CALL, EMMA KATHRYN. Sortase and Sortase-Dependent Cell Surface Proteins of Probiotic and Commensal Lactobacilli. (Under the direction of Dr. Todd Klaenhammer).

Lactic acid bacteria (LAB) are Gram-positive organisms which have been safely consumed by humans for thousands of years, and are thus generally recognized as safe (GRAS). These microbes are used in a vast array of fermented foods including cheeses and yogurts. Some LAB, including members of the *Lactobacillus* genus, are considered probiotic microbes for their recognized role in promoting health, most notably in the gastrointestinal tract (GIT). Additionally, the GRAS status of these microbes and their ability to survive passage through the stomach and interact with the intestinal mucosa make them attractive candidates for the delivery of oral vaccine antigens and adjuvants.

Sortase enzymes are responsible for the display of a variety of cell surface proteins, and covalently coupling a subclass of these structures, known as sortase-dependent proteins (SDPs), to the cell wall of Gram-positive organisms. In LAB, both the house keeping sortase (SrtA) and pilus sortase (SrtC) have been identified. SrtA couples anywhere between two and 27 SDPs to the cells of various LAB. Additionally, deficiency of sortase and thus SDPs has been implicated in negating mucus binding and host immune modulation in *Lactobacillus salivarius* UCC118 and *L. plantarum* WCFS1. Furthermore, SrtC has been shown to assemble functional adhesion promoting pilus structures in *Lactobacillus rhamnosus* GG.

*Lactobacillus acidophilus* NCFM, a widely distributed probiotic culture, and *Lactobacillus gasseri* ATCC 33323, a common human commensal in the mouth, GIT and vagina, were the focus of this study. Both beneficial microbes possess 12 SDPs, 8 with predicted functionality in *L. acidophilus* and 6 with predicted functionality in *L. gasseri*. 
Importantly, mucus-binding proteins and adhesion exoproteins are represented in those SDPs. In this study, the gene encoding sortase in both these organisms was deleted using a upp-based counterselective gene replacement. The affect of sortase deficiency in these lactobacilli was evaluated for adhesion to mucin and Caco-2 cells, persistence in the GIT of germ-free mice, and immunomodulation. ΔsrtA mutants showed decreased adhesion to porcine mucin in vitro. In addition, in vivo co-colonization using gnotobiotic 129Sv/Ev mice did not show significant difference in the ability of the sortase-deficient L. acidophilus strain to persist in the gastrointestinal tract (GIT). Murine dendritic cells (DCs) exposed to either ΔsrtA mutant of L. acidophilus or L. gasseri showed lower levels of induced proinflammatory cytokines (IL-6, IL-12, and TNF-α) when compared to the parental Srt+ wildtype strains. This study suggested that sortase-dependent proteins (SDPs) do not play an important role in survival or retention in the GIT, but may play a very specific role in the modulation of host immune responses.
Sortase and Sortase-Dependent Cell Surface Proteins of Probiotic and Commensal Lactobacilli

by

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DEDICATION

To my mom, Diana Call, and dad, Jeff Call, for their constant and enduring love.

Thank you for all the nights of math tutoring, the weekend trips to science fairs and swim meets, and giving Haley and me every chance to succeed. And, to my sister, Haley Call, for always making me laugh and always reminding me to not take the little things too seriously.
BIOGRAPHY

Emma Call was born in Fredricksburg, VA, but spent most of her childhood in Baltimore, MD where she lived with her mom, dad, and younger sister, Haley. Emma graduated from Case Western Reserve University (CWRU) in Cleveland, OH in Spring 2011 with a B.A. in both Biochemistry and Nutrition. While attending CWRU, Emma was a member of the varsity swim team and co-captain of the team her junior and senior years. Additionally, from September 2009 till her graduation in May 2011, Emma also worked in Dr. Clifford Harding's research laboratory where she investigated the pathogenesis of human immunodeficiency virus (HIV). In the summer of 2009, Emma became interested in the field of food science and was accepted as a summer scholar in the Cornell University Food Science Summer Scholars program where she preformed research under the mentorship of Dr. Kathryn Boor and Dr. Martin Wiedman. At Cornell, Emma research methods to detect spoilage organisms in fluid milk. Since participating in the Cornell Summer Scholars Program, Emma has continued to pursue her interest in food science by first interning with PepsiCo in the summer of 2011 and then beginning her M.S. in Food Science at North Carolina State University. Emma still enjoys swimming, but has taken to running and rock climbing in her spare time.
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CHAPTER 1. LITERATURE REVIEW

Relevance and Application of Sortase and Sortase-Dependent Proteins in Lactic Acid Bacteria

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1.1 Abstract

Lactic acid bacteria (LAB) are a diverse group of Gram-positive bacteria found in a vast array of environments including dairy products and the human gastrointestinal tract. In both niches, surface proteins play a crucial role in mediating interactions with the surrounding environment. The sortase enzyme is responsible for covalently coupling a subset of sortase dependent proteins (SDPs) to the cell wall of Gram-positive organisms through recognition of a conserved C-terminal LPXTG motif. Genomic sequencing of LAB and annotation has allowed for the identification of sortase and SDPs. Historically, sortase and SDPs were predominately investigated for their role in mediating pathogenesis. Identification of these proteins in LAB has shed light on their important roles in mediating nutrient acquisition through proteinase P as well as positive probiotic attributes including adhesion, mucus barrier function, and immune signaling. Furthermore, sortase expression signals in LAB have been exploited as a means to develop oral vaccines targeted to the gastrointestinal tract. In this review, we examine the collection of studies which evaluate sortase and SDPs in select species of dairy associated and health promoting LAB.
1.2 Introduction

In Gram-positive bacteria, the cell wall is a crucial cellular component affecting a bacterium's fitness and survival. The cell wall is responsible for maintaining structural stability, providing a barrier to osmotic pressures, and facilitating interactions with the surrounding environment. Cell walls of Gram-positive bacteria are decorated with a vast array of macromolecular structures which facilitate these interactions. These structures include teichoic acids, lipoteichoic acids, exopolysaccharides, Surface (S)-layer proteins, enzymes, and other cell surface proteins, such as adhesins and pili-like structures which are directly involved in host attachment (Marraffini et al., 2006; Weidenmaier and Peschel, 2008). The ecological niche of the microbes often dictates the mosaic-like surface display of macromolecules. The surface proteins of pathogenic microbes, such as internalin A in Listeria monocytogenes and protein A in Staphylococcus aureus, play a crucial role in establishing pathogenicity and infection (Mazmanian et al., 2000; Cabanes et al., 2002; Clancy et al., 2010). Alternately, in probiotic microbes, which confer health benefits upon the host, surface structures may play essential roles in eliciting these benefits.

Surface display is a two-fold process composed of both protein targeting and protein attachment to the cell exterior. Protein targeting to the cell exterior is typically achieved through either the secretory (Sec) pathway or the twin-arginine translocation (TAT) pathway. The Sec pathway recognizes unfolded protein targets containing an N-terminal leader peptide, a hydrophobic core, and a C-terminal sequence that promotes binding of Sec machinery. Depending on the peptide sequence in the C-terminal region, the proteins are either exported out of the cell or N-terminally anchored in the membrane. Proteins that are N-
terminally anchored in the membrane and processed by the Sec pathway represent a large proportion of membrane-anchored proteins in lactobacilli (Kleerebezem et al., 2010). Unlike the Sec pathway, the TAT pathway serves to transport folded protein to the cell's exterior. This pathway appears to be much more uncommon in species of lactic acid bacteria (LAB). To date this pathway has only been identified in Streptococcus thermophilus and not in lactococci or lactobacilli (Hols et al., 2005). Further association of these proteins targeted to the membrane and cell exterior can either be achieved through covalent linkages or non-covalent interactions. The non-covalent interactions which may allow for protein association with the cell wall following export by Sec or TAT machinery have been reviewed (Schaffer and Messner, 2005; Kleerebezem et al., 2010).

One class of proteins covalently associated with the peptidoglycan of the cell wall after Sec targeting are the LPXTG-anchored proteins. These proteins contain a C-terminal cell wall sorting signal with the sequence of amino acids leucine (L), proline (P), X (representing any amino acid substitution), threonine (T), and glycine (G), and are linked to the cell wall by the housekeeping sortase, sortase A (SrtA). Successful linkage of sortase-dependent proteins (SDPs) to the cell wall is facilitated by the presence of not only the aforementioned LPXTG motif, but also a proceeding C-terminal hydrophobic region and a positively charged tail (Figure 1A). The N-terminal region of SDPs contains a signal peptide. This signal peptide enables secretion of the sortase substrate by the secretory pathway, while the C-terminal charged tail anchors the substrate once it reaches the cell membrane. Anchoring in the cell membrane by the C-terminal tail brings the SDP and the sortase enzyme, also embedded in the cell membrane, into proximity so that the enzyme may
carry out the transpeptidation reaction required for cell wall anchoring. The first step in the transpeptidase reaction is the cleavage of the sortase substrate between the glycine and the threonine residue forming a sortase enzyme/SDP complex. The resulting thioester acyl bond between these two proteins is then subjected to nucleophilic attack and subsequent linkage to lipid II. Although lipid II is composed of both the peptidoglycan precursors N-acetylglucosamine and N-acetylmuramic acid, as well as the pentapeptide peptidoglycan cross bridge, SDPs have been shown to link specifically to the pentapeptide (Maresso and Schneewind, 2008; Kleerebezem et al., 2010; Spirig et al., 2011). Once linked to the cross bridge, SDPs are incorporated into the cell wall with lipid II as it is translocated to the outer surface of the cell (Figure 1B).

Although these class A sortases which couple sortase substrates to the cell wall are the most well characterized, other classes of sortase enzymes have been identified. Class B sortases have been implicated in heme acquisition from the host, while class D, E, and F sortases have been identified and functionally analyzed to a lesser extent (Spirig et al., 2011). Class C sortases are better studied and play a critical role in pilus assembly. Class C sortases are transpeptidases, like class A sortases; however, they recognize a different sorting motif of (Isoleucine [I]/L)(P/Alanine [A]) XTG (Hendrickx et al., 2011). Functionally, class C sortases catalyze linkages between the Spa proteins to elongate the pilus shaft. Once elongation is complete, the pilus is anchored to the cell wall by either the class C sortase itself or by a class A sortase (Spirig et al., 2011). Similar to proteins coupled to the cell wall by class A sortases, the pili formed by class C sortases represent another mechanism of bacterial interaction with the environment. Pili are responsible for adherence to epithelial
cells and extracellular matrix proteins, interaction with the host immune system, as well as biofilm formation (Danne and Dramsi, 2012).

Sortase proteins have been most extensively studied in the context of pathogens. Sortase A was first identified in \textit{S. aureus} where it is responsible for coupling between 18 and 22 substrates to the cell wall depending on the species (Marraffini et al., 2006). In \textit{S. aureus}, as well as in \textit{L. monocytogenes} and other Gram-positive pathogens, the deletion of the gene encoding the sortase enzyme showed attenuated virulence in animal models (Mazmanian et al., 2000; Bierne et al., 2002; Garandeau, 2002). This decrease in infective capability is attributed to the loss of adhesins, internalins, clumping factors, or host evasion molecules such as protein A from the cell surface of \textit{S. aureus}. Not surprisingly, the product of sortase C action, the pilus, has also been implicated in pathogenicity through adhesion and host cell invasion in, but not limited to, \textit{Corynebacterium diphtheriae}, \textit{Streptococcus pneumoniae}, \textit{Streptococcus pyogenes}, and Actinomyces naeslundii (Ellen et al., 1978; Ton-That and Schneewind, 2003; Mora et al., 2005; Barocchi et al., 2006; Gaspar and Ton-That, 2006; Telford et al., 2006; Mishra et al., 2007). Taken together, these findings were suggestive that sortase inhibition could function as an anti-infective therapy as thoroughly reviewed by Anthony W. Maresso and Olaf Schneewind (Maresso and Schneewind, 2008).

Sortase enzymes are found in all Gram-positive microbes, including food grade and health-relevant microbes of the lactic acid bacteria (LAB). Members of the LAB have a history of safe use and consumption of these microbes has been associated with health benefits, including competitive inhibition of pathogens, maintenance of epithelial barrier function, and a reduction in the symptoms of irritable bowel syndrome (Ventura et al., 2009).
Nonetheless, in many cases the mechanisms associated with these effects are unknown. The sortase enzyme and SDPs in LAB are of interest in delineating the molecular mechanisms of host-bacterial interaction. While sortase A enzymes have been identified in a handful of LAB members, the sortase C enzyme has only been functionally characterized in *Lactobacillus rhamnosus* GG (Kankainen et al., 2009). Furthermore, the sortase cell wall anchoring machinery in LAB has been explored in the development of vaccines which could be administered orally as strains generally recognized as safe (GRAS).

### 1.3 Comparative Analysis of Sortase in LAB

As previously stated, the sortase protein is ubiquitous among Gram-positive bacteria, and members of the LAB family are no exception. Sequencing of various LAB genomes has allowed for the identification of bacterial genes, including those that code for sortase. In 2002, a collaborative effort between the Department of Energy - Joint Genome Institute and the scientists of the Lactic Acid Bacteria Genome Consortium announced an elaborate sequencing project which aimed to make LAB genomes available to the public. Four years after the announcement of the initiative, 18 genomes of LAB were publically available (Makarova et al., 2006). Approximately a decade later, over 26 LAB genomes are available (Zhou et al., 2010). Encompassed in this collection of LAB are probiotic strains which include *Lactobacillus acidophilus, Lactobacillus gasseri*, and *Lactobacillus plantarum*. The genome assemblies have shed light on the presence of cell surface-associated structures which are suggested to modulate the microbe-host response.
The role of sortase enzymes in the attachment of proteins to the cell wall makes it an attractive target for genome mining of structures involved in bacterial-host interactions. Genes encoding housekeeping sortase enzymes (srtA) have been identified in the genomes of LAB including *L. acidophilus* (Buck et al., 2005), *Lactobacillus salivarius* UCC118 (van Pijkeren et al., 2006), *Lactobacillus johnsonii* NCC533 (Denou et al., 2008), *L. rhamnosus* GG (Kankainen et al., 2009), *Lactococcus lactis* IL1403 (Dieye et al., 2010), *Lactobacillus casei* BL23 (Munoz-Provencio et al., 2012), *L. plantarum* (Remus et al., 2012), and *Lactobacillus crispatus* ST1 (Edelman et al., 2012). Additionally, genes encoding class C sortase proteins (srtC) have been identified in both *L. rhamnosus* GG (Kankainen et al., 2009) and *L. casei* BL23 (Munoz-Provencio et al., 2012). The genomic context of the srtA locus varies widely among species (Figure 2A); however, srtC genes have been found to cluster with their targets (spa genes) as was observed in both *L. rhamnosus* GG (Figure 2B) and *L. casei* BL23 (Munoz-Provencio et al., 2012). An unrooted phylogenetic tree shows the relationships between the sortase protein sequences identified in the aforementioned species of LAB as well as those present in other LAB species including *L. lactis* subsp. *cremoris* MG1463 and *S. thermophilus* LMD-9 (Figure 3). As expected based on differences in target proteins, distinct clusters formed indicating divergence in amino acid sequences of the housekeeping sortase, SrtA, and the pilin sortase, SrtC. Additionally, the SrtA proteins from group A members of the acidophilus complex cluster independently from those of the group B acidophilus complex, and those LAB (i.e. *L. lactis* and *S. thermophilus*) whose 16s rRNA are divergent from those of lactobacilli also possess SrtA enzymes which cluster independently.
Further analysis of the genomes of LAB has identified those substrates that the sortase enzyme covalently links to the cell wall. SDPs can be identified based on the presence of a C-terminal LPXTG motif as well as an N-terminal signal peptide followed by a series of hydrophobic residues, as previously discussed. SDPs were first identified in sequenced genomes using a hidden Markov model which predicted 732 sortase targets in 49 different prokaryotes (Boekhorst et al., 2005). Years later, those LPXTG containing proteins found in prokaryotic LAB were compiled and combined with other cell wall anchored and secreted structures to form the LAB secretome database (http://www.cmbi.ru.nl/lab_secretome/index.php) (Zhou et al., 2010). This database currently contains twenty six LAB and is publicly available to aid in the identification of SDPs as well as other secreted structures. Although the software is capable of identifying SDPs, it is unable to distinguish between those SDPs which are pseudo or truncated genes, and those SDPs which are fully functional genes and contain the three domains described above. The presence of the signal peptide and other protein domains can be determined using InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/), while the functionality and cleavage site within the signal peptide can be determined using SignalP 4.0 software (http://www.cbs.dtu.dk/services/SignalP/). A combination of these tools and a survey of the current literature was used to develop a table summarizing the prevalence of sortase enzymes, SDPs, and the functionality of SDPs in some common LAB (Table 1).
1.4 Functional Analysis of the Housekeeping Sortase and Sortase-Dependent Proteins (SDPs) in LAB

Prior to genome mining for SDPs, Roos and Jonsson first described the functional role of a protein in the SDP family in *Lactobacillus reuteri* 1063 (Roos and Jonsson, 2002). The previously uncharacterized putative cell surface protein (NCBI Reference Sequence: AF120104) contained repeat regions homologous to mucus-binding (mub) domains and a C-terminal sortase recognition LPQTG motif. Furthermore, the protein contained an N-terminal secretion signal consistent with the conserved structure of functional sortase targets. The protein was called "Mub" and recombinant forms of the protein showed adherence to mucin derived from different animal species (Roos and Jonsson, 2002). Although the publication did not identify a sortase protein itself, the genome sequence of a different strain of *L. reuteri* (DSM 20016) with an available genome indicated the presence of a sortase enzyme (NCBI Reference Sequence: YP_001270843.1). This study asserted the first suggestion of an adhesive protein in a *Lactobacillus* species, and furthermore, the first suggestion that LPXTG anchored motifs may play a role in mucus binding in the GIT of the host.

Similar adhesion factors were identified in *L. acidophilus* NCFM after its genome sequence became available in 2005 (Altermann et al., 2005). Three proteins, LBA1633, LBA1634, and LBA1392, were identified for their putative adhesive capacity based on their sequence homology with R28 protein from *S. pyogenes* (Buck et al., 2005). In *S. pyogenes*, R28 plays a role in binding to a cervical epithelial cell line (Stalhammar-Carlemalm et al., 1999). Additionally, the *L. acidophilus* protein, LBA1392, was found to have 25% protein homology to the Mub identified in *L. reuteri* (Buck et al., 2005). Interestingly, all three of
these proteins contained a C-terminal LPXTG sorting signal. The presence of the LPXTG signal in these three putative Mubs as well as in nine other open reading frames in the *L. acidophilus* genome were suggestive of the presence of a sortase protein identified as LBA1244. Deletion and functional analysis of the sortase-linked Mub, LBA1392, showed significant impairment in adherence to human Caco-2 epithelial cells, but this observation was not reproducible for LBA1633 or LBA1634 (Buck et al., 2005). The nine other predicted sortase targets remained uninvestigated in this study.

The aforementioned studies in *L. reuteri* and *L. acidophilus* NCFM implicated surface proteins in specific adhesive roles of probiotic bacteria in the GIT and directed attention to the sortase protein and SDPs as an important mediator of this positive probiotic attribute. Further comparative analysis of cell surface structures associated with probiotic bacteria revealed that LPXTG-anchored targets could be found in common probiotic species including *L. plantarum* WCFS1, *L. johnsonii* NCC 533, *Lactobacillus sakei* 23K, and *L. salivarius* UCC118 (Table 1). Furthermore, the number of targets present varied from species to species, with the highest number found in *L. plantarum* WCFS1 (Kleerebezem et al., 2010). While the role of sortase and SDPs in *L. plantarum* was evaluated at a later time (Remus et al., 2012), the initial focus was placed on determining the functional role of sortase and SDPs in *Lactobacillus salivarius* UCC118. Publication of its genome revealed the presence of ten SDPs consisting of four intact targets and six pseudogenes (Claesson et al., 2006; van Pijkeren et al., 2006).

With the genome sequence available, functional analysis of sortase and SDPs in *L. salivarius* UCC118 were greatly expedited. The sortase gene, identified as LSL_1606, was
deleted while the four functional SDPs were insertionally inactivated (van Pijkeren et al., 2006). In two separate in vitro adhesion assays, one using Caco-2 and one using HT-29 adenocarcinoma epithelial cells, the sortase-deficient strain showed significant decreases in adhesion. One of the SDPs, named LspA (LSL_0311), was shown to contribute to this phenotype, while two others (LspB and LspD) did not appear to significantly reduce adhesion in these model systems. Not surprisingly, the SDP LspA contained a series of mucus-binding domains as were previously described as involved in adhesion in both L. reuteri and L. acidophilus NCFM (van Pijkeren et al., 2006). Current annotations of this protein describe it as Mbp2 since Lsp is used to denote lipoproteins structures unrelated to sortase.

A collection of SDPs from other probiotic microbes, including the mannose-specific adhesin (msa) in L. plantarum 299v (Gross et al., 2008) and the Lactobacillus epithelial adhesin (LEA) isolated from L. casei BL23 (Edelman et al., 2012) have been shown to contribute to bacterial adhesion. In L. casei BL23, twenty three SDPs were predicted; however, none were specifically targeted for investigation. Alternately, each of the four sortases genes (two srtA and two srtC) were inactivated and the adhesion phenotype examined. In this study, inactivation of both SrtA enzymes was required to functionally impact binding to colonic epithelial cell lines, while inactivation of either of the class C sortases did not impact adhesion (Edelman et al., 2012). The latter finding was unexpected since in L. rhamnosus GG this gene is essential in constructing pili which directly contribute to epithelial cell adhesion (Kankainen et al., 2009). It is unclear which of the twenty three predicted SDPs plays a specific role in the adhesion phenotype as none share homology with
Mubs found in *L. acidophilus* or *L. reuteri* (Edelman et al., 2012). However, one SDP (LCABL_23040) shares homology with a mucus-binding factor (MBF) in *L. rhamnosus* GG and may provide insight into SDPs mediating adhesion in *L. casei* BL23 (Munoz-Provencio et al., 2012).

Recent interest in sortase and SDPs has begun to focus on the role of these proteins in the immunomodulatory capacity of probiotic bacteria. These bacteria have the capacity to influence immune signaling of the colonic epithelium directly and through modulation of NF-κB signaling pathways. As an example, *L. rhamnosus* GG and *L. plantarum* BFE have been shown to enhance innate immune signals by increasing the expression of Toll-like receptors (TLRs) in HT-29 cells (Pinto et al., 2009). Additionally, antagonists of the potent proinflammatory transcriptional regulator, NF-κB, were upregulated after one hour exposure of Caco-2 cells to *L. acidophilus* NCFM based on microarray analysis (O’Flaherty and Klaenhammer, 2012). This same technique was used to investigate transcriptional responses of Caco-2 cells exposed to both the sortase-deficient mutant of *L. salivarius* UCC118 and the wild type (O’Callaghan et al., 2012). Although the wild type strain showed down regulation of the NF-κB antagonist as well as induction of some innate immune regulators such as chemokines, the immune signaling pathways did not appear to be different after exposure to ΔsrtA *L. salivarius* UCC118. However, epithelial mucin genes were significantly down regulated after exposure to ΔsrtA *L. salivarius* UCC118 (O’Callaghan et al., 2012). Mucin is important in the colonic epithelium for maintaining lubrication and barrier functionality of the GIT as well as preventing pathogen penetration into epithelial cells of the GIT (Shirazi et al., 2000; Moran et al., 2011). Down regulation of the mucin genes after exposure to *L.*
*salivarius* UCC118 lacking sortase and thus SDPs, implicate SDPs in stimulating mucin production to maintain barrier function as well as in adhesion, as previously described (van Pijkeren et al., 2006).

The role of sortase in non-pathogenic species has been focused, but not limited to, probiotic LAB. Initial studies using lactococci as a vaccine carrier suggested sortase machinery was functional due to the ability to display known sortase-anchored proteins from other species (e.g. M6 from *S. pyogenes*) on the surface of *L. lactis* ssp. *cremoris* (Norton et al., 1996; Piard et al., 1997). Additionally, amino acid sequence comparison of the *L. lactis* ssp. *cremoris* genes including the sex-factor aggregation gene (*cluA*) and proteinase P (*ptrP*) showed regions of homology to the LPXTG domains of cell wall anchored proteins (Vos et al., 1989; Godon et al., 1995). This provided evidence for localization of PrtP at the cell wall and its display outside the cell envelope in a location important for nutrient acquisition in a dairy environment (Vos et al., 1989). Further investigation into sortase machinery in the industry-relevant strains has been accomplished in *L. lactis* ssp. *lactis* IL1403. This specific *L. lactis* has two sortase genes, *srtA* and *srtC*. The *srtC* gene is only expressed at low levels and does not appear to build functional pili under normal growth conditions (Oxaran et al., 2012). In contrast, SrtA couples at least five proteins to the cell wall of *L. lactis* ssp. *lactis* IL1403, some of which were shown to contain mucus-binding domains homologous to those present in some lactobacilli. This finding is suggestive of potential binding capacity to cellular components of the human GIT although this has not been demonstrated experimentally. In addition, PrtP is not found in this particular species although it can be found in *L. lactis* ssp. *cremoris* MG1363 (Dieye et al., 2010).
Variation in the sortase gene in species of *S. thermophilus* can also be observed. For example, the genome of *S. thermophilus* LMD-9 appears to encode an intact sortase, while the genomes of *S. thermophilus* CNRZ1066 and *S. thermophilus* LMG 13811 both harbor truncated enzymes (Goh et al., 2011). Furthermore, the latter two species do not contain genes encoding SDPs (Bolotin et al., 2004), while *S. thermophilus* LMD-9 potentially contains three SDPs, as predicted by the LAB secretome database (Zhou et al., 2010; Goh et al., 2011). In the case of *S. thermophilus* CNRZ1066 and *S. thermophilus* LMG 13811 this further substantiates the hypothesis of genomic decay during adaptation to milk and loss of gene features, including cell surface proteins, shown to contribute to virulence in related streptococcal pathogens (Bolotin et al., 2004).

This collection of studies represents the state of functional analysis of the housekeeping SrtA and its targets in probiotic species of lactobacilli as well as the limited investigation of this enzyme in food-associated *L. lactis* and *S. thermophilus*. While the studies published on srtA in probiotic lactobacilli are not exhaustive, they suggest an important role for this enzyme and SDPs in adhesion to the intestinal mucosa of the host. Moreover, the functional role of sortase in adhesion is predicted to be mediated through its role in linking mucus-binding proteins or similar protein structures, such as the mucus-binding factor found in *L. rhamnosus* GG (Munoz-Provencio et al., 2012), to the cell wall. Additionally, although not investigated in great detail at this point in time, the adhesive capacity of different probiotic strains to mucus and the GIT may function to allow interactions with the local immune system in the GIT. Beyond investigating gene expression of epithelial cell lines such as Caco-2 cells after probiotic exposure, it may be prudent to
explore the responses of dendritic cells (DCs) to such treatment. Dendritic cells are resident immune cells in the GIT with the capacity to sample antigens and signal the immune system through cytokines. This approach to investigating immune stimulation by probiotic bacteria has been employed with regard to a sortase-deficient mutant of *L. plantarum* WCFS1, although significant changes in the amounts of anti-inflammatory IL-10 and pro-inflammatory IL-12p70 were not detectable when DCs were cultured with the individual strains (Remus et al., 2012). Studies such as these with other strains, both wild type and sortase-deficient, will help unravel the mechanisms behind probiotic functionality. Namely, as suggested by O'Callaghan *et al.* (2012), the combined adhesive capacity provided by some SDPs and the immune stimulation induced by probiotics, acting together, may condition the GIT for potential pathogen exposure.

**1.5 The Pili Sortase: Characterization of SrtC in *L. rhamnosus* GG**

Like the housekeeping sortase, originally discovered in the Gram-positive pathogen *S. aureus*, the sortase responsible for pili assembly, sortase C, was first described in the pathogen *Corynebacterium diptheriae* (Ton-That and Schneewind, 2003). It was shown to play a key role in assembling subunits, namely SpaA and SpaC, to form the pili found to protrude from the surface of *C. diptheriae*. Pili are filamentous structures, approximately 1-2 µm in length, and usually numerous in their display on the bacterial surface. Since their discovery in *C. diptheriae*, pili have been described in many more Gram-positive pathogens as key components involved in host tissue colonization; however, until 2009 these structures remained undiscovered in commensal lactobacilli (Kankainen et al., 2009)
In 2009, the presence of mucus-binding pili displayed on the surface of *L. rhamnosus* GG was described (Kankainen et al., 2009). *L. rhamnosus* GG is a probiotic bacterial strain which has been in use for over two decades. Additionally, *L. rhamnosus* GG shows exemplary ability to adhere to Caco-2 cells as compared to other probiotic strains (Jacobsen et al., 1999). The genome sequence of *L. rhamnosus* GG revealed two potential clusters of pilus-encoding genes in tandem with a *srtC* gene (Figure 2B). The first cluster identified contained genes for *spaA* (LGG_00442), *spaB* (LGG_00443), and *spaC* (LGG_00444) clustered with *srtC*1 (LGG_00441), while the second cluster contained genes for *spaD* (LGG_02370), *spaE* (LGG_02371), and *spaF* (LGG_02372) clustered with *srtC*2 (LGG_02369) (Kankainen et al., 2009). Furthermore, demonstration of the expression and presence of pilin-like structures on the surface of *L. rhamnosus* GG was accomplished using immunogold transmission electron microscopy, as was first described by Ton-That, H. and Schneewind, O. in *C. diptheriae* (2003). Double labeling, first with primary antibodies to the SpaC subunit of the pili and subsequently with a secondary antibody containing gold nanoparticles, allowed the pili to be detected under transmission microscopy. Remarkably, the pili were not only identifiable, but they were relatively numerous at approximately 10-50 pili per cell (Kankainen et al., 2009).

The *spa* genes found in *L. rhamnosus* GG have been further characterized with regard to their function in assembling pili and in their ability to adhere to mucin. The protein product of the first gene following *srtC*1, the SpaA subunit, forms the backbone of the pili in *L. rhamnosus* GG. SpaB is found at the base of the pilin structure and is likely attached to the cell wall through the action of the sortase A enzyme, which is also encoded in the genome.
Finally, SpaC can be found flanking the pilin shaft. SpaB, and to some extent SpaC, contribute to the adhesive capacity of *L. rhamnosus* GG to mucin through different mechanisms (Reunanen et al., 2012). Insertional inactivation of the spaC gene essentially abolishes binding to human intestinal mucus, and expression and further purification of this pilus subunit from *E. coli* showed significant binding to immobilized human intestinal mucus. The SpaB protein showed even the greatest degree of binding to human intestinal mucus which was attributed to its net positive charge, facilitating binding with negative residues present in human mucus (von Ossowski et al., 2010).

To date, pili have not been functionally identified in other species of food-adapted or probiotic lactobacilli. Gene clusters with homologous structures to those found in *L. rhamnosus* GG have been described in *L. casei* BL23. In *L. casei* BL23, the gene cluster encoding spaA, spaB, spaC and a class C sortase appear to be genetically intact; however, functional pili on the surface of *L. casei* BL23 have not been reported (Munoz-Provencio et al., 2012). Furthermore, the gene cluster encoding spaD, spaE, and spaF appears to be present, but with truncations in spaE and spaF. Recently, a gene encoding a sortase C homolog flanked by three genes with LPXTG motifs was identified in *L. lactis* IL1403. The expression of the genes and the formation of pili could not be detected under normal growth conditions; however, cloning and overexpression of the gene cluster under a high copy lactococcal promoter led to pili display (Oxaran et al., 2012).
1.6 LPXTG motif and biotherapeutic application in lactic acid bacteria

The conserved C-terminal anchor motif recognized by sortase in Gram-positive microbes has been suggested as a means of antigen display in vaccine development (Norton et al., 1996; Bermudez-Humaran et al., 2003; Cortes-Perez et al., 2003; Cortes-Perez et al., 2005; Fredriksen et al., 2010; Kajikawa et al., 2011). Specifically, the use of the LPXTG motif has been investigated for in vitro vaccine delivery using food grade and probiotic lactobacilli as the presentation vector for the antigen. Unlike vaccine delivery vehicles which rely on attenuated strains of pathogenic bacteria, food grade LAB and notably probiotic lactobacilli present an alternative delivery vehicle as they have a safe history of use in foods and dietary supplements, are GRAS, and are able to survive passage through the GIT for vaccine delivery to the mucosal immune system. In addition, LAB have been shown to also have secretory and C-terminal cell wall anchoring machinery which can be exploited for antigen immobilization. To date, LAB and sortase-mediated cell wall anchoring have been explored in the display of potential vaccine antigens including tetanus toxin fragment C (Norton et al., 1996), human papillomavirus (HPV) type 16 E7 antigen (Bermudez-Humaran et al., 2003; Cortes-Perez et al., 2003; Cortes-Perez et al., 2005), the oncofetal antigen (Fredriksen et al., 2010), and Salmonella enterica serovar Typhimurium flagellin (FilC) (Kajikawa et al., 2011).

The functionality of sortase-mediated cell wall localization in LAB was demonstrated through display of the M6 protein, a LPXTG-anchored virulence factor of S. pyogenes, in L. lactis. The M6 protein was also successfully displayed on the cell wall of other LAB including L. fermentum LEM83, L. sakei 23K, and S. thermophilus CNRZ302. This was
achieved through cloning of the gene encoding the M6 protein (*emm6*) into the aforementioned LAB and then examining the distribution of the M6 protein using Western blot analysis. The authors were able to demonstrate the conserved nature of sortase anchoring among Gram-positive organisms; however, they noted differences in anchoring efficiency between different LAB. For example, M6 protein could be detected in the supernatant from the cocci, while it was not readily detected in the supernatant collected from the rod-shaped lactobacilli (Piard et al., 1997). The authors attributed the differences in successful anchoring to cell wall composition or cell wall turn over. This finding was reproduced by Dieye et al. (2001) who showed inefficient cell wall localization of their reporter protein, staphylococcal nuclease A, in *L. lactis* when it was coupled to the M6 protein cell wall anchor and signal peptide. By switching the signal peptide to one of lactococcal origin (Usp45), M6 was able to be efficiently displayed in *L. lactis* as well as in other LAB including *L. casei, L. sakei,* and *L. plantarum*. These studies suggest that the sortase machinery is functionally different across LAB and have the capacity to recognize substrates from an unrelated microbe (Piard et al., 1997; Dieye et al., 2001).

Demonstration of the cross functionality of LPXTG cell wall anchoring across Gram-positive species pointed to new directions in vaccine design. Localization of antigen to the cell wall had been shown to not only be effective, but also substantially increases immune responses compared to the intracellular or secreted form of the antigen (Reveneau et al., 2002). In an initial study of the effectiveness of vaccine delivery of the tetanus toxin fragment C (TTFC) in *L. lactis*, anchoring of TTFC using the proteinase P (PrtP) cell wall anchor was found to elicit the most robust anti-toxin immunoglobulin G (IgG) response as
compared to the toxin expressed in the soluble form in a murine model (Norton et al., 1996). In this case, the LPXTG anchor from the PrtP gene used to couple the TTFC antigen to the cell wall was of endogenous origin, as opposed to an exogenous anchoring motif from *S. pyogenes* used to display the M6 protein in *L. lactis*. The enhanced immune response against TTFC can be attributed to successful localization of the antigen to the cell wall due to sortase specificity for the endogenous anchor.

Two oncogenes, the E7 antigen from HPV type-16 and the oncofetal antigen, have also been expressed in LAB as potential vaccine candidates (Bermudez-Humaran et al., 2003; Cortes-Perez et al., 2003; Cortes-Perez et al., 2005; Fredriksen et al., 2010). The E7 antigen is specific to cervical cancer, while the oncofetal antigen has been found on all mammalian tumors (Fredriksen et al., 2010). In both studies, the probiotic LAB species, *L. plantarum*, was used as a model vaccine vector. As was shown in display of TTFC, successful presentation of both the E7 and oncofetal antigen was achieved using a species-specific cell wall anchor. The consensus sequence for cell wall anchoring by sortase in *L. plantarum* differs from that of LPXTG found in lactococci and streptococci as it has been shown to recognize the motif, LPQTXE (Kleerebezem et al., 2003). The use of this *L. plantarum* sortase consensus sequence did not only enhance the efficiency of surface display as indicated by Western blot, but also, in the case of oncofetal antigen, promoted IgG oncofetal-specific immune responses in mice after oral immunization (Fredriksen et al., 2010).

One issue arising from studies of the efficacy of oral administration of vaccines, is that differences in IgG responses to the specific antigen were lower when lactobacilli were
fed orally as opposed to administered through the nasal route (Reveneau et al., 2002). Kajikawa et al. (2011) examined this phenomenon when they engineered a recombinant strain of *L. acidophilus* NCFM expressing the salmonella flagellin (FliC), which was covalently linked to the cell wall using an LPXTG motif. The goal of the study was to evaluate the potential of FliC to serve as a vaccine adjuvant for LAB vaccines; however, it can be argued that the major finding of this research was that antigen display on the cell surface is susceptible to degradation by gastric juices. In order to protect the FliC fragment on the surface of *L. acidophilus*, the recombinant *L. acidophilus* cell suspensions were supplemented with either sodium biocarbonate or soybean trypsin inhibitor (SBTI). Both of these treatments were found to protect the antigen from degradation when incubated in simulated gastric juices; however, SBTI has a greater protective effect likely due to its sequestration of bile away from the bacterial cells, thus contributing to increased viability and robust antigen production (Kajikawa et al., 2011).

Taken together these studies indicate that vaccine delivery by LAB using LPXTG or LPXTG-like cell wall anchors has great potential. Additionally, these studies highlight some important considerations in the development of LAB vaccine vectors. Although cell wall anchoring and surface display functions vary across Gram-positive species, they also indicate sortase specificity for its target domain, which leads to differences in the efficiency of antigen display. The study conducted by Kajikawa *et al.* (2011) indicates that despite efficient surface display, protection of antigens from GIT juices may be crucial in achieving the most robust immune response. Finally, these studies further validate the presence of functional sortase proteins in LAB which operate in protein anchoring to the cell wall.
1.7 Conclusion

Interest in sortase and sortase protein substrates have extended beyond the arena of pathogens and promotion of intimate associations and infection. Rather, sortase is clearly an important mechanism for display of cell surface proteins, a significant niche-related trait of commensal and probiotic microbes associated with the intestinal mucosa. It is not difficult to envision further use of sortase machinery present in LAB to present cell surface oral vaccines, given the success of antigen display discussed in this review. The hypothesis that sortase enzymes may play crucial roles in bacterial physiology (as in the case of PtrP in *L. lactis* ssp. *cremoris* MG1363) as well as mediating bacterial-host interactions has accelerated the study of this enzyme in different species of LAB. The ability to access and examine sortase enzymes and their targets using genomic analysis tools has been crucial. The mechanisms of sortase action and covalent linkage of SDPs to the cell wall is a successful tactic of surface display in Gram-positive bacteria which has enabled some pathogenic organisms to gain advantage of their host, while allowing others, namely commensal and probiotic bacteria, to adhere and interact with their hosts in positive way.

1.8 Acknowledgements

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1.9 References


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proteins, confers protective immunity and promotes binding to human epithelial cells.

*Molecular Microbiology* 33, 208-219.


Table 1.1 Comparative genomic analysis of sortase-dependent proteins in select species of LAB

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Sortase Protein</th>
<th>Presence (+) or Absence (-)</th>
<th>Number of Targets</th>
<th>Predicted Functional Targets</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. salivarius</td>
<td>srtA</td>
<td>+</td>
<td>10</td>
<td>4</td>
<td>Van Pijkeren J-P., et al., 2006</td>
</tr>
<tr>
<td></td>
<td>srtC</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L. casei BL23</td>
<td>srtA</td>
<td>+ (2)</td>
<td>17</td>
<td>13</td>
<td>Munoz-Provenicio, D. et al., 2012d</td>
</tr>
<tr>
<td></td>
<td>srtC</td>
<td>+ (2)</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>L. plantarum</td>
<td>srtA</td>
<td>+</td>
<td>32</td>
<td>27</td>
<td>Remus D.M., et al., 2009</td>
</tr>
<tr>
<td>WCFS1</td>
<td>srtC</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L. lactis IL1403</td>
<td>srtA</td>
<td>+</td>
<td>11</td>
<td>9</td>
<td>Dieye, Y. et al., 2010</td>
</tr>
<tr>
<td></td>
<td>srtC</td>
<td>+</td>
<td>3</td>
<td>Not expressed under normal conditions</td>
<td></td>
</tr>
<tr>
<td>L. rhamnosus GG</td>
<td>srtA</td>
<td>+</td>
<td>Unknownc</td>
<td>N/A</td>
<td>Kankainen, M. et al., 2009 and von Ossowski, I. et al., 2010</td>
</tr>
<tr>
<td></td>
<td>srtC</td>
<td>+ (2)</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>L. gasseri ATCC</td>
<td>srtA</td>
<td>+</td>
<td>12</td>
<td>6</td>
<td>Kleerebezem et al., 2010</td>
</tr>
<tr>
<td>33323</td>
<td>srtC</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>srtA</td>
<td>+</td>
<td>12</td>
<td>8</td>
<td>This review</td>
</tr>
<tr>
<td>NCFM</td>
<td>srtC</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

a Targets containing an LPxTG motif were predicted using the hidden Markov model described by Boekhorst, J. et al. and are now currently compiled in the LAB secretome database (Boekhorst et al., 2005; Zhou et al., 2010).

b Functional targets were determined based on the presence of a functional signal peptide (described in the literature or as determined by SignalP 4.0) and a LPXTG or LPXTG-like motif.

c L. rhamnosus GG is not a part of the LAB secretome database developed by Zhou, M. et al., 2010.

d 23 total SDPs were identified and described by this group. In this review, predicted SDP’s were further clarified based on the targeting by SrtA or SrtC as well as their predicted functionality based on the parameters above.
Figure 1.1 Sortase anchoring in Gram-positive bacteria. (A) Sortase substrates are recognizable due to the presence of an N-terminal signal peptide and a C-terminal LPXTG sorting signal followed by a series of hydrophobic and positively changed residues facilitating membrane anchoring. (B) Covalent linkage of sortase substrates to the cell wall is accomplished in a series of 5 steps: (1) Sec machinery recognizes the signal peptide on the sortase substrate and exports it to the cell's exterior. The substrate remains embedded in the membrane due to the presence of a hydrophobic region terminated by a charged tail. (2) Once sortase and the sortase substrate are in proximity, sortase cleaves the target between the glycine and threonine residues via a transpeptidase reaction. (3) Nucleophilic attachment by lipid II disassociates the sortase/SDP complex and (4) forms a lipid II intermediate through interaction with the pentapeptide cross bridge. (5) In the final stages of sortase anchoring, the sortase substrate is incorporated into the cell wall as part of normal cell wall construction (Adapted from Hendrickx, et al., 2011).
Figure 1.2 Genomic context of genes encoding sortases in LAB. (A) The genomic context of sortase A (green arrow) as found in *L. acidophilus* NCFM, *L. plantarum* WCFS1, *L. rhamnosus* GG, and *L. lactis* subsp. *lactis* IL1403 vary greatly. The sortase in *L. lactis* is not yet annotated as such; however, the gene annotated *ylcC* was identified to be the putative sortase enzyme by Dieye, Y., et al. (Dieye et al., 2010). These species were chosen to represent species from members of the acidophilus complex, other probiotic lactobacilli, and food-associated LAB. (B) Unlike sortase A whose targets are dispersed throughout the genome, sortase C enzymes cluster with their pili-subunit targets (Adapted from Kankainen et al., 2009).
Figure 1.3. Unrooted phylogenetic tree of sortase enzymes in different LAB. Sortase enzymes were identified using available genome sequences in the NCBI database. Protein sequences were aligned in ClustalX v.2 and then imported into MEGA 4 for phylogenetic tree construction.
CHAPTER 2. PHENOTYPIC EVALUATION OF LACTOBACILLI DEFICIENT IN SORTASE DEPENDENT PROTEINS
2.1 Abstract

Surface proteins of probiotic microbes, including *Lactobacillus acidophilus* and *Lactobacillus gasseri*, are believed to promote retention in the gut and mediate host-bacterial communications. Sortase, an enzyme that covalently couples a subset of extracellular proteins containing an LPXTG motif to the cell surface, is of particular interest in delineating bacterial adherence and communication with the mucosal immune system. Using annotated genome sequence data, a sortase gene was identified in *L. acidophilus* NCFM (LBA1244) and *L. gasseri* ATCC 33323 (LGAS_0825). Additionally, twelve sortase targets (cell surface proteins, adhesion exoproteins, and mucus-binding proteins) containing a C-terminal LPXTG motif were predicted in both organisms using the lactic acid bacteria (LAB) secretome database. Due to the role of sortase in coupling these targets to the cell wall, ΔsrtA mutants of *L. acidophilus* and *L. gasseri* were created. The sortase-deficient mutants of *L. acidophilus* and *L. gasseri* were evaluated for phenotypes associated with growth, adhesion, persistence in a murine model, and immune signaling. Absence of sortase did not cause significant alteration in growth or survival in simulated gastrointestinal juices. Both ΔsrtA mutants showed decreased adhesion to porcine mucin *in vitro*. Meanwhile, an *in vivo* co-colonization experiment using gnotobiotic 129Sv/Ev mice did not show significant difference in the ability of the sortase-deficient *L. acidophilus* strain to persist in the gastrointestinal tract (GIT). Interestingly, murine dendritic cells (DCs) exposed to either the ΔsrtA mutant of *L. acidophilus* or *L. gasseri* showed lower levels of induced proinflammatory cytokines (IL-6, IL-12, and TNF-α) compared to the parent strains as measured by enzyme-linked
immunosorbent assay (ELISA). These data suggest that sortase-dependent proteins (SDPs) may play a very specific role in the modulation of host immune responses.
2.2 Introduction

Surface proteins have been implicated in the ability of bacteria to communicate and interact with their host. Investigation of these proteins is important to the understanding of the mechanisms underlying the health benefits of commensal and probiotic microbes, including competitive inhibition of pathogens, immunomodulation, and the maintenance of the gastrointestinal tract (GIT) epithelial barrier. In *Lactobacillus acidophilus* NCFM, deficiency of the surface protein lipoteichoic acid (Lta) has been shown to modulate proinflammatory signals present in a murine colon cancer model such that they return to a baseline, protective level (Khazaie et al., 2012). Additionally, surface layer protein (S-layer) A of *L. acidophilus* NCFM has been characterized for its direct interaction with the DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) receptor on dendritic cells (DCs), and thus the modulation of homeostatic cytokine signals to potentially abrogate pathogen-induced immune signaling (Konstantinov et al., 2008). These reports emphasize the potential of genetically engineered probiotic microbes for immunomodulatory roles.

The sortase protein, first identified in *Staphylococcus aureus* in 1999, covalently couples a subset of surface proteins, referred to as sortase-dependent proteins (SDPs), to the cell wall peptidoglycan of Gram-positive microorganisms (Mazmanian et al., 1999). Although multiple classes of sortase enzymes exist which function in pili assembly and iron acquisition, the class A sortase (SrtA) enzyme is solely responsible for coupling surface proteins to the cell wall. SrtA recognizes targets exported by the secretory pathway that contain a C-terminal LPXTG or LPXTG-like motif proceeded by a hydrophobic region and positively changed tail. The sortase enzyme then cleaves between the threonine (T) and
glycine (G) residues at which point the sortase and SDP form a complex linked by a thioester acyl bond. This bond is then subjected to nucleophilic attack, the SDP is subsequently linked to lipid II, and incorporated as a part of normal cell wall biosynthesis (Maresso and Schneewind, 2008; Kleerebezem et al., 2010; Hendrickx et al., 2011; Spirig et al., 2011). In *S. aureus*, the sortase enzyme couples between 18 to 22 SDPs to the cell surface, many of which are associated with virulence and host adhesion (Marraffini et al., 2006). The finding that SDPs play a role in pathogenesis is conserved across other species of pathogenic Gram-positive organisms, namely *Listeria monocytogenes* and *Streptococcus pneumoniae*; however, the role of these proteins has only recently begun to be investigated in species of commensal and probiotic microbes. To date, SrtA enzymes have been identified in eight of these microbes including *L. acidophilus* NCFM (Buck et al., 2005), *Lactobacillus salivarius* UCC118 (van Pijkeren et al., 2006), *Lactobacillus johnsonii* NCC533 (Denou et al., 2008), *Lactobacillus rhamnosus* GG (Kankainen et al., 2009), *Lactococcus lactis* IL1403 (Dieye et al., 2010), *Lactobacillus casei* BL23 (Munoz-Provencio et al., 2012), *Lactobacillus plantarum* (Remus et al., 2013), and *Lactobacillus crispatus* ST1 (Edelman et al., 2012).

In the current study, the sortase enzyme was examined in the context of a widely used commercial probiotic, *L. acidophilus* NCFM, and the commensal GIT and genital tract inhabitant, *L. gasseri* ATCC 33323. The genomes of both *L. acidophilus* NCFM and *L. gasseri* ATCC 33323 have been sequenced enabling targeted gene deletion and phenotypic analysis (Altermann et al., 2005; Azcarate-Peril et al., 2008). Each of the two genomes harbor one sortase protein and twelve predicted SDPs. A sortase-deficient mutant of each strain was generated and assessed with regard to key probiotic traits including survival in
gastrointestinal juices, adhesion capacity, and immune signaling. Both mutants showed impaired adhesion to procine mucin and alteration of the proinflammatory signaling. These changes were more profound in L. gasseri ATCC 33323. This findings suggest that despite sharing approximately 83% nucleotide homology, key difference in cell surface topology, namely the lack of a traditional S-layer by L. gasseri ATCC 33323, may be responsible for differences observed in immune signaling.

2.3 Materials and Methods

2.3.1 Bacterial Culture and Growth Conditions

The bacterial strains used are listed in Table 1, and plasmids are listed in Table 2. Lactobacillus strains were propagated in deMan, Rogosa, and Sharpe (MRS) medium (Becton, Dickinson, and Company [BD], Franklin Lakes, NJ) at 37°C statically under anaerobic conditions and Escherichia coli strains were maintained in Brain Heart Infusion medium (BHI) (BD) at 37°C aerobically with shaking. Plating was performed on solid medium with the addition of 1.5% (w/v) agar to either MRS or BHI medium. Antibiotics including erythromycin (Em, 5µg/mL), kanamycin (Kn, 40 µg/mL), chloramphenicol (Cm, 5µg/mL), rifampicin (Rif, 250µg/mL), and streptomycin (Stp, 1000µg/mL) (Sigma Aldrich, St. Louis, MO) were aseptically added to sterilized MRS/ BHI media or agar to maintain selective pressure. Reagents were sourced from Fisher Scientific (Pittsburg, PA) unless otherwise noted. All the bacterial strains and plasmids generated or used in this study are part of the NCK culture and TRK plasmid collection at North Carolina State University.
2.3.2 DNA manipulation strategies

The DNA manipulation and cloning methodology used to construct ΔsrtA mutants followed those previously described by Goh et al. (2009). All primers (Table 2) were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). PCR amplifications for gene deletions were generated using the Expand High Fidelity Plus PCR system (Roche Molecular Biochemicals, Indianapolis, IN) and were digested for cloning purposes using BamHI and SacI (Roche Molecular Biochemicals). Ligation was performed using NEB Quick Ligase (New England BioLabs, Ipswich, MA). Chemically competent E. coli cells (Hanahan, 1985) were used to maintain plasmids before harvesting for electroporation into L. acidophilus or L. gasseri as described by Walker et al (1996). PCR screening of recombinants was performed using Choice-Taq Blue DNA polymerase (Denville Scientific Inc., Metuchen, NJ).

2.3.4 Construction of sortase-deficient derivatives of L. acidophilus NCFM and L. gasseri ATCC 33323 mutants

In-frame deletions of srtA in L. acidophilus NCFM (LBA1244) were constructed using upp-counterselective gene replacement as described by Goh et al. (2009). Similarly, an in-frame deletion of srtA in L. gasseri ATCC 33323 (LGAS_0825) was constructed using upp-counterselective gene replacement as established by Selle et al. (unpublished). Artemis software was used to visualize the specific chromosomal region associated with each of the sortase gene, and primers (Table 2) were designed to amplify homologous regions of approximately 600 base pairs upstream and downstream of the target. Once amplified using
PCR, the two flanking regions were then linked together using splicing by overlapping extension PCR (SOE-PCR) (Horton et al., 1989). The vector (pTRK935) and the SOE-PCR product were both digested with *BamHI* and *SacI*, ligated, and transformed into chemically competent *E. coli* EC 101 cells. Recombinant plasmids obtained from positive clones were sequenced by Davis Sequencing, Inc. (Davis, CA) to confirm fidelity.

Following confirmation of sequence fidelity, the recombinant plasmids were electrotransformed into their respective *Lactobacillus* background hosts, *L. acidophilus* NCK1910 and *L. gasseri* NCK2253. Transformants were isolated based on their resistance to both Em and Cm conferred by the presence of pTRK669 and the pTRK935-based deletion plasmid, respectively. These transformants were then passed three times in a 42°C water bath causing loss of the pTRK669 helper plasmid and facilitating selection of integrants resulting from homologous recombination of either pTRK1059 or pTRK1066 into the *L. acidophilus* or *L. gasseri* chromosome, respectively. Chromosomal integration renders the transformants Cm^r^, Em^t^, and 5-flourouracil (5-FU) (Sigma) sensitive. Replica plating on MRS agar with Em and MRS agar with Cm identified chromosomal integrants. Selected integrants were passed three times in MRS broth, without Em, at 24hour intervals at 37°C. Following the third passage, cultures were plated on glucose semi-defined medium (GSDM) (Kimmel and Roberts, 1998) supplemented with 5-FU to select for 5-FU^R^ colonies in which excision had occurred, resulting in either reversion to the wild type or the ΔsrtA genotype. Colonies were screened using primers flanking the deletion region (*Table 2*). Deletions were confirmed by DNA sequencing. ΔsrtA *L. acidophilus* and *L. gasseri* were designated NCK2232 and NCK2256, respectively (*Table 1*).
2.3.5 Growth and Survival Assays

*L. acidophilus* NCK2232 and *L. gasseri* NCK2256 were evaluated for growth in MRS and survival in simulated gastric juices (Charteris et al., 1998; Frece et al., 2005) as compared to the parent strains *L. acidophilus* NCK1909 and *L. gasseri* NCK2253, respectively. For growth studies, a 2% inoculum from a stationary phase culture was followed for 12h at OD (600 nm) and colony forming units (CFU) were enumerated. For survival assays, 1 mL of a 16h overnight culture was spun down, washed twice in Phosphate Buffered Saline (PBS) (Invitrogen, Carlsbad, CA), and resuspended in 1 mL of sterile water. This cell suspension (0.2 mL) was combined with another 1 mL of simulated gastric juice (SGJ) [0.5% sodium chloride, 3 mg/mL pepsin, pH = 2.0] or 1 mL of simulated small intestinal juice (SIJ) [0.5% sodium chloride, 1g/L pancreatin, and 3 g/L oxgall, pH = 8.0] and incubated at 37°C. Survival was measured over the course of 1h in SGJ and 4h in SIJ as determined by CFU/mL on MRS agar. Sodium chloride and pepsin were both obtained from Fischer Scientific. Pancreatin is a product of MP Biomedicals (Santa Ana, CA) and oxgall was obtained from Difco (Franklin Lakes, NJ).

2.3.6 Mucin and Caco-2 Adherence Assays

Adherence to porcine mucin (Sigma) and to Caco-2 adenocarcinoma cells (ATCC HTB-37) was performed as described previously (Goh and Klaenhammer, 2010). Briefly, Type III porcine gastric mucin (Sigma) was immobilized in 96-well flat bottom microtiter plates for 18h at 4°C. Excess mucin was washed with PBS (7.4) and blocked with 2% bovine serum albumin (BSA, Invitrogen) for 2h at 4°C. Wells were washed 2X with PBS to remove
excess BSA. An overnight culture was washed in PBS and adjusted to an OD$_{600nm}$ = 0.6. The bacterial suspension (100 µL) was added to each well and incubated for 1h at 37°C. Adherent cells were recovered following treatment of the wells with 0.05% Triton X-100 (Fisher) and plating on MRS. For Caco-2 adherence assays, Caco-2 monolayers (between passage 26 and 45) were incubated (37°C) with bacterial strains resuspended in PBS at 1 X 10$^8$ CFU/mL for 1.5h. Following incubation, Caco-2 monolayers were washed 5 times with PBS and disrupted with Triton X-100. Adherent bacterial cells were enumerated on MRS.

**2.3.7 Dendritic Cell (DC) Assays**

Bacterial strains were co-incubated with marrow-derived murine immature dendritic cells (DCs) and supernatants from the DCs were evaluated for production of four cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, and IL-12 using Single-Analyte ELISAArray Kits according to manufacturing instructions (Qiagen). DCs from Balb/c mice were purchased from Astarte-Biologics LLC (Redmond, WA) and stored in liquid nitrogen until use. On the day of the experiment, DCs were thawed, transferred to a 50 mL conical tube containing DNaseI (Stem Cell Technologies Inc., Vancouver, Canada), and resuspended in Rosewell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS). Cells were pelleted once (200 x g, 15 minutes, room temperature) (Centrifuge 5810R, Eppendorf International, Hauppauge, NY) and resuspended in RPMI with 10% FBS. Following enumeration of viable cells using Trypan Blue (Sigma) staining, DCs were diluted to a final concentration of 1X10$^6$ DCs/mL and 100 µL of
standardized cell suspension was pipetted into wells of 96-well polypropylene plates. DCs were incubated at 37°C, 5% CO₂ while bacterial strains were prepared.

Stationary phase cultures of *L. acidophilus* NCK1909 and NCK2253, and *L. gasseri* NCK2232 and NCK2256 were pelleted using a bench top swing arm rotor centrifuge (3000 x g, room temperature) and washed with PBS. Supernatants were discarded and each stain was resuspended in PBS to a final OD (600 nm) corresponding to 1 X 10⁸ CFU/mL, centrifuged in a microcentrifuge (10,000 x g, 1 minute), and resuspended in 1 mL of RPMI with 10% FBS. A standardized bacterial suspension (200 µL) was combined in the 96-well plate with the DCs so that the final ratio of bacteria to DCs was approximately 10:1. Co-incubation was carried out for 24h (37°C, 5% CO₂). Supernatants were collected after centrifugation of the 96-well plates and stored at -80°C until assayed by ELISA according to the manufacturer’s instructions (Single-Analyte ELISArray Kit, Qiagen).

### 2.3.8 Selection of Naturally Antibiotic Resistant Strains of *L. acidophilus* NCK1909 and NCK2232

Antibiotic-resistant mutants of *L. acidophilus* NCK1909 and NCK2232 were selected on MRS agar supplemented with 250 µg/mL Rif or 1000 µg/mL Str (Sigma Aldrich), respectively. Resistant mutants of *L. acidophilus* NCK1909 were designated NCK2271 (Rif<sup>R</sup>) and streptomycin-resistant NCK2232 was stored as NCK2272 (Str<sup>R</sup>) (Table 1). Both mutants were evaluated for growth in MRS broth and resembled that of the parental strain. Briefly, a 1% inoculum of overnight culture of each strain was placed in 200 µL of MRS medium in a 96-well plate. Growth was monitored over 24h at 37°C at 600 nm in a microtiter
plate reader (FLUROstar OPTIMA, BMG Labtech, Cary, NC). Each strain was represented in triplicate and the entire experiment was performed on three separate occasions. Additionally, both mutants were evaluated for their ability to maintain their antibiotic resistance markers without antibiotic pressure in MRS broth over the course of 28 days. Plating on both MRS and MRS with the corresponding antibiotic was performed daily for the first 7 days, and on day 14 and day 28 thereafter.

2.3.9 Co-colonization of L. acidophilus NCK2271 and ΔsrtA L. acidophilus NCK2272 in a Germ-free Mouse Model

Cultures of L. acidophilus NCK2271 and NCK2272 were propagated overnight in MRS broth (37°C) and harvested at stationary phase (~16 hrs of growth). Bacteria were harvested by centrifugation in a bench top swing arm rotor centrifuge (3000 x g, 4 minutes, room temperature), washed once in PBS, and resuspended in PBS to OD corresponding to 5 X 10^8 CFU/mL for each strain. Equal volumes of L. acidophilus NCK2271 (Rif^R) and NCK2272 (Str^R, ΔsrtA) were combined to represent a 1:1 ratio in the bacterial suspension for administration to mice.

Six (5 male, 1 female), 12-14 week old germ-free 129Sv/Ev mice were gavaged once with 200 µL of the above bacterial suspension so that each mouse received a total of 1 X 10^8 CFU/mL containing a 1:1 ratio of NCK2271 to NCK2272. Fecal samples were collected approximately 24h following the gavage (Day 1) and continued to be collected daily for a one week period. After 8 days following the initial gavage, all 6 mice were exposed to the microbiota from conventional, specific pathogen free (SPF) 129Sv/Ev mice. This was
achieved by soaking a sterile swab containing a resuspended fecal pellet in 1 mL of PBS from a SPF 129Sv/Ev mouse and then exposing the gnotobiotic mice through swabbing both the oral and anal routes. Cell counts of *L. acidophilus* NCK2271 and NCK2272 (CFU/g) recovered from the stool of each of the 6 mice were enumerated using the corresponding selective media.

### 2.4 Results

#### 2.4.1 *L. acidophilus* and *L. gasseri* each harbor 12 SDPs

The genomes of both *L. acidophilus* NCFM and *L. gasseri* ATCC 33323 encode a single class A sortase identified as LBA1244 and LGAS_0825, respectively. Additionally, each microbe possesses twelve SDPs as identified using the LAB secretome database and a domain search for the LPXTG cell wall anchoring motif (Zhou et al., 2010). Each target was then further assessed for its potential functionality based on the presence of a signal peptide as determined using InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and SignalP4.1 (http://www.cbs.dtu.dk/services/SignalP/). In total, 9 intact SDP were identified in *L. acidophilus* NCFM (Table 3) and 6 intact SDPs in *L. gasseri* ATCC 33323. Of the 9 SDPs in *L. acidophilus* NCFM, 3 were predicted to be mucus-binding proteins (Mubs) or mucus binding precursors. Similarly, in *L. gasseri* ATCC 33323, 2 of the 6 intact SDPs were annotated as adhesion exoproteins, each containing varying numbers of Mub repeats (Azcarate-Peril et al., 2008).
2.4.2 Construction of sortase-deficient mutants

\( \Delta srtA \) mutants of \textit{L. acidophilus} NCK1909 (\( \Delta upp \)) and \textit{L. gasseri} NCK2253 (\( \Delta upp \)) were generated using \( upp \)-based counterselection as described previously. The \( \Delta srtA \) mutant of each strain was confirmed by PCR and with DNA sequencing using primers specific to the upstream and downstream flanking genes of the \( srtA \) deletion target (\textbf{Table 2}). The \( \Delta srtA \) derivative \textit{L. acidophilus} NCFM was designated NCK2232 and the \( \Delta srtA \) deletion in \textit{L. gasseri} ATCC 33323 was designated NCK2256. These mutants contain in-frame deletions of approximately 90% of the sortase gene, and are also expected to lack the surface display of predicted SDPs due to the requirement of the sortase enzyme for their surface display (Dieye et al., 2010; Remus et al., 2013). When the \( \Delta srtA \) mutants were examined for growth characteristics in culture media (MRS) and survival in SGJ and SIJ, neither mutant showed impairment when compared to their respective parent strains (data not shown).

2.4.3 Adhesion of mutants to porcine mucin and epithelial cells in vitro

To determine the effects of sortase deletion on the adhesive capacity of \textit{L. acidophilus} NCK2232 and \textit{L. gasseri} NCK2256, mutants and their respective parent strains were incubated with immobilized porcine mucin, and Caco-2 epithelial monolayers. Both \textit{L. acidophilus} NCK2232 and \textit{L. gasseri} NCK2256 showed significantly reduced adhesion to porcine mucin (27.08 ± 5.52\%, \( p=0.033 \) and 36.90 ± 10.11\%, \( p=0.023 \) respectively) as compared to the parental strains (\textbf{Figure 1}). For Caco-2 adhesion, both of the \( \Delta srtA \) mutants did not show significant differences in adherence capacity as compared to their parent strains (\( p>0.05 \) for both mutants). These results indicate that deficiency of sortase and SDPs in \textit{L.}
*Lactobacillus acidophilus* and *L. gasseri* does not impact adherence *in vitro* to this particular cell line. The observation that adherence to mucin was impacted points to the specific binding capacity of some of the SDPs, namely Mubs and adhesion exoproteins, to cellular components or specific receptors not presented in Caco-2 cells.

2.4.4 Sortase mutants elicit anti-inflammatory signals from murine DCs

Immune cells residing in the GIT communicate with the microbiota and elicit specific responses through cytokine production. The interaction of microbial cell surface proteins and their role in communication with the GIT is crucial to understanding of microbe-host signaling. To this end, production of IL-6, IL-10, IL-12, and TNF-α were quantified from the supernatants of murine DCs co-incubated with ΔsrtA *L. acidophilus* NCK2232 and *L. gasseri* NCK2256, as well as the respective parent strains. The DCs exposed to the ΔsrtA mutant of *L. acidophilus* showed a marked decrease in the production of the proinflammatory cytokine TNF-α as compared to the parent *L. acidophilus* strain (Figure 2.2D). Additionally, DCs incubated with the ΔsrtA mutant of *L. gasseri* produced significantly less of the proinflammatory cytokine IL-12 than did the parent *L. gasseri* strain (Figure 2.2C). No significant changes in IL-10 (Figure 2.2A) or IL-6 (Figure 2.2B) production were observed between the ΔsrtA mutant of either *L. acidophilus* or *L. gasseri*.

Clear differences in the cytokine profiles of DCs were observed between *L. acidophilus* and *L. gasseri*. Namely, cytokine production, with the exception of IL-12, was significantly higher in the supernatants of DCs incubated with *L. acidophilus* than with *L. gasseri*. These results point to a cell surface structure in *L. acidophilus*, which may not only
stimulate cells of the immune system to a greater degree, but may also shelter SDPs from direct interactions with DCs and resulted in a muted response when SDPs are not displayed.

2.4.5 **Sortase-deficiency in a 1:1 co-colonization does not affect competitive colonization with the parent strain in vivo**

Prior to co-colonizationing germ free mice with a 1:1 ratio of the parental and \( \Delta \text{srt}A \) derivative of \( L.\ acidophilus \), both strains were individually examined for their ability to persist in the GIT of germ free 129Sv/Ev mice. When monoassociated, both strains were found to persist at similar levels in 6 mice for a period of 28 days (**Figure 2.3**), and thus co-colonization was pursued to further evaluate the effect of sortase deficiency on \( L.\ acidophilus \) persistence in a competitive colonization experiment.

In order to examine the selectively enumerate the \( L.\ acidophilus \) parent (NCK1909) and \( \Delta \text{srt}A \) derivative of \( L.\ acidophilus \) NCFM (NCK2232) when co-colonized in 129Sv/Ev mice, a Rif\textsuperscript{R} of NCK1909 (i.e. NCK2271) and a Str\textsuperscript{R} of NCK2232 (i.e. NCK2272) were generated using natural selection. Each resulting antibiotic-resistant strain was cultured for 28 days without selection and showed that the mutations were stable over this time period (**Figure 2.4**). These strains were used in a competitive model of colonization using 129Sv/Ev germ-free mice. \( L.\ acidophilus \) NCK2271 and NCK2272 were combined in a 1:1 ratio and introduced into six 129Sv/Ev gnotobiotic mice through gavage. Fecal pellet collection began twenty-four hours following gavage, and continued to be collected every day for 7 days thereafter (**Figure 2.5A**). Over the course of the first week of the experiment, persistent colonization at a similar level was observed for both strains (**Figure 2.5B**). At this point in
time (Figure 5B, black arrow), the mice were exposed to further competition with the introduction of microbiota from SPF 129Sv/Ev mice. The relative levels of *L. acidophilus* NCK2271 and NCK2272 continued to be enumerated from the stool samples, but again no differences were observed between the strains. Although lower levels of *L. acidophilus* NCK2272 as compared to NCK2271 were observed on day 12 and day 14, these differences were found to be statistically insignificant (Figure 2.5C). Furthermore, less than 1% of the original inoculum of each strain could be detected in the stool on day 14 (6 days following the introduction of the normal microbiota), and neither strain was detectable in the stool of the mice on day 21.

### 2.6 Discussion

Lactic acid bacteria have a long history of safe consumption, and a subset of these microbes with proposed probiotic properties, have been studied for their health-promoting benefits. A current survey of the literature indicates increased interest in understanding the cell surface molecules associated with probiotic action (Bron et al., 2012). Sortase and SDPs have been shown to have varying roles in LAB, but that has yet to be investigated in a widely distributed commercial probiotic strain, *L. acidophilus*, or contrasted to a common human commensal of the GIT such as *L. gasseri*.

In this work, four desirable probiotic phenotypes were examined: survival in simulated GIT juices, adherence to mucin and intestinal epithelial cells, persistence and colonization in a murine model, and immunomodulatory capacity. In *L. acidophilus* NCFM and *L. gasseri* ATCC 33323, sortase-deficiency did not alter survival in simulated juices of
the GIT consistent with previous observations in *L. plantarum* WCFS1 (Remus et al., 2013). Additionally, alterations in the ability to adhere to *in vitro* models of adhesion, namely Caco-2 intestinal epithelial cells, were not affected by the ΔsrtA mutations. In contrast, in *L. salivarius* UCC118, the absence of sortase did appear to significantly lower adherence to Caco-2 cells (68%, *P* = 0.007) (van Pijkeren et al., 2006). Interestingly, both ΔsrtA mutants of *L. acidophilus* and *L. gasseri* exhibited a decreased ability to adhere to porcine mucin, likely due to the loss of SDPs classified as Mubs. The difference in the overall mucin populations of the models used may explain why a deficiency in SDPs did not consistently affect adhesion to both mucin and Caco-2 cells. As an example, MUC2 is the predominant secreted mucin in the intestinal tract, and Caco-2 cells have recently been shown to exhibit low mRNA levels of MUC2. Rather, as compared to MUC2 mRNA levels, Caco-2 cells express high levels of MUC5AC, a mucin predominately found in the stomach, pancreas, and hepatobiliary system (Bu et al., 2011). Additionally, Caco-2 cells may lack specific binding receptors for the SDPs represented in the strains examined, and other bacterial surface or bacterial cell proteins may compensate binding to epithelial cells when SDPs are not displayed in *L. acidophilus* and *L. gasseri*.

Persistence of the sortase-deficient strain of *L. acidophilus* NCFM was evaluated in a gnotobiotic mouse model, and to our knowledge this study is the first in which a mutant and a parent probiotic strain were co-colonized simultaneously. Prior to co-colonizing the ΔsrtA mutant and the parent strain, each strain was introduced alone into the GIT of 129Sv/Ev mice. The ΔsrtA derivative of *L. acidophilus* persisted for 28 days at levels of approximately 1 X10⁹ CFU/g, mirroring the persistence capacity of the wild type strain (*L.*
Competitive colonization of the ΔsrtA derivative was therefore pursued, and modeled after challenge studies typically used to assess the ability of probiotic microbes to competitively exclude pathogens. When challenged simultaneously in germ free mice, no difference in persistence was observed. Additionally, the introduction of the microbiota from SPF Sv129Sv/Ev mice did not further enhance any differences in persistence of the ΔsrtA derivative as compared to the parent.

The finding that sortase-deficiency does not affect GIT persistence in a co-colonization experiment has been reported for a sortase-deficient strain of *L. plantarum* WCFS1; however, this was determined using only two mice in a conventional model (Remus et al., 2013). Nonetheless, despite differences in mouse models, sortase-deficiency did not affect the persistence of either *L. acidophilus* (this study) or *L. plantarum* WCFS1 (Remus et al., 2013). Specifically in *L. acidophilus*, the majority of SDPs found are classified as Mubs and these proteins may not be the sole proteins mediating adherence in GIT. Rather uninvestigated cell structures may play a more significant role (Lukic et al., 2012). In addition, although it has been shown that sortase is required for the surface display of SDPs, we cannot out rule the possibility of some residual surface exposure of SDPs resulting from an unknown compensatory mechanism.

Another finding of this study was that, in general, DCs co-incubated with *L. acidophilus* NCFM produce greater amounts of IL-10, IL-6, and TNF-α than did those cultured with *L. gasseri* ATCC 33323. *L. acidophilus* NCFM is a member of the acidophilus complex A homology group of lactobacilli which are characterized by the presence of a thick, electrostatically-associated, outer shell of proteins known as the surface-layer.
on DNA hybridization with specific probes to surface layer protein A (slpA) and slpB of L. acidophilus NCFM, members of the acidophilus complex A homology group including L. crispatus, L. amylovorous, and L. gallinarum showed homologus S-layer structures (Boot et al., 1996). Unlike L. acidophilus NCFM and members of the acidophilus complex A homology group, L. gasseri ATCC 33323 does not produce an S-layer. Taxonomically, L. gasseri groups with other lactobacilli such as L. johnsonii, which also does not express an S-layer. Furthermore, components of the S-layer of L. acidophilus have been shown to be inflammatory in nature and bind to a specific ligand for the dendritic cell receptor, DC-SIGN (Konstantinov et al., 2008), which has yet to be demonstrated for L. gasseri. This particular difference in surface topology is thought to be crucial in the inflammatory nature of the strains examined. Our finding also suggests that the absence of a classical S-layer as in L. gasseri may aid in the display of SDPs to DCs, and thus explains the more pronounced difference that was observed in the immune response elicited by the ΔsrtA mutant of L. gasseri, compared to the respective mutant of L. acidophilus.

In summary, this work has shown that SDPs impact mucin binding, while leaving the capacity of these strains to adhere to epithelial cells un tarnished. In addition, sortase-deficiency in L. acidophilus did not appear to hinder the ability of this probiotic microbe to persist in the GIT of germ-free mice in a monocolonization experiment or in a co-colonization experiment with the parent strain. Although SDPs play a role in modulating the immune response of DCs in both L. acidophilus and L. gasseri, the removal of these proteins from L. gasseri led to a more noticeable anti-inflammatory reaction. This further reinforces
the need to closely examine the cell surface displays of probiotic lactobacilli that are likely important to both gastrointestinal survival and immune activation.

2.7 Acknowledgements

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Table 2.1 Strains used or resulting from this study.

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Table 2.2 Primers and plasmids used in or resulting from this study.

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<td>LGAS_0825</td>
<td>(Excision Screening)</td>
<td>F: ACT ATT GGT GCT TCA GGG CTT AAT CGG CTT AGC C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Host</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTRK669</td>
<td>NCK1831</td>
<td>Heat sensitive helper plasmid, provides RepA in trans, Cm'</td>
</tr>
<tr>
<td>pTRK935</td>
<td>NCK1911</td>
<td>pORI-based vector used in counterselective gene replacement, P-\textit{upp}, \textit{lacZ'}, Em'</td>
</tr>
<tr>
<td>pTRK1059</td>
<td>NCK2231</td>
<td>pTRK935 containing deletion construct for LBA1244 (\textit{srtA}), Em'</td>
</tr>
<tr>
<td>pTRK1066</td>
<td>NCK2255</td>
<td>pTRK935 containing deletion construct for LGAS_0825 (\textit{srtA}), Em'</td>
</tr>
</tbody>
</table>

* Forward and reverse primers are denoted by F and R, respectively.
Table 2.3 Sortase-dependent proteins identified in the genome of *L. acidophilus* NCFM and *L. gasseri* ATCC 33323.

<table>
<thead>
<tr>
<th>Predicted Function</th>
<th>ORF Designation</th>
<th>Protein Length (number of amino acids)</th>
<th>Signal Peptide (+/-)</th>
<th>Signal Peptide Cleavage Site</th>
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</thead>
<tbody>
<tr>
<td>Putative Mucus Binding Protein</td>
<td>LBA1018</td>
<td>346</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Putative Mucus Binding Protein</td>
<td>LBA1019</td>
<td>2659</td>
<td>+</td>
<td>VHA/DEINI</td>
</tr>
<tr>
<td>Surface Protein</td>
<td>LBA1611</td>
<td>2539</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Surface Protein</td>
<td>LBA1633</td>
<td>1659</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Surface Protein</td>
<td>LBA1634</td>
<td>1924</td>
<td>+</td>
<td>QA/ATEEE</td>
</tr>
<tr>
<td>Mucus Binding Protein Precursor, mub</td>
<td>LBA1652</td>
<td>1174</td>
<td>+</td>
<td>PVKA/TSS</td>
</tr>
<tr>
<td>Mucus Binding Protein Precursor, mub</td>
<td>LBA1709</td>
<td>1208</td>
<td>+</td>
<td>VQA/DSVE</td>
</tr>
<tr>
<td>Mucus Binding Protein Precursor, mub</td>
<td>LBA1392</td>
<td>4326</td>
<td>+</td>
<td>VHA/ENIDN</td>
</tr>
<tr>
<td>Putative fibrinogen binding protein</td>
<td>LBA1496</td>
<td>991</td>
<td>-</td>
<td></td>
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<tr>
<td>Hypothetical Protein</td>
<td>LBA0036</td>
<td>435</td>
<td>+</td>
<td>TYA/ANLSD</td>
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<tr>
<td>Putative Membrane Protein</td>
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<td>1376</td>
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<td>VNA/DEMT*</td>
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<tr>
<td>Hypothetical Protein</td>
<td>LBA1793</td>
<td>438</td>
<td>+</td>
<td>HA/DKGST</td>
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### Table 2.3 Continued

<table>
<thead>
<tr>
<th>Protein Type</th>
<th>L. gasseri ATCC 33323</th>
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<tr>
<td><strong>Adhesion Exoprotein</strong></td>
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<tr>
<td>LGAS_0045</td>
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<tr>
<td>LGAS_0143</td>
<td>2823</td>
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<tr>
<td>LGAS_0146</td>
<td>967</td>
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<tr>
<td>LGAS_0383</td>
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<tr>
<td>LGAS_0410</td>
<td>2457</td>
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<tr>
<td>LGAS_0866</td>
<td>268</td>
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<tr>
<td>LGAS_0942</td>
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<tr>
<td>LGAS_0943</td>
<td>979</td>
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<td>LGAS_1067</td>
<td>765</td>
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<td>LGAS_1663</td>
<td>2449</td>
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<td>LGAS_1671</td>
<td>2552</td>
</tr>
<tr>
<td>LGAS_1725</td>
<td>1993</td>
</tr>
</tbody>
</table>

*a* The presence of a signal peptide was determined using InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/).

*b* Signal peptide for *L. acidophilus* (this study) and *L. gasseri* (Kleerebezem et al., 2010) were determined using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The predicted cleavage sites (indicated by the backslash) for both microbes were also determined using SignalP 4.1 (this study).

* Signal peptide of YSIRK origin detected by InterProScan; however, this signal peptide was not detected within the cutoff restraints (D=0.500) of SignalP 4.1. The sequence listed is the most likely cleavage site determined using SignalP 4.1 (D=0.450).
Figure 2.1 Relative adherence of stationary phase ΔsrtA mutants of *L. acidophilus* NCFM (NCK2232) and *L. gasseri* ATCC 33323 (NCK2256) to (A) porcine mucin and (B) Caco-2 cells. Relative adherence was determined as the percent adherent mutant bacterial cells as compared to that of the parent strain (black bar) using the viable count method. Results shown are representative of three independent experiments (four technical replicates each) with error bars representing one standard deviation of the median. Asterisks indicate a significant reduction in adherence (*p*<0.05) as determined by a two tailed Student's *t*-test.
Figure 2.2 Cytokine expression of murine DCs exposed to both the wild-type (gray bars) and ΔsrtA mutants (hashed white bars) of both *L. acidophilus* NCFM and *L. gasseri* ATCC 33323. Actual concentrations of (A) IL-10, (B) IL-6, (C) IL-12, and (D) TNF-α found in supernatant of DCs incubated with the parent strain or ΔsrtA mutant of *L. acidophilus* or *L. gasseri* as measured by ELISA are shown. The bacterial cell to DC ratio was targeted at 10:1 (1 X 10⁶ CFU/ml bacteria: 10⁵ DC). The data shown are the average cytokine concentration of each cytokine from three independent ELISA assays containing two technical replicates each. Error bars represent one standard deviation of the mean, and statistically significant changes are indicated by either a single or double asterisk denoting *p* < 0.05 and *p* < 0.01, respectively.
Figure 2.3 Enumeration (CFU/g) of *L. acidophilus* NCFM, wild type strain, and *L. acidophilus* NCK2232 (ΔsrtA) when monoassociated with 129Sv/Ev germ free mice. Each point represents the average of six 129Sv/Ev mice and the error bars represent one standard deviation of the mean.
Figure 2.4 Stability of the natural rifampicin and streptomycin resistance markers in NCK1909 and NCK2232, respectively. Marker stability was monitored by plating in duplicate on both MRS medium and MRS medium with the corresponding antibiotic over the course of 28 days.
Figure 2.5 Persistence of ΔsrtA L. acidophilus NCFM as compared to the parent strain in a 1:1 co-colonization. (A) Experimental workflow used to assess the persistence of sortase-deficient L. acidophilus NCFM (NCK2272, StpR) as compared to the parent strain (NCK2271, RifR) in a 1:1 co-colonization. Naturally antibiotic resistant strains of each bacterium were combined in a 1:1 ratio (Day 0). Six, germ-free 129Sv/Ev mice were gavaged with the mixture and stool samples were collected for bacterial enumeration for 28 days. (B) Enumeration of each strain (CFU/g) was performed by antibiotic selective plating. Inoculation of the mice with microbiota from SPF 129Sv/Ev mice is indicated by the black arrow. (C) Point plot showing the variation among six mice on Day 8 (immediately before colonization with SPF 129Sv/Ev microbiota), Day 12, and Day 14. A p=0.05 is considered significant.
APPENDICES
APPENDIX I. DELETION OF THE SORTASE-DEPENDENT PUTATIVE MEMBRANE PROTEIN (LBA1740) IN *L. ACIDOPHILUS* NCFM
A.1. Deletion of LBA1740 in *L. acidophilus* NCFM

In addition to the deletion of sortase from *L. acidophilus* NCFM, a sortase-dependent protein, LBA1740, was also deleted. LBA1740 is predicted to be a putative membrane protein of approximately 4.15 kilobases (kb) comprising 1376 amino acids. The gene is flanked on the 5' end by a cell-wall hydrolase (LBA1741) and on the 3' end by a hypothetical protein (LBA1739) ([Figure 1A](#)). LBA1740 was deleted using those protocols described in this thesis (Chapter 2, Material and Methods) as well as in the publication by Goh *et al.* (2009). The primers used to amplify a region of homology flanking LBA1740 are shown in [Table 1](#). The helper plasmid, backbone vector, and deletion construct and strains used to maintain plasmids or supplement the deletion of LBA1740 are shown [Table 1](#). Ultimately, one mutant harboring a deletion of LBA1740, following a double homologous recombination event was screened using PCR ([Figure 1B](#)) and DNA sequencing (UC Davis). Following confirmation of the deletion, the strain was submitted and stored in the NCK culture collection as NCK2251.
**Figure AI.1 Construction and confirmation of LBA1740 deletion in *L. acidophilus* NCFM** (A) Genetic context of LBA1740. Red arrows indicate where upstream and downstream primers anneal in order to generate regions of homology flanking LBA1740 to generate the deletion construct. (B) PCR screen to confirm the deletion of LBA1740 using Taq-Blue polymerase and visualization with ethidium bromide. Lane 1 is the kilobase ladder, lane 2 is the wild type (*L. acidophilus* NCFM), and lane 3 is a putative mutant harboring an excision of LBA1740. Due to the size of LBA1740 in the wild type stain (4.15 kb), it could not be amplified using the polymerase and primers described. However, when the gene was deleted, an expected product of approximately 1300 bp was observed in the putative mutant in lane 3.
### Table AI.1 Primers, plasmids, and strains used in the construction of ΔLBA1740 *L. acidophilus* NCFM.

#### Primers

<table>
<thead>
<tr>
<th>Primer Target</th>
<th>Primer Sequence (5' to 3')</th>
</tr>
</thead>
</table>
| LBA1740 (Upstream) | F: GTA ATA GGA TCC CTT GCT AAA GAG CAG GTG  
|                  | R: CTG AAG CAG CAC CAA TAG T                                                          |
| LBA1740 (Downstream) | F: ACT ATT GGT GCT TCA GGG CTT AAT CGG  
|                  | R: TAA AGT AGA GCT CGT AGA TAC AAA AAG AGT CTG GG                                     |
| LBA1740 (Excision Screening) | F: GGC TAA TCA AAT CGT TAA GAC  
|                  | R: CCA CAA CGA TCA CGT ATC G                                                           |

#### Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Host</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTRK669</td>
<td>NCK1831</td>
<td>Heat sensitive helper plasmid, provides RepA in trans, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>pTRK935</td>
<td>NCK1911</td>
<td>pORI-based vector used in counterselective gene replacement, <em>upp</em>, <em>lacZ</em>, and Em&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>pTRK1064</td>
<td>NCK2250</td>
<td>pTRK935 containing deletion construct for LBA1740, Em&lt;sup&gt;+&lt;/sup&gt;</td>
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</tbody>
</table>

#### Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host</th>
<th>Background</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCK2250</td>
<td>E. coli</td>
<td>EC101</td>
<td>pTRK1064 host</td>
</tr>
<tr>
<td>NCK 1911</td>
<td>E. coli</td>
<td>EC 101</td>
<td>pTRK935 host, pORI-based plasmid used for counterselective gene replacement</td>
</tr>
<tr>
<td>NCK1909</td>
<td><em>L. acidophilus</em></td>
<td>NCFM</td>
<td>Δ<em>upp</em></td>
</tr>
<tr>
<td>NCK 1910</td>
<td><em>L. acidophilus</em></td>
<td>NCFM</td>
<td>Δ<em>upp</em>, pTRK669 helper plasmid host</td>
</tr>
<tr>
<td>NCK2251</td>
<td><em>L. acidophilus</em></td>
<td>NCFM</td>
<td>Δ<em>upp</em>, ΔLBA1740</td>
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</tbody>
</table>