Salmonella enterica* serotype Typhimurium	*in vitro:* HT-29-16E cell line *in vivo:* murine ligated ileal gut loops	Searle et al., 2010

Note: Gal, Galactose; GalNAc, N-Acetylgalactosamine; Glc, Glucose; GlcNAc, N-acetyl-D-glucosamine; Neu5Ac, N-acetylneuraminic acid (sialic acid); OS, oligosaccharides; GOS, galactooligosaccharides; chito-oligosaccharides, COS; PD, polydextrose; EPS, exopolysaccharides

Excellent examples of glycan receptor analogs serving as effective anti-adhesion therapeutics for infection prevention and treatment also include the previously mentioned globo-series oligosaccharides. Salminen et al. (2007) demonstrated that chemically synthesized multivalent galabiose derivatives were able to inhibit P-fimbriated *E. coli* adherence *in vitro*. Using both *in vitro* and *in vivo* studies, monovalent globotriose (Galα1-4Galβ1-4Glc) was also able to inhibit adherence and colonization of uropathogenic *E. coli* (Leach et al., 2005). Furthermore, the chemically derived globotriose–chitosan conjugate showed high efficacy in neutralizing cytotoxic effects that were induced by Shiga-like toxins in both Vero cell cytotoxicity assays and mouse models (Li et al., 2012). Moreover, globotetraose (GalNAcβ1-3Galα1-4Galβ1-4Glc) was found to have anti-haemagglutination and anti-adhesion effects as the ability of uropathogenic *E.coli* to agglutinate human and guinea pig erythrocytes and to attach to mouse bladder epithelial cells was inhibited (Edén et al., 1982).

Milk is one of the most important and abundant natural sources of oligosaccharides with anti-adherence activities for human and other mammals (Gopal & Gill, 2000; Kunz & Rudloff, 2008; Kunz et al., 1999; Urashima et al., 2001). Human milk oligosaccharides (HMO) account for more than 50 g/L in colostrum and 10-15 g/L in mature milk (Kunz et al., 1999; Kunz & Rudloff, 2008). They are principally comprised of five monosaccharide units of d-glucose, d-galactose, N-acetylglucosamine, l-fucose, and sialic acid, which collectively give rise to highly diverse and complex structures with more than 200 isomers and varying degrees of polymerization of between DP 3 and DP 20 (Bode, 2006;

Han et al., 2011). Recently Hickey (2012) extensively reviewed plethoras of anti-adhesive oligosaccharides and related glycoconjugates that are capable of inhibiting a wide variety of pathogens. One example of anti-adhesion studies conducted on HMO include the use of α1,2-linked fucosyloligosaccharides to inhibit the binding of *Campylobacter jejuni* in cell lines, mice and human illeum mucosa (Ruiz-Palacios et al., 2003). Other studies examined the efficacy of fractionated neutral oligosaccharides from HMO in inhibiting the binding of *Listeria monocytogenes,* EPEC serotype O119, *Salmonella fyris, Vibrio cholera* to the Caco-2 cells (Coppa et al., 2003; Coppa et al., 2006). Additional anti-adhesion studies on HMO and other sources of anti-adhesive glycans, which include commercial prebiotics as well as glycans synthesized by microorganisms, are further exemplified in **Table 1-6**.

1.8. LAB Derived Glycans as Potential Anti-adhesive Therapeutics

Although chemical methods have been traditionally employed to synthesize specific anti-adhesive carbohydrates as previously listed, such production processes are highly expensive, complex and time-consuming, and the resultant low product yields make industrially scaled-up production inconceivably difficult and infeasible (Maitin & Rastall, 2005). Conversely, economical production of oligosaccharides at the industrial scale can be achieved in one-step reactions and with little or no by-products by primarily utilizing naturally occurring enzymes such as glycosyltransferases and glycosidases (Thiem, 1995). Numerous strains of LAB are ideal sources for producing both these enzymes and respective glycan products because of their relatively short generation time,

generally regarded as safe (GRAS) status and potential probiotic properties (Holzapfel & Schillinger, 2002; Stern & Frazier, 1941).

LAB glycosyltransferases are critical enzymes that participate in the formation of heterooligosaccharide (HeOS) types of EPS, since they form glycosidic linkages by transfering a variety of activated donor monosaccharides on to specific acceptor carbohydrates (De Vuyst & Degeest, 1999; Jolly et al., 2002). While LAB EPS have been commercially valuable for enhancing rheological properties in food products for quite some time according to Cerning (1990), very little studies have been conducted on the potential anti-adhesive properties of these EPS. Consequently, anti-haemagglutination properties of EPS on porcine specific ETEC will be further explored in the subsequent chapter.

Unlike microbial glycosyltransferases, glycosidases were originally thought hydrolyze glycosidic bonds in carbohydrates; but glycosidases such as beta-galactosidases (β-Gal) have been found to synthesize β-GOS via transglycosylation reactions if the concentrations of acceptor carbohydrates are sufficiently high (Boon et al., 2000). In fact, the hydrolase activities are actually resulting from the presence of excessive water as it can act as an acceptor in transglycosylation reactions. Although enzymatic synthesis of oligosaccharides using different acceptor sugars via β-Gal have been extensively studied, very little information is available on the formation of alpha-galactooligosaccharides (α-GOS), especially in regards to the use of acceptor sugars and linkage types in the oligosaccharides (Gänzle, 2012; Schwab et al., 2011). Therefore, synthesis of α-GOS using different acceptor sugars via cloned LAB α-Gal will be further

discussed in the chapter following the EPS studies. Although not directly examined, anti-adhesive properties of α-GOS also warrant much research in the future.

1.9. Hypothesis and Objectives

This thesis aimed to test the hypothesis that LAB and their metabolites can prevent infections and promote health in farm animals. Using animal models, my studies have been specifically focused on developing novel probiotic LAB applications for treating and preventing metritis in dairy cattle, and evaluating LAB synthesized glycans for preventing and treating post-weaning diarrhoea (PWD) in swine.

The characterization of vaginal microbiota of dairy cows and the isolation and selection of pediocin-producing *Pediococcus acidilactici* for probiotic applications will be described and discussed in chapter two. Chapter three will describe the use of exopolysaccharide synthesized by *Lactobacillus reuteri* to decrease the binding of enterotoxigenic *Escherichia coli* to porcine erythrocytes. Chapter four pertains to the characterization of α-Galactooligosaccharides formed via heterologous expression of α-Galactosidases from *Lactobacillus reuteri* in the *Lactococcus lactis* expression system. Lastly, general discussion and conclusion will be presented in Chapter five.

1.10. References

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Chapter 2

Characterization of Vaginal Microbiota of Dairy Cows and Isolation of Pediocin-producing *Pediococcus acidilactici*

A Version of this chapter has been submitted for publication in BMC Microbiology

2.1. Introduction

Infection of the uterine tract, also known as metritis, has a significant impact on the profitability of the dairy industry because of lowered reproductive efficiency, decreased milk production, and increased costs associated with treatment and culling of animals due to infertility (Lewis, 1997; Ross, 2002; Sheldon et al., 2006). Uterine infections in dairy cows are associated with predisposing factors such as calving difficulty, retained placenta, and the age of the cows, along with the overgrowth of pathogenic microorganisms in the reproductive tract (Coleman, Thayne, & Dailey, 1985). Immediately after calving, the dilated state of the cervix allows microorganisms from the environment, cow's skin, and faecal material to enter through the vagina into the uterus and initiate inflammation of the endometrium, which is highly associated with infertility (Sheldon & Dobson, 2004). Metritis associated bacteria have been classified as pathogens, potential pathogens, or opportunistic pathogens (Sheldon et al., 2002; Williams et al., 2005). Recognised uterine pathogens that are associated with severe endometrial inflammation and clinical endometritis include *Escherichia coli*, *Arcanobacterium pyogenes*, *Fusobacterium necrophorum*, *Prevotella melaninogenica* and *Proteus* species. Williams et al. (2007) considered high cell counts of *E. coli* as the basis for the onset of uterine infection.

In a healthy female reproductive tract of humans, mice, or monkeys, lactobacilli are the predominant organisms (Herthelius et al., 1989; Redondo-Lopez et al., 1990; Vintiñi et al., 2004). Vaginal lactobacilli inhibit the growth of genitourinary pathogenic microorganisms through mechanisms of competitive exclusion of pathogens, stimulation of the host immune system, and production of specific antibacterial compounds such as acetic and lactic acids, hydrogen peroxide, and

antimicrobial peptides (Charteris et al., 2004; Eschenbach et al., 1989). A contribution of bacteriocin production by vaginal probiotics to probiotic activity has not been demonstrated experimentally**,** but production of the bacteriocin Abp118 by *Lactobacillus salivarius* UC118 conferred resistance to hepatic and splenic infections by *Listeria monocytogenes* in mice (Corr et al., 2007b).

The microbial flora of a healthy bovine reproductive tract consists of a combination of aerobic, facultatively anaerobic, and obligately anaerobic microorganisms. Lactobacilli were found to be present in low numbers in the bovine vaginal microbiota (Otero et al., 1999); additionally, *Enterobacteriaceae* are among the dominant populations (Otero et al., 2000). However, alterations in the vaginal microbiota composition in the first weeks after parturition, i.e. the time during which metritis develops, remain poorly documented. The aim of our study is to characterize the vaginal microbiota of both healthy pregnant and infected post-partum cows by culture-dependent analysis. In addition, culture independent quantitative PCR (qPCR) analyses was used to characterize the vaginal microbiota of metritic cows two weeks before and two weeks calving. Isolates were studied with regards to Shiga-like toxin and pediocin production.

2.2. Materials and Methods

2.2.1. Animals

Fifteen lactating Holstein dairy cows were used in our study for characterizing the vaginal microbiota of heathy pregnant and metritic postpartum cows. For characterizing the vaginal microbiota of metritic cows two weeks before calving and two weeks after calving, ten animals were selected. All animals were maintained at the Dairy Research and Technology Centre of the University of Alberta. Metritis or uterine infections were diagnosed on the basis of watery reddish-brown, purulent, or

mucopurulent discharges with or without fetid odour. Rectal temperatures of 39.5°C or higher and impaired general condition as expressed in a lowered feed intake or milk production were also taken into consideration for diagnosis. Protocol number A5070-01 was obtained upon ethics approval.

2.2.2. Samples

For culture-dependent analyses, vaginal swab samples were obtained from seven healthy pregnant cows and eight infected post-partum cows. The vulvar area was thoroughly cleaned with water and then disinfected with 30% (vol/vol) iodine solution (Iosan, WestAgro, Saint Laurent, Canada) prior to sampling. A stainless steel vaginal speculum was gently inserted into the vagina, opened, and a long-handled sterile cotton swab was introduced to obtain a sample from the anterolateral vaginal wall. Each sample was collected in 4 mL of 0.1% (w/v) sterile peptone water with 0.85% (w/v) NaCl and 0.05% (w/v) L-cysteine-HCl x H_2O. The cotton swab was moistened by immersion in the peptone water immediately before sampling.

For culture-independent analyses, vaginal mucus samples were collected using syringes fitted with an approximately 30 cm long collection tube without the use of a vaginal speculum. The weight of mucus in each sample was determined by recording the total weight of each sample collection tube with 1 ml of peptone water before and after each mucus sample was collected. All samples were stored at temperatures between -20 °C to -80 °C.

2.2.3. Isolation of Microorganisms

Ten-fold serially diluted samples were plated on Reinforced Clostridial Medium (RCM) with 5% animal blood, Endo agar (Difco, Sparks, USA), and modified MRS (mMRS) agar (Stolz et al., 1995). Representative colonies from each type of plates were purified by repeated streak-plating until uniform colony

morphology was obtained. Isolates from mMRS and RCM with blood were streaked on mMRS agars whereas isolates from Endo plates were streaked on Luria-Bertani (LB) agars. Frozen stock cultures of each isolate were prepared from a single colony and stored in 60% glycerol at -70°C.

2.2.4. General Molecular Techniques

General DNA manipulations and agarose gel electrophoresis were performed as described by Sambrook et al. (1989). Chromosomal DNA of isolated strains was extracted from 1 ml cultures using a DNeasy® Blood and Tissue Kit (Qiagen, Mississauga, Canada). Unless otherwise stated, PCR amplifications were performed in GeneAmp® PCR System 9700 (Applied Biosystems, Streetsville, Canada) by using Taq DNA polymerase and deoxynucleoside triphosphates (Invitrogen, Burlington, Canada). The PCR products were purified using the QIAquick PCR purification kit (Qiagen). DNA sequence of each PCR product was compared with 16S rRNA gene sequences of type strains in the Ribosomal Database Project II (RDP-II; Michigan State University, East Lansing, USA).

2.2.5. Random Amplified Polymorphic DNA-PCR (RAPD-PCR) Analysis

DNA template was isolated as described above. DAF4 primer was used to generate RAPD patterns for isolates from Endo agar and M13V primer was used for RAPD typing of all other strains (**Table 2-1**). The reaction mixture contained 10 µL of 5x Green GoTaq® Reaction Buffer (Promega, San Luis Obispo, USA), 3 µL of 25 mM $MgCl_2$ (Promega), 150 pmol primer (**Table 2-1**), 1 µL of 10 mM dNTP (Invitrogen, Burlington, Canada), 1.5 U GoTaq® DNA Polymerase (Promega), and 1 µL of template DNA suspension or autoclaved water filtered with Milli-Q water purification system as the negative control (Millipore Corporation, Bedford, Massachusetts, United States). The PCR program comprised of an initial denaturation

step at 94°C for 3 minutes, followed by 5 cycles of denaturation, annealing and extension steps at 94°C for 3 minutes, 35°C for 5 minutes, and 72°C for 5 minutes. An additional 32 cycles of denaturation, annealing and extension steps were also performed at 94°C for 1 minute, 35°C for 2 minutes, 72°C for 3 minutes, followed by a final extension step at 72°C for 7 minutes. RAPD PCR products were electrophoresed in a 1.5% agarose gel with 0.5x TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.0). A 2-log molecular size marker (New England Biolabs, Pickering, Canada) was included on all gels.

2.2.6. Partial 16S Ribosomal rRNA Gene Amplification and Sequencing

Clonal isolates were eliminated on the basis of their origin and RAPD patterns, and remaining isolates were identified by partial sequencing of 16S rRNA genes. PCR reaction was performed in a master mix with a final volume of 50 µL containing 1.5 U Taq DNA Polymerase (Invitrogen), 5 µL of 10X PCR Reaction Buffer (Invitrogen), 1.5 µL of 25 mM $MgCl_2$ (Invitrogen), 25 pmol of universal bacterial primers 616V and 630R (**Table 2-1**), 1 µL of 10 mM dNTP, and 1 µL of template DNA. PCR product was electrophoresed in 1.0% (w/v) agarose gel, with a 2-log ladder (New England Biolabs). All sequencing data were obtained from sequencing services provided by Macrogen (Rockville, USA).

2.3.7. Identification of *E. coli* with Species-specific PCR and API 20E Test System

PCR amplification of the hypervariable regions of the *E. coli* 16S rRNA gene used primers described by Sabat et al. (2000). The PCR reaction mix (final volume 50 µL) consisted of 1.25 U Taq DNA Polymerase (Invitrogen), 5 µL of 10X PCR Reaction Buffer (Invitrogen), 1.5 µL of 25 mM $MgCl_2$ (Invitrogen), 100 pmol of ECP79F and ECP620R (**Table 2-1**), 1 µL of 10 mM dNTP, and 1.5 µL of template

DNA. Reference strains used as positive and negative controls are listed in **Table 2-2**. The API 20E test system (bioMérieux, Saint Laurent, Canada) was used to confirm identification to the species level. PCR-based detection of Shiga-like toxin producing *E. coli* (STEC) was conducted with 50 µL reaction mixes that contained 1.25 U Taq DNA Polymerase (Invitrogen), 5 µL of 10X PCR Reaction Buffer (Invitrogen), 1.5 µL of 25 mM $MgCl_2$ (Invitrogen), 1 µL of 10 mM dNTP (Invitrogen), 25 pmol SLTI-F and SLTI-R (**Table 2-1**), or 25 pmol SLTII-F and 25 pmol SLTII-R. Positive controls are listed in **Table 2-2**.

2.2.8. Deferred Inhibition Assay for Bacteriocin Detection

Overnight cultures of test strains were prepared in MRS broth that contained 2 g/L glucose. Test strains used in this study included *Lactobacillus sakei* FUA3089, and *Ped. acidilactici* FUA3072, FUA3138 and FUA3140. MRS plates with 2 g/L glucose were spotted with 3 µL of each overnight culture and the plates were incubated overnight under anaerobic conditions at 37°C. *Ped. acidilactici* FUA3072 was used as a positive control.

Cultures of indicator strains (**Table 2-2**) grown in overnight MRS broth with 2 g/L glucose were used to inoculate MRS soft agar at an inoculation level of 1% and the soft agar was overlayered over the MRS plates with test strains. Indicator strains included *E. coli* FUA1036, *E. coli* FUA1063, *E. coli* FUA1064, *Listeria innocua* ATCC33090, and *Enterococcus faecaelis* FUA3141.

The deferred inhibition assay was repeated with the addition of 20 g/L proteinase K in 100 mM Tris-Cl, pH 8.5, which was spotted adjacent to test strain colonies and plates were incubated for four hours at 55°C to maximize proteinase activity before overlaying was conducted.

2.2.9. PCR-DGGE Analysis and Identification of Library Clones via Sequencing

Culture-independent DGGE-PCR analysis was performed for vaginal swab samples. Total bacterial DNA extracted from samples via both phenol chloroform extraction and Wizard MagneSil® Tfx™ System (Promega). PCR-DGGE procedures were adapted from Walter et al. (2000). Nested PCR was conducted to maximize DNA amplification by amplifying with 616V and 630R primers before using HDA primers (**Table 2-1**). The DCode electrophoresis system (Bio-Rad Laboratories, Mississauga, Canada) was used to perform DGGE analysis of PCR products. Tris-acetate-EDTA buffer was used for 6% polyacrylamide gels with denaturation gradient of urea and formamide between 22% - 55%.

DGGE patterns could be obtained only for samples from animal # 2409 animal # 2373. Using these DNA samples, PCR products were obtained using HDA primers and subsequent DGGE analyses were conducted. The single band in the DGGE pattern of the sample from animal # 2409 matched the band obtained with isolates from the same sample. For the sample from cow # 2373, a clone library was constructed using the TOPO TA Cloning® Kit (Invitrogen). PCR products associated with cow # 2373 were cloned into the pCR 2.1-TOPO vector according to manufacture's instructions. The Promega's Wizard® Plus SV Minipreps DNA Purification System was used for plasmid isolation. To confirm the cloning of the inserts, sequencing of the amplified insert was performed on four out of sixteen samples according to the Invitrogen TOPO TA Cloning® Kit manual.

2.2.10. Quantitative PCR

Quantitative PCR was conducted with thawed vaginal mucus samples collected from metritic cows two weeks before calving and two weeks after calving. Total bacterial DNA was extracted using the Wizard MagneSil® Tfx™ System (Promega) and DNA concentrations were measured using the NanoDrop spectrophotometer system ND-1000, software version 3.3.0 (Thermo Fisher Scientific Inc., Wilmington, USA). All dagger-marked primer pairs that are listed in **Table 2-1** were used in the preparation of standards and qPCR analyses. Standards were prepared using purified PCR products, which were serially diluted ten-fold. Diluted standards (10^{-3} to 10^{-8}) were used to generate standard curves. TaqMan probes were used for the pedA gene and the total bacteria qPCR experiments. In both cases, each probe was labeled with 5'-FAM and 3'-TAMRA as fluorescent reporter dye and quencher respectively. The total reaction volume was set to 25 µL, which contained 12.5 µL TaqMan Universal PCR Master Mix (Applied Biosystems), 2.5 µL of template DNA extracted from vaginal mucus and 5 µM of each primer (**Table 2-1**), and 0.2 µM of the TaqMan probe. SYBR green assays were used for all remaining target-group primer pairs. The total reaction was also set at 25 µL containing 12.5 µL Fast SYBR Green Master Mix (Applied Biosystems), 1 µM primer, and 1 µL DNA template. Amplification conditions generally followed an initial denaturation at 95ºC for 5 min for 1 cycle; 40 cycles of denaturation at 95ºC for 30 sec, annealing with listed annealing temperatures in **Table 2-1** for 1 min, and extension at 72ºC for 2 min. Quantitative PCR was executed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions were performed in triplicates in MicroAmp Fast Optical 96-well reaction plates, sealed with MicroAmp Optical Adhesive Film (Applied Biosystems).

Table 2-1. Primers used in the study

Target/Specificity	Primer/Probe Sequence (5'→ 3')	Annealing Temperature (°C)	Reference(s)
†*Lactobacillus –Pediococcus-Leuconostoc-Weissella* (*Lactobacillus* group) (341 bp)	**Lac1:** AGC AGT AGG GAA TCT TCC A **Lab667r:** CAC CGC TAC ACA TGG AG	62	Heilig et al., 2002; Walter et al., 2001
†*Enterococcus* spp. (144 bp)	**Ent-F:** CCC TTA TTG TTA GTT GCC ATC ATT **Ent-R:** ACT CGT TGT ACT TCC CAT TGT	60	Rinttilä et al., 2004
†*Enterobacteriaceae* (195 bp)	**Enterobac-F:** CAT TGA CGT TAC CCG CAG AAG AAG C **Enterobac-R:** CTC TAC GAG ACT CAA GCT TGC	63	Bartosch et al., 2005
†*Staphylococcus* spp. (370 bp)	**TStaG422:** GGC CGT GTT GAA CGT GGT CAA ATC **TStaG765:** TIA CCA TTT CAG TAC CTT CTG GTA A	55	Martineau et al., 2001
†*Bacillus* spp. (995 bp)	**BacF:** GGGAAACCGGGGCTAATACCGGAT **BacR:** GTC ACC TTA GAG TGC CC	55	Garbeva et al., 2003
†*E. coli* (544 bp)	**ECP79F:** GAA GCT TGC TTC TTT GCT **ECP620R:** GAG CCC GGG GAT TTC ACA T	54	Sabat et al., 2000
†SLT-I (614 bp)	**VT1 (SLTI-F):** ACA CTG GAT GAT CTC AGT GG **VT2 (SLTI-R):** CTG AAT CCC CCT CCA TTA TG	55	Gannon et al., 1992
†SLT-II (779 bp)	**VT3 (SLTII-F):** CCA TGA CAA CGG ACA GCA GTT **VT4 (SLTII-R):** CCT GTC AAC TGA GCA CTT T	55	
16S rDNA Sequencing (~1500 bp)	**616V:** AGA GTT TGA TYM TGG CTC **630R:** AAG GAG GTG GAT CCA RCC CAKAAAGGAGGTGGATCC	52	Juretschko et al., 1998

†Selected primer pairs were also used in quantitative PCR analyses
Sizes of amplication products are indicated in brackets

Table 2-1. Continued: Primers used in the study

Target/Specificity	Primer/Probe Sequence (5'→ 3')	Annealing Temperature (°C)	Reference(s)
Random Primer for RAPD	**DAF4:** CGG CAG CGC C	35	Vogel et al., 2000
	M13V: GTT TTC CCA GTC ACG ACG TTG	35	Meroth et al., 2003
Universal Primers for DGGE	**HDA1:** ACT CCT ACG GGA GGC AGC AG	52	Walter et al., 2000
	HDA2: GTA TTA CCG CGG CTG CTG GCA	52	
	HDA1+GC: CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GGC ACG GGG GGA CTC CTA CGG GAG GCA GCA G		Walter et al., 2000 Yanisch-Perron et al., 1985
TA Cloning	**M13Forward (-20):** GTA AAA CGA CGG CCA G	55	
	M13Reverse: CAG GAA ACA GCT ATG AC		
†Pediocin Structural Gene pedA (100 bp)	**pedA2RTF:** GGC CAA TAT CAT TGG TGG TA	55	Yanisch-Perron et al., 1985
	pedA2RTR: ATT GAT TAT GCA AGT GGT AGC C	60	Mathys et al., 2007
	TqM-pedA: FAM-ACT TGT GGC AAA CAT TCC TGC TCT GTT GA-TAMRA		
†Total Bacteria (727 bp)	**TotalBac-F785:** GGA TTA GAT ACC CTG GTA GTC	52	Greisen et al., 1994; Lyons et al., 2000
	TotalBac-R1512r: TAC CTT GTT ACG ACT T		
	TaqMan 1400r Probe: 6-FAM-TGA CGG GCG GTG TGT ACA AGG C-TAMRA		

†Selected primer pairs were also used in quantitative PCR analyses
Sizes of amplication products are indicated in brackets

Table 2-2. Reference strains used in the study

Strain	Description
Lactobacillus plantarum FUA3099	Positive control for RAPD with M13V primer
Shigella boydii ATCC4388 *Shigella dysenteriae* ATCC188 *Shigella flexneri* ATCC62	Negative control for species specific PCR of *E. coli* 16S rRNA gene
E. coli O157:H7 ATCC43888	Positive control for species specific PCR of *E. coli* 16S rRNA gene
E. coli O157:H7 ATCC43889	SLT-II positive control
E. coli O157:H7 ATCC43890	SLT-I positive control
Pediococcus acidilactici FUA3072 *Pediococcus acidilactici* FUA3100	Bacteriocin-producing strains expressing the pediocin AcH/PA-1 operon
Lactobacillus sakei FUA3089	Non-bacteriocinogenic meat isolate
Listeria innocua ATCC33090	Indicator strains used in deferred inhibition assay for bacteriocins detection

2.2.11. Gene Accession Numbers of 16S rRNA Gene Sequences Obtained in This Study

Sequences of 16S rRNA genes of isolates obtained in this study were deposited in GenBank® with the following accession numbers: FUA3086 (GQ222397), FUA3087 (GQ222398), FUA3088 (GQ222399), FUA3089 (GQ222408), FUA1167 (GQ205673), FUA1035 (GQ222390), FUA1037 (GQ222410), FUA3137 (GQ222393), FUA3140 (GQ222392), FUA3141 (GQ222407), FUA3226 (GQ222394), FUA3136 (GQ205672), FUA1062 (GQ222401), FUA2027 (GQ205674), FUA2028 (GQ222400), FUA3251 (GQ222395), FUA1046 (GQ222387), FUA3135 (GQ222404), FUA2023 (GQ205670), FUA2024 (GQ205671), FUA1036, (GQ222389), FUA3139 (GQ222406), FUA1063 (GQ222403), FUA3227 (GQ205669), FUA3138 (GQ222409), FUA1049 (GQ222388), FUA1070 (GQ222391), FUA1064 (GQ222405), FUA3180 (GQ222402), FUA2029 (GQ222396).

2.3. Results

2.3.1. Composition of Microbiota in Healthy and Infected Dairy Cows: Isolation and Identification of Bacterial Species

Analysis of the microbiota of the reproductive tract of dairy cows was initially based on a qualitative, culture-dependent approach. Bacterial isolates were obtained from healthy, pre-partum animals (n=7) or infected, post-partum animals (n=8). Clonal isolates were eliminated by RAPD-PCR analysis and isolates representing different RAPD profiles were identified on the basis of the sequence of approximately 1400 bp of the 16S rRNA genes. Strain identification to species level was based on 97% or greater sequence homology to type strains. Strains of the species *E. coli* could not be identified on the basis of 16S rRNA sequences alone because of the high homology of rDNA sequences to closely-related species such as *Shigella* spp.

and *Escherichia fergusonii*. Classification of all *E. coli* isolates was verified with species-specific PCR and API-20E test strips. The biochemical characteristics of isolates matched properties of *E. coli* (99.8%) in the API-20E database. The identity of thirty isolates and their origin is listed in **Table 2-3**.

Bacilli, staphylococci, and lactic acid bacteria of the genera *Enterococcus*, *Lactobacillus*, and *Pediococcus* were present in both healthy and infected cows. *Escherichia coli* was also frequently isolated, particularly from infected animals. Isolates were screened for the presence of SLT-I and SLT-II genes, sample results for their PCR detection in *E. coli* isolates are shown in **Figure 2-1a** and **Figure 2-1b**, respectively. *E. coli* FUA1064 isolated from cow #2507 harboured the SLT-I gene, while *E. coli* FUA1037 and FUA1062, isolated from cow #2373 and #2374, respectively harboured the SLT-II gene (**Table 2-3**).

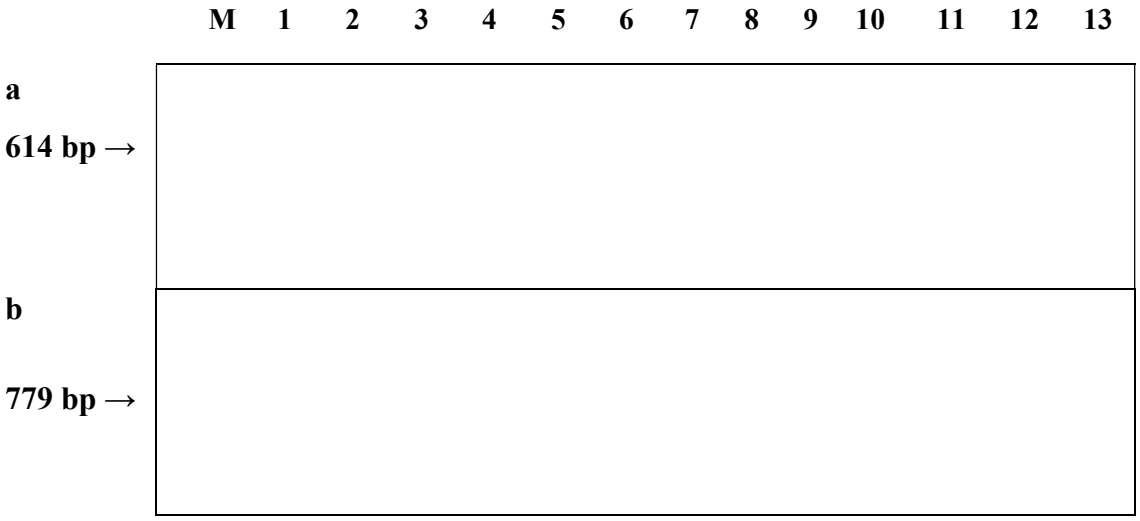

Figure 2-1. PCR-based detection of shiga-like toxins. Panel a. PCR-based detection of shiga-like toxin I (SLT-I)-producing *E. coli* FUA1064 (lane 7). DNA extracted from *E. coli* O157:H7 ATCC43890 was used as positive control for SLT-I (lane 12). **Panel b.** PCR based detection of SLT-II-producing *E. coli* FUA1037 (lane 3), and *E. coli* FUA1062 (lanes 9 and 10). DNA extracted from *E. coli* O157:H7 ATCC 43889 was used as positive control for SLT-II (lane 11).

Table 2-3. Qualitative characterization of the vaginal microbiota of dairy cows

Animal #	FUA #	Identified Species	% Identity to Type Strain [(a)]	Shiga -like Toxin Gene	Pediocin Immunity Gene
2102 (Healthy)	3086	*Staphylococcus epidermidis*	0.990	n.d.	n.d.
	3087	*Staphylococcus epidermidis*	0.991	n.d.	n.d.
	3088	*Staphylococcus warneri*	0.985	n.d.	n.d.
	3089	*Lactobacillus sakei*	0.986	n.d.	n.d.
2151 (Healthy)	1167	*Proteus mirabilis*	0.995	n.d.	n.d.
2363 (Healthy)	1035	*Escherichia coli*	0.980 (*Shigella flexneri*)	-	n.d.
	1037	*Escherichia coli*	0.930	SLT-II	n.d.
	3137	*Pediococcus acidilactici*	0.990	n.d.	+
	3140	*Pediococcus acidilactici*	1.000	n.d.	+
	3141	*Enterococcus faecalis*	0.990	n.d.	n.d.
	3226	*Pediococcus acidilactici*	0.990	n.d.	-
2367 (Healthy)	3136	*Staphylococcus warneri*	0.993	n.d.	n.d.
2374 (Healthy)	1062	*Escherichia coli*	0.976 (*Shigella flexneri*)	SLT-II	n.d.
	2027	*Bacillus licheniformis*	0.982	n.d.	n.d.
	2028	*Bacillus licheniformis*	0.978	n.d.	n.d.
	3251	*Streptococcus pluranimalium*	0.990	n.d.	n.d.
2409 (Healthy)	1046	*Escherichia coli*	0.978 (*Shigella flexneri*)	-	n.d.
	3135	*Staphylococcus hominis* subsp. *hominis*	0.991	n.d.	n.d.
2426 (Healthy)	2023	*Bacillus altitudinis*	0.998	n.d.	n.d.
	2024	*Bacillus pumilus*	0.981	n.d.	n.d.
*2211-A (Infected)	1036	*Escherichia coli*	0.981(*Shigella flexneri*)	-	n.d.
	3139	*Enterococcus faecalis*	0.980	n.d.	n.d.
*2211-B (Infected)	1174	*Escherichia coli*	0.980	-	n.d.

Healthy, pregnant animals and those diagnosed with post-partum uterine infections at the time of sampling are indicated in brackets

[(a)] % identity of partial 16S rDNA to type strain or closest relative; +: positive PCR results; -: negative PCR results; n.d.: data not determined

*Cow #2211-A and 2211-B represent two different animals that were assigned the same number at different times

Table 2-3. Continued: Qualitative characterization of the vaginal microbiota of dairy cows

Animal #	FUA #	Identified Species	% Identity to Type Strain [(a)]	Shiga -like Toxin Gene	Pediocin Immunity Gene
	1176	*Escherichia coli*	0.980	-	n.d.
	2044	*Bacillus licheniformis*	0.998	n.d.	n.d.
	2045	*Bacillus galactosidilyticus*	0.990	n.d.	n.d.
	2049	*Bacillus oleronius*	0.990	n.d.	n.d.
	2052	*Rummeliibacillus pycnus*	0.970	n.d.	n.d.
2312 (Infected)	2039	*Bacillus licheniformis*	0.982	n.d.	n.d.
	2047	*Lysinibacillus fusiformis*	0.970	n.d.	n.d.
	2048	*Sporosarcina contaminans*	0.980	n.d.	n.d.
	2050	*Streptococcus thoraltensis*	0.990	n.d.	n.d.
	2051	*Rummeliibacillus pycnus*	0.970	n.d.	n.d.
	3308	*Lactobacillus mucosae*	0.996	n.d.	n.d.
2373 (Infected)	1063	*Escherichia coli*	0.987 (*Shigella flexneri* / *Escherichia fergusonii*)	-	n.d.
2429 (Infected)	3227	*Staphylococcus warneri*	0.990	n.d.	n.d.
	3138	*Pediococcus acidilactici*	0.990	n.d.	+
2435 (Infected)	1049	*Escherichia coli*	0.980 (*Shigella flexneri* / *Escherichia fergusonii*)	-	n.d.
2436 (Infected)	1070	*Escherichia coli*	0.973 (*Escherichia fergusonii*)	-	n.d.
2507 (Infected)	1064	*Escherichia coli*	0.960 (*Shigella flexneri*)	SLT-I	n.d.
	3180	*Streptococcus pluranimalium*	0.990	n.d.	n.d.
	2029	*Bacillus licheniformis*	0.995	n.d.	n.d.

Healthy, pregnant animals and those diagnosed with post-partum uterine infections at the time of sampling are indicated in brackets

[(a)] % identity of partial 16S rDNA to type strain or closest relative; +: positive PCR results; -: negative PCR results; n.d.: data not determined

*Cow #2211-A and 2211-B represent two different animals that were assigned the same number at different times

2.3.2. Pediocin Production

PCR screening revealed that *Ped. acidilactici* FUA3137, FUA3140, and FUA3138 harboured the pediocin AcH/PA-1 immunity gene (data not shown). Pediocin production was investigated via deferred inhibition assays. *Ped. acidilactici* FUA3140 and the positive control meat isolate *Ped. acidilactici* FUA3072 produced inhibition zones against *Enterococcus faecalis* FUA3141 (**Figure 2-2a**). Inhibition zones of comparable diameter were observed with *L. innocua* (data not shown). Further tests with proteinase K verified that the antimicrobial agent is a protein (**Figure 2-2b**).

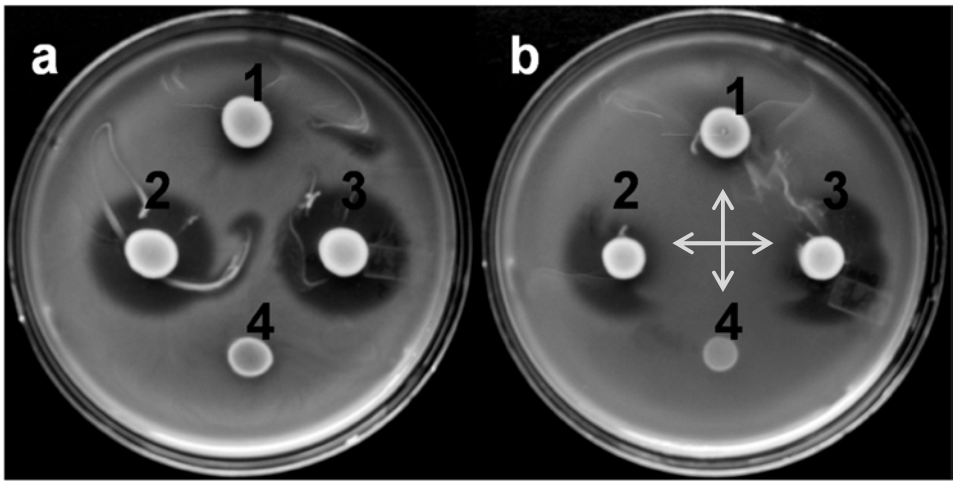

Figure 2-2. Deferred inhibition assay for bacteriocin production. Test strains were grown on mMRS and overlaid with *Enterococcus faecalis* FUA3141, which was as an indicator strain. **Panel a**, no addition of proteinase; **panel b**, addition of proteinase K adjacent to colonies of test strains. Arrows indicate the site of proteinase K application. The following test strains were used, 1, *Ped. acidilactici* FUA3138; 2*, Ped. acidilactici* FUA3072; 3, *Ped. acidilactici* FUA3140; 4, *Lb. sakei* FUA3089. Comparable results were observed with *Listeria innocua* ATCC33090 used as an indicator strain (data not shown). The indicator strains of *E. coli* FUA1036, FUA1063 and FUA1064 were also used but no inhibition was observed (data not shown).

Although *Ped. acidilactici* FUA3138 was positive for the pediocin AcH/PA-1 immunity gene, the isolate consistently produced smaller inhibition zones and yielded negative results with proteinase K tests. This suggested that *Ped. acidilactici* FUA3138 was not likely to produce any active pediocin. Other vaginal isolates including *E. coli* FUA1036, FUA1063, and FUA1064 were also used as indicator strains but no inhibition was observed (data not shown).

2.3.3. Quantification of Bacterial Groups, SLT and Pediocin Structural Genes

PCR-DGGE analysis was initially carried out to characterize bovine vaginal microbiota by a culture-independent approach. The DNA concentration of samples from most healthy cows, however, was below the detection limit of PCR-DGGE analysis and DGGE patterns could be obtained only for two samples (data not shown). A clone library was constructed using PCR products that were amplified with HDA primers from one of two animals. Results confirmed that all bacteria present in the bovine vagina were accounted for by culturing (data not shown). To overcome limitations of PCR-DGGE analysis, quantitative PCR was employed as sensitive and quantitative tool for culture-independent analysis of the composition of vaginal microbiota before and after parturition instead. Primers were selected to quantify bacterial groups isolated from healthy, pre-partum or post-partum animals, as well as SLT genes and the pediocin structural gene (pedA) (**Table 2-2** and **2-3**). Ten animals were sampled two weeks pre-partum and two weeks post-partum. To account for the large individual differences in the vaginal microbiota of different animals, results were calculated as differences

(post-partum – pre-partum) between the least squares means of log rDNA or DNA copy numbers for each target group (**Figure 2-3**).

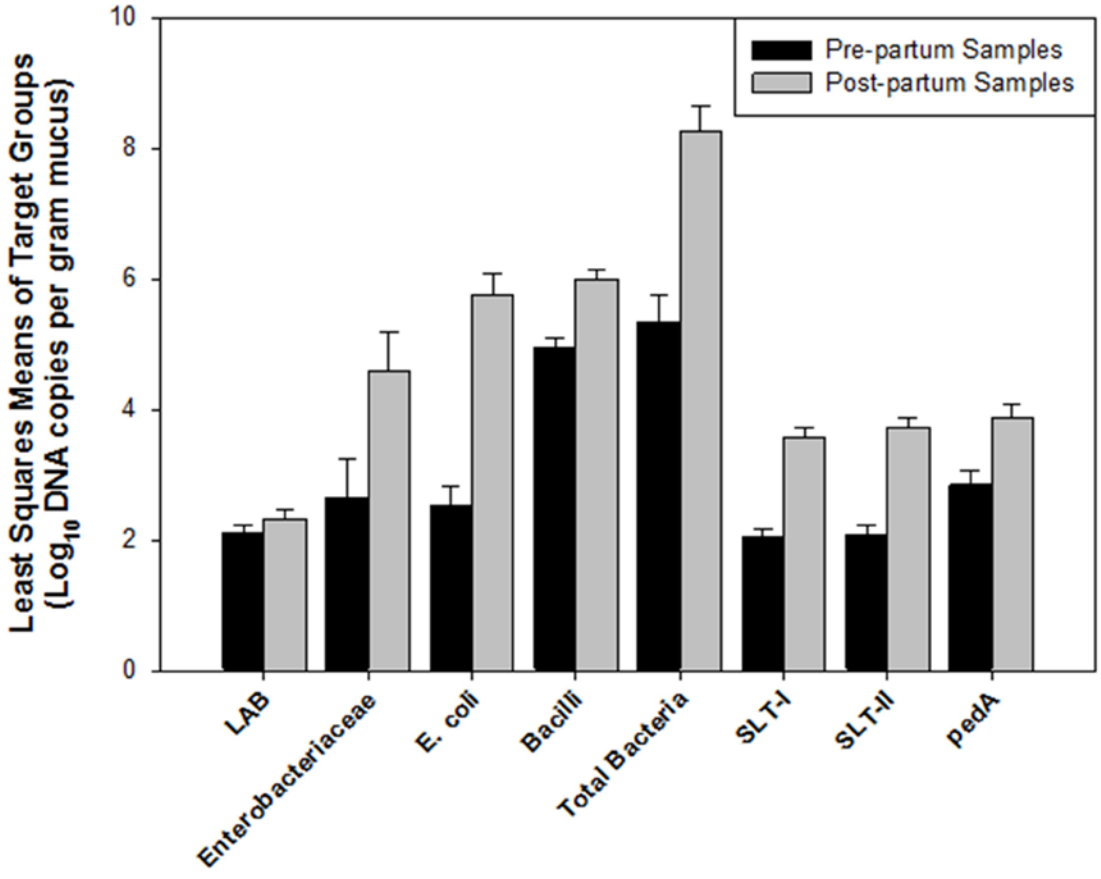

Figure 2-3. Differences in least squares means of log rDNA or DNA copy numbers of target groups. Vaginal mucus was sampled from ten animals before and after calving, and bacterial rDNA, shiga-like-toxin genes, and the pediocin structural gene were quantified by qPCR. The figure depicts the differences in least squares means of the target groups. Statistically significant differences between pre-partum and post-partum periods were observed in all groups (as indicated by *) except for the lactic acid bacteria group.

Number of rDNA copies of the *Lactobacillus* group relatively stable in the observation period with no statistically significant changes between the pre-partum and post-partum periods. In all other cases, the postpartum gene copy values are higher than the prepartum values. The pediocin structural gene was consistently detected in low numbers. Approximately a 3 log difference between the total bacteria values was observed. This increase was predominantly attributable to increased numbers of *E. coli* and *Enterobacteriaceae*. *E. coli* increased on average by more than 3 log. Genes coding for SLT-I and SLT-II increased by less than 2 log. *Enterococcus* spp. and *Staphylococcus* spp. were below the detection limit.

2.4. Discussion and Conclusions

This study provides a comparison of the vaginal microbiota of healthy, pregnant dairy cows, and infected postpartum cows by using culture, PCR, and qPCR. In contrast to the stable commensal microflora observed in humans and other mammals, total bacterial numbers in vaginal mucus were low and the composition of the bovine vaginal microbiota on species level was highly variable. Bacteria found within the microbiota are thus likely to be contaminants from the environment, the cow's skin, and or fecal material, rather than representing a stable flora autochthonous to the reproductive tract. Quantitative PCR confirmed the presence of lactic acid bacteria, staphylococci, *E. coli*, and bacilli in the vagina of pregnant dairy cows. Moreover, counts of *Enterobacteriaceae* and *E. coli* were found one thousand fold higher in infected,

post-partum cows compared to samples from the same animals obtained pre-partum.

Overall, our data indicated that vaginal bacterial flora in cows affected by metritis was dominated by strains of *E. coli*, supporting previous observations (Ambrose et al., 1986). This study extends previous results by documenting changes of the vaginal microbiota in individual animals in the first two weeks after calving. Both the *Enterobacteriaceae* and *E. coli* showed marked increase in mucus samples collected from infected post-partum cows. The amplification of *Shigella* rDNA with *E. coli* species-specific primers is not surprising because *Shigella* spp. and *E. coli* are indistinguishable on the basis of rDNA sequences (Christensen et al., 1998). In keeping with the recognition of *Shigella* spp. as human-adapted pathovar of *E. coli*, all isolates were identified as *E. coli* by biochemical tests. Culture-based analysis and qPCR demonstrated presence of STEC in both healthy and infected animals. Three out of eleven *E. coli* isolates were found to carry genes coding for SLT-1 or SLT-II. Moreover, SLT-genes were consistently detected by qPCR in samples from metritic cows; STEC accounted for about 1-10% of the total *E. coli* population. SLT production causes diarrhoea in calves (Wani et al., 2003), but the role of STEC in the pathogenesis of metritis in adult animals warrants further clarification.

Bacilli are present in the environment and they frequently contaminate the bovine uterine lumen (Williams et al., 2008). However, pediococci have not yet been described as part of the bovine vaginal microbiota. The genus *Pediococcus* is closely related to the genus *Lactobacillus*. Pediococci produce antimicrobial

compounds such as organic acids, hydrogen peroxide, and antimicrobial peptides such as pediocin AcH/PA-1 (Eijsink et al., 2002). *Ped. acidilactici* are applied as starter cultures for meat fermentation and are additionally used as probiotic cultures, or as protective cultures to inhibit food-borne pathogens such as *L. monocytogenes* or *Staphylococcus aureus* (Hudson et al., 2000). *Ped. acidilactici* was isolated from the GI tract of poultry, ducks, and sheep, and pediocin AcH/PA-1 producing strains have been isolated from human infant faeces (Hudson et al., 2000; Juven et al., 1991; Kurzak et al., 1998; Mathys et al., 2007).

The synthesis of pediocin AcH/PA-1 was initially described for the strains *Ped. acidilactici* PAC1.0 and *Ped. acidilactici* H, but synthesis has also been observed in other *Ped. acidilactici* strains as well as *Lactobacillus plantarum* WHE92, *Pediococcus parvulus* ATO34, and ATO77 (Bennik et al., 1997; Ennahar et al., 1996; Gonzalez & Kunka, 1987). Pediocin AcH/PA-1 production is a plasmid-borne trait (Ray et al., 1989). The pediocin AcH/PA-1 operon consists of pediocin AcH/PA-1 gene (*pedA/papA*), a specific immunity gene (*papB*), and genes responsible for processing and secretion (*papC* and *papD*) (Marugg et al., 1992).

Majority of *Ped. acidilactici* isolates harboured the pediocin AcH/PA-1 operon, and qPCR analyses consistently detected the operon in both pre-partum and post-partum vaginal samples. However in addition to PCR, sequencing and qPCR analyses, deferred inhibition assays are required to confirm pediocin production since *Ped. acidilactici* FUA 3140 but not *Ped. acidilactici* FUA 3138

was found to produce active pediocin, despite positive PCR, sequencing and qPCR results that were obtained from both isolates.

Studies on the isolation of bacteriocin-producing lactic acid bacteria from the human vagina and their antimicrobial activities against human vaginal pathogens are well established (Aroutcheva et al., 2001; Juarez Tomás et al., 2002; Sobel, 1999). Bacteriocin-producing *Lactobacillus* strains inhibited vaginal pathogens including *Gardnerella vaginalis* and *Pseudomonas aeroginosa* (Alpay-Karaoğlu et al., 2003). Although prior research by Otero et al. (2000), Sobel and Chaim (1996) indicated that bovine vaginal microbiota have much lower total cell counts and lactobacilli populations in comparison to the human vaginal microbiota and that Hammes and Hertel (1998) demonstrated that pediocin was not active against *E. coli*, which is the dominant organisms in the vaginal microbiota of infected animals, bacteriocin such as pediocin may influence the microbial ecology in the reproductive tract of dairy cattle if the producer strains are administered in high numbers. Therefore, additional research is need for determining the potential impact of pediocin-producing *Ped. acidilactici* on the bovine vaginal microbiota, and their efficacy in preventing and treating metritis.

In conclusion, culture-dependent analysis of vaginal microbiota of dairy cows, supported by PCR and qPCR analyses, allowed the characterization of the bovine vaginal microbiota of healthy pregnant and infected post-partum cows and the identification of Shiga-like-toxin producing strains of *E. coli*. Identification of pediocin-producing pediococci in the bovine vaginal microbiota may encourage the development of novel prophylactic interventions against metritis by

application of bacteriocin-producing probiotic bacteria into the vaginal tract of dairy cows during the transition period.

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Chapter 3

Exopolysaccharide Synthesized by *Lactobacillus reuteri* Decreases the Ability of Enterotoxigenic *Escherichia coli* to Bind to Porcine Erythrocytes

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CHAPTER 3

3.1. Introduction

Lactic acid bacteria (LAB) are important in food production due to their positive contribution to flavor and preservation of the final product. Some of these food-grade microorganisms are also valuable for their ability to synthesize exopolysaccharides (EPS). EPS are high-molecular-weight sugar polymers, which remain attached to the microorganism as capsular EPS or become excreted into the environment in the form of slime or ropy EPS (Sutherland, 1972; van Hijum et al., 2006). LAB utilize two distinct biosynthetic pathways to produce EPS. Heteropolysaccharides (HePS) comprised of two to eight repeating units of monosaccharides are assembled by cell wall-bound glycosyltransferases in low quantities from intracellular sugar nucleotide precursors (de Vuyst et al., 2001). Extracellular glycansucrases (glucan- or fructansucrases) synthesize homopolysaccharides (HoPS) consisting of either glucose or fructose from sucrose, and their yield can be as high as 40 g/L (Korakli et al., 2003; Monsan et al., 2001). EPS formation by glycansucrases has been reported for lactobacilli of the species *Lactobacillus reuteri*, *Lb. pontis*, *Lb. panis*, *Lb. acidophilus*, and *Lb. frumenti* (Tieking, Kaditzky, et al., 2003; Tieking, Korakli, et al., 2003). HoPS synthesis and the corresponding genes have been especially well characterized in *Lb. reuteri* (Tieking & Gänzle, 2005). All EPS used in this study were HoPS formed in the presence of sucrose. In addition to HoPS synthesis, glycansucrases are capable of producing oligosaccharides (OS). Several OS are found to have prebiotic properties as they can benefit host health by acting as non-digestible food ingredients and by enhancing the growth of desired microbial

members of the gastrointestinal microbiota (Gibson & Roberfroid, 1995). Emerging research efforts have investigated potential applications of OS as anti-adhesive agents in preventing pathogen colonization. Shoaf et al., (2006) suggested that commercial oligo- and polysaccharides reduce the adherence of enteropathogenic *Escherichia coli* (EPEC) to cell lines. Martín-Sosa et al., (2002) reported antiadhesive properties of human milk oligosaccharides against human strains of enterotoxigenic *Escherichia coli* (ETEC) and uropathogenic *E. coli*. Similarly, Martín et al., (2002) confirmed the binding of milk oligosaccharides to ETEC isolated from calves. In contrast, adhesion studies with EPS are limited.

The inhibition of ETEC is of special interest to the swine industry because ETEC is the primary cause for diarrhea in neo- and postnatal piglets and results in substantial economic losses. We therefore aimed to test for anti-adhesive properties of EPS synthesized by LAB and commercially available prebiotics against porcine ETEC strains. Hemagglutination assays were used; these assays are generally accepted as an effective model system for testing ETEC adherence as hemagglutination resembles the attachment of K88-positive bacteria to the gut wall (Jones & Rutter, 1974).

3.2. Materials and Methods

3.2.1. Preparation of EPS

EPS was obtained from *Lb. reuteri* strains TMW1.656, LTH5794, and FUA3048 and *Weissella cibaria* strains 10M and W58. *Lb. reuteri* TWM1.656, LTH5794, and FUA3048 formed reuteran, levan, and an uncharacterized glucan, respectively (**Table 3-1**).

Table 3-1. Strains used for EPS production.

Strains used for EPS production	Origin	EPS Formation Visible on Modified MRS	Glycosyltransferase Gene(s) (PCR)	HoPS Monosaccharide Component	EPS	Reference(s)
Lactobacillus reuteri						
TMW1.656	Sourdough	+	*gtf*A, *inu*	Glucose	Reuteran	Kaditzky et al., 2008; Schwab & Gänzle, 2005
LTH5794	Human intestine	+	*gtf*B	Fructose	Levan[b]	Gänzle & Schwab, 2009; Schwab & Gänzle, 2005
FUA3048	Mouse instestine	+	ND[c]	Glucose	Glucan	This study
Weissella cibaria						
10 M	Sourdough	+	*dexWc*[a]	Glucose	Dextran	This study; Kang et al., 2009; Schwab et al., 2008
W58	Sourdough	+	*dexWc*	Glucose	Dextran	This study; Kang et al., 2009; Schwab et al., 2008

[a] Amplification of partial sequence using primer: GTFWcFor (5'-GCATCTTTCAATACTTGAGG-3') and GTFWcRev (5'-CATGACTTGTTGGCATAGC-3') obtained from accession no. ACK28203; dexWc is the dex gene from *W. cibaria*.
[b] D. Bundle, personal communication.
[c] ND, not determined.

W. cibaria 10M and W58 formed a dextran, and the corresponding dextransucrase genes were amplified by PCR using primers GTFWcFor (5'-GCATCTTTCAATACTTGAGG-3') and GTFWcRev (5'-CATGACTTGTTGGCATAGC-3'). The amplified 993- and 981-bp sequences of *W. cibaria* 10M and W58, respectively, were 97% homologous to the dextransucrase dsrWc gene of *Weissella cibaria* CMU (ACK38203) (Kang et al., 2009; Clarissa Schwab et al., 2008). Strains were grown in modified MRS medium containing 100 g/L sucrose as the sole carbon source (Stolz et al., 1995). EPS was harvested via ethanol precipitation (Tieking, Korakli, et al., 2003), dialyzed, and further purified by hot phenol extraction (Westphal & Jann, 1965). Mono-, di-, and oligosaccharides were removed by dialysis using Spectra/Por 2 molecular porous membrane tubing (molecular weight cutoff [MWCO], 12,000 to 14,000) (Spectrum Laboratories Inc., Rancho Dominguez, USA) at 4 °C with frequent changes of ultrapure water. UltraPure buffer-saturated phenol (Invitrogen, Burlington, Canada) was added to each sample at an equal volume. The samples were incubated in a water bath at 70 °C for 70 min, cooled on ice for 30 min, and centrifuged at 3,000 × g for 20 min at 4 °C. The aqueous layer was collected and dialyzed to remove phenol for 4 days. Samples were freeze-dried. SDS-PAGE was used to confirm removal of proteins. Commercially available dextrans (dextran, 1×10^5 to 2×10^5 Da; dextran HM, 5×10^6 to 4×10^7 Da; Sigma Aldrich, Oakville, Canada), isomalto-oligosaccharides (IMO) (VitaSugar; BioNeutra Inc., Edmonton, Canada), cellobiose (Sigma Aldrich), inulin from chicory (Sigma Aldrich), raffinose (Difco, Mississauga, Canada), and Raftiline

ST (Orafti, Tienen, Belgium) were included for comparison. Sizes of bacterial EPS were determined by size-exclusion chromatography using a Superdex 200 column (GE Healthcare, Baie d'Urfe, Canada). Water was used as a solvent at a flow rate of 0.4 ml/min. EPS was detected with a refractive index (RI) detector.

3.2.2. Haemagglutination Assays

Four porcine ETEC strains positive for the K88 (F4) antigen were obtained from the *Escherichia coli* Laboratory at the University of Montréal. The strains used were *E. coli* ECL13086 (O149, virotype STa:STb:LT:EAST1:F4), ECL13795 (O149, virotype STb:LT:EAST1:F4), ECL13998 (O149, virotype STa:STb:LT:EAST1:F4:Paa), and ECL14048 (O149, virotype STb:LT:EAST1:F4:Paa). Overnight cultures were recovered from Minca agar with 1 ml of phosphate-buffered saline (PBS; 150 mM, pH 7.2) (Guinée et al., 1977). For preparation of erythrocytes, porcine whole blood (Innovative Research, Novi, Michigan, USA) was washed three times in PBS and resuspended in PBS at 5%. Hemagglutination tests were conducted based on the protocol used by Martín et al., (2002). Briefly, ETEC suspensions, which contained between 2.5×10^{11} and 2.5×10^{12} CFU of bacterial cells in 25 µl, were diluted 2-fold in V-bottomed 96-well polystyrene microtiter plates (Corning, New York, USA). Twenty-five microliters of PBS or PBS containing EPS was added and incubated for 5 min. Finally, 25 µl of 5% erythrocyte suspension in PBS was applied to the wells and mixed gently. Microtiter plates were inspected visually after 2 h of incubation at 4 °C.

3.3. Results and Discussion

EPS produced by *Lb. reuteri* LTH5794 and FUA3048 were slightly larger than EPS synthesized by *Lb. reuteri* TMW1.656 and *W. cibaria* W58 and 10M (**Figure 3-1** and data not shown). Purified bacterial EPS and commercial oligo- and polysaccharides were tested at concentrations of 2.5, 5, and 10 mg/mL in hemagglutination assays. All four porcine ETEC strains agglutinated porcine erythrocytes and were resistant to mannose at 10 mg/mL (**Figure 3-2)**. Glucan, levan, and reuteran consistently inhibited hemagglutination at 10 mg/mL for all ETEC strains (**Table 3-1**). Anti-hemagglutination activities for these EPS were also observed at 5 mg/mL in a few strain-dependent cases but were less pronounced. In contrast, anti-hemagglutination activities were not observed at 10 mg/mL using commercially available oligo- and polysaccharides; tests with lower sugar concentrations were not conducted. Overall triplicate tests were conducted with three different EPS preparations, fresh ETEC cultures, and blood.

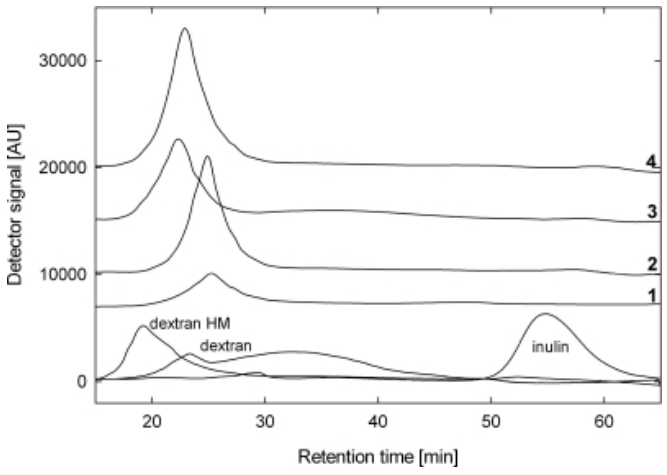

Figure 3-1. Size-exclusion chromatography of EPS produced by *W. cibaria* W58 (line 1) and *Lb. reuteri* TMW1.656 (line 2), LTH5794 (line 3), and FUA3048 (line 4) in comparison to commercially available dextran, dextran HM, and inulin.

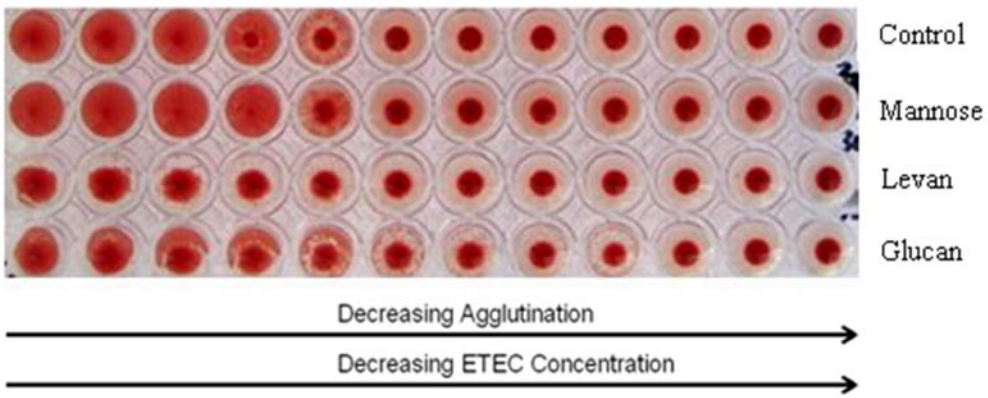

Figure 3-2. Hemagglutination of porcine erythrocytes in the presence of mannose, levan (*Lactobacillus reuteri* LTH5794), and glucan (*Lactobacillus reuteri* FUA3048) at 10 mg/mL, using enterotoxogenic *E. coli* ECL14048, which was serially diluted 2-fold. PBS (150 mM at pH 7.2) was used as a negative control.

Table 3-2. Inhibition of erythrocyte agglutination of four porcine ETEC strains tested against bacterial EPS and commercially available prebiotics.

	EPS Source and Oligosaccharides/ Polysaccharides	Agglutination of ETEC at conc. (mg/mL)[a]: 10	5	2.5
Bacterial				
	Reuteran (*Lactobacillus reuteri* TMW 1.656)	+	+/–	–
	Levan (*Lactobacillus reuteri* LTH 5794)	+	+/–	–
	Glucan (*Lactobacillus reuteri* FUA 3048)	+	+/–	–
	Dextran (*Weisella cibaria* 10M)	–	ND	ND
	Dextran (*Weisella cibaria* W58)	–	ND	ND
	Dextran (Sigma Aldrich)	–	ND	ND
	Dextran HM (Sigma Aldrich)	–	ND	ND
Commercial				
	IMO (VitaSugar^{TM}, BioNeutra Inc.)	–	ND	ND
	Cellobiose (Sigma Aldrich)	–	ND	ND
	Inulin from chicory (Sigma Aldrich)	–	ND	ND
	Raffinose (Difco)	–	ND	ND
	Raffiline ST (Orafti)	–	ND	ND

[a]ND, not determined; +, agglutination observed; –, agglutination not observed; +/–, strain dependent agglutination.

Our results have indicated that LAB EPS can interfere with ETEC adhesion and therefore have the potential to benefit the swine industry. Rapid proliferation of ETEC is attributed to fimbria-triggered attachment to specific receptors on intestinal enterocytes and subsequent secretion of heat-labile and heat-stable toxins (Gross et al., 1976). In particular, K88 fimbriae were found to be associated with the colonization of the small intestines of neonatal and postnatal piglets by interacting with glycoproteins in ileal mucus and receptors on porcine intestinal epithelial cells (Conway et al., 1990; Jones & Rutter, 1974; Metcalfe et al., 1991; Sellwood, 1980; Wilson & Francis, 1986). K88 fimbriae are composed of a single adhesin major protein subunit and minor subunits that are suspected to regulate fimbrial expression (Bakker et al., 1992; van Zijderveld et al., 1990). K88 fimbriae bind to cell surface receptors that contain carbohydrate structures, with β-D-galactose being an essential component (Bijlsma & Frik, 1987; Gibbons et al., 1975; Sellwood, 1980). Galactose-containing glycoprotein in pig gastric mucin, glucosamine, and chondrosine inhibited hemagglutination in ETEC K88 strains (Meng et al., 1998; Payne, 1994). Inhibition of adherence was suspected to be nonspecific as the presence of large hydrophobic glucoside molecules likely disrupted hydrophobic interactions between fimbriae and the receptor (Payne, 1994). Bacterial EPS successfully decreased the adherence of ETEC strains. The presence of aggregatory compounds, receptor analogues, or competitive exclusion might contribute to binding inhibition (Korakli & Vogel, 2006). Interestingly, our results indicated that among glucans, the α-(1-4)-, α-(1-6)-linked reuteran and a glucan of undetermined linkage type had

antihemagglutination activity but the predominately α-(1-6)-linked dextran did not (van Hijum et al., 2006). In the case of fructans, the β-(2-6)-linked levan was found to have antiadherence properties but the β-(2-6)-linked inulins (Sigma Aldrich and Orafti) did not (van Hijum et al., 2006). Although the glucans and fructans tested were identical in composition, they differ greatly in molecule weight and structure. Yet, whereas no correlation between molecule weight and antiagglutination activity could be observed, our findings suggest that antiadherence requires a certain degree of structural specificity. Further studies on the structure/function relationships among EPS, ETEC adhesins, and host cell receptors are needed to gain a deeper understanding of the antiadhesive properties of bacterial EPS and to determine the mode of interaction of ETEC and EPS.

Currently, antibiotics are widely used in pig production at subtherapeutic and therapeutic levels. However, increasing public health concerns have led to efforts to reduce antibiotic use. The application of bacterial EPS might be an alternative to prevent and treat diarrhea caused by ETEC. EPS can be produced in cereal fermentations and can be easily incorporated into feed in liquid and dry form (Kaditzky et al., 2008; Schwab et al., 2008; Tieking & Gänzle, 2005).

3.4. Acknowledgements

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3.5. References

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Chapter 4

Characterization of α-Galactooligosaccharides Formed via Heterologous Expression of α-Galactosidases from *Lactobacillus reuteri* in *Lactococcus lactis*

This chapter includes experimental work of Brenna Black, performed under the supervision of Dr. Michael G. Gänzle and Dr. Jonathan Curtis. A version of this chapter will be submitted for publication in Applied Microbiology and Biotechnology

4.1. Introduction

As the interactions between the surface glycans of eukaryotic cells and the adhesion mechanisms of viruses, pathogenic bacteria and their toxins become more elucidated, therapeutic applications of using oligosaccharides to treat infections in farm animals and humans have significantly increased in recent times (Arslanoglu et al., 2007; Bruzzese et al., 2009; Liu et al., 2008; Rozeboom et al., 2005). For example, human milk oligosaccharides (HMO) have shown to inhibit bacterial adhesion to epithelial surfaces in infants (Kunz et al., 2000). Furthermore, β-galacto-oligosaccharides (β-GOS) prevented the adherence of *Salmonella enterica* in models that were *in vitro* and *in vivo* (Searle et al., 2010). A number of commercial β-GOS were able to also prevent the adhesion of enteropathogenic *Escherichia coli* to tissue culture cells (Searle et al., 2010; Shoaf et al., 2006). In addition, specific glycan configurations in the β-anomer forms, such as Gal-β-(1→4)-Glc and Gal-β-(1→4)-Fuc-α-(1→3)-Glcβ, have been identified as binding targets for toxins such as heat labile enterotoxin (Bertozzi & Kiessling, 2001). Similar α-anomers forms of glycan such as Gal-α-(1→4)-Galβ and Gal-α-(1→4)-Gal-β-(1→4)-Glc have also been identified in the targets of P-frimbriae of *E. coli* and Shiga toxin I respectively.

Through exploiting the transgalactosylation properties of microbial β-galactosidases (β-Gal) with different acceptor carbohydrates and sugar alcohols, numerous studies have achieved enzymatic production of β-GOS and heterooligosaccharides (HeOS) with potential applications in food and therapeutics (Bridiau et al., 2006; Lu et al., 2009; Park & Oh, 2010). Studies by

Schwab et al. (2011) specifically produced β-GOS and β-HeOS using lactic acid bacteria (LAB) and bifidobacteria that potentially mimicked the receptor glycan structures of bacterial adhesins and toxins and terminal structures of human milk oligosaccharides.

Alpha-galactosidases [EC 3.2.1.22] hydrolyze α-(1→6) galactosidic linkages present in alpha-galactooligosaccharides (α-GOS). When acceptor sugars are present in high concentrations, the formation of α-GOS via transferase activities of α-Gal can occur. Alpha-GOS formation via α-Gal in the LAB strain *Lactobacillus reuteri* has been previously investigated by Alazzeh et al., (2009) and Tzortzis et al. (2003). Additionally, according to Teixeira et al. (2012), the use of LAB levansucrase to produce α-GOS from raffinose family oligosaccharides (RFO) has recently been found to be an effective process. However, the formation of α-GOS via transferase activities of α-Gal with different acceptor carbohydrates has not yet been examined. Furthermore, there was no such data obtained for cloned α-Gal from lactic acid bacteria. Moreover, not much information has been provided on the degrees of polymerization and the linkage types of the oligosaccharides that were produced during acceptor reactions.

Therefore, the aim of this study was to characterize the α-Gal from *Lb. reuteri* 100-16 and the genome-sequenced *Lb. reuteri* 100-23 by cloning the gene (*aga*) into the α-Gal negative model strain *Lactococcus lactis*. Transgalactosylation reactions of α-Gal were achieved using the crude cell extracts (CCE) of the transformed α-Gal-active *Lc. lactis* and the structures of the

α-GOS were determined using electrospray ionisation tandem mass spectrometry (ESI-MS/MS). These findings will contribute to support the future incorporation of α-GOS as therapeutic functional food ingredients to reduce the incidence of gastrointestinal infections and to improve the health of farm animals and humans alike.

4.2. Materials and Methods

Asterisks (*) indicate contributions by Brenna Black.

*4.2.1. Chemicals and standards

Oligosaccharide standards including melibiose, raffinose, and stachyose were purchased from Sigma Aldrich (Oakville, Canada) and globotriose was purcchased from Carbosynth (Berkshire, United Kingdom). Fisher Scientific (Ottawa, Canada) supplied HPLC grade acetonitrile, methanol, and ammonium acetate. All other solvents were of analytical grade unless otherwise specified.

4.2.2. Bacterial strains and growth conditions

All LAB strains *Lb. reuteri* 100-23, *Lb. reuteri* 100-16, and *Lactococcus lactis* MG1363 were obtained from the food microbiology laboratory strain collection at the University of Alberta (Edmonton, Canada). *Escherichia coli* TG1 was purchased from Stratagene (Amsterdam, the Netherlands). *Lb. reuteri* strains were grown in modified deMan-Rogosa-Sharp (mMRS) media (10 g/L trypton, 5 g/L beef extract, 5 g/L yeast extract, 10 g/L malt extract, 10 g/L maltose, 5 g/L glucose, 5 g/L fructose, 4 g/L dipotassium phosphate, 2 g/L potassium phosphate, 3 g/L ammonium chloride, 0.5 g/L L-cysteine hydrochloride monohydrate, 0.2 g/L magnesium sulphate, 0.05 g/L manganese sulphate, 1 g/L Tween 80, pH 6.5).

Lc. lactis were grown in M17 media with the addition of 0.5% glucose (mM17). *E. coli* was grown in LB. All agar plates contained 15 g/L agar for each medium and incubation temperature for all strains were at 37 °C.

4.2.3. Cloning the α-Gal and transformation of *E. coli* and *Lc. lactis*

The *E. coli – Lc. lactis* expression shuttle vector pAMJ586 previously provided by Søren Madsen (Bioneer A/S, Hørsholm, Denmark) was used for cloning the α-Gal gene (*aga*). The α-Gal genes from *Lb. reuteri* 100-23, *Lb. reuteri* 100-16 were amplified by PCR using primers listed in **Table 4-1**.

Table 4-1. Primers used in α-galactosidase amplification.

Primer Name	5' – 3'	Reference Sequence Accession Number	Restriction Enzyme Site Included
LR-agaF-Sma+RBS	TCCCCCGGGTCTAGATTAGGGTAACTTTGAAAGGATATTCCTC**ATG**ATTACATTTGATGAACAGC	NZ_AAPZ02000002 (Region: 241775..243964)	*Sma*I
LR-agaR-Sal	ACGCGTCGAC*CTA*TTCACCTTTAAAGTAATGC		*Sal*I

Underlined: restriction site; bold: start codon; italics: stop codon; highlighted: α-galactosidase (*aga*) gene sequence.

Restriction digest of the PCR products was then conducted to generate the insert with FastDigest® restriction enzymes *Sma*I and *Sal*I (Fermentas, Burlington, Canada). The same restriction digest was done for the pAMJ586 shuttle vector and all *Sma*I and *Sal*I restriction fragments were gel purified using the PureLink™ Quick Gel Extraction Kit (Life Technologies Inc., Burlington, Canada) after electrophoresing in a 1% agarose gel at 90 V for 45 minutes.

Ligation using the T4 DNA ligase (Fermentas) was done to produce the final constructs, which were named as pAMJ586-*aga*23 and pAMJ586-*aga*16.

The recombinant plasmid was transformed into *E. coli* TG1 via electroporation to first increase the yield of pAMJ586-*aga*LR, then it was subsequently transformed into the α-Gal negative wild-type host strain *Lc. lactis* MG 1363. The electroporation conditions used were 25 μF, 1.7 kV, and 200 Ω in 0.1 cm Gene Pulser® cuvettes (Biorad, Mississauga, Canada). Electroporated *E. coli* cells were recovered in SOC medium (Life Technologies) and electroporated *Lc. lactis* cells were recovered in mM17. Both strains were incubated for at least 2 hours at 37 °C. After recovery, transformed strains were grown on their respective media with erythromycin as the selective agent at (5 mg/L in LB) for *E. coli* and (100 mg/L in mM17) for *Lc. lactis*. Competent *Lc. lactis* was prepared as described by Schwab et al (2010), except overnight cultures were inoculated 2% in 500 mL mM17 supplemented with 1% glycine. Successful cloning of the gene was confirmed by PCR and sequencing by Macrogen (Macrogen, Rockville, USA). Gene expression was confirmed with the observation of blue coloured transformants growing on mM17 agar supplemented with erythromycin at 5 mg/L and 20 μl of 20 mg/ml chromogenic compound 5-Bromo-4-chloro-3-indoxyl-α-D-galactopyranoside (X-α-D-Galactoside) (Gold Biotechnology Inc., St. Louis, USA) that forms the blue colouration upon enzymatic degradation by α-Gal. The new transformants, which carries *aga*23 and *aga*16 (and expresses AGA23 and AGA16) from *Lb. reuteri* 100-23 and

Lb. reuteri 100-16, were incorporated into the strain collection and were respectively designated as FUA 3376 and FUA 3377.

4.2.4. Preparation of crude cell extract (CCE)

Single colonies of wild-type *Lc. latis* and transformed *Lc. latis* (FUA 3016, FUA 3376, FUA 3377) were individually used to inoculate 10 ml of mM17 with 0.0274% manganese sulphate added (Ibrahim et al., 2010). The cultures were incubated overnight and were used to inoculate 500 ml of the same medium at 2%. The cultures were then incubated until the pH reaches between pH 5 and pH 5.2 before cells were harvested by centrifugation at 5525 x g for 20 minutes. Each cell suspension was washed once in McIlvaine Buffer (a mixture of 0.1 M citric acid and 0.2 M disodium phosphate), which was adjusted to pH 5.66 (McIlvaine, 1921). The pellets were then resuspended in the same buffer, but additionally supplemented with 10% glycerol and 0.0274% manganese sulphate. The final cell suspensions were approximately 50x more concentrated than the original culture and they were disrupted using a bead beater at 4 °C. Crude cell extracts were collected after centrifuging at 12, 000 x g for 10 minutes at 4 °C.

4.2.5. Determination of α-Gal activity of AGA23 and AGA16 in CCEs

Enzymatic assays were conducted using a protocol modified from (Church, Meyers, & Srinivasan, 1980) by adding 5 µL of each CCE to 95 µL of 4 mg/mL 4-nitrophenyl-α-D-galactopyranoside (PNPG). The reaction was stopped every minute by the addition of 130 µL of 1 M sodium carbonate (Na_2CO_3) until 5 minutes was reached. Absorption at 400 nm was determined using a Varioskan Flash Multimode Reader (Fisher Scientific Limited, Ottawa, Canada). Relative α-

Gal activity of each CCE was defined as the liberation of 1 mmol PNPG per minute per mg of CCE at 35 °C. Using the Bio-Rad Protein Assay reagent (Bio-Rad) and bovine serum albumin (New England Biolabs, Missisauga, Canada) as standards, protein contents of all CCEs were determined. The optimum pH was determined using McIlvaine buffer with PNPG dissolved at 4 mg/mL; pH was adjusted to 3.34, 3.64, 4.03, 4.47, 4.64, 4.75, 5.48, 6.10, 6.67, and 7.03. For optimum temperature determination, temperatures between 20 °C to 55 °C with increments of five degrees were used. In all cases, no α-Gal activity was detected for the wild-type *Lc. lactis* FUA3016 CCE.

4.2.6. Acceptor sugar reactions and high performance liquid chromatography analyses

Crude cellular extracts (CCE) of *Lc. lactis* with active α-gal (178 ± 10 μmol•min-^{1}mg^{-1} most active at ambient room temperature and pH 4.75; or 151 ± 39 μmol•min-^{1}mg^{-1} at 135 °C and pH 4.7) were incubated at 30 °C for 24 hours with meliobiose (600 mg/mL), raffinose (600 mg/mL), meliobiose (300 mg/mL) + lactose (300 mg/mL), melibiose (300 mg/mL) + fucose (300 mg/mL), meliobiose (300 mg/mL) + N-acetylglucosamine (NAG) (300 mg/mL), raffinose (300 mg/mL) + lactose (300 mg/mL), raffinose (300 mg/mL) + fucose (300 mg/mL) or raffinose (300 mg/mL) + NAG (300 mg/mL) at 1:1 (v/v). Negative controls without any CCE added, and FUA 3016 CCE without apparent α-gal activity were also included. All carbohydrate solutions were buffered to allow reactions to occur at pH 4.7.

Sugar standards of glucose, galactose, melibiose, raffinose, fucose, NAG and lactose were prepared by dissolving 600 mg/mL of each sugar in distilled

water. Standards together with acceptor sugar reactions were first diluted 1:1000 before the samples were analysed and quantified with high performance anion exchange chromatography coupled to pulsed amperometric detection using a CarbopacPA20 column (Dionex, Oakville, Canada). Water (A), 200 mM NaOH (B) and 1 M sodium acetate (C) were used as solvents. The flow rate was set to 0.25 mL/min and the concentrations of glucose and galactose were determined using the gradient with 30.4% B, 1.3% C at 0 min, 30.4% B, 11.34% C at 22 min, which was followed by washing and regeneration. In order to avoid co-elusion of the NAG standard with glucose and galactose standards, acceptor reactions with the presence of NAG were analysed and quantified using the Aminex HPX 87H column (Bio-Rad), with 5 mM H_2SO_4 as solvent at a flow rate of 0.4 mL/min. A refractive index detector was used for signal detection as previously described by (Schwab et al., 2007). Quantification was achieved using standard curve constructed from the peak area integration results and known sugar concentrations.

*4.2.7. Combined liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS)

Underivatized oligosaccharide samples were analysed by LC/ESI-MS/MS. Separations were conducted on an Agilent 1200 series LC system (Agilent Technologies; Palo Alto, USA) at 25°C using a Supelcosil LC-NH_2 column (250 mm x 4.6 mm I.D., 5 μm) (Sigma Aldrich, Oakville, Canada). Samples preparations were identical to those previously mentioned for HPLC analyses. Working solutions were injected onto the column at 3 μL each time, using an isocratic flow of acetonitrile/water 80:20 (v/v) at a rate of 1 mL/min. The effluent from the column was split at a ratio of 1:3 (v/v) so that the flow rate to the mass

spectrometer was 0.3 mL/min. A post-column addition of ammonium acetate (40 mM in methanol) at 0.03 mL/min was delivered by an Agilent 1200 series isocratic pump that was added to the ESI source.

Negative ion ESI-MS and collision induced dissociation tandem mass spectrometry (CID-MS/MS) were performed on a QStar® Elite hybrid orthogonal Q-TOF mass spectrometer coupled to a TurboIon Spray® source with Analyst® QS 2.0 software (Applied Biosystems/MDS Sciex, Concord, Canada). The source conditions were: nebulizer gas 50 (arbitrary units), auxillary gas 60 (arbitrary units), ionspray voltage -4500V, curtain gas 25, declustering potential (DP) -50V, focusing potential -150V, DP2 -10V, and a source temperature of 400°C, scanning over a mass range of *m/z* 100-1000. Fragmentation was achieved using nitrogen as a collision gas at a collision energy that varied between -10 to -35eV and optimised for each saccharide.

4.3 Results

4.3.1. Enzyme Sequence Alignment

The α-Gal cloned from *Lb. reuteri* 100-16 and *Lb. reuteri* 100-23 into *Lc. lactis* were first studied in terms of their protein sequences. After aligning the putative protein sequences of the active sites of α-Gal using the CLUSTAL 2.1 Multiple Sequence Alignment software, the two protein sequences (AGA23 AND AGA16) were found to have a sequence alignment score of 97 (data not shown). AGA23 had a 62% match with the well characterized *melA* from *Lb. plantarum* (Silvestroni et al., 2002); 42% match with the α-Gal in *Lb. brevis* (Q03PP7), 37% match with *melA* in *Lb. fermentum* (Q6IYF5), and 51% match with *aga*A in

Carnobacterium maltaromaticum (AAL27305.1) (Alignment data shown in **Figure 4-1**). Overall, AGA23 and AGA16 were highly similar in sequence, had comparable enzyme activities, and acceptor reaction results. While CCE containing both types of enzymes were used in most cases during our study, results either one of the enzymes are presented.

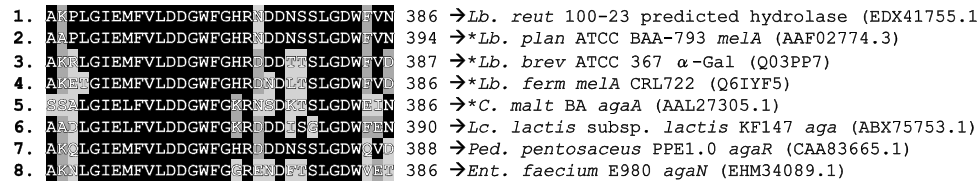

Figure 4-1. Multiple sequence alignment analyses of putative α-Gal active sites in lactobacilli and other selected LAB species, adapted from (Silvestroni et al., 2002). **1**: *Lb. reuteri* 100-23 predicted hydrolase (EDX41755.1); **2**: *Lb. plantarum* ATCC BAA-793 *melA* (AAF02774.3); **3**: *Lb. brevis* ATCC 367 α-Gal (Q03PP7); **4**: *Lb. fermentum melA* CRL722 (Q6IYF5); **5**: *C. maltaromaticum* BA *agaA* (AAL27305.1); **6**: *Lc. lactis* subsp. *lactis* KF147 *aga* (ABX75753.1); **7**: *Ped. pentosaceus* PPE1.0 *agaR* (CAA83665.1); **8**: *Ent. faecium* E980 *agaN* (EHM34089.1). The number at the right end of each sequence indicates the last amino acid number and asterisks indicate characterization of α-Gal at the protein level

4.3.2. Enzyme Assays

In order to determine the optimal conditions for acceptor sugar reactions, enzymes assays were conducted. Using PNPG as a substrate, optimum α-Gal activity was found to occur at pH 4.7, and temperatures between 30°C - 35°C (**Figure 4-2**). Remarkably, the temperature optimum of the enzyme was below the growth optimum of *Lb. reuteri*, 37°C - 42°C (Gänzle et al., 1995; van Hijum et al.,

2002). In order to determine whether the presence of sugars can alter the optimum temperature of α-Gal, sucrose and melibiose were incorporated into enzyme assays. While the presence of sucrose and melibiose initially inhibited the enzyme at temperatures lower than 35 °C when compared to the control, the enzyme activities were increased in the presence of the sugars at higher temperatures. Furthermore, the addition of melibiose was observed to shift the optimum temperature of α-Gal from 35°C to 40°C.

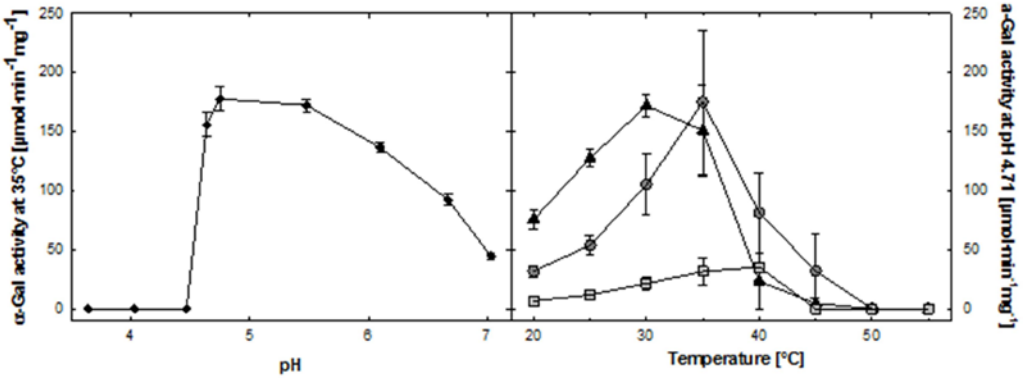

Figure 4-2. Optimum pH and temperature of AGA16. **A:** Optimum pH of AGA16 in *Lc. lactis* at ambient temperature. Symbols (♦) are larger than error bars. **B:** Optimum temperature of AGA16. **(**▲absolute activity of α-Gal degrading PNPG at varying temperatures and a constant pH of pH4.71; ● absolute activity of α-Gal degrading PNPG with the addition of 300 mg/mL sucrose and a constant pH of 4.71; ■ absolute activity of α-Gal degrading PNPG with the addition of 300 mg/mL melibiose and a constant pH of 4.71**).** Data were collected in replicates of three.

4.3.3. Acceptor Reactions

Acceptor reactions results from HPLC analyses were obtained with AGA16 and were qualitatively compared in **Table 4-2**. Since liberation of glucose represent the total enzyme activities and the liberation of galactose represented the hydrolytic activities of the enzyme, transferase activities was able to be

calculated by subtracting the values for hydrolysis from the total enzymatic activities. The melibiose concentration in its acceptor reaction with AGA16 was two times higher than the other three acceptor sugar reactions. Therefore, the reaction with only melibiose was excluded from statistical analyses. For the acceptor reactions with fucose, lactose and NAG, one-way analysis of variance (ANOVA) analyses was used to analyze their percent hydrolase activities, percent transferase activities and the molar quantities of glucose liberated. However, no statistically significant differences were found among the reactions ($P > 0.05$).

4.3.4. Formation of Potential Acceptor Sugars

When comparing the HPLC chromatograms that were obtained from the acceptor reactions of AGA23 with only melibose or raffinose, melibose with lactose, melibiose with fucose, and melibiose with NAG, formation of potential acceptor sugars were observed in all cases (**Figure 4-3**).

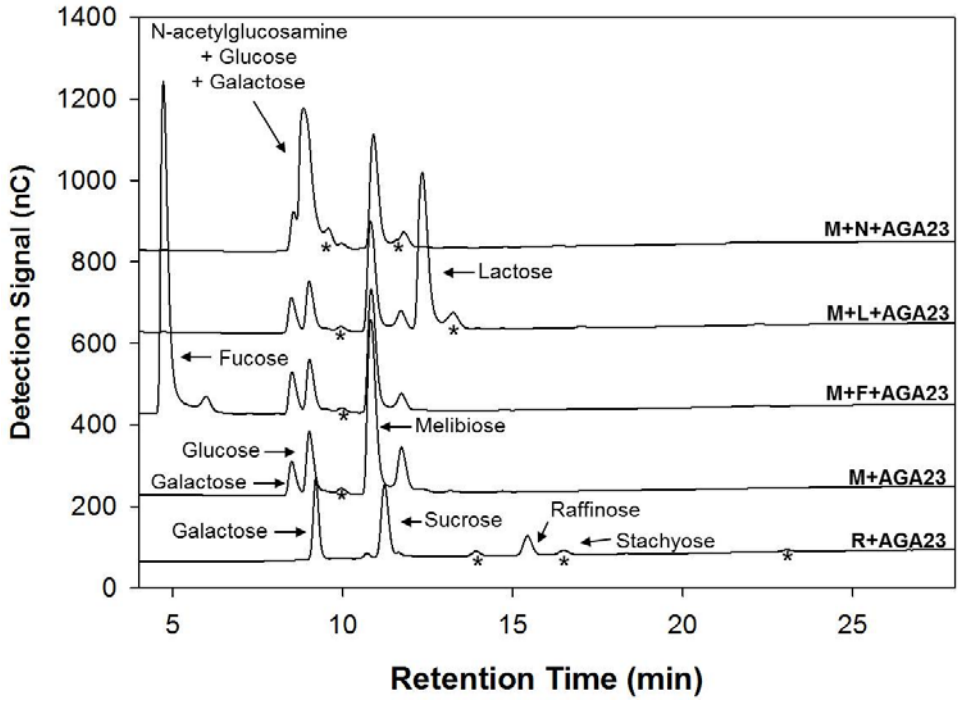

Figure 4-3. HPLC chromatograms of acceptor reactions with AGA23. M, melibiose, N, N-acetylglucosamine; L, lactose; F, fucose; potential acceptor carbohydrates are indicated by asterisks and sugar standards are indicated by arrows. Acceptor reactions were buffered at pH 4.7, and allowed to occur at 30 °C for 24 hours.

Table 4-2. Comparisons between enzyme activities among different acceptor reactions.

Acceptor Reactions [n = 3]	Glucose liberated (mol/L)	Percent Hydrolase Activity	Percent Transferase Activity
M + AGA16	0.52 ± 0.17	55.1 % ± 0.5	44.9 % ± 0.5
M + F + AGA16	0.26 ± 0.13	80.2 % ± 6.3	19.8 % ± 6.3
M + L + AGA16	0.36 ± 0.12	65.0 % ± 1.1	35.0 % ± 1.1
M + N + AGA16	0.22 ± 0.03	77.9 % ± 13.7	22.1 % ± 13.7

Note: M + AGA16 values are separated from the others because the initial concentration of melibiose present in the reaction is two times higher than the other reactions.

***Table 4-3.** Mass accuracy of deprotonated molecules and retention times of all possible GOS or HeOS formed between samples with either NAG, fucose or lactose added as acceptor carbohydrates.

Acceptor	Compound	Retention Time (min)	Measured Mass (Da)	Error (mDa)
		Melibiose		
Melibose Only	$C_{12}H_{21}O_{11}$	16.3; 18.2; 21.9	341.1099	1.0
	$C_{18}H_{32}O_{16}$	39.9; 53.3	503.1637	1.9
+ Fucose	$C_{12}H_{21}O_{10}$	11.5; 14.0	325.1156	1.6
	$C_{12}H_{21}O_{11}$	21.9	341.1076	-1.3
	$C_{18}H_{31}O_{15}$	25.1; 39.7; 52.8	487.1689	2.0
	$C_{18}H_{32}O_{16}$	39.9; 53.0	503.1609	-0.9
+ Lactose	$C_{12}H_{21}O_{11}$	18.7; 21.8	341.1081	-0.8
	$C_{18}H_{32}O_{16}$	37.5; 53.0	503.1632	1.4
+ NAG	$C_{14}H_{24}O_{11}N$	15.6	382.1375	2.0
	$C_{12}H_{21}O_{11}$	18.6; 21.8	341.1099	1.0
	$C_{18}H_{32}O_{16}$	39.5; 53.1	503.1638	2.0
		Raffinose		
Raffinose Only	$C_{18}H_{32}O_{16}$	24.2; 31.2; 33.0	503.1629	1.1
	$C_{24}H_{41}O_{21}$	48.5; 75.7	665.2164	1.8
		Standard		
N/A	Lactose	18.8	341.1082	-0.7
	Melibiose	21.9	341.1095	0.6
	Raffinose	31.3	503.1628	1.0
	Globotriose	33.9	503.1626	0.8
	Stachyose	75.8	665.2155	0.9

*4.3.5. Compositional analysis of oligosaccharides with Electrospray – Mass Spectrometry

ESI-MS was employed to identify the presence of oligosaccharides formed by the transferase reaction of alpha-galactosidase with either melibiose or raffinose. Samples with additional acceptor carbohydrates including fucose, lactose and NAG in addition to melibiose were also analyzed using ESI-MS. All exact masses of observed compounds are shown in **Table 4-3**. In order to confirm the structural composition, tandem mass spectrometry (MS/MS) was performed. The MS2 step allows for the additional confirmation of matching fragmentation patterns to known standards, as well as matching retention times from MS separation and detection (**Figure 4-4**). Exact masses and fragmentation patterns confirmed that a total of four oligosaccharides were formed from alpha-galactosidase supplemented with melibiose. Two of the four disaccharides respectively eluted at 16.3 and 18.2 minutes, while the other two trisaccharides eluted at 39.9 and 53.3 minutes (data not shown). A total of four products were also formed with raffinose in the presence of alpha-galalactosidase, in which two trisaccharides had retention times of 24.2 and 33.0 minutes, and other two tetrasaccharides eluted at 48.5 and 75.7 minutes (data not shown). When lactose, fucose or NAG was introduced to melibiose and alpha-galactosidase as an acceptor in the place of water, additional HeOS were able to form as a result.

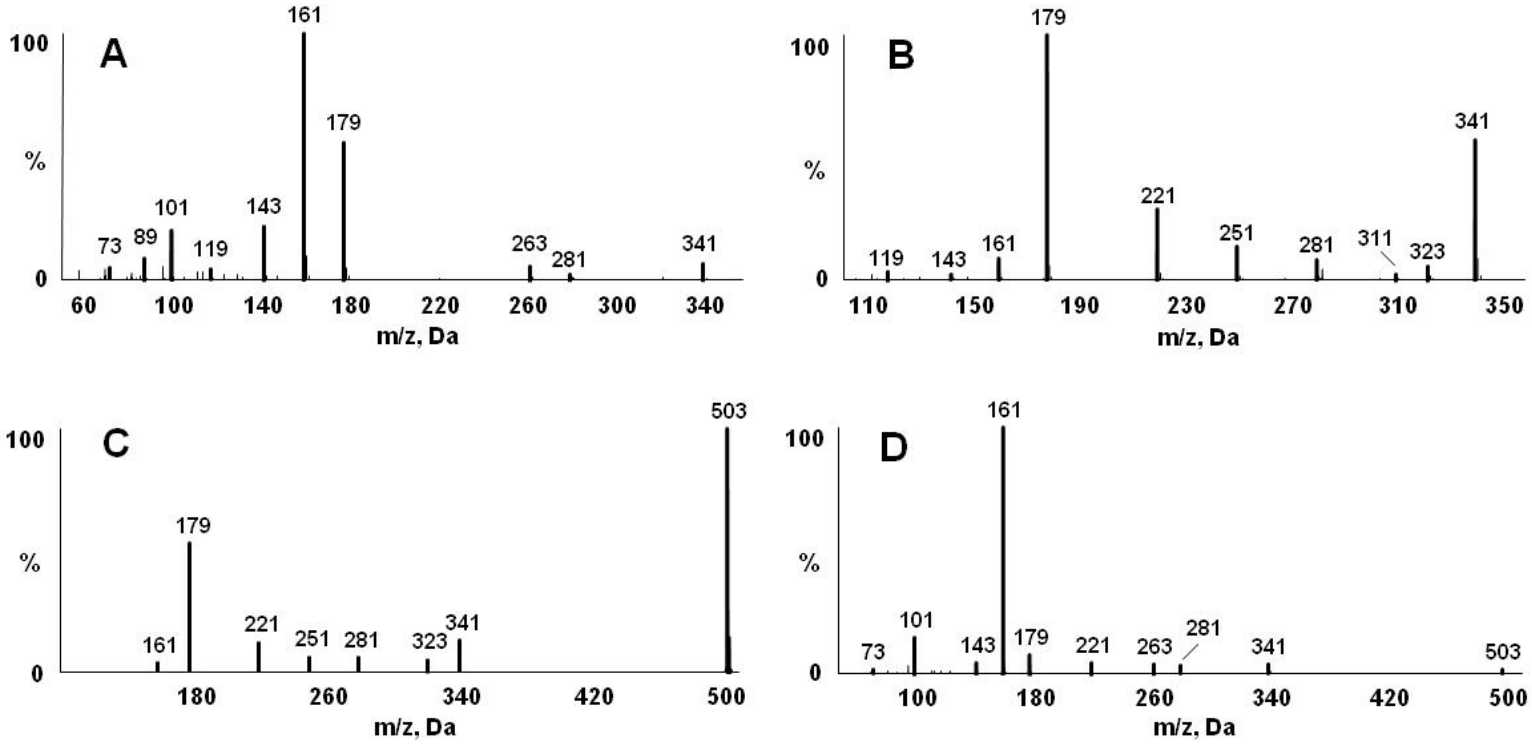

***Figure 4-4.** ESI-MS/MS spectra of [M-H]- ions representative of authentic standards. (A) lactose; (B) melibiose; (C) raffinose; (D) globotriose.

In the presence of fucose, a total of seven oligosaccharides were obtained. Two additional disaccharides eluted at 11.5 and 14.0 minutes and the three additional trisaccharides eluted at 25.1, 39.7 and 52.8 minutes (data not shown). It was noted that when fucose was present, disaccharides were formed only with the transgalactosylation of fucose but not melibiose as no disaccharide was formed from melibiose alone. This suggested that fucose is a preferred acceptor for disaccharide formation over galactose or glucose from melibiose. However in terms of trisaccharides generation, preference for acceptors was not observed as oligosaccharides were able to form from both melibiose and fucose. In the presence of lactose, one additional trisaccharide was formed and eluted at 37.5 minutes. This suggested that it displaced one of the glucose/galactose trisaccharide adducts within the melibiose trangalactosylated sample, which eluted at 39.9 minutes (**Figure 4-5**). Finally, in reactions with NAG, one new disaccharide was formed and eluted at 15.6 minutes. It also placed one disaccharide from the melibiose trangalactosylated products (data not shown).

The standards that were commercially available were analyzed under identical conditions as all samples. Stachyose production by α-galactosidase from raffinose was confirmed as analogous retention times and identical fragmentation patterns between the authentic standard and sample compound were obtained (**Figure 4-6**). Other authentic standards were also analyzed, but none presented corresponding data to any known compounds in the samples.

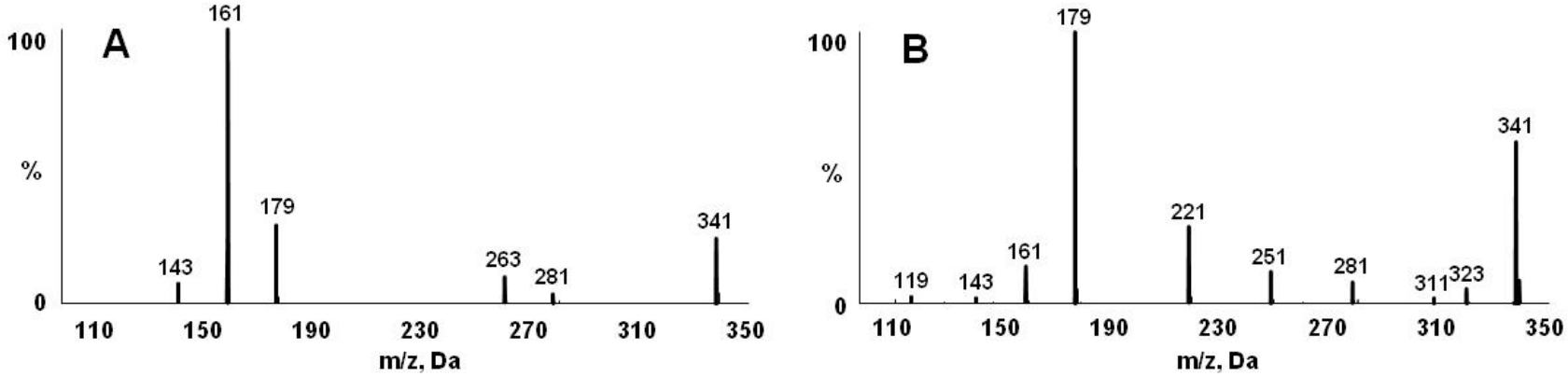

***Figure 4-5.** ESI-MS/MS spectra of $[M-H]^-$ ions of sample with α-gal acting on melibiose and lactose as an acceptor. (A) lactose reactant; (B) melibiose reactant.

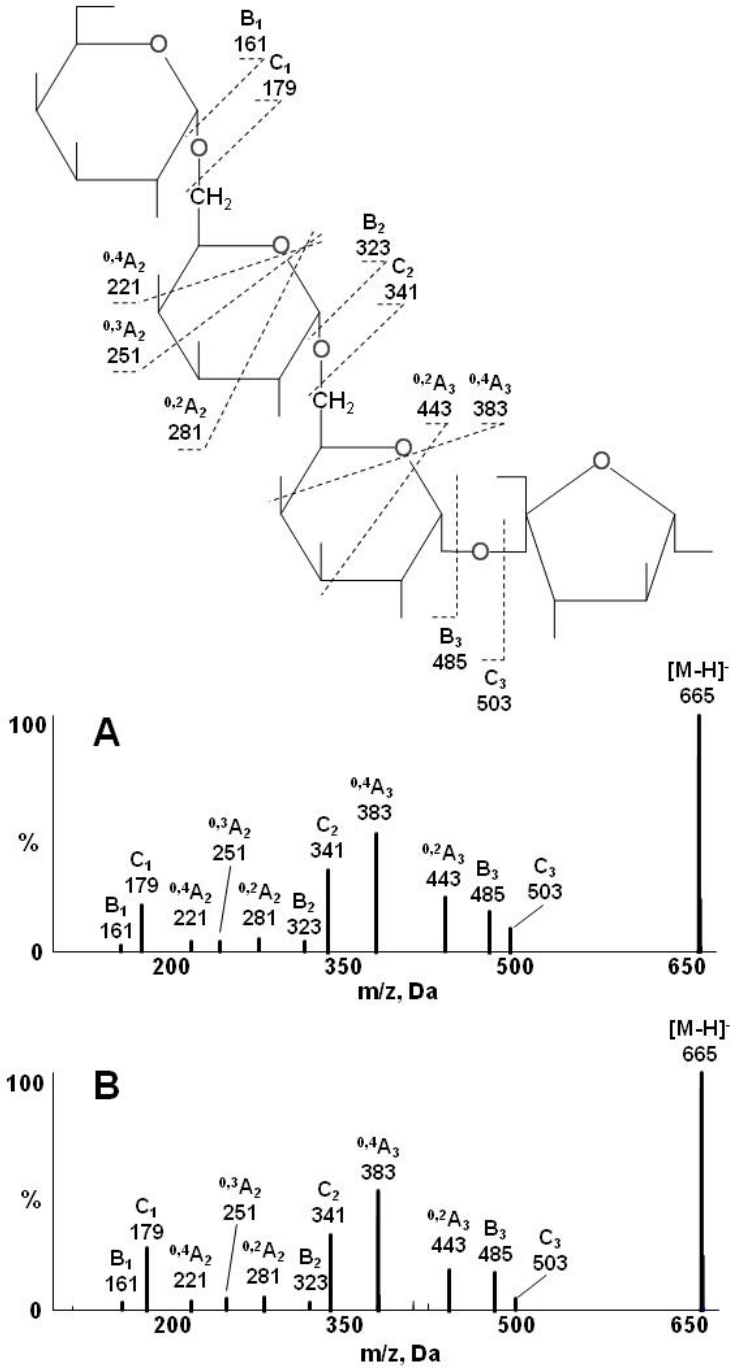

***Figure 4-6.** ESI-MS/MS spectra of $[M-H]^-$ ions representative of stachyose. (A) stachyose standard; (B) sample compound.

4.4. Discussion

LAB are excellent sources for producing food grade α-Gal (Alazzeh et al., 2009; Donkor et al., 2007). Although α-Gal gene sequences are present in many LAB genomes, the production of active α-Gal was only observed among a few strains within the *Lactobacillus* genus, including *Lactobacillus reuteri*, *Lb. plantarum*, *Lb. fermentum*, *Lb. brevis*, and *Lb. buchneri* (Garro et al., 1993; Mital et al., 1973; Tamura & Matsushita, 1992). Tzortzis et al. (2003) reported α-Gal activity and oligosaccharide formation in *Lactobacillus reuteri*, but the connection between activity and DNA and protein sequences has not been made. Silvestroni et al. (2002) have previously aligned the putative active site for the biochemically and physiologically well characterized α-Gal in *Lb. plantarum* with available LAB α-Gals protein sequences and found significant homology among the sequences. By adapting such alignments in combination with the characterization data that were obtained from enzyme assays, it was confirmed that the uncharacterized hydrolases of *Lb. reuteri* 100-16, and *Lb. reuteri* 100-23, which were previously predicted by genome sequencing, were indeed functional.

For most lactobacilli, the temperature optimum of α-Gal in lactobacilli ranges between 38 °C to 42 °C (Mital et al., 1973). However, the optimum temperature range for the cloned α-Gal from *Lb. reuteri* was between 30 °C to 35 °C, which was lower. Interestingly, although *Lb. fermentum* and *Lb. reuteri* are closely related based on their genome sequences, *Lb. fermentum* CRL722 has a thermostable α-Gal which remains active at 55°C and stable at wide temperature and pH ranges, the overall protein sequence alignment results of α-Gal from

Lb. fermentum CRL722 (AY612895) only had a 37% match with the α-Gal from *Lb. retueri* 100-23 as previously indicated (Carrera-Silva et al., 2006). Furthermore, the optimum temperature range for the α-Gal was also much lower than the optimal temperature range for *Lb. reuteri* growth, which is between 37 °C and 42 °C (Gänzle, 1998; Gänzle & Vogel, 2003).

The observed shifts in the optimum temperature of α-Gal when melibiose and sucrose were incorporated into the enzyme reactions suggested that the presence of these sugars were inhibitory for the enzyme at suboptimal temperatures, but have stabilizing effects at temperatures above the optimal temperature range. Therefore, melibiose and sucrose may behave as compatible solutes for microorganisms in a physiological context (Brown & Simpson, 1972). The optimum pH of the cloned α-Gal from *Lb. retueri* 100-23 at around pH 4.7 was in agreement with Mital et al. (1973) and Tzortzis et al. (2003)

HPLC analyses not only demonstrated the ability of α-Gal from *Lactobacillus reuteri* to produce oligosaccharides, which can become potential acceptor sugars, but they also allowed hydrolase and transferase activities of the enzyme to be quantified; albeit no statistically significant differences were observed among the different acceptor sugars. Nevertheless, as seen in **Figure 4-2**, non-substrate sugars such as sucrose markedly decreased the enzyme activity of α-Gal, and data from **Table 4-2** seems to suggest that percent transferase activities decrease with increasing the quantities of glucose liberated.

From analysing ESI-MS/MS results, the composition, sequence and degrees of polymerization of the α-GOS that were formed were determined as data for the retention times and exact masses of the products formed in the reactions were collected. Adducts with degrees of polymerization ranging from 2 to 4 were formed. Information with regards to the linkage types of reaction products was achieved with samples that contained the transglycosylation products of raffinose, namely stachyose with α-(1→6) linkages, due to the availability of a matching standard. There were three retention times obtained from compounds with the formula $C_{12}H_{21}O_{11}$, which were formed from only melibiose **Table 4-3**. This suggested that in addition to melibiose being one of the three compounds, there were at least two linkage types present for compounds either formed as Gal-Gal or Gal-Glc disaccharides. Evidence of the presence of exactly two linkage types was observed in compounds that were formed from using fucose as an acceptor sugar, in which two retention times were obtained for galactosylated fucose ($C_{12}H_{21}O_{11}$), which are solely composed of galactose and fucose.

The formation of oligosaccharides using acceptor sugars such as fucose, lactose and NAG have been previously characterized for LAB β-Gal by Schwab et al. (2011) and Black et al. (2012). Based on previous study with β-gal in which β-(1→6) linkages have formed in β-GOS regardless of the acceptor sugars that were involved in the reaction, it is likely that α-GOS formed without the presence of raffinose, such as those formed with fucose, would also contain α-(1→6) linkages. Further support for the formation of α-(1→6) linkages were provided by

Mital et al. (1973) as they reported the formation of manninotriose [Gal-α-(1→6)-Gal-α-(1→6)-Glc] from transgalactosylation reactions with melibiose as well as from differential hydrolysis of stachyose. Nevertheless, additional studies are needed to confirm the linkage types of α-GOS formed with other acceptor sugars.

Globo-series carbohydrates are abundantly present in receptors in animal and human hosts and are widely targeted by bacteria and their toxins for establishing adherence (Jacewicz et al., 1986; Leach et al., 2005; Samuel et al., 1990; Stromberg et al., 1990). We initially suspected the formation of globotriose [Gal-α-(1→4)-Gal-β-(1→4)-Glc] from acceptor reaction with lactose [Gal-β-(1→4)-Glc]. However, both HPLC and MS data excluded the formation of this compound because products with exact masses that match globotriose were not detected. This led to our conclusion that α-(1→4) linkages are not formed by α-Gal. Nevertheless, comparable to results obtained by Black et al. (2012) with LAB β-Gal, LAB α-Gal also formed products with two linkages types and that there seems to be a preference for the formation of the α-(1→6) linkages.

In conclusion, this study demonstrated that HeOS can be produced by LAB α-Gal using different acceptor sugars. Supporting data for the presence of at least two different linkage types were obtained. The presence of α-(1→6) linkages were confirmed in the case of stachyose and the absence of α-(1→4) were confirmed when globotriose was not detected from reactions using lactose as an acceptor sugar. Additional studies will be needed to produce potentially novel HeOS as well as to provide further support the current findings. Moreover, potential anti-adhesive properties of novel α-GOS produced by LAB α-Gal also

warrants more future investigations for pathogen adhesion studies, especially for those to be conducted *in vivo*.

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Chapter 5

General Discussion and Conclusions

5.1. General Discussion

This thesis research achieved the characterization of the vaginal microbiota of healthy pregnant dairy cows as well as infected postpartum cows, and the isolation of putative probiotic LAB, including pediocin producing *Ped. acidilactici* FUA3140, non-pediocin producing *Ped. acidilactici* FUA 3138, and *Lactobacillus sakei* FUA3089 from healthy dairy cows. The isolation of putative probiotic LAB subsequently led to the confirmation of their probiotic properties and the development of prophylactic intravaginal infusions for preventing and treating metritis in dairy cows.

In addition, the characterization of anti-adhesive LAB EPS, and the production of potentially anti-adhesive and novel α-GOS from *Lb. reuteri* derived α-Gal were also achieved. These studies were conducted to support the development of potential applications for mitigating the problem of PWD in swine production.

5.1.1. Probiotic Preparations and Challenges in Maintaining Strain Stability

Under collaborative efforts with Dr. Ametaj's research group, intravaginal infusions were prepared from a mixture of *Ped. acidilactici* FUA 3140, *Ped. acidilactici* FUA 3138, and *Lb. sakei* FUA 3089. Probiotic bacteria cultures were prepared by separately growing each strain overnight and reconstituting the strains in 10% skim milk, which functioned as a cryoprotectant. Frozen aliquots of the probiotic mixture were prepared at -20°C and samples were then freeze dried at -70°C. Using a sterile insemination pipettes and plastic syringes, Holstein

dairy cows before and after calving were treated with either the freeze dried probiotic mixture, which was reconstituted in 1 mL sterile 0.9% saline just before treatment, or control treatments, which were thawed 10% skimmed milk samples that were frozen at -20 °C. Intravaginal infusions were conducted once a week, from two weeks before calving to one week after calving and up to four weeks after calving. As indicated by Sharma et al. (2011), overall improvements in the reproductive health and performance of the cows were observed after parturition. Although there are no other data on the effects of intravaginal infusion of probiotics on the reproductive health of dairy cows that were published to date, additional investigations such as those associated with bacteriocin production are still needed to elucidate the health-promoting properties and mechanisms of these probiotics.

In order to successfully commercialize such probiotic mixtures as intravaginal infusions to prevent and treat metritis in dairy cows, potential challenges that are associated with maintaining strain viability must be addressed. During probiotic product manufacture and storage, the stability of the probiotics can be affected by many environmental conditions such as pH, temperature, water activity (a_w), oxygen content, the presence of chemicals and or other microorganisms (Gueimonde, de los Reyes-Gavilán, & Sánchez, 2012). As emphasized by (Gaggia et al., 2011), successful probiotic administrations not only require probiotic strains to remain viable and functional throughout the preservation, storage and administration processes, but more importantly, probiotic treatments must also remain effective by overcoming adverse conditions

at their target sites. Strain viability was especially important during the development of probiotic vaginal infusions as samples with the presence of cryoprotectants but were not properly preserved during the freeze drying procedure showed decreased efficacy in promoting the reproductive health of treated dairy cows. Although freeze drying allows for easy shipping and handling of microbial cultures and it is currently considered to be the standard preservation method, improvements are much needed to reduce the overall cost associated with large energy consumption, and to increase bacteria survival during the process (Morgan et al., 2006). Therefore as reviewed by Gueimonde & Sánchez (2012) and (Heidebach et al., 2012), further research in alternative cost-effective preservation strategies such as enhancement of stress tolerance in probiotic strains and microencapsulation can be conducted to overcome the challenges with maintaining stable probiotic preparations.

5.1.2. EPS Production with Regards to Fermentation Technologies and Future Animal Studies

As detailed in Chapter 3, ethanol precipitation, hot phenol extraction and dialysis for the removal of mono-, di-, and oligosaccharides were all necessary steps needed to prepare pure EPS for *in vitro* anti-adhesion tests. Unlike the relatively simple preparation of LAB cultures for probiotic treatments, the isolation and purification of EPS from LAB is lengthy, costly, and difficult to achieve high yields for large-scale production. However, EPS can remain stable and effective in a dry environment for longer periods of time during storage, and fermentation technologies, particularly those associated with cereal fermentations, allow cost-effective *in situ* production of EPS at large quantities without the need

for purification procedures (Franz & Feuerstein, 1997; Welman, 2009). For example, the yield of reuteran produced by *Lb. reuteri* VIP in a control wheat sourdough can be up to 5.8 g/kg (Galle et al., 2012). Furthermore, the incorporation of fermentation techniques especially those involving the use of LAB in the production of fermented feed for swine production have been prevalent and widely accepted for approximately two decades (Lindgren & Dobrogosz, 1990; Olstorpe et al., 2008). Thus, animal feeding trials and *in vivo* studies would be necessary to confirm the current *in vitro* results that were obtained with regards to the anti-adhesive properties of LAB produced EPS.

Feeding trials in conjunction with ligated intestinal loops models in pigs have been well established, and would thus serve as a good method for testing anti-adhesion properties. This is because adhesin-mediated adherence of ETEC to small intestinal epithelial cells and the subsequent release of heat stable and heat-labile enterotoxins increase fluid and electrolyte secretion, which allow the detection of these effects in the small intestine of test animals to be easily achieved (Casey et al., 2012; Nagy & Fekete, 2005). Furthermore, this model would not only allow the anti-adhesive properties against ETEC to be tested, anti-adhesive properties against secreted toxins would also be able to be verified. Similar to the studies conducted with EPS from LAB, screening novel α-GOS for anti-adhesive properties would also require *in vitro* assays such as haemagglutination assays, as well as *in vivo* studies when promising *in vitro* results are obtained.

5.1.3. Promising Applications of *Lactobacillus reuteri* as Novel Probiotic and Starter Culture Bacteria

One promising research area that can stem from this research is the notion that EPS and or α-GOS producing *Lb. reuteri* strains TMW1.656, LTH5794, and 100-23 can be further evaluated for applications as novel probiotic strains. *Lactobacillus reuteri* TMW1.656 is a sourdough isolate and LTH5794 is a human intestinal isolate (Schwab & Gänzle, 2005). These two strains were previously selected for the respective production of reuteran and levan. By virtue of being naturally present in an accepted food fermentation such as sourdough or as harmless commensals that are members of the human intestinal microbiota, these two strains are safe for use in food, and they are highly suitable for being developed into human probiotics. In addition to potential production of anti-adhesive EPS, *Lb. reuteri* TMW1.656 has also been found to produce reutericyclin, which is an antimicrobial compound that is especially potent for inhibiting diarrhoea inducing *Clostridium difficile* (Gänzle & Vogel, 2003; Hurdle et al., 2011).

Lactobacillus reuteri 100-23, which expresses functional α-Gal that can be used for the production of potentially novel α-GOS, is a well characterized strain that was first isolated in rats (Wesney & Tannock, 1979). Interestingly, recent comparative genomic hybridization studies that were conducted by Su et al. (2011) showed that the rodent strain 100-23 is phylogenetically closely related to the sourdough strain LTH2584, and that both strains are equally well adapted for both the intestinal and sourdough milieu.

As demonstrated by research results obtained by Galle (2011a) and Galle et al. (2010; 2011b; 2012a; 2012b), LAB produced EPS, particularly dextran and reuteran, can be used to replace existing hydrocolloids to improve bread quality; especially in sourdough bread made with wheat or gluten free flours. Therefore, *Lb. reuteri* strains that are capable of producing EPS and thriving in the sourdough environment can be potentially used as starter culture for improving sourdough bread quality. Furthermore, the potential ability of *Lb. reuteri* to inhibit pathogens or spoilage microorganisms through its production of antimicrobial metabolites, such as reutericyclin, and potential anti-adhesive EPS and α-GOS can further expand their uses as protective cultures for food products, far beyond their expected applications as probiotics.

5.2. Conclusions

Overall, the promising results obtained from this thesis research will help to spark the development of probiotics and novel therapeutic glycans for applications in improving the health of farm animals. Implications of these results will also facilitate the development of comparable applications for improving human health. As ongoing research efforts in evaluating and validating probiotic applications of selected strains such as *Lb. reuteri* continue, it is foreseeable that novel and advanced functional foods, beverages, dietary supplements and nutraceuticals, which rely on versatile probiotic cultures to improve sensory qualities and extend shelf-life of the food products will be developed in the near future to ultimately confer health benefits in their animal and human consumers alike.

5.3. References

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