ABSTRACT

SELLE, KURT MICHAEL. Evaluation of the Role of *ltaS* in the *in vitro* Adhesion and Immunomodulatory Capacity of *Lactobacillus gasseri* ATCC 33323. (Under the direction of Dr. Todd Klaenhammer.)

Lactobacillus gasseri ATCC 33323 is a member of the acidophilus-complex group, microbes of human origin with significant potential for impacting human health based on niche-specific traits. In order to facilitate functional analysis of this important species, a *upp*-based counterselective chromosomal integration system was established and employed for knockout mutagenesis of the lipoteichoic acid synthase gene in *L. gasseri* ATCC 33323. The *ltaS* deficient derivative exhibited significantly reduced ability to adhere to Caco-2 intestinal cell monolayers, relative to the wild-type parent strain. The *ltaS* knockout mutant also induced increased secretion of IL-10 and decreased IL-12 secretion from murine immature dendritic cells upon co-incubation, *in vitro.* Therefore, *ltaS* appears to have a significant role in mediating immunomodulation by *L. gasseri* ATCC 33323 to dendritic cells and adhesion to intestinal epithelial cells.

Evaluation of the Role of *ltaS* in the *in vitro* Adhesion and Immunomodulatory Capacity of *Lactobacillus gasseri* ATCC 33323

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Food Science

Raleigh, North Carolina

2013

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DEDICATION

To my brothers, Paul and Jacob Selle, who have thus far provided me with a lifetime worth of humor, conscience, and camaraderie. You have both driven me to be exemplar and I would be remiss to fall short of what you have inspired me to achieve.

BIOGRAPHY

Kurt Selle grew up and attended High School in West Bend, WI. After graduating in 2006, he attended the University of Wisconsin Madison and received a B.S. in Microbiology in 2010. During his undergraduate career he worked in the lab of Dr. Jim Steele on several projects, spurring his interest in food science and probiotic microbes, while affording him experience working with lactic acid bacteria in a research setting. From there, Kurt was accepted into graduate school at North Carolina State University under the direction of Dr. Todd Klaenhammer. Kurt is currently pursuing an M.S. in Food Science, and will enter the Functional Genomics Ph.D. program to continue his work with Dr. Klaenhammer.

ACKNOWLEDGEMENTS

I cannot stress enough the benefit of the experiences that were afforded to me by Dr. Todd Klaenhammer's efforts in mentoring me as a student. I am truly privileged to have been given the opportunity to work with him and in his lab. The education and guidance I have received on his part are invaluable and will continue to shape my professional and personal career.

Also tantamount to my success were the several individuals I met working in the lab and through the Department of Food, Bioprocessing, and Nutrition Sciences. I have had the privilege and honor of working with Jon Baugher, Emma Call, Rosemary Sanozky-Dawes, Grace Douglas, Evelyn Durmaz, Dr. Yong Jun Goh, Brant Johnson, Dr. Akinobu Kajikawa, Dr. Sarah O'Flaherty, who were both friends and colleagues alike. The environment in the lab was highly educational, cooperative, friendly, and humorous, all due to the colorful individuals I was grateful to be considered one of.

Chad Jordan and Quin Patton have been valued friends during the course of my M.S. and have provided great humor, counsel, and heated social discourse. I am truly blessed to have had the immeasurable, unequivocal, and unyielding care and support of Margaret Schneider.

I also thank the Southeast Dairy Foods Research Center and Du Pont Nutrition and Health for financial support.

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Chapter I. Lactobacillus acidophilus complex species

Kurt Selle, W Michael Russell, and Todd R. Klaenhammer

Abstract

Lactobacillus acidophilus has a long history of safe human consumption in fermented milk products. Advances in 'omics' technologies have further driven our understanding of the mechanisms contributing to health benefits that have been associated with this species. The phylogenetic relationships of the members of the *acidophilus* complex have been structured by 16S rRNA sequencing, which has greatly facilitated identification and differentiation of the species within the acidophilus complex.

Introduction

Lactobacillus acidophilus, first isolated by Moro (1900) from infant faeces, has undergone many transformations in the description of its metabolic, taxonomic and functional characteristics. The acidophilus (meaning 'acid-loving') bacterium is isolated from the intestinal tract of humans and animals and is also reported in the faeces of milk-fed infants and older persons consuming high milk-, lactose- or dextrin diets. Historically, *L. acidophilus* is the *Lactobacillus* species most often implicated as an intestinal probiotic capable of eliciting beneficial effects on the microbiota of the gastrointestinal tract (GIT). Metchnikoff's 1906 publication "The Prolongation of Life: Optimistic Studies", implicated a lactic acid bacillus in Bulgarian yogurts as the agent responsible for preventing intestinal putrefaction and aging. Later, it was discovered that Metchnikoff's bulgarian strain did not survive passage through the gastrointestinal tract, prompting substitution of *Lactobacillus acidophilus* as the most likely candidate to fulfill the primary criteria expected of an

intestinal probiotic. It has since been discovered that a variety of homofermentative and heterofermentative lactobacilli inhabit the GIT, mouth and vagina and each may elicit a variety of benefits as constituents of the normal microbiota. The most predominant among these are six species of homofermentative lactobacilli that now constitute the group known as the *L. acidophilus* complex. The six species shown in Table 1 collectively demonstrate the metabolic and functional properties that have typically been assigned to the bacteria called *L. acidophilus* over the last century.

Table 1. Species of the *Lactobacillus acidophilus* complex

	DNA Homology Groups			
Species	Johnson et al	Lauer et al	G+C %	Type strain
Lactobacillus acidophilus	A1	Ia	34–37	ATCC 4356
Lactobacillus crispatus	A2	Ic	35–38	ATCC 33820
Lactobacillus amylovorus	A3	Ib	40–41	ATCC 33620
Lactobacillus gallinarum	A4	Id	36–37	ATCC 33199
Lactobacillus gasseri	B1	IIa	33–35	ATCC 33323
Lactobacillus johnsonii	B2	IIb	33-35	ATCC 33200

Taxonomy

The *L. acidophilus* species are Gram-positive rods (dimensions are in the range 0.5–1 × 2–10 μm), with rounded ends, occurring in pairs or short chains. The group was initially

categorized in the thermobacteria classification of lactic acid bacteria based on their homofermentative metabolism and ability to grow at 45°C. In 1980, *L. acidophilus* was recognized as a heterogeneous group by DNA hybridization studies and separated into two main DNA homology groups (A and B or I and II), eventually forming six distinct species composed of *L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. gallinarum*, *L. gasseri* and *L. johnsonii*.

Figure 1 shows the phylogenetic relatedness of the *L. acidophilus* group based on analysis of their 16S ribosomal RNA sequences. *L. acidophilus* is most closely related to *L. helveticus*, a milk fermenting *Lactobacillus*, and the other members of the A-homology group, *L.* cri*spatus* and *L. amylovorus*. *L. gasseri* and *L. johnsonii* are related in the acidophilus phylogenetic group, but are found more distant from *L. acidophilus* than either of the fermentative strains of *L. helveticus* or *L. delbrueckii*. The genetic relationship between the gastrointestinal lactobacilli of the *L. acidophilus* complex and the milk-fermenting species *L. helveticus* and *L. delbruckeii* ssp. *bulgaricus* has been elucidated by genome sequencing of these species. Comparative genomics has yielded important disparities between the probiotic and milk fermenting strains of the acidophilus complex, elaborating upon their phenotypic and genotypic differentiation following adaptation to their respective niches, notably milk and the gastrointestinal tract.

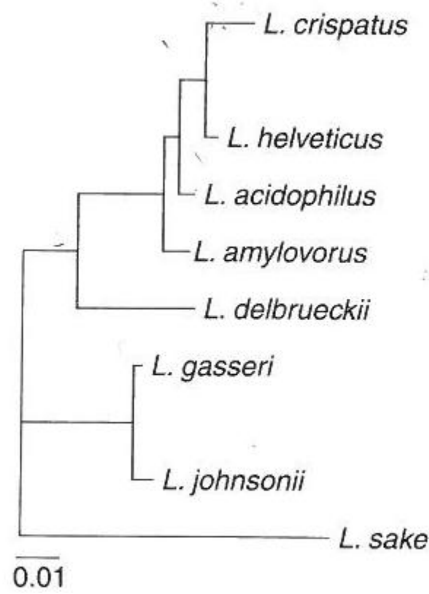

Figure 1. 16S rRNA phylogeny of *L. acidophilus* complex members.

Phylogenetic relationships between members of the *L. acidophilus* group based on an analysis of aligned 16S rRNA gene sequences. The tree was rooted with *L. sake* and created by applying the neighbour-joining method of Saitou and Nei (1987), to a matrix of pairwise distances. The bar indicates 0.01 fixed mutations per nucleotide position. Phylogenetic analysis and tree construction by M Kullen.

Metabolism and End Products

Members of the *L. acidophilus* complex are classified as obligate homofermenters. Hexoses are fermented primarily to lactic acid (> 85%) by the Embden–Meyerhof–Parnas

(EMP) pathway. They possess aldolase and lack phosphoketolase. Neither gluconate nor pentoses are fermented. All species produce both D and L isomers of lactic acid. *L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. gallinarum*, and *L. johnsonii* all possess β-galactosidase, whereas *L. gasseri* lacks β-galactosidase, but has phospho-β-galactosidase, and some strains of *L. gasseri* also have β-glucuronidase activity. Fermentation patterns and key distinguishing characteristics of species within the *L. acidophilus* complex are listed in Table 2. All species possess a Lys-D -Asp type peptidoglycan. Analysis of cell wall components and genetic analysis for slp-related sequences have generally shown that members of the A homology group possess an S-layer, whereas members of the B group do not.

In 1995, Hammes and Vogel grouped lactobacilli and related genera based on both their fermentation patterns and phylogenetic relatedness. The *L. acidophilus*-complex species belong to group Aa, being defined as obligately homofermentative organisms closely related to *L. delbrueckii*. In addition to the six species in the *L. acidophilus* complex, there are five other recognized species in the Aa group: *L. delbrueckii*, *L. jensenii*, *L. helveticus*, *L. amylophilus* and *L. kefiranofaciens*. Based on the difference in G+C content between *L. delbrueckii* (49–51 mol%) and the rest of the species in the group (33–41 mol%), the '*L. delbrueckii* group' was renamed the '*L. acidophilus* group' because of the more typical G+C content (34–37 mol%) for the majority of species defined in this group.

Table 2. Distinguishing characteristics and fermentation patterns of species in the *Lactobacillus acidophilus* complex

	acidophilus	*crispatus*	*amylorous*	*gallinarum*	*gasseri*	*johnsonii*
Growth at 30°C	−	−	−	−	−	−
Growth at 45°C	+	+	+	+	+	+
Lactic acid isomers	DL	DL	DL	DL	DL	DL
S-layer	+	+	+	+	−	−
Aesculin	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+
Glycogen	+	+	−	+	−	−
Galactose	+	+	+	+	+	+
Lactose	+	+	−	v	v	V
Maltose	+	+	+	+	v	+
Mannitol	−	−	−	−	−	−
Mannose	+	+	+	+	+	+
Melibiose	v	−	−	+	v	V
Raffinose	−	−	−	+	v	
Rhamnose	−	−	−	−	−	−
Salicin	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
Trehalose	v	−	+	−	v	V
Xylose	−	−	−	−	−	−

+, >90% of strains are positive.

−, >90% of strains are negative.

v, variable.

The *L. acidophilus* group also produces a variety of antimicrobial compounds, including lactic acid (> 85%), hydrogen peroxide, and numerous peptide bacteriocins. In assays screening for antimicrobial activities, the impacts of acid and hydrogen peroxide must

be eliminated by neutralization of the culture supernatant to pH 6.0-7.0 or addition of catalase (3%), respectively. *L. acidophilus* cultures are known to produce hydrogen peroxide, but the amount produced varies considerably between strains. The evolutionary role of bacteriocins is considered to be a competitive advantage in an ecological setting and is substantiated by the likely strong competitive roles for bacteriocins in bacteria endogenous to the gastrointestinal tract. Bacteriocins synthesized by LAB are largely heterogeneous, as they vary in structural properties and mechanisms of inhibition as well as target range. A list of peptide bacteriocins produced by members of the *L. acidophilus* complex is shown in Table 3. Most of the bacteriocins are class II peptide bacteriocins, such as lactacin F, lactacin B and acidocin A, which are active against other lactobacilli and enterococci. However, an unusual *L. acidophilus* bacteriocin, acidocin B, was isolated which showed activity against *Listeria monocytogenes*, *Clostridium sporogenes* and *Brochothrix thermosphacta*, but not other lactobacilli.

Table 3. Bacteriocin production by probiotic lactobacilli

Bacteriocin	Producer
Lactacin B	*L. acidophilus*
Acidophilucin A	*L. acidophilus*
Acidocin 8912	*L. acidophilus*
Acidocin A	*L. acidophilus*
Acidocin B	*L. acidophilus*
Acidocin JCM1132	*L. acidophilus*
Acidocin J1229	*L. acidophilus*
Lactobin A	*L. amylovorus*
Lactacin F	*L. johnsonii*
Gassericin A	*L. gasseri*
Gassericin T	*L. gasseri*

Identification and Differentiation

It is critically important to have rapid and accurate methods available to identify and differentiate species within the *L. acidophilus* complex from each other, as well as from closely related lactobacilli. Identification based on traditional phenotypic criteria has been unreliable due to the physiological and biochemical diversity of this genus. Molecular techniques targeting highly conserved rRNA sequences are rapid, accurate and reliable. Differentiation of species within the *L. acidophilus* group has been reported through the use of specific rRNA oligonucleotide probes for hybridization, generation of Random Amplified Polymorphic DNA (RAPD) patterns, and the use of species-specific oligonucleotide primers in polymerase chain reactions to amplify 16S rRNA sequences. A list of probes and primers compiled for the species of the *L. acidophilus* complex is given in Table 4. These protocols are highly sensitive, require very specific temperatures and conditions, and can be difficult to reproduce between laboratories. Therefore, the identities of unknown organisms are often difficult to resolve. In these cases, it is recommended that unknown lactobacilli be identified by sequencing the 16S or 23S rRNA regions that define the species.

Table 4. Oligonucleotide probes and primers reported for the differentiation of species of the *L. acidophilus* complex

Probes	Target (positions)	Reference
AGAGTTTGATCCTGGCTCAG GGCTGCTGGCACGTAGTTAG	V1 and V2 regions of 16S rDNA sequence all *L. acidophilus* group	Kullen et al. (2000) J. App. Microbiol. Vol89. 3:511-516
TCTTTCGATGCATCCACA	23S – *Lactobacillus acidophilus* (1159-1180)	Pot et al. (1993) J. Gen. Microbiol 139:513-517
CAATCTCTTGGCTAGCAC	23S - *L. crispatus*	Ehrmann et al (1992) Syst. Appl Microbiol 15:453-455

Table 4 Continued

GTAAATCTGTTGGTTCCGC	16S - *L. amylovorus*	Ehrmann et al (1994) FEMS Microbiol Lett 117: 143-149
None	*L. gallinarum*	
TCCTTTGATATGCATCCA	23S - *L. gasseri* (1160-1178)	Pot et al (1993) J. Gen. Microbiol 139:513-517
ATAATATATGCATCCACAG	23S - *L. johnsonii* (1158-1179)	Pot et al. (1993) J. Gen. Microbiol 139:513-517
Specific primers		
Aci 1 - TCTAAGGAAGCGAAGGAT Aci II - CTCTTCTCGGTCGCTCTA	16S-23S intergenic spacer region, *L. acidophilus* ATCC 4356	Tilsala-Timisjarvi and Alatossava (1997) Inr J. Food Microbiol 35: 49-56
HE1- AGCAGATCGCATGATCAGCT SS2 - CACGGATCCTACGGGTACC TTGTTACGACTT	16S - *L. acidophilus*[a]	Drake et al (1 996) J. Food Protection 59: 1031 -1036
GCATTAGTGTGCAACCCATCT GG GATCTGCTGGATTGCTTCTAC CG	CRISPR sequence- *L. acidophilus* strains	Russell, W. M., Barrangou, R. & Horvath, P. US Patent Application 20060199190 (2006).
RAPD primers		
OPL-1 GGCATGACCT	All *L. acidophilus* group	Du Plessis and Dicks (1995) Curr Microbiol 31: 114-188
OPL-4 GACTGCACAC	All *L acidophilus* group	
AGCAGCGTGG	Differentiates *L. acidophilus* from other lactobacilii	Cocconcelli et al (1995) Lett. Appl Microbiol 21: 376-376

a Also generates an amplicon in *L. helveticus* which can be differentiated by restriction fragment length polymorphism analysis

Becoming more common is the need to differentiate a strain within a given species. Traditional methods of strain typing include plasmid profiles and total soluble protein patterns. Recent efforts have resulted in two methods that generate simple and reproducible DNA restriction fragment patterns that allow differentiation of strains of lactobacilli. Ribotypes are the patterns of an rRNA-oligonucleotide probe following its Southern

hybridization to the DNA restriction digests of the strain being characterized. Pulsed field gel electrophoresis (PFGE) uses alternating currents to separate large restriction fragments (> 50 kb) which are generated with restriction enzymes that cut infrequently in the genome. For lactobacilli, the preferred PFGE methods use mutanolysin, to carry out in-block cell lysis, followed by lengthy digestions with enzymes such as SmaI (5′ CCCGGG 3′) or ApaI (5′ GGGCCC 3′). The Sma I banding patterns for the ATCC neotype strains of the six species of the *L. acidophilus* complex are shown in Figure 2. Analysis of large DNA fragmentation patterns can discriminate effectively between strains on the basis of overall genomic organization. However, PFGE cannot detect minor genetic differences within the large fragments (e.g. point mutations, minor additions, deletions and rearrangements) that can occur between clonal variants. These small genetic changes can impact the phenotypic characteristics, activities and behavior of strains that may appear identical by PFGE. Therefore, descriptions of any member of the lactobacilli should include both genetic and phenotypic information. Another method for distinguishing the species and strains of the acidophilus group employs repetitive element PCR fingerprinting. Repetitive elements distributed throughout the genome are amplified using primers specific to the related group of organisms. The primer 5'-GTG GTG GTG GTG GTG-3' has previously been employed to differentiate lactic acid bacteria. The resulting fingerprint is analyzed using agarose gel electrophoresis or microfluidics and the banding patterns are often strain specific. Rep-PCR methods are fast, efficient and when used in combination with microfluidics, highly sensitive.

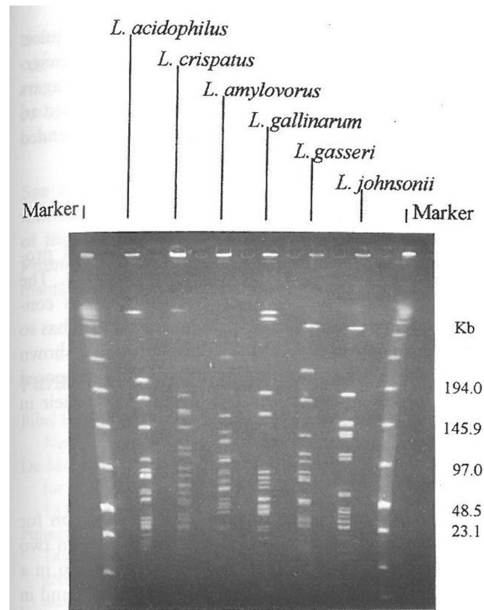

Figure 2. Pulsed field gel electrophoresis fingerprints of acidophilus complex members. SmaI fragmentation patterns for the type strains comprising the *L. acidophilus* group. Samples of 18 h digestions were run in 1.1% agarose at 200 V for 22 h at 14°C. Lanes 1 and 8, molecular weight marker; lane 2, *L. acidophilus*; lane 3, *L. crispatus*; lane 4, *L. amylovorus*; lane 5, *L. gallinarum*; lane 6, *L. gasseri*; lane 7, *L. johnsonii*.

Genomics

Comprehensive genome sequencing on four members of the acidophilus group has been accomplished, namely the species *L. acidophilus*, *L. gasseri*, *L. crispatus* and *L. johnsonii*. The size of their respective genomes ranges from 1.9 Mb to 2.04 Mb, with the relatively small genome size indicative of evolutionary adaptation to the nutrient rich niche

of the mammalian intestinal tract. This is evident in the analysis of metabolic pathways encoded in the genomes. For example, members of the *L. acidophilus* group are largely deficient in amino acid biosynthetic pathways, but compensate by expressing proteinase PtrP, the oligopeptide transporter *opp* and multiple other peptide transporters to effectively process and acquire nitrogenous metabolites exogenously. Additionally, species of the *L. acidophilus* group retain a myriad of saccharide transporters, enabling catabolism of a diverse array of sugars present in the GI tract, including prebiotic compounds such as fructo-oligosaccharides by ABC transporters and galacto-oligosaccharides by the lacS permease. Monosaccharides are transported by sugar specific phosphoenolpyruvate phosphotransferase systems, the translocated phosphate groups activating them for glycolysis. In total, approximately 13-18% of these genomes encode amino acid and carbohydrate transport proteins. The genomes of the *L. acidophilus* species also highlight genes facilitating survival during gastric passage, transient colonization of the GI tract, and means of probiotic action. Acid tolerance mechanisms include F_1 F_0 ATPase proton pumps and ornithine decarboxylases contribute to maintenance of a stable intracellular pH. Synthesis of sterols and peptidoglycan decrease membrane permeability. Chaperones and additional intracellular proteins can repair oxidatively damaged cellular constituents. *L. gasseri* encodes multiple bile salt hydrolases and transporters, but their role in tolerating bile *in vivo* has yet to be determined conclusively. Genes facilitating adherence to the epithelial layer of the intestinal tract encode for mucus binding proteins, fibronectin binding proteins, and mannose-specific adhesion proteins. These adhesion proteins can facilitate competitive exclusion with other

commensals or pathogenic microorganisms for attachment to the epithelial layer and transient colonization of the desirable niche. Adhesion also increases the propensity of these microorganisms to transiently colonize the GI tract and interact with immune system cells located in the gastrointestinal tract. The *L. acidophilus* genome also harbors sequences of clustered regularly interspaced short palindromic repeats, or CRISPR sequences. The presence of CRISPR sequences in *L. acidophilus* offers a DNA signature with the potential for use in strain identification and differentiation. S-layer proteins have been observed in several members of the group, including *L. acidophilus*, *L. crispatus* and *L. helveticus*, and *L. gallinarum*. S-layers have been demonstrated to participate in immune system modulation, adherence to epithelial cells, and also have been implicated in inhibition of GI tract pathogens. Interestingly, *L. acidophilus* encodes three S-layer proteins in the genome, the predominant slpA, minimally expressed slpX, and slpB, which is not expressed under normal culture conditions. The high expression and self-assembling crystalline lattice structure are properties that result in a high potential for applications in biotechnology and nanotechnology.

Genomic comparison of *L. acidophilus* to the closely related organisms *L. helveticus* and *L. bulgaricus* ssp. *delbruckeii* show up to 75% homology in open reading frames, but certain disparities are obvious which highlight evolutionary divergence of the organisms with regard to long term niche adaptation. Notably, the high prevalence of pseudogenes in *L. helveticus* and *L. bulgaricus* ssp. *delbruckeii* compared to *L. acidophilus* is indicative of genome decay by selective inactivation of genes ill-suited for these species' adapted

ecological niche, milk. This is reflected in gene loss associated with diverse sugar catabolism and preferential catabolism of lactose, the primary fermentable sugar in milk. Interestingly enough, other functions lost in the genome decay of *L. helveticus* include those responsible for survival in the gastrointestinal tract, namely bile salt hydrolases as well as mucin binding proteins, further evidence of delineation between the phylogenetic groups.

Growth and Culture Conditions

Lactobacillus acidophilus cultures are microaerophilic and capable of aerobic growth in static cultures without shaking. Anaerobic conditions are preferable and growth is stimulated in broth or agar under a standard anaerobic gas mixture of 5% carbon dioxide, 10% hydrogen, and 85% nitrogen. The nutritional requirements of *L. acidophilus* reflect the fastidious nature of these bacteria. Media for standard propagation are rich in amino acids and vitamins (peptones, tryptones, yeast/beef extracts), and usually contain sorbitan monooleate (Tween 80), sodium acetate and magnesium salts which stimulate growth. The primary propagation medium is MRS (de Man, Rogosa and Sharpe) broth which is sold commercially (Difco Laboratories, Detroit, MI). Derived from the original selection medium, using high levels of acetate, a number of media are now available for selection of the *L. acidophilus* complex species from food products and biological samples (intestinal, vaginal, mouth, faeces) where mixed bacterial populations exist. Three primary selective agents can be used, individually or in combinations: sodium acetate (15–25 $g\ l^{-1}$), tomato juice and bile

(ranging from 0.15% to 1.0% oxgall). The primary selection media for lactobacilli are Rogosa SL media (Difco Laboratories, Detroit, MI) and Lactobacillus Selection Agar (LBS, Becton Dickenson, Cockeysville, MD). For isolation of *L. acidophilus* from biological or food samples, LBS + 20 % centrifuged tomato juice can be used. Plates are incubated for 48–72 h at 37°C under anaerobic conditions. Either MRS (Difco) or LBS (BBL) agars containing 0.15% oxgall (BBL) have also been used to assess injury of *L. acidophilus* populations suspended in dried or frozen cultures.

Probiotic capacity of Lactobacilli

The current and most widely cited definition of probiotics is that they are "live microorganisms, which when administered in adequate amounts confer a health benefit upon the host". The majority of probiotic cultures are of the genera *Lactobacillus* and *Bifidobacterium*, with some notable exceptions. Members of the *L. acidophilus* complex are generally considered to facilitate the establishment of the normal gastrointestinal microbiota, represented by a complex population of microorganisms that are known to exert beneficial influences on the host. Probiotic lactobacilli have further been implicated in a variety of beneficial roles with both prophylactic and therapeutic capabilities listed in Table 5. Within the last decade, the health benefits associated with probiotic cultures have been increasingly supported by a plethora of studies, including a number of human clinical trials. Investigation of the interaction of probiotic bacteria with the host using 'omic' technologies have further

elucidated the complex mechanisms inherent in the microbe-host crosstalk and how they are manifested in human health. Despite this recent increase in research activity, further studies are still necessary to clarify strain, dose, and site specific health benefits elicited by probiotics *in vivo*.

Table 5. Health benefits of probiotic microbes

Benefits of Probiotics	Reference
Protection against infection	(Corr et al., 2007)
Lowered incidence of diarrhea	(Lonnermark et al., 2009)
Lowered levels of cold and influenza-like symptoms in children and reduction in missed school days	(Leyer et al., 2009)
Antimicrobial activity	(Ryan et al., 2009)
Competitive exclusion of pathogens	(Lee et al., 2003)
Immune tolerance	(van Baarlen et al., 2009)
Reduction in colorectal cancer biomarkers	(Rafter et al., 2007)
Return to pre-antibiotic baseline flora	(Engelbrektson et al., 2009)
Epithelial barrier function	(Mennigen et al., 2009)
Increased cellular immunity (e.g. increased natural killer cell activity)	(Takeda and Okumura, 2007)
Increased humoral response (e.g. IgA secretion)	(Viljanen et al., 2005)
Lowering of blood cholesterol levels	(Ataie-Jafari et al., 2009)
Prevention of necrotizing enterocolitis in infants	(Lin et al.,2008)
Amelioration of colitis symptoms and maintenance of epithelial integrity	(Mohamadzadeh and Klaenhammer., 2010)
Prevention of atopic disease in infants	(Kalliomäki et al, 2001)
Reduction in irritable bowel disease symptoms	(Macfarlane et al., 2009)
Delivery of therapeutics	(Wells and Mercenier, 2008)

The health benefits of probiotics listed in Table 5 can be categorized into host-microbe and microbe-microbe interactions. Probiotics confer a protective effect against GI

infection by competitive exclusion of pathogens by production of antimicrobials, adhesion to the epithelial cell layer, nutrient competition and similar microbe-microbe interactions. Members of the acidophilus group are well known to synthesize antimicrobial compounds such as lactic acid, hydrogen peroxide and bacteriocins (Table 3) which have the capacity to inhibit pathogens *in vivo*. *L. gasseri* has demonstrated improved treatment of *H. pylori* in humans. *L. acidophilus*, *L. crispatus* and *L. helveticus* have demonstrated inhibitory activity against GI pathogens *in vitro*. Probiotics also influence the host and potentiate epithelial barrier integrity by inducing upregulation of tight junction proteins, increased production of mucin and defensin proteins as well as prevention of cellular apoptosis. Immunomodulation occurs by direct interaction of probiotics with resident immune system cells in the GI tract. It is known that probiotic lactobacilli influence the immune system through conserved molecular patterns of peptidoglycan, teichoic acids, S-layers and secreted proteins. The pro and anti-inflammatory responses to probiotics depends on the interaction of these molecular patterns with immune cell pattern recognition receptors, and are species, strain, dose and host specific. The mucosal immune system cells sample luminal antigens which skew cytokine profiles that dictate the overall immune response by T-cell differentiation and proliferation. In healthy individuals, activation of the mucosal immune system by probiotic cultures also enhances the systemic immune system and confers protection against infection. Feeding of *L. acidophilus* demonstrated decreased incidence, duration, and need for treatment of influenza like symptoms in children. In inflammatory mediated diseases such as IBS, the downregulation of the immune system by probiotics results in the alleviation of the

symptoms associated with the disease state. *L. acidophilus* has also demonstrated potential in the reduction of colitis symptoms in *in vivo* models. Additionally, probiotic lactobacilli have potential for the delivery of biotherapeutics. *L. gasseri* successfully delivered *Bacillus anthracis* vaccine and conferred a protective effect against a lethal challenge in a murine model. Human health and disease are complex states of homeostasis and morbidity, and the use of probiotic cultures in improving human health must be optimized for each.

Selection criteria for comparison of potential probiotic lactobacilli have been widely reported. The collective list of desired characteristics grows continuously, but no consensus has been reached about key characteristics. A list of selection criteria is shown in Table 6. The question marks identify proposed criteria that remain subject to debate as to their *in vivo* significance.

Table 6. Selection criteria for intestinal probiotics

Selection criteria
Species specific origin: human isolates for human probiotics
GRAS
Acid tolerant
Bile tolerance
Bile-salt hydrolase activity?
Adherence – cell type in vivo or in vitro
Antimicrobial
Immunogenic
Non-invasive
Non-pathogenic
Coaggregation?
Reduces cholesterol?
Survival in food and host GI tract
Fermentation compatible
Genetically amenable
Bioprocessing and storage stability

Conclusions

L. acidophilus type strains under consideration for use as probiotics are often expected to serve in two very contrasting roles: survival or fermentation in a food or delivery vehicle, followed by survival and *in vivo* activity during passage into the gastrointestinal tract. In this regard, a number of major challenges continue to face the production of *Lactobacillus* cultures targeted for use as probiotics. First, the stability of cultures in dried, frozen, or suspended states remains a critical issue and efforts to improve the stress tolerance of *L. acidophilus* type species is needed. Second, the potential impact of culture propagation and storage conditions on the *in vivo* conditioning of the probiotic must be considered. Third, understanding the genetic components and controls that direct probiotic properties will be the key to uncovering the *in vivo* activities and will ensure expression of vital characteristics during production, storage and, ultimately, in the gastrointestinal tract. Therefore, as molecular biology has now elucidated the taxonomic relationships of the species defined within the *L. acidophilus* complex, genetic information is now revealing the activities and interactions of these bacteria with the host, immune system and the microbiota of the gastrointestinal tract.

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Chapter II. Genomic and phenotypic evidence for probiotic influences of *Lactobacillus gasseri* on human health

Kurt Selle and Todd R. Klaenhammer

Abstract

Certain lactic acid bacteria have the capacity to occupy mucosal niches of humans, including the oral cavity and gastrointestinal tract. Among commensal lactic acid bacteria are the species within the acidophilus complex, which have proven to be a substantial reservoir for microorganisms with probiotic attributes. Specifically, *Lactobacillus gasseri* is an autochthonous microorganism which has been evaluated for probiotic activity based on the availability of genome sequence and species-specific adaptation to the gastrointestinal tract. *L. gasseri* displays potential probiotic applications based on safety, genomic and empirical analysis, as well as clinical trial data.

Introduction

Probiotics are "live microorganisms, which when administered in adequate amounts, confer a health benefit upon the host" (33). Probiotic microbes are associated with several health benefits related to the maintenance of gut homeostasis and the immune system, which have the potential for improving human health through prophylactic and therapeutic applications (16). Many of the well characterized probiotic strains are lactic acid bacteria (LAB), which are a phylogenetically diverse clade of Gram-positive eubacteria that ferment glucose with lactate as a primary metabolic end product. LAB are primarily adapted to nutrient rich environments, including plant and food ecosystems as well as the mammalian

mucosa. They are among the first bacteria to colonize the gastrointestinal tract (GIT) along with *Bifidobacterium*, although the latter is predominant in early colonization (101). The oral cavity is also considered a reservoir for intestinal bacteria and is a common mucosal niche for LAB (23). Probiotic bacteria are predominantly of the genera *Lactobacillus* and *Bifidobacterium*, with some notable exceptions (26). *Lactobacillus gasseri* is a prolific autochothonus microorganism that colonizes the GIT, oral cavity, and vagina in humans. *L. gasseri* is classified as a group B acidophilus complex microbe, and can be differentiated from group A by genetic determinants and the apparent absence of major surface-layer proteins (100, 15). The niche-related phenotypes involved in colonization of the human mucosa, including the mouth, gastrointestinal tract (GIT), and vagina exhibited by LAB such as *L. gasseri* may contribute to and potentiate probiotic activity.

Certain commercial probiotic cultures belonging to the acidophilus group, such as *Lactobacillus acidophilus*, a group A microbe with three S-layer proteins (43), have been used as probiotics for over 85 years. *L. acidophilus* NCFM is a widely consumed and well established probiotic microorganism, for which double-blinded randomized, placebo-controlled clinical trials have substantiated its safety and propensity to positively influence human health (79, 96, 102, 73). The genomic sequence of *L. acidophilus* NCFM has facilitated functional analysis of key genetic determinants that contribute to its survival and activity in the human GIT, which adds further credence to its status as a probiotic (3). Specifically, probiotic microorganisms must survive GIT passage to dominate the small intestine, where adhesion of probiotic microbes to the host epithelium facilitates differential

gene expression in intestinal epithelial cells and immunomodulation (13, 123). Microbe-host crosstalk by other established probiotic microorganisms has also been demonstrated to increase epithelial barrier integrity by up-regulation of tight junction proteins, eliciting increased defensin production by paneth cells and increased mucin production by goblet cells (81, 111, 112, 4, 58). The ability of probiotics to transiently dominate in the small intestine may also induce differential expression in the host microbiota contributing to GIT homeostasis, and may cause direct antagonism of enteric pathogens (21, 73).

For the application of new probiotic species, proper identification and considerable *in vitro*, *in vivo*, and clinical evidence must predicate their addition to foods for safe consumption by the greater population (106). Several criteria for the selection of probiotic microorganisms have been established by collaborations though the lactic acid bacteria industrial platform (LABIP), as outlined in Table 1. There is an increasing body of evidence that indicate *L. gasseri* has significant potential for probiotic application by fulfilling these criteria.

Table 1. Probiotic Selection Criteria

Human Origin
Non-pathogenic, non-toxigenic and non-invasive
Devoid of transmissible antibiotic resistance genes

Table 1 Continued

Resistance to technological processes
Demonstrated acid and bile tolerance
Adhesion to epithelial tissue
Transient persistence in the GIT
Produce antimicrobial substances
Antagonize pathogens and prevent infection
Modulate immune responses
Positively influence metabolic activity

Modified from Dunne et al., 1999

Safety

There are several criteria for the selection of probiotic microorganisms, the primary foci of which are safety and efficacy (38, 106). Proper identification of probiotic microorganisms is essential because of the strain-specific nature of the health benefits associated with probiotic cultures and the intrinsic safety issues associated with erroneous identification (108). Initially *L. gasseri* was indistinguishable from *L. acidophilus* by phenotypic and metabolic analysis and was not classified as a separate organism until after the disparity in characterizing lactate dehydrogenases and cell wall autolysins from *L.*

acidophilus (39, 40). The difficulty in distinguishing *L. acidophilus* and *L. gasseri* by cell morphology and biochemical means necessitated the development of practical hybridization techniques that could differentiate these two species (80, 100). *L. gasseri* was defined as a separate organism based on DNA-DNA hybridization techniques (76, 77). Since then, other nucleic acid based methods have been developed to distinguish among closely related members of the acidophilus complex, including comparison of the variable region (V1 and V2) sequences within the 16S rRNA gene and repetitive element-PCR (Rep-PCR) using primers specific for lactobacilli (41, 72). However, the utility of 16S rRNA sequences in distinguishing *L. gasseri* from *L. acidophilus* and *L. johnsonii* is limited due to the high sequence similarity of the 16S rRNA genes between the species. Instead, the specific design of primers for generating amplicons unique to *L. gasseri*, enabled by whole genome sequence analysis, offers a greater distinction from other acidophilus complex microbes (70).

Following proper identification of a potential probiotic microbe, the specific strain should be evaluated for the traits listed in Table 1, notably, antibiotic resistance profiles, safety, and absence of unsavory genes. Putative probiotic strains should be non-pathogenic, non-invasive, non-toxigenic and contain no transmissible antibiotic resistance genes. Toxicological assays and epidemiological tracking demonstrate that commercial probiotic microbes have a low propensity to cause septicemia or adverse side effects when consumed in large doses in healthy individuals (110, 30, 105). The widespread colonization of mucosal niches by *L. gasseri* indicates its prevalence as a commensal in healthy individuals and an absence of any association with a health detriment (49, 28, 68, 25, 48). In fact, *L. gasseri* is

commonly found in infants and is one of the predominant species involved in early colonization of the GIT and is persistent throughout adulthood (125). The colonization of the vagina by *L. gasseri* and its early colonization of the infant GIT seem to indicate a potential route of transfer to neonates (17, 32). The genomic sequence of *L. gasseri* ATCC 33323 revealed the absence of transmissible antibiotic resistance genes and other unsavory genes, beyond niche related genes that facilitate adherence or toxin and drug efflux systems (7). Despite this, it cannot be assumed that all strains of *L. gasseri* are devoid of transmissible antibiotic resistance genes, necessitating the evaluation of antibiotic resistance assays and genome sequencing of each putative probiotic strain prior to commercialization.

With regard to metabolic activity, one study evaluated the toxicity of *L. gasseri* CECT5714 when orally administered to Balb/c mice at 10^{10} cfu/day for 30 days. Bacterial translocation and mucosal barrier integrity were assayed, as well as serum parameters for stress. The extended treatment did not increase bacterial translocation and did not significantly change any biochemical or hematological parameters, including degradation of mucin (75). The strain exhibited no significant resistance to clinically relevant antibiotics. The results from the toxicological mouse assays with *L. gasseri* were corroborated with feeding studies in humans with the same dose but a duration of five days (50). Markers for gut homeostasis, hepatic, and kidney function were not significantly affected by the treatment, nor were blood parameters.

Despite the established safety of this microorganism in healthy humans it is pertinent to note that probiotic microbes used for therapeutic purposes may be administered to individuals with compromised epithelial barrier function and immune systems, as in the elderly, neonates and those predisposed to autoimmune diseases (108). These individuals may be more susceptible to septicemia or adverse side effects of probiotic administration. It is of interest, however, that even in individuals with compromised barrier integrity or immune system function, therapeutic administrations of probiotic strains did not increase translocation or incidence of septicemia (79, 108). Due to the inherent complexity of human health and the ecosystem of the GIT it is difficult to predict the safety of a particular probiotic culture, especially intended for compromised individuals. Moreover, studies indicating clinical safety cannot be extrapolated to closely related strains or across different host systems.

Genomic basis for *in vitro* survival and activity

Probiotic cultures must remain viable in both the disparate ecosystems of the delivery vehicle during shelf life and also during transit through the GIT to effectively retain probiotic activity. Growth conditions, processing parameters, vehicle characteristics, and the stress response of probiotic microbes dictate viability in these ecosystems. Survival and transient colonization of probiotic microbes in the GIT depends on acid tolerance, bile salt resistance, adhesion capacity, metabolic capacity and several host dependent factors. In this regard, it is

necessary to highlight the role of genomic sequencing in the selection process for probiotic microbes. Genome sequences are indispensible for taxonomic identification, analysis of safety, and evaluation of efficacy of potential probiotic candidates. Analysis of the genome offers insights into the presence of virulence and antibiotic resistance genes, as well as genes and regulatory networks involved in probiotic mechanisms. However, the influence of probiotic microorganisms on human health is also dependent on activity in the host, so expression analysis and *in vitro* and *in vivo* testing of key genotypes are necessary to gauge the relevance of genomic features. The genomic sequence of *L. gasseri* ATCC 33323 reveals a mechanistic basis for its survival in GI passage as well as potential probiotic genotypes.

Acid and bile exposure during GI passage constitute significant hurdles for probiotic microorganisms to survive and retain activity in the GIT (109). LAB encode several acid tolerance mechanisms including F_1F_0 ATPases as well as ornithine decarboxylases to maintain stable intracellular pH in acidic environments. *L. gasseri* ATCC 33323 encodes GroEL, GroES, DnaK, DnaJ, and Clp protease chaperones to protect against intracellular aggregation of proteins during stress (7). *L. gasseri* also encodes putative stress response proteins with homology to CbsD and UspA, which have yet to be phenotypically analyzed for their role in the general stress response. Bile salt hydrolases and bile salt transporters have been shown to confer bile tolerance to commensal microbes in the GIT and also represent important attributes for probiotic survival (9, 10). The genome of *L. gasseri* contains an operon containing two bile salt transporters and a bile salt hydrolase with considerable homology to the corresponding proteins in *L. acidophilus* NCFM and *L.*

johnsonii NCC533. *L. gasseri* also contains a bile salt hydrolase homologue that specifically hydrolyzes bile salts conjugated to taurine and an operon similar to one in *L. acidophilus* that is demonstrated to influence bile tolerance (86, 99, 7). Interestingly, the *in vitro* bile tolerance of *L. gasseri* and *L. acidophilu*s was pH-dependent, with *L. gasseri* demonstrating an order of magnitude higher bile tolerance at pH 6 than *L. acidophilus.* Analysis of several *L. gasseri* strains for survival in simulated gastric juice yielded strain-specific viability, but in general *L. gasseri* strains were acid and bile tolerant (5, 116, 34, 7).

Adhesion and possible penetration of the mucus layer are important probiotic attributes that may contribute to transient colonization of the GIT and competitive exclusion of pathogens. The capacity to penetrate the mucus layer may also promote direct interaction with intestinal epithelial cells and resident immune system cells of the lamina propria (107). Adhesion to mucus, the extracellular matrix and intestinal epithelial cells is mediated by several factors on the cell surface of Gram-positive microorganisms, including lipoteichoic acid, mucus-binding proteins, fibronectin-binding proteins and surface-layer (S-layer) proteins (19, 44). Although they lack genes encoding S-layer proteins, *L. gasseri* and *L. johnsonii* encode proteins annotated as “aggregation promoting factors” (Apf), which share several features to S-layer proteins of *L. acidophilus*, alluding to the possibility of shared functionalities (124). Specifically, S-layer proteins of lactobacilli have a high predicted pI, low sulfur amino acid content and high hydrophobic amino acid content, similarly observed in the Apf proteins of *L. gasseri.* The genetic structure of Apf proteins is conserved across multiple strains of *L. gasseri*, encoded as two tandem genes spaced by a short intergenic

region, characteristic of the arrangement of S-layer genes in lactobacilli. The S-layer proteins of *L. acidophilus* NCFM are implicated in modulation of the immune system and adhesion to Caco-2 cells, possibly suggesting similar roles for the Apf proteins in *L. gasseri* (43, 71). Additionally, *L. gasseri* ATCC 33323 contains 14 mucus-binding proteins, six of which exhibit a signal peptide and four of which are predicted to be covalently linked to the membrane via sortase A cleavage of the LPTXG motif (7). The proteins contain multiple mucus-binding domains that show homology with demonstrated functional mucus-binding proteins in *L. reuteri* 1063 and *L. acidophilus* NCFM. *L. gasseri* also contains a putative fibronectin-binding protein, which may mediate adhesion to the extracellular matrix of mammalian cells. The protein has homology to similar genes in *L. johnsonii* NCC 533 and *L. acidophilus* NCFM, although their roles in adhesion have not been confirmed. The adhesion of multiple *L. gasseri* strains to Caco-2 intestinal cells was evaluated and indicated strain-specific adherence capacity, but was generally less than *L. acidophilus* NCFM (7). In comparison to other strains of lactobacilli, *L. gasseri* ATCC 33323 exhibited slightly higher adhesion capacity to Caco-2, HT-29 and LS-174T cell lines, but less than that of *L. reuteri* strains. The relevance of each of the individual mucus-binding and fibronectin-binding proteins to adhesion of *L. gasseri* ATCC 33323 has yet to be investigated, *in vitro* or *in vivo*.

The broad metabolic capabilities observed in *L. gasseri* ATCC 33323 may facilitate transient colonization of the upper GIT. However, while encoding 20 various phosphoenolpyruvate-dependent phosphotransferase systems (PTS) for sugar uptake and the capability of fermenting several monosaccharides, *L. gasseri* ATCC 33323 does not ferment

five-carbon sugars and does not encode a β-galactosidase (7). Instead, it encodes two PTS and four putative phospho-β-galactosidases for the metabolism of lactose, while some strains (ranging from11-89%) of *L. gasseri* do not ferment the disaccharide (45). Interestingly, even though *L. gasseri* is a prevalent microorganism in early gut colonization, ATCC 33323 is unable to ferment breast milk oligosaccharides or fructo-oligosaccharides, unlike other closely related lactobacilli (126).

Antimicrobial activity and bacteriocin production

Many LAB demonstrate antimicrobial activity towards a broad range of other bacteria by means of producing several antagonistic compounds, including organic acids, hydrogen peroxide and bacteriocins (Figure 1). Bacteriocins are classified as proteinaceous antimicrobial compounds that kill closely related microbes. In Gram-positive bacteria, a select few bacteriocins (e.g. nisin, pediocin, and lacticin) have a broad spectrum of activity against Gram-positive bacteria in general, including pathogens (29, 103, 87, 1). The classification system categorizes bacteriocins into four classes (I) lantibiotics, (II) small heat-stable peptides not containing lanthionine residues, (III) large heat-labile proteins and (IV) peptides complexed with carbohydrate or lipid moieties (69). The acidophilus complex microorganisms are well documented as bacteriocin producers, and many of the bacteriocins exert activity specifically toward closely-related species and some enteric pathogens. *L. gasseri* has been reported to produce a number of bacteriocins, with the most well

characterized being gassericin A from *L. gasseri* LA39, isolated from infant feces (119). Gassericin A is a small cyclic peptide with 74% of its amino acids being hydrophobic (59, 60). It is heat stable and resistant to degradation by proteases, as is common for most class II bacteriocins, due to the compact secondary structure mainly consisting of α-helices (59, 61, 83). The bacteriocin production and immunity genes are encoded by seven *gaa* genes in the 33 kb conjugative plasmid pLgLA39, including structural genes for putative transport and immunity (51). Gassericin A has similarities with acidocin B from *L. acidophilus* M46 and reutericin 6 from *L reuteri* LA6, with homologies at 98% and 100%, respectively. The high sequence similarities of the conjugative plasmids pLgLA39 and pLrLA6 encoding the bacteriocins gassericin A and reutericin 6, respectively, between the two species indicate that the bacteriocin genes likely were disseminated by horizontal gene transfer. This is corroborated by the fact that microbial isolates producing gassericin A and reutericin 6 both originated from the same infant (52). The ability of the pLgLA39 plasmid to conjugate between closely-related strains was demonstrated and the plasmid conferred both bacteriocin synthesis and immunity in the transconjugant. The *in vitro* activity of *L. gasseri* LA39 was demonstrated against Gram-positive pathogens, namely *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* in a strain-specific manner. Reutericin 6 was observed to have a narrower spectrum which was thought to be due to the difference of one D-alanine amino acid, resulting in disparate secondary structure and function (52, 63), but this hypothesis was later disproved when the structures were found to be identical (5). The *in vivo* antagonism of enteric pathogens by gassericin A has yet to be established but would be

prudent to investigate, as bacteriocins of lactobacilli have been demonstrated to confer protection to infection in *vivo* (21).

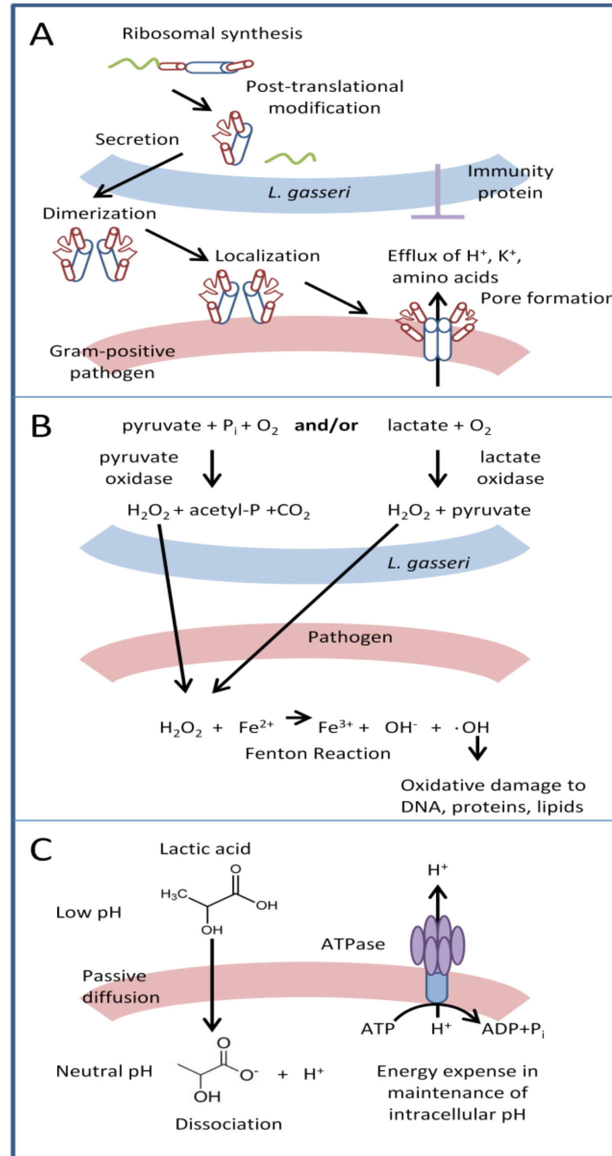

Figure 1. Potential mechanisms of antagonism of pathogens by *L. gasseri* through production of antimicrobial metabolites.

Figure 1 depicts the synthesis and potential mode of action of circular bacteriocin gassericin A. Bacteriocins are ribosomally synthesized and often undergo post-translational processing such as hydrolysis of leader peptides. In class II bacteriocins, hydrolysis occurs at a conserved gly-gly motif that is absent in circular bacteriocins, which are predicted to have a different processing site. Secretion of bacteriocins is achieved using a dedicated ABC transporter and circularization occurs enzymatically. The host cell synthesizes an immunity protein containing transmembrane domains that is predicted to localize cationic residues on the cell surface and sterically inhibit deposition of the bacteriocin on the host cell membrane, preventing permeabilzation of the membrane of the producer cell. Circular bacteriocins are thought to dimerize and localize on the cell membrane of Gram-positive pathogens, where they undergo conformational changes and integrate hydrophobic α-helices into the cell membrane, resulting in pore formation and cell death by dissipation of the proton motive force (PMF), as well as efflux of potassium and amino acids. Activity of bacteriocins is limited to Gram positive bacteria due to the exclusion barrier of the outer membrane in Gram-negative microbes. Two potential pathways were identified in the *L. gasseri* ATCC33323 genome for hydrogen peroxide production. Both pyruvate oxidase and lactate oxidase require oxygen and the metabolic end products of glycolysis for which they are named. Production of hydrogen peroxide by lactobacilli is hypothesized to be a non-specific antimicrobial, but has been demonstrated to contribute to killing of pathogens *in vitro* with potential implications for urogenital health. Susceptibility of microbes varies largely among organisms, among both catalase-positive and catalase-negative targets. In some cases,

hydrogen peroxide alone is not sufficient for killing of target organisms but cumulative effects of antimicrobials produced by lactobacilli augment its lethality. The mechanisms for cell death by hydrogen peroxide is largely facilitated by passive diffusion into the cell and reaction with Fe^{2+} by the Fenton reaction to produce highly reactive hydroxyl radicals, which cause DNA damage, denature proteins and disrupt the cell membrane. The antimicrobial activity of organic acids is largely dependent on their respective pKa and the pH o the environment, since passive diffusion into the cell is achieved only by the protonated acid. Once in the neutral pH of the cytoplasm, organic acids deprotonate and contribute to intracellular acidification. The cell must expend energy to drive proton efflux by ATPase to maintain a neutral intracellular pH.

L. gasseri LF221, previously identified as *L. acidophilus,* encodes two chromosomally located bacteriocin sequences for acidocin A and acidocin B (82). Biochemical analysis indicated that acidocin A and B represent class II bacteriocins, being small, hydrophobic and heat stable. The genetic structure of acidocin A indicates a putative operon containing three open reading frames encoding the structural genes, the putative immunity protein and a putative secondary component for the active mature acidocin A. The open reading frames for acidocin B share moderate homology with the functional two component bacteriocin lactacin F from *L. johnsonii*, indicating that acidocin A and acidocin B in *L. gasseri* LF221 are independent on a mechanistic basis, contributing to the broad

spectrum of activity from LF221. *L. gasseri* LF221 inhibited *Listeria innocua*, *S. aureus*, and several species of *Clostridium*, demonstrating the potential to antagonize pathogenic as well as spoilage microorganisms (12). Interestingly, gassericin T from *L. gasseri* SBT2055 has high homology with acidocin B from *L. gasseri* LF221, with 100% sequence identity to the structural and immunity genes. However, their differential activity spectrum suggest post-translational modifications of the bacteriocin peptides that impact host sensitivity (61, 60). Another bacteriocin characterized from *L. gasseri* is gassericin KT7 from an infant isolate *L. gasseri* KT (133). Gassericin KT7 is a small, heat stable peptide sensitive to proteases that has demonstrated bactericidal activity against a number of Gram-positive foodborne pathogens, including *B. cereus*, *L. monocyotogenes, Clostridium botulinum Clostridium perfringens*, and *S. aureus*, but not against Gram-negative bacteria. The activity of gassericin KT7 was stable across a broad pH range (2.5 - 9.0) and 59% of the amino acids were hydrophobic. Other than sharing some biochemical characteristics with class II bacteriocins, it is unknown how this bacteriocin relates to others that have been classified previously on nucleotide/amino acid sequence alone. These studies highlight the potential of bacteria within the acidophilus-complex to provide a source of novel bacteriocins with unique activity and applications.

The specific role of bacteriocins in probiotic-mediated antagonism of pathogens *in vivo* was recently demonstrated in a mouse model challenged with *L. monocytogenes*. The study showed that the protective effect conferred by *L. salivarius* UCC118 was due to the bacteriocin salivaricin by preventing infection and mortality. A bacteriocin-negative mutant

deficient in salivaricin production failed to confer protection (21). In light of these results, it remains essential to investigate the potential of novel bacteriocins in preventing infection from Gram-positive enteric pathogens *in vivo*. Bacteriocins isolated from *L. gasseri* may also be applied in food preservation, as the high heat stability, extensive pH range, and broad-spectrum activity constitute suitable longevity and application against spoilage microorganisms. Additionally, studies identifying novel bacteriocins from microorganisms within the acidophilus group are an indication of the inherent strain-specific disparities and phenotypic diversity of the group, and necessitates a thorough investigation of the activity of each novel bacteriocin.

Evidence for persistence in the GIT

Survival and activity in the GIT are requisites for probiotic activity and accordingly, it is necessary to establish the viability of potential probiotic candidates empirically. Genomic analysis of *L. gasseri* reveals niche-related genes that may contribute to survival during GI transit, *in vivo*, which have been substantiated by studies designed to detect and quantify levels of *L. gasseri* following consumption and GIT passage. In some cases, detection of *L. gasseri* post-consumption in humans was associated with a positive influence on certain health parameters of the host, indicating a potential role in maintenance of host homeostasis.

In one study, individuals fed with *L. gasseri* OLL2716 had detectable PCR amplification of DNA specific to *L. gasseri* OLL2716 from the mucosal layer 1 hour after consumption, indicating that this strain penetrates the human mucosa (35). In a separate investigation, two groups of elderly individuals, one healthy and one with atrophic gastritis were fed 10^{10} cfu of *L. gasseri* ADH twice daily for 3 days (98). *L. gasseri* ADH was isolated in samples from 11 of 12 individuals collected from the stomach, small intestine, and feces. In the healthy individuals, *L. gasseri* ADH was not isolated from the small intestine or stomach, but was isolated from individuals with atrophic gastritis. In both treatment groups *L. gasseri* ADH was isolated from the feces at levels of 10^7 cfu/g. In another study, *L. gasseri* SBT2055 was administered at levels of 10^9, 10^{10} and10^{11} cfu to individuals in treatment groups for each dosage (36). Following consumption, levels of the bacterium in the feces of each individual were recorded over time. The results indicated dose-dependent and host-dependent levels of detection in each of the treatment groups. Twelve of the 26 individuals receiving the low and middle dosage had detectable *L. gasseri* SBT2055, achieving maximum log values of 10^4 and 10^6 cfu/g in the feces, respectively. The high dosage group displayed a maximum of 10^7 cfu/g in the feces. Consumption of *L. gasseri* SBT2055 at the high dosage was associated with a decrease in the putrefactive marker p-cresol in the feces and a decrease in the resident *Staphylococcus* population of the microbiota. In a separate study, consumption of *L. gasseri* SBT0255 was accompanied by an increase in fecal *Lactobacillus* counts and detection of SBT0255 by PCR with specific primers, up to two weeks following feeding (117). Taken together, these studies highlight

the ability of *L. gasseri* to transiently colonize in the GIT of humans, although comparison among strains is impossible due to the different feeding regimens, experimental design, and detection methods.

Maintenance of gut homeostasis

Overall gut health is a complex spectrum of homeostasis, regulation, health and morbidity, which varies greatly on an individual basis due to genetic and environmental factors. Probiotic microorganisms have been suggested to contribute to maintenance of gut homeostasis in a multifaceted manner. Certain probiotic bacteria have the capacity to decrease GI transit time, regulate host metabolism, and improve epithelial barrier properties (93, 113). One example of how gut microorganisms have been suggested to regulate host metabolism is through the production of short chain fatty acids (6). Consumption of *L. gasseri* CECT5714 and *L. coryniformis* CECT5711 daily for 4 weeks was implicated in increasing butyrate concentration in the human intestine. The difference between the yogurt control group and the probiotic treatment group occurred at 2 weeks into the treatment but was not observed at 4 weeks, indicating that a time dependent response was responsible for the increase in butyrate (94). The consistency and volume of feces as well as intestinal function were reported to significantly improve with the treatment. In a separate study, gut health parameters were compared between two groups, one that received conventional yogurt and one that received a probiotic yogurt containing the above strains (74). Consumption of

the probiotic yogurt increased IgA secretion in the mucosal layer, was implicated in reducing fecal cytotoxicity, and decreased *Salmonella choleraesuis* adhesion to intestinal mucins in the feces. A randomized, placebo controlled double-blind multicenter study involving 169 members compared the efficacy of using a combination of *L. gasseri* and *Bifidobacterium longum* versus *E. faecium* in mitigating acute diarrhea. The combination treatment decreased the duration and severity of acute self-limiting diarrhea, suggesting that consumption of *L. gasseri* contributed to maintenance of gut homeostasis (84). Combined, these studies substantiate the potential probiotic roles of *L. gasseri, in vivo*.

L. gasseri may potentially play a role in maintaining serum and kidney homeostasis through the metabolic capacity to degrade oxalate in the GIT (Figure 2). Oxalate is a toxic component in certain foods that is not degraded by host mechanisms, but rather by oxalate-degrading bacteria in the GIT. Excessive levels of oxalate can result in kidney failure. The genome of *L. gasseri* ATCC 33323 encodes enzymes involved in oxalate catabolism, specifically formyl-coenzyme A transferase (*frc)* and oxalyl-coenzyme A decarboxylase *(oxc)* (7). Expression analysis indicated that both *frc* and *oxc* were co-expressed in an operon during oxalate degradation, *in vitro*. While oxalate degradation varies by strain (7), the results illustrated that *L. gasseri* might be used to prophylactically or therapeutically lower oxalic acid toxicity or renal complications. *L. gasseri* Gasser AM63 demonstrated the ability to persist and degrade oxalate in a continuous culture simulator of the human colon microbiota without changing the overall population (78).

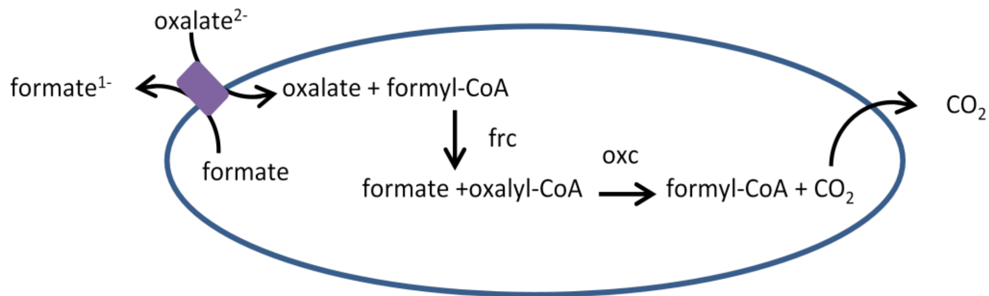

Figure 2. Putative pathway by which *L. gasseri* ATCC 33323 degrades oxalate. Oxalate is imported into the cell via a formate-oxalate antiporter which may contribute to establishing a PMF in some oxalate degraders. Once internalized, oxalate is activated with Coenzyme A through the action of formyl transferase, resulting in the release of formate. Degradation of oxalate occurs via enzymatic activity of oxalate decarboxylase, the products of which are CO_2 and formyl-CoA. *In vitro* expression of *frc* and *oxc* are pH dependent (8).

Probiotic yogurt containing *L. gasseri* OLL2716 was demonstrated to decrease the ulcer index in mm^2 of acute HCl-induced gastric ulcers in rats, whereas non-fermented milk was shown to have no effect at the same dose. The inhibitory effect of the probiotic yogurt on ulcer development was abrogated, but not abolished with pre-treatment of the rats with indomethacin, indicating a possible role for prostaglandin E_2. Prostaglandin E_2 causes increased secretion of mucus and decreased secretion of gastric acid, which may contribute to abatement of ulcers. Prostaglandin E_2 expression was upregulated in the treatment group, further suggesting the capacity of OLL2716 to promote healing of ulcers in this manner (120). Live *L gasseri* OLL2716 was similarly shown to accelerate the healing of acetic

acid-induced ulcers in rats (121). Interestingly, both live and irradiated *L. gasseri* cells inhibited development of HCl-induced lesions and increased expression of prostaglandin E_2. These studies highlight a potential role for *L. gasseri* in maintaining gastric homeostasis and promoting healing of gastric lesions.

Immune system regulation and alleviation of allergic response

There has been a recent increase in research activity surrounding the mechanisms of immunomodulation by probiotic microbes. This is justified given that the ability of probiotic microbes to regulate both systemic and mucosal immunity provides a unique opportunity to mediate afflictions related to both systems (Figure 3). Probiotic microbes modulate the immune system through the interaction of microbe-associated molecular patterns (MAMPs) with PRRs on APCs. The MAMPs of probiotic microbes that govern the immune response include lipoteichoic acids, peptidoglycan, S-layer proteins, and nucleic acids (16). The PRRs that probiotic microbes interact with include the dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), nucleotide-binding oligomerization domain-containing protein 2 (NOD2), toll-like receptors (TLR)-2 and TLR-6 (16). The resulting cytokine profiles elicited from APCs govern the proliferation and differentiation of effector T-cells, resulting in both the ephemeral and perennial immune responses to the bacterium. Lactobacilli commonly induce T-cell polarization towards a T-helper (Th)-1 response by eliciting IFN-γ and IL-12 secretion by APCs during antigen presentation to $CD4^+$ T-cells

(89, 90). *L. gasseri* ATCC 33323 has been well characterized in its ability to interact with specific TLRs on the macrophage cell line HEK-293, as well as the cytokine profiles elicited when co-incubated with myeloid differentiated DCs (115). *L. gasseri* ATCC 33323 was reported to have preferentially interact with a TLR-2/6 heterodimer, with less activation of TLR-2 alone. Interestingly, TLR-2 interacts with a diverse array of MAMPs, including lipoteichoic acids, glycolipids and peptidoglycan, suggesting these components of *L. gasseri* may be active in immune signaling (132). The cytokine profile elicited from DCs when co-incubated with *L. gasseri* ATCC 33323 indicated a pro-inflammatory response to the bacteria, as increased expression of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ IL-6, IL-10 and granulocyte-macrophage colony-stimulating factor (GM-CSF) occurred. Similar results were obtained upon co-incubation of DCs with *L. acidophilus* NCFM, indicating that the immune responses to both microorganisms were largely dictated by conserved MAMPs of lactobacilli (115). *L. gasseri* TMC0356, previously mis-identified as *L. acidophilus* TMC0356, was exposed to a murine macrophage cell line and the cytokines elicited were quantified by ELISA (91). *L. gasseri* TMC0356 elicited high levels of IL-10, IL-6, IL-12, and TNF-α, in accordance with the observation of cytokines elicited from DCs when exposed to *L. gasseri* ATCC 33323 (115). In another study, heat-killed *L. gasseri* TMC0356 was demonstrated to increase the pulmonary mRNA expression of IFN-α, IFN-β and IL-2 in senescence-accelerated mice (67). These cytokines may contribute to the proliferation and differentiation of natural killer (NK) cells, which was observed in the analysis of splenic NK cells from mice fed *L. gasseri* TMC0356. In a separate study, heat-

killed *L. gasseri* OLL2809 cells had the capacity to alter T-cell differentiation by suppressing $CD4^+$ T-cells in a MyD88 dependent manner. Purified microbial RNA also suppressed $CD4^+$ T-cells, suggesting that nucleic acids of *L. gasseri* may play a role in immunomodulation (131).

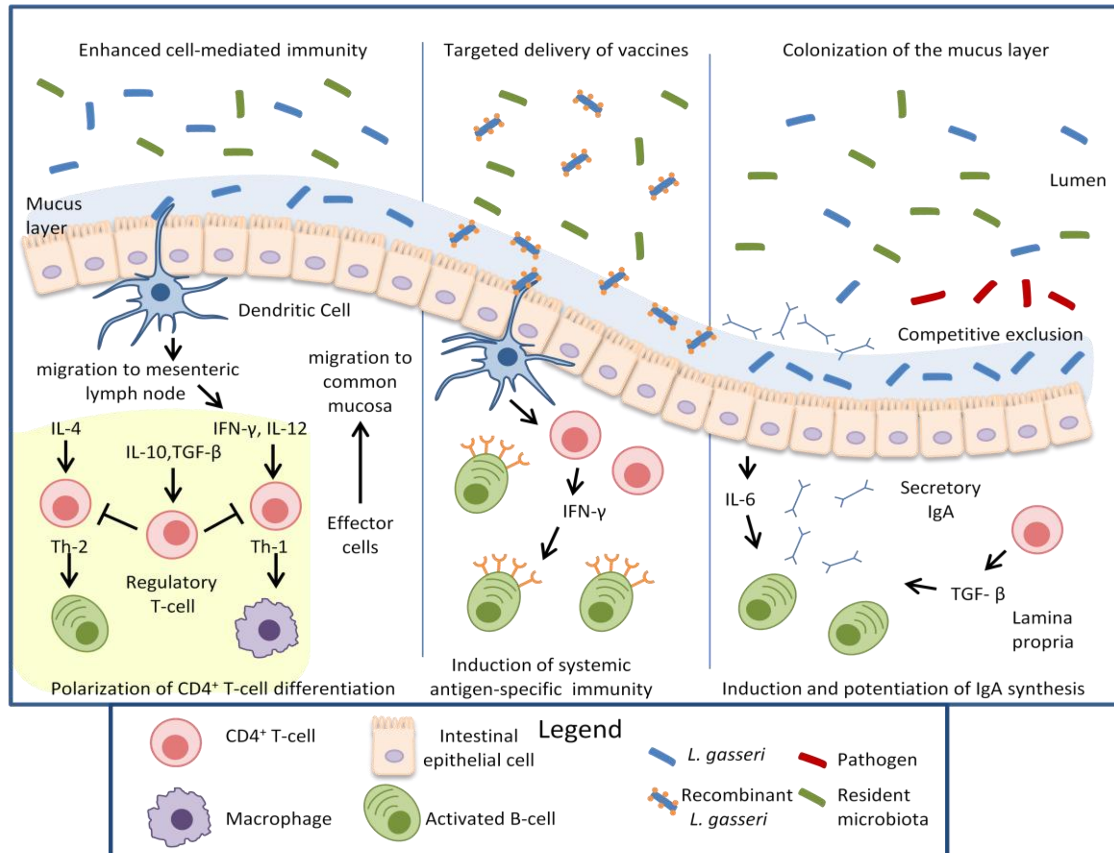

Figure 3. Activity of *L. gasseri* in the GIT.

Figure 3 depicts attributes and activity of various strains of *L. gasseri* discussed throughout the review, and accordingly, is not comprehensive for probiotic action. The complete mechanisms by which probiotic microbes elicit health benefits remain to be fully elucidated,

but potential routes for *L. gasseri* to influence the GIT are depicted. Probiotic microbes modulate the mucosal and systemic immune systems through the interaction of microbial surface components with pattern recognition receptors (PRRs) on antigen presenting cells (APCs). Cytokines secreted by the APCs and intestinal epithelial cells activate resident lymphocytes and macrophages in the lamina propria, enhancing the mucosal immune response and potentiating immunoglobulin A (IgA) synthesis. Furthermore, APCs migrate to mesenteric lymph nodes and induce T-cell differentiation through presentation of microbial antigens and secretion of cytokines. The capacity to modulate the immune system also contributes to the innate adjuvanticity of *L. gasseri* in the delivery of vaccines. *L. gasseri* may also contribute to homeostasis through the production of compounds antagonistic towards pathogens, potentially inhibiting their activity in the GIT.

The immune response of murine Peyer's patch (PP) cells to *L. gasseri* TMC0356 and *L. rhamnosus* GG was compared *in vitro* and *in vivo* (47). Interestingly, when co-incubated with cells from the PP *in vitro, L. gasseri* TMC0356 elicited higher levels of IL-6, IL-12, IFN-γ, and IgA than did *L. rhamnosus* GG. In contrast, the immune response of PP cells to the intragastrically administered probiotic strains indicated that *L. rhamnosus* GG elicited higher levels of IFN-γ, IL-6, and IgA. The disparate results between the *in vitro* and *in vivo* comparison of these strains may arise from the different capacity of each to adhere to the epithelium and interact with PP cells, as well as the inherent strain-specific expression of immunomodulatory MAMPs that may manifest in differential immune responses *in vivo*. In humans, a combination of *L. gasseri* CECT5714 and *L. coryniformis* CECT5711 was

demonstrated to stimulate NK cells and also increase IgA levels secreted, but this effect was not seen for *L. gasseri* ATCC 33323 or *L. gasseri* TMC0356 (95, 115). Taken together, these studies highlight the potential of both live and heat-killed *L. gasseri* to influence the host immune system. The differential experimental methodologies and strain-specific nature of immunomodulation make strain to strain comparison challenging. Nonetheless, disparate *in vitro* and *in vivo* results signify the necessity of thorough investigation of immunomodulation by probiotic microbes *in vivo* to confirm the validity and relevance of results obtained from cell culture.

The factors involved in the development of allergic response are not well understood, but there is evidence suggesting that probiotic microorganisms may confer a protective effect against the immunological imbalances contributing to this condition (56, 57). Notably, neonates genetically predisposed to hypersensitivity may fail to undergo environmentally driven Th-1 responses mediated by the microbiota or exposure to pathogens. The result of which is a predominant Th-2 response that promotes IgE expression, as well as the activation of eosinophils and mast cells active in the allergic response (97). It is also thought that allergy is exacerbated by decreased or ineffective modulation of the immune system by regulatory T-cells, which normally act to suppress Th-1 and Th-2 responses alike (37). Consequently, the potential mechanisms by which probiotics may prophylactically and therapeutically ameliorate the development of allergy is multifaceted. The propensity of probiotic microbes to increase epithelial barrier integrity and potentiate the expression of IgA may decrease epithelial permeability to antigens. Moreover, the ability of probiotic microbes

to modulate the immune system and skew towards a Th-1 cellular response holds potential for abrogating the development of allergic responses in hypersensitive individuals, since the expression of Th-1 associated cytokines counter-regulates Th-2 responses. Furthermore, probiotic microbes that induce expression of IL-10 and TGF-β from regulatory T-cells may contribute to downregulation of inflammation in established hypersensitivity. Lastly, recombinant DNA technologies may advance oral immunotherapy strategies by targeted expression of allergens with designed immunomodulatory surface components to induce tolerance or skew towards a Th-1 response (130). It is reported that allergic individuals may have abnormal GI microbiota compositions, notably some low in lactobacilli albeit that any causal relationship in this association is unclear (11, 114, 54).

L. gasseri has been evaluated in eliciting immunological changes both in allergen sensitized models and hypersensitive individuals. In one study, peripheral blood mononuclear cells (PBMCs) were purified from allergic and healthy individuals, co-incubated with allergens and the cytokine profiles analyzed. Pre-incubation of the PBMCs with *L. gasseri* PA16/8 diminished Th-2 response associated cytokines IL-4 and IL-5, and increased the Th-1 associated IFN-γ (42). Interestingly, the effects were greater in the PBMCs from healthy rather than allergic individuals, suggesting that the predisposed immunological state of humans may influence the degree to which probiotic microbes can modulate their responses. The ability of *L. gasseri* TMC0356 combined with *L. rhamnosus* GG to alleviate perennial allergic rhinitis was evaluated in ovalbumin sensitized guinea pig and Norway rat models. The administration of the probiotic combination abrogated the

increase of vascular permeability caused by local inflammation in both rats and guinea pigs. In the guinea pig model, a non-statistically significant decrease in leukocytes in the nasal cavity was observed and, in both models, a non-statistically significant decrease of serum IgE was observed (65, 66). Individuals with high levels of serum IgE and perennial allergic rhinitis were administered milk fermented with *L. gasseri* TMC0356 daily for a duration of four weeks (92). Following the treatment, the individuals exhibited decreased total serum IgE levels and decreased antigen specific serum IgE compared to the baseline control. An increase of Th-1 associated PBMCs was also associated with the treatment. A double-blind randomized clinical trial compared the immunological response of allergic children to conventional yogurt with a probiotic yogurt containing *L. gasseri* CECT5714 and *L. coryniformis* CECT5711 (85). The treatment group consumed 200g of a probiotic yogurt with 10^6 cfu/g of each strain daily for four months and immunological parameters were observed. The probiotic treatment group exhibited a decrease in serum IgE and an increase in IgA when compared to the conventional yogurt group. Furthermore, the probiotic yogurt group experienced an increase in $CD4^+$ $CD25^+$ T-regulatory cells and NK cells, although basophils and eosinophils were unaffected.

L. gasseri has been insufficiently studied in the avenue of preventing allergic diseases, but nevertheless shows potential in reducing Th-2 and IgE related immunological responses, possibly through promotion and stimulation of the Th-1 associated cytokines and cell types. These studies further contribute to the basis of probiotic-mediated regulation of

the mucosal and systemic immune systems, although effective application through clinical therapy requires considerable work to be implemented.

Inhibition of *Helicobacter pylori*

Helicobacter pylori is a common gastric pathogen with infection resulting in acute mucosal damage and the possible clinical manifestation of peptic ulcers, gastritis, and gastric cancer due to chronic infection. Current treatments include administration of antibiotics, which often result in the alleviation of symptoms, but not necessarily eradication of *H. pylori* in the host. Consequently, remission can occur and the subsequent infection of *H. pylori* can be related to the antibiotic resistance of the infectious strain. *L. gasseri* OLL2716 demonstrated a direct inhibitory effect on the *in vitro* and *in vivo* growth and colonization of several strains of *H. pylori*, consisting of clarithromycin-sensitive and resistant subtypes of the pathogen (122). Interestingly, gnotobiotic mouse studies have further indicated the potential of lactobacilli to eradicate *H. pylori in vivo*, or to prevent infection entirely when colonized with lactobacilli (2, 55). IL-8 has been implicated in playing a major role in the development of mucosal inflammation and injury in *H. pylori* infections as it is a chemotactic factor for neutrophils. Chronic expression of IL-8 due to recurrent *H. pylori* infection may exacerbate mucosal inflammation and tissue damage (22). In this regard, co-incubation of *L. gasseri* OLL2716 with a MK45 cell line infected with *H. pylori* inhibited expression of IL-8 when compared with the control (122). Further investigation of the

ability of *L. gasseri* OLL2716 to inhibit IL-8 yielded similar results, but indicated that the probiotic strain neither inhibited adhesion of *H. pylori* to infected cells nor interfered with TNF-α induced IL-8 expression (118). This suggests the decrease in *H. pylori*-induced IL-8 expression was mediated by a mechanism independent of adhesion of the pathogen. The study also reported that the decrease in IL-8 secretion observed in the cell culture line occurred similarly in humans infected with *H. pylori.*

The ability of *L. gasseri* OLL2716 to inhibit *H. pylori* infection in cell culture lines was also observed in human clinical trials. A trial with 31 participants infected with *H. pylori* were fed yogurt containing *L. gasseri* OLL2716 at a level of 10^7 cfu/g daily for 16 weeks, and the urea breath test was performed as a measure of infection. The probiotic yogurt decreased markers for *H. pylori* infection two weeks after consumption but not during the treatment. The probiotic yogurt treatment group exhibited decreased *H. pylori* in antral biopsies as well as the infection marker of serum pepsinogen levels (104). Another study evaluated the efficacy of *L. gasseri* in both prevention and treatment of *H. pylori* infection in children (14). *L. gasseri* OLL2716 was administered to 82 asymptomatic *H. pylori* infected children for one year, who were subsequently assayed for a *H. pylori* stool antigen to monitor infection. In the treatment group, 42% of the participants had remission six months after cessation of treatment, but twenty-four of the participants were free from infection after the one year. The consumption of *L. gasseri* as a preventative measure was not associated with a decrease in the incidence of *H. pylori* infection. Data from *in vitro* assays and animal models suggest a potential role for mitigation and treatment of *H. pylori* infection by

administration of *L. gasseri* OLL2716, but more human trials will be needed to substantiate its clinical relevance.

Alleviation of symptoms due to viral infection

The mechanisms by which probiotic microorganisms limit duration of viral infection and the associated symptoms is increasingly being elucidated by *in vitro* and *in vivo* animal studies. Conventional Balb/c mice were intra-nasally administered *L. gasseri* TMC0356 and challenged by H1N1 influenza infection. Mice receiving the *Lactobacillus* treatment experienced decreased morbidity from infection and upregulated expression of mucosal cytokines, including IL-1β, TNF-α, IL-10 and monocyte chemotactic protein 1 (64). Similarly, Balb/c mice were prophylactically fed heat-killed *L. gasseri* TMC0356 and challenged with influenza virus H1N1 (46). Pulmonary virus titers, activation of NK cells and the expression of cytokines were recorded after sacrificing the animals. In the TMC0356 treatment group, weight loss associated with infection was abrogated and pulmonary virus titers were decreased when compared with the control group. The mucosal epithelium of the treatment group also retained higher barrier integrity when compared with the control. The pulmonary expressions of several cytokines were upregulated, including those associated with the activity of NK cells. Specifically, increases in expression of IFN-γ, TNF-α, and IL-12 were observed (62). Heat-killed TMC0356 conferred a similar protective effect against H1N1 as live cells. Although this was the case, different cytokines were analyzed in each

study. Therefore, it is difficult to note any differential immunological responses that might have occurred due to heat-treatment of the cells. In addition, it is challenging to associate any particular cytokines with the protective phenotype.

Human clinical trials are needed to determine the clinical relevance of administering probiotic microbes as a preventative measure to limit viral infection. Notably, a combination of *L. acidophilus* NCFM and *Bifidobacterium animalis* ssp. *lactis* Bi-07 decreased severity and duration of acute flu-like symptoms in children when compared to the control group (79). Similarly, *L. gasseri* has been reported to decrease the severity of symptoms in a human clinical trial, possibly due to similar mechanisms as the related *L. acidophilus* NCFM. Induction of viral defense genes in cell culture indicate a possible mechanism for the action of *L. acidophilus* NCFM in limiting viral infections in humans. *L. acidophilus* NCFM was demonstrated to induce viral defense genes via TLR-2, causing increased expression of IFN-β, and subsequently inducing IL-12 and TLR-3 in bone marrow derived DCs (128). In this regard, a combination of *L. gasseri* PA16/8, *B. longum* SP 07/3 and *B. bifidum* MF20/5 was administered in a multicenter randomized, placebo-controlled study during two sessions of the flu season (27). Symptom scores, duration of infection, as well as the cellular immune response and fecal counts were recorded. Although there was no difference in incidence of viral infection, the treatment group had reduced severity of bronchial, pharyngeal and nasal symptoms, as well as reduced the duration of symptoms and days with fever. The cellular immune response in the treatment group showed a significantly higher amount of cytotoxic T-cells and $CD8^+$ T cells when compared to the control, while other immune parameters

remained the same. The study indicates that *L. gasseri* has human clinical relevance in abrogating symptoms and the duration of upper respiratory viral infections, but additional human trials are needed to further substantiate the protective effect.

Delivery of Biotherapeutics

The generally recognized as safe (GRAS) status of certain probiotic microbes and the genetic tools available will likely accelerate the use of designed probiotic microbes for the targeted delivery of biotherapeutics (129). Specifically, due to the propensity of probiotic microbes to survive GI passage and adhere to intestinal epithelial cells, they are able to effectively deliver targeted recombinant biotherapeutics that benefit the host, either metabolically or immunologically (Figure 3). Knowledge and use of the inherent genetic systems available for transformation were employed in the recombinant production of CC chemokines by *L. gasseri* ADH, which was augmented by the use of the φ-ADH phage to transduce the gene and insert multiple copies into the host chromosome, achieving high expression (24). The genetic tools available in *L. gasseri* have been demonstrated to facilitate recombinant expression of biotherapeutics, including introducing biosynthetic genes for folate synthesis, as well as the expression of manganese superoxide dismutase (Mn-SOD) (127, 18). Recombinant Mn-SOD may contribute to GI homeostasis by neutralizing reactive oxygen species (ROS), which play an integral role in tissue damage associated with colitis. Expression of Mn-SOD in the GIT ameliorated the histological

inflammatory scores in an IL-10 deficient murine colitis model, indicating potential for the therapeutic use of recombinant *L. gasseri* in abrogating colitis symptoms (20).

Probiotic microbes with an inherent capacity to modulate the systemic and mucosal immune systems are positioned to mitigate and potentiate the host response to particular antigens in the delivery of mucosal vaccines (115). In a promising experiment, mice were prophylactically fed with *L. gasseri* ATCC 33323 expressing recombinant protective antigen for anthrax toxin fused to a DC targeting peptide. Those mice challenged with *B. anthracis* exhibited a 100% survival rate, whereas the control mice did not survive (88). Oral delivery of the recombinant protective antigen expressed by *L. gasseri* increased the protective antigen specific antibody in the mice and upregulated cytokines associated with T-cell proliferation and immunity, indicating a strong potential for the innate adjuvanticity of this strain to potentiate targeted delivery of vaccines.

Conclusion

L. gasseri is a widespread commensal bacterium that inhabits human mucosal niches and demonstrates potential probiotic applications by fulfilling many desirable probiotic attributes. Due to recent advances and cost effectiveness of genome sequencing technology, candidate strains that are considered for probiotic or therapeutic applications should be comprehensively sequenced, due to the necessity of proper identification and evaluation of

safety. Genome sequencing facilitates pan-genome species and strain comparison that identify niche-specific factors as well as conserved genotypes between lactobacilli. Genome sequences enable the use of genetic tools for functional analysis of key genotypes involved in the mechanisms of probiotic activity and expedites the utility of recombinant applications in strains of interest. Consequently, each putative probiotic strain of *L. gasseri* should be sequenced and thoroughly characterized since safety and clinical efficacy cannot be extrapolated between strains. Increasingly, *in vivo* gene expression analysis of probiotic cultures and host tissues will continue to be used to determine relevant genotypes and regulatory networks involved in eliciting health benefits, but has yet to be performed with regard to *L. gasseri*. Furthermore, novel cell surface proteins specific to *L. gasseri* and other *L. acidophilus* B-complex members, such as the Apf proteins and other cell surface factors should be characterized to determine their roles in adhesion, immunomodulation or other host-microbe crosstalk. Comprehensive understanding of host-microbe crosstalk opens up avenues for future probiotic applications through effective tailoring of probiotic microorganisms for amelioration of specific immunological conditions and physiological maladies. The empirical evidence suggesting probiotic application for *L. gasseri* must be substantiated with randomized, double-blind placebo-controlled human clinical trials to establish the efficacy of specific strain related health benefits.

Acknowledgements

Research efforts in the NCSU Klaenhammer group are supported by the North Carolina Dairy Foundation, Dupont Nutrition & Health, the Southeast Dairy Foods Research Center, and Dairy Management, Inc as administered by the Dairy Research

Institute. The authors thank colleagues that contributed to this work through peer-review and discussions, Yong Jun Goh, Sarah O'Flaherty, Emma Call, and Brant Johnson.

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Chapter III. Utility of a *upp*-based counterselective gene replacement system in the mutagenesis of *ltaS in Lactobacillus gasseri* ATCC33323

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Introduction

Lactobacillus gasseri ATCC 33323 is a commensal bacterium that is commonly found in the human microbiome (3). It is a prolific autochthonous microorganism that colonizes the human mucosa, including the oral cavity, vagina, and gastrointestinal tract (GIT) in healthy individuals (30, 14, 34, 11, 29, 9). *L. gasseri* is also among the predominant microorganisms in the initial colonization of the neonatal GIT following childbirth (49). Niche-related phenotypes have been defined that likely confer its capacity to colonize the mucosa and potentially contribute to the ability of *L. gasseri* to positively impact health. Specifically, *L. gasseri* exhibits bile resistance, adhesion to a Caco-2 intestinal epithelial cells, and immunomodulatory properties (43, 51, 3, 54). Consumption of *L. gasseri* has been associated with health benefits substantiated by randomized human clinical trials, such as the capacity to reduce severity and duration of symptoms associated with acute diarrhea and upper respiratory viral infections alike, as well as suppression of *H. pylori* infection (12, 42, 5). Given the considerable potential of *L. gasseri* for probiotic applications, this species warrants genetic efforts to correlate genotypes with phenotypic traits potentially impacting health and well-being.

The published genome sequence of *L. gasseri* ATCC 33323 facilitates identification and subsequent functional analysis of relevant genes involved in survival and activity in the GIT, such as mucus-binding proteins, putative adhesion factors, and immunomodulatory proteins (3). This study established a deletion and/or knockout strategy for genes in *L. gasseri* using a *upp* (uracil phosphoribosyltransferase) based markerless gene replacement

strategy (23). This system was used to investigate the role of the immunomodulatory component lipoteichoic acid (LTA), a conserved Gram-positive macroamphiphile, on immature dendritic cell (iDC) signaling by *L. gasseri.* Previously, elimination of LTA from the surface of *L. acidophilus* was correlated with a shift to anti-inflammatory cytokine profiles from (iDCs) and mitigation of both colitis and colon cancer outcomes in mouse models (44, 32). The effect of a *ltaS* insertional knockout on adhesion and immunomodulation was functionally analyzed to elucidate how LTA potentially contributes to immune signaling by *L. gasseri.*

Materials and Methods

Bacterial Strains

All bacterial strains and plasmids are listed in Table 1. Bacterial cultures were cryopreserved in their respective media with a 15% glycerol concentration (vol/vol) and stored at -80˚C. *L. gasseri* was propagated in de Mann, Rogosa and Sharpe (MRS) (Difco Laboratories, Inc., Detroit, MI) broth under statically aerobic conditions at 37˚C, or on MRS agar (1.5% wt/vol agar, Difco) incubated anaerobically at 37˚C for 48 hours. Concentrations of 2 µg/mL of erythromycin (Em) (Sigma-Aldrich, St. Louis, MO) and 5 µg/mL of chloramphenicol (Cm) (Sigma) were used for plasmid selection in *L. gasseri* ATCC 33323, when appropriate. Selection for 5-FU resistant *L. gasseri* was performed by supplementing

glucose semi-defined (GSDM) (33) agar with a final concentration of 100 μg/mL of 5-FU (Sigma). *E. coli* EC1000 was propagated aerobically in Luria-Bertani (Difco) broth at 37˚C, or on brain-heart infusion (BHI) (Difco) solid medium supplemented with 1.5 % agar. Antibiotic selection of *E. coli* was maintained with 40 μg/mL kanamycin (Kn) and 150 μg/mL of Em for recombinant *E. coli*, when appropriate.

DNA Isolation, Manipulation, and Transformation

All kits, enzymes, and reagents were used according to the manufacturers' instructions. DNA purification and cloning were performed as previously described (23). Purification of genomic DNA from *L. gasseri* employed a ZR Fungal/Bacterial MiniPrep kit (Zymo Research Corp., Orange, CA). Plasmid DNA was isolated from *E. coli* using Qiagen Spin miniprep kit (Qiagen Inc., Valencia, CA). High fidelity PCR amplification of DNA was performed with PFU HS II DNA polymerase (Stratagene Corp., La Jolla, CA). Routine PCRs were conducted with Choice-*Taq* Blue polymerase (Denville Scientific Inc., Meutchen, NJ). Primers for PCR amplification were purchased from Integrated DNA Technologies (Coralville, IA). DNA amplicons were separated using 0.8 % agarose gel electrophoresis and stained with ethidium bromide for visualization. DNA extraction from agarose gels was performed with a Zymoclean DNA gel recovery kit. Restriction endonucleases BamHI and SacI and corresponding buffers were acquired from Roche Molecular Biochemicals (Indianapolis, IN). Ligations were performed with New England Biolabs (Beverly, MA)

quick T4 ligase. Sequencing was performed by Davis Sequencing Inc. (Davis, CA). Rubidium chloride competent *E. coli* cells were prepared as previously described and frozen at -80˚C (27). The ligation mixture was combined with competent cells which were then subjected to heat shock at 42˚C for 1 minute. Super optimal broth (Difco) was added and the cells incubated at 37˚C, while shaking (250 rpm) for 90 minutes. The cells were then plated on selective BHI with Kn, for transformants, which were subsequently screened by PCR for inserts using primers flanking the multiple cloning site (MCS) of pORI28. Potential positive clones were propagated in broth cultures, plasmids isolated and screened for presence of the insert by restriction mapping. Plasmids putatively containing inserts were sequenced across the MCS to ensure fidelity. Newly constructed integration plasmids were electroporated into competent *L. gasseri* ATCC 33323 containing the temperature-sensitive helper plasmid, pTRK669, according to methods described previously (56, 51). Penicillin G at a concentration of 10 µg/mL was employed in the preparation of the competent cells to promote electroporation efficiency (57).

Construction of *upp* deletion in *L. gasseri*

Primers were designed for amplification of genomic regions flanking the putative *upp* gene in *L. gasseri* ATCC 33323. The upstream and downstream regions were 579 bp and 580 bp, respectively, resulting in a 571 bp in frame deletion. The flanking amplicons were purified and subsequently combined using splicing overlap extension (SOE)-PCR (31),

resulting in two homologous sequences to regions flanking *upp* in the host chromosome. Restriction endonuclease sites were incorporated into the 5-prime ends of the primers to enable forced directional cloning into pORI28. The integration vector, pTRK1065 was electroporated into competent *L. gasseri* ATCC 33323 already containing the temperature-sensitive helper plasmid pTRK669 with a Cm marker and repA, provided in trans for replication of the pORI-based integration vector. Transformants were selected on MRS agar with 2 µg/mL Em and 5 µg/mL Cm and confirmed by PCR with plasmid specific primers. Confirmed transformants were inoculated into broth cultures with the appropriate antibiotic selection and then subcultured three times at a 1% inoculum into MRS with Em selection for the integration vector, but without Cm selection for pTRK669. Loss of the helper plasmid occurred upon propagation at the non-permissive plasmid replication temperature of 45˚C. The culture was subsequently diluted and plated for isolation on MRS +2 µg/mL Em, after which colonies were replica plated on MRS + 2 µg/mL Em and MRS + 5 µg/mL Cm. Clones devoid of the helper plasmid which exhibited a Em^R, Cm^S phenotype, were subsequently screened by PCR for the absence of a repA amplicon from pTRK669. Colonies exhibiting a Em^R, Cm^S phenotype and no repA amplicon were inoculated into broth culture containing Em and their genomic DNA purified. Chromosomal integration of pTRK1065 was confirmed by PCR amplification of the integration junctions using primers rooted in the genome and the Em gene on the plasmid. Confirmed integrants were subsequently subcultured into MRS without Em selection to allow resolution and excision of pTRK1065. Clones were replica plated onto MRS and MRS + Em to screen for Em^S colonies, which were then screened by

PCR with primers flanking the deletion junction to confirm loss of the sequence. Positive clones were sequenced at the deletion junction to ensure fidelity.

Knockout mutation of *ltaS* in *L. gasseri*

Construction of the integration vector for deletion of *ltaS* was performed essentially as described above, with minor exceptions. Specifically, the homologous regions in the integration plasmid were amplified from within the *LtaS* gene, rather than flanking it, such that any recombination would result in an insertional knockout. The two regions within the gene that were amplified were 631 bp on the 5' and 640 bp on the 3' end, respectively, resulting in an in frame 786 bp deletion. The construct was cloned into the previously created pTRK935 to create pTRK1070, which was electroporated into NCK2254. Following integration of the plasmid into the host chromosome by site-directed recombination, the Em^R culture was propagated without antibiotic selection, and subsequently plated on GSDM supplemented with 5-FU to select for excision recombinants.

Adhesion to Caco-2 epithelial cell line

All cell culture media and reagents were obtained from Gibco (Gibco-Invitrogen Corp., Carlsbad, CA). Caco-2 cell line ATCC HTB-37 was purchased from the American Type Culture Collection (Rockville, MD) and maintained in minimal essential medium

(MEM) supplemented with 1mM sodium pyruvate, 0.1 mM nonessential amino acids, 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, 100 µg/mL penicillin and 0.25 µg/mL amphotericin. Adherent cells were cultivated in 5% CO_2 at 37˚C by seeding 2 mL of MEM per well in 12 well plates. MEM was replaced every two days and the monolayers were used for assays 21 days post-seeding. Cells were used between passage 23 and 40. Prior to the addition of bacterial cells, the monolayers were washed twice with 1 mL of 37˚C phosphate-buffered saline (PBS) (pH 7.4) after which 1 mL of warm MEM with no antibiotic supplementation was added to each well. The monolayers were incubated at 37˚C and 5% CO_2 prior to co-incubation with bacterial cells. Lactobacilli were cultivated from a frozen stock and subcultured once overnight at a 1% inoculum, subsequently subcultured and incubated for 16 hrs. Stationary phase cells were harvested by centrifugation at 10,000 rpm for 2 min, the supernatant discarded and washed once in room temperature PBS . Cultures were centrifuged again and resuspended in PBS. The optical density of the cell suspension was measured at 600 nm, and diluted in PBS to achieve approximately 1 x 10^8 cfu/mL for each strain. One mL of the bacterial suspension was then added to the monolayers and co-incubated for 1 h at 37˚C and 5% CO_2. The monolayers were then washed five times with 37˚C PBS. Finally, 1 mL of 0.05% (vol/vol) Triton X-100 was added to each well and incubated for 10 min with agitation. Monolayers were disrupted by pipetting, removed, and vortexed. The cell suspension was plated using a Whitley automatic spiral plater (Don Whitley Scientific Ltd., West Yorkshire, England) and a ProtoCOL (Synoptics Ltd., Cambridge, UK) colony counter to enumerate cfu.

Cytokine profiling

Cryopreserved Balb/c bone marrow-derived murine iDCs were acquired from Astarte-Biologics LLC (Redmond, WA) and stored in liquid nitrogen. On the day they were used, the cell suspension was thawed in a 37°C water bath and subsequently transferred to a 50 mL conical tube containing 100 µg of DNase I (Stem Cell technologies Inc., Vancouver, Canada) to prevent clumping. The cell pellet was resuspended in 25 mL of Rosewell Park Memorial Institute (RPMI)-1640 + 10% FBS and centrifuged in a swing arm rotor in a benchtop centrifuge at 200 x g at room temperature for 15 minutes. The supernatant was decanted, 50 µL of DNase I was added, and the pellet resuspended in 25 mL of RPMI-1640 + 10% FBS. The suspension was centrifuged at 200 x g at room temperature for 15 minutes and 50 µL of DNase I was added. The pellet was resuspended in RPMI-1640 + 10% and a 20 µL aliquot of cell the suspension was removed from the vial for enumeration of viable cells, using Trypan Blue (Sigma) and the Invitrogen Countess, according to the manufacturer's instructions. Based on the viable cell concentration, cells were diluted to a final concentration of 10^6/mL in RPMI-1640 + 10% FBS + 75 µg/mL streptomycin (Sigma). One hundred µL of the standardized cell suspension was aliquoted in each well of a round bottom, polypropylene 96 well plate and incubated in 5% CO_2 at 37°C. Bacterial strains were inoculated from frozen stocks, subcultured twice in MRS and then grown to stationary phase (16 hrs). The cells were harvested by centrifugation (10,000 rpm for 2 min) and washed once in PBS, then resuspended in PBS and the OD_{600} measured. The cultures were standardized to an OD_{600} corresponding to 1 x 10^8 cfu/mL, determined previously for each

strain. From each cell suspension, 3 x 10^6 cfu was centrifuged and decanted. The cell pellet was resuspended in a final volume of 600 µL of RPMI-1640 +10% FBS and duplicate 200 µL aliquots were combined with 100 µL of RPMI-1640 + 10% FBS+ 75 µg/mL containing 10^5 viable DCs in each well of a 96 polypropylene plate, resulting in a final bacterial to DC ratio of 10 to 1. The bacterial cells and iDCs were co-incubated for 24 hours in 5% CO_2. After the co-incubation, the cell suspension was centrifuged at 4000 rpm for 10 minutes and the supernatant harvested. Supernatants were stored at -80˚C until tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10 and IL-12 were quantified using sandwich-based ELISA kits, according to the manufacturer's instructions (Qiagen). Each standardized cell suspension was diluted and plated for enumeration of the viable bacterial cfu/mL.

Results

Construction of *L. gasseri* NCK2253 Δ*upp*, 5-FU^R

In microbes with a functional uracil ribonucleotide salvage pathway, the toxic uracil analogue 5-FU is imported and incorporated into the pathway, resulting in formation of 5-fluoro-deoxy-uracil-monophosphate, lethally acting as a thymidylate kinase inhibitor (Figure 1). *L. gasseri* ATCC 33323 was evaluated for sensitivity to 5-FU by plating approximately 10^8 cfu of a 16 hour stationary phase culture onto GSDM supplemented with a final concentration of 100 µg/mL of 5-FU. Approximately 10 colonies appeared, indicating that

spontaneous mutation rates to a 5-FU^R phenotype occurred at a frequency of 10^{-7}. The typical excision rate of integrated pORI-based plasmids from lactobacilli has been observed to be roughly 0.2% to 1% (23). Therefore, 5-FU^R colonies arising from spontaneous mutation would occur at levels of <0.01 clones per plate, making the number of spontaneous mutants relative to the excision recombinant clones negligible.

The 5-FU^S of the wild-type strain indicated that *L. gasseri* ATCC 33323 contained an active uracil ribonucleotide salvage pathway. Analysis of the genome confirmed that *L. gasseri* ATCC 33323 lacked a complete pathway for synthesis of uracil *de novo,* but encoded a putative uracil phosphoribosyltransferase which enzymatically catalyzes the phosphorylation of imported uracil (3). *L. gasseri* ATCC 33323 chromosomally encodes LGAS_1245, a putative *upp* gene with 78% sequence identity and 82% predicted amino acid identity to the confirmed *upp* gene in *L. acidophilus* NCFM. The gene was subsequently deleted by the allelic replacement method using the pTRK1065 integration vector (Figure 2). PCR using primers flanking the deletion junction were used to screen the excision recombinants, which yielded a putative *upp* deletion genotype in an Em^S clone. The potential positive clone was sequenced at the deletion, which confirmed fidelity of the flanking regions and loss of the 571 bp sequence (Figure 3).

The deletion clone was designated NCK2253 and was plated on GSDM supplemented with 5-FU to confirm that deletion of LGAS_1245 conferred the 5-FU resistance phenotype. NCK2253 was electroporated with pTRK669, which was designated as

NCK2254, as a host for subsequent gene deletion experiments in *L. gasseri*. NCK2254 was electroporated with pTRK935 to determine if the P-*upp* cassette from *L. acidophilus* NCFM could complement the *upp* deletion in *L. gasseri* ATCC 33323, and in doing so, restore the 5-FU^S phenotype. Complementation was assessed by plating NCK2254 containing pTRK669 and pTRK935 on GSDM + 5-FU supplemented with Cm and Em to maintain selection for the plasmids. The pTRK935 containing clones of NCK2254 were sensitive to 5-FU and therefore, the *L. acidophilus upp* construct successfully complemented, in trans, the *upp* deletion in *L. gasseri*. Complementation of the deletion and restoration of the 5-FU^R phenotype following curing of the pTRK935 plasmid confirmed the utility of the integration vector by facilitating positive selection for plasmid-free excision recombinants in *L. gasseri* ATCC 33323.

Construction of *L. gasseri* NCK2270 Δ*upp ltaS*$^-$, 5-FU^R, Em^R

In order to abolish LTA biosynthesis in *L. gasseri* NCK2254 for phenotypic analysis, the pTRK935 integration vector was employed to delete *ltaS*. LTA in lactobacilli structurally consists of a long hydrophobic tail, a carbohydrate moiety and a polymeric glycerol phosphate (poly-Gro-P) chain that is primarily substituted with D-alanine and glycosyl residues (39). The biosynthesis of LTA begins on the cytoplasmic side of the cell membrane, where glycosyltransferases attach various saccharides dependent on the species to diacylgycerol (48) (Figure 4). The glycolipid is then flipped to the extracellular face of the

cell membrane by the putative transmembrane ltaA, where ltaS catalyzes the polymerization of the poly-Gro-P chain. On a separate locus in the chromosome, the *dlt* operon encodes enzymes that substitute D-alanine on the completed poly-Gro-P backbone to yield canonical LTA. In *L. acidophilus* NCFM and *L. gasseri* ATCC 33323 the genes encoding the LTA biosynthetic enzymes are organized within a putative operon flanked by two putative rho-independent terminators. The putative operons in *L. gasseri* and *L. acidophilus* share conserved structural synteny (73% sequence homology), containing two putative glycosyltransferases, a putative *ltaA* transmembrane "flippase" and a phosphoglycerol transferase gene (Figure 5). LGAS_1586, annotated as a phosphoglycerol transferase, shared 78% predicted amino acid homology to *ltaS* in *L. acidophilus* NCFM and was targeted for deletion by allelic replacement to create a putative LTA deficient *L. gasseri* ATCC 33323 mutant.

The integration plasmid pTRK1070 was constructed by cloning an SOE-PCR construct of flanking regions within the LGAS_1586 gene. Consequently, any homologous recombination of pTRK1070 in the chromosome resulted in an insertion knockout of the putative phosphoglycerol transferase gene. Gene replacement by excision of the plasmid was expected to result in an in frame 786 bp deletion. Plasmid integrants were successfully recovered and PCR amplification of the junctions confirmed the insertional knockout of *ltaS* (LGAS_1586). Extensive screening for 5-FU^R excision recombinants for allelic replacement failed to recover an excision/deletion mutant. Interestingly, clones screened were 5-FU^R, plasmid cured and Em^s, but all contained a wild-type *ltaS* allele. This suggests that although

knockout of *ltaS* by integration is not lethal, the selective pressure to maintain the wild-type genotype results in heavy skewing of the double recombinants to maintain the wild-type allele.

Relative adhesion of NCK2270 to Caco-2 cell monolayers

The *ltaS* knockout mutant NCK2270 was phenotypically analyzed for adhesion to Caco-2 cell line monolayers to determine whether the *ltaS* knockout would alter the capacity of *L. gasseri* ATCC 33323 to adhere to intestinal epithelial cells *in vitro.* Approximately 1 x 10^8 cfu stationary phase bacterial cells were incubated with Caco-2 monolayers for 1 hour at 37˚C and 5% CO_2. The *ltaS* knockout mutant exhibited a 41% decrease in adhesion to the Caco-2 cell monolayer (Figure 6). The decrease in adhesion observed in the NCK2270 *ltaS* knockout indicates that LTA plays a role in adhesion of wild-type *L. gasseri* ATCC 33323 to intestinal epithelial cells, *in vitro*.

Cytokine profiling of NCK2270 using murine iDCs

The cytokines elicited from antigen presenting cells (APCs) influence GI homeostasis in a multi-faceted manner. Secretion of cytokines in the lamina propria may contribute to activation of resident effector cells and lymphocytes, whereas cytokines secreted during presentation of antigens in mesenteric lymph nodes influence polarization of $CD4^+$ T-cells

(54). Consequently, the effect of the *ltaS* knockout in *L. gasseri* on immunomodulation was assessed by quantifying cytokine levels elicited from Balb/c murine iDCs upon *in vitro* co-incubation. Specifically, iDCs and bacterial cells were co-incubated at a 1:10 ratio in RPMI + 10% FBS + 25 μg/mL streptomycin for 24 hours at 37˚C, and in 5% CO_2. The supernatant was harvested by centrifugation and subsequently ELISA was performed to quantify IL-10 (Figure 7), IL-12 (Figure 8), IL-6 (Figure 9), and TNF-α (Figure 10). In particular, IL-6 and TNF-α were selected for analysis as general markers of inflammation in the acute-phase immune response to bacteria, whereas IL-12 was assessed due its capacity to polarize $CD4^+$ T-cells to the Th-1 phenotype. IL-10 is considered a regulatory cytokine that contributes to homeostasis in the GIT by suppression of inflammatory responses in both Th-1 and Th-2 pathways, as evidenced by spontaneous development of inflammatory bowel disease in IL-10 knockouts (10). The *ltaS* knockout mutant elicited similar levels of IL-6, and TNF-α when compared to the wild-type parent strain, but exhibited an increase in the level of IL-10 and a decrease in IL-12. Therefore, knockout of the *ltaS* gene was demonstrated to significantly influence the quantity of IL-10 and IL-12 induced upon co-incubation with iDCs *in vitro*. The *ltaS* knockout had no significant impact on the levels of IL-6, and TNF-α elicited.

Discussion

L. gasseri is an autochthonous microbe inhabiting the human mucosa which likely exerts benefits to health as a member of the commensal microbiota. As a member of the

acidophilus complex, this *Lactobacillus* species has been used extensively in a number of industrial applications as both a dairy starter culture, and a probiotic. Comparative genome analysis of *L. gasseri* ATCC 33323 offers insights into the genotypes that potentially may contribute to colonization of the human GIT and serves to guide targeted functional genomic analysis for investigating genes of interest.

In this study a deletion within the *upp* gene (LGAS_1245) of *L. gasseri* was accomplished in order to develop a genetic platform for generating knockout and deletion mutants in this important commensal species. Previously, the validity of using a *upp* deficient parent strain for phenotypic analysis was thoroughly investigated in *L. acidophilus* NCFM. The *upp* deficient *L. acidophilus* NCFM derivative was not phenotypcially disparate from the parent wild-type in growth, cell morphology, or resistance to stress (23). Furthermore, microarray expression analysis confirmed that the Δ*upp* mutant did not exhibit any significant differential expression. Genes successfully deleted in *L. acidophilus* NCFM include surface-layer protein X, (LBA0512), aggregation promoting factor (LBA0493), myosin cross-reactive antigen, (LBA0649) and lactose permease (LBA1463) (23, 24, 47, 2). Establishing this system in *L. gasseri* provides numerous advantages, notably the ability to positively select for excision recombinants yielding gene deletions or replacements. Markerless allelic replacement facilitated by the *upp* system also allows for stable chromosomal expression of recombinant proteins, with potential applications for food grade expression of biotherapeutics or subunit vaccines (15). Site-directed homologous recombination of gene cassettes downstream of specific promoters allows design for

constitutive or inducible expression of the encoded protein at various levels (16). This method offers advantages over plasmid-based expression of recombinant proteins, which requires selection and yields unstable expression *in vivo*.

Modulation of the mucosal and systemic immune systems by probiotic microbes has increasingly become an area of great interest with significant potential application for improving human health (35). Specifically, there is emphasis on characterizing strain-specific immunomodulatory components of probiotic microbes and employing animal models to establish their potential therapeutic application in various immune-related conditions (45, 32). LTA is a major microbial immunomodulatory surface component that elicits a predominantly inflammatory response from APCs, and may be partially responsible for the Th-1 polarized response elicited by lactobacilli (22, 43). In lactobacilli, the general conserved structure of LTA consists of a long hydrophobic tail with a carbohydrate moiety and a polymeric Gro-P backbone that is typically substituted with D-alanine or glycosyl residues (39). LTA is well known to interact with toll-like receptors (TLR)-2/6 heterodimers with co-receptors CD14 and CD36 on APCs (22). Knockout of lipoteichoic acid synthase, a phosphoglycerol transferase enzyme catalyzing the polymerization of the Gro-P backbone abolishes the activity of LTA, resulting in pleiotropic effects on bacterial physiology and bacterial-mammalian cell interactions (44). The *ltaS* gene was previously deleted in *L. acidophilus* NCFM to create the NCK2025, which exhibited a marked reduction in adherence to Caco-2 intestinal epithelial cell line monolayers when compared to the wild-type parent strain (O'Flaherty and Klaenhammer, unpublished). Furthermore, the mutant

elicited decreased inflammatory cytokines and increased regulatory signals upon in vitro co-incubation with iDCs (44).

The counterselective system established in this study was employed in the knockout of *ltaS* in *L. gasseri* ATCC 33323 to investigate the effects on intestinal cell adhesion and immunomodulation. This genetic platform in *L. gasseri* was used successfully to delete the gene encoding sortase (LGAS_0825; Call, Selle, Goh, O'Flaherty, and Klaenhammer, unpublished). However, extensive efforts to delete the *ltaS* gene were unsuccessful. Therefore, in this study a knockout insertional mutant was created in *ltaS* in order to investigate phenotypic outcomes of this mutation in *L. gasseri*, comparatively against a similar LTA mutation made previously in *L. acidophilus* (44). The *L. gasseri* *ltaS* knockout mutant did not exhibit altered morphological or growth phenotypes.

The *ltaS* integration knockout mutant derivative of *L. gasseri* ATCC 33323 exhibited a decrease in adhesion to Caco-2 cell line monolayers relative to the wild-type parent strain. This is in accordance with previous studies indicating the role of LTA in adhesion of lactobacilli to mammalian cells and the results observed in adhesion of *L. acidophilus* NCFM Δ*ltaS* to Caco-2 cells (25, O'Flaherty and Klaenhammer, unpublished). LTA may contribute to adhesion to epithelial cells by lactobacilli through a few proposed mechanisms. In group A streptococci, M protein-anchored LTA contributes to cell surface hydrophobicity by orientation of the glycolipid moiety such that it interacts with fibronectin on epithelial cells and brings surface adhesins into contact with the cells (7, 8). However, a genome search of *L.*

gasseri ATCC 33323 and *L. acidophilus* NCFM indicated no clear homologous proteins, conserved domains, or genes with predicted amino acid identity to proteins M1 and M3 from *Streptococcus pyogenes*. Electrostatic interaction as a general mechanism of adhesion is supported by the decrease of adhesion observed with increased anionic charge of *dlt* mutants deficient in D-alanine substitution (46). Inactivation of the *dlt* operon abrogated D-alanine substitution in *L. plantarum* NCIMB8826, which is compensated for by increases in glycosyl residue substitution on the poly-Gro-P backbone, thus decreasing the presence of cationic charges (26). However, deletion of the phosphoglycerol transferase gene, which potentially abolishes the poly-Gro-P backbone would eliminate both the cationic and anionic charges associated therewith. Thus, the inhibited adhesion phenotype observed in *ltaS* knockout mutants could be attributed to the lowered cation content on the cell surface or be suitably explained by the general removal of charged molecules from the cell surface. Moreover, pathogenic *dlt* knockout derivatives exhibited decreased adhesion to mammalian cells which further substantiates the role of D-alanine substitution in adherence capacity (1, 21). Interestingly, the random distribution of D-alanine observed in other lactobacilli precludes the notion of localized areas of net cationic or anionic nature that would facilitate electrostatic attraction (4, 6). This is in contrast with other organisms that display a gradient of substitution based on chain length that may exhibit localized regions of net negative or net positive charges (40, 19, 20). Considerable work in elucidating the relationship of cell surface molecules and the structure of LTA as they relate to adhesion is required to

adequately explain the mechanisms responsible for the inhibited adherence phenotype in organisms expressing modified LTA.

Due to the significant biotherapeutic potential of lactobacilli expressing modified LTA, NCK2270 was investigated for the cytokine profile elicited from murine Balb/c bone marrow derived iDCs to evaluate any immunological shift associated with the *ltaS* knockout. Inactivation of *ltaS* in *L. gasseri* resulted in significant alterations of selected cytokines induced from APCs when exposed to the mutant versus the parent. Notably, IL-10 was induced at higher levels and IL-12 at lower levels in response to the mutant, whereas IL-6 and TNF-α were unaffected. Previously, in *L. acidophilus* NCFM, the phosphoglycerol transferase (LBA0447) gene was targeted for deletion, which putatively abolishes the formation of the poly-Gro-P backbone of LTA (44). Co-incubation of NCK2025 with murine bone marrow derived iDCs *in vitro* yielded an altered cytokine profile when compared to the wild-type strain. IL-10 was significantly upregulated whereas TNF-α and IL-12 were significantly decreased. In contrast to the previously reported shift observed in NCK2025 (44), an upregulation of IL-10 was not elicited by NCK2025 in this study. However, the decrease of IL-12 was in accordance with previously reported trends. NCK2270 exhibited similar levels of the cytokines IL-6, and TNF-α to the wild-type parent strain, but increased levels of IL-10 and decreased levels of IL-12 compared to the wild-type parent strain were observed. Previous studies have indicated the necessity of the glycolipid component and D-alanine substitution for optimum immune signaling by LTA, suggesting that the altered cytokine profiles observed with deletion of phosphoglycerol transferase may stem from the

lack of a poly-Gro-P backbone to substitute (13, 45, 26). Interestingly, treatment of LTA with cationic defensins decreases its capacity to interact with TLR-2, suggesting that either the presence of cationic compounds inhibit immune signaling, or that neutralization of the anionic charges of the poly-Gro-P backbone could also potentially inhibit this interaction (52). Several potential mechanisms may contribute to the disparate results between the respective *ltaS* deficient mutants.

L. acidophilus and *L. gasseri* express disparate cell surface proteins and glycolipids, which will likely predominate immune signaling in the absence of LTA. Specifically, *L. acidophilus* expresses surface layer (S-layer) proteins that exhibit immunomodulatory capacity by binding DC-SIGN (36). In contrast, *L. gasseri* lacks an S-layer but expresses surface displayed aggregation promoting factor proteins whose role in immunomodulation, if any, has yet to be investigated (55). LTA is known to exhibit competitive binding of TLR-2, which may limit immunomodulation from peptidoglycan and other potential ligands (17, 58). Consequently, TLR-2 ligands may be more prolific in immunomodulation in the absence of LTA. Moreover, murein hydrolases are upregulated in the absence of LTA, due to its role in regulation of these enzymes (18). Increased murein hydrolase activity could increase liberation of surface displayed immunomodulatory proteins, further potentiating their propensity to induce cytokine expression from APCs and amplifying differences in immune signaling between *ltaS* deficient *L. gasseri* and *L. acidophilus* strains associated with these components.

Disparate LTA structures from *L. gasseri* and *L. acidophilus* may also have separate affinities for TLR-2, since LTA from various species have been shown to be variable in eliciting TNF-α and may be dependent on poly-Gro-P chain length and degree of D-alanine substitution (28). Thus, species-specific structural disparities in the LTA molecule may cause differential cytokine profiles to be elicited from DCs when co-incubated with the wild-type parent strains, which may result in differential cytokine profiles upon removal of LTA. Furthermore, it has been demonstrated that various ligands of TLR-2 result in differential temporal expression in macrophages (41), which further substantiates the concept that structural differences between the LTA from *L. gasseri* and *L. acidophilus* could potentially elicit disparate expression of cytokines upon interaction with TLR-2 and consequently, disparate cytokines in the absence of LTA for each species.

Conclusions

Insertional knockout mutagenesis of the phosphoglycerol transferase gene responsible for LTA biosynthesis in *L. gasseri* ATCC33323 resulted in phenotypic effects on adhesion and immunomodulation. Adhesion of NCK2270 to Caco-2 intestinal epithelial cells *in vitro* was reduced and the mutant elicited increased IL-10 and decreased IL-12 from murine iDCs.

Acknowledgements

This research was supported by the North Carolina Dairy Foundation and Danisco USA/Dupont Nutrition & Health. We gratefully acknowledge Yong Jun Goh, Sarah O'Flaherty, Akinobu Kajikawa, Emma Call, Rosemary Sanozky-Dawes, Evelyn Durmaz, and Brant Johnson for their counsel, technical support and scientific discussions.

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Table 1. Strains and Plasmids used in this study

Strain designation	Description	Original Reference
E. coli EC1000	Host for pORI plasmids, chromosomal $repA^{+}$ (pWVO1), Km^{R}	38
L. gasseri ATCC 33323	Human intestinal isolate, neotype strain	37
NCK2253	*L. gasseri* ATCC 33323 Δ*upp*, $5\text{-}FU^{R}$	This study
NCK2254	NCK2253 with pTRK669	This study
NCK2070	NCK2253 with pTRK1070, LTA^{-}	This study
Plasmids		
pORI28	Broad range non-replicative vector, Em^{R}	38
pTRK669	Ts-helper plasmid $repA^{+}$, Cm^{R}	51
pTRK935	pORI-based counterselective integration vector, P-*upp*, Em^{R}	23
pTRK1065	pORI28 Δ*upp*, Em^{R}	This study
pTRK1070	pTRK935 Δ*ltaS,* Em^{R}	This study

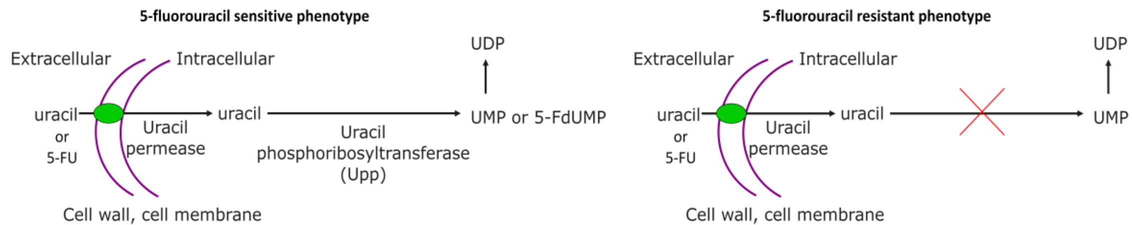


Figure 1. Uracil ribonucleotide salvage pathway. An active uracil salvage pathway in wild-type *L. gasseri* imports and utilizes exogenous uracil or toxic analogue 5-fluorouracil from the environment, exhibiting a 5-FU sensitive phenotype. Deletion of *upp* abolishes the uracil salvage pathway, conferring a 5-FU resistant phenotype. Complementation of the *upp* deletion with a P-*upp* cassette on a pORI-based integration plasmid results in 5-FU sensitive phenotype. Resolution and excision of the integration plasmid restores the 5-FU resistant

phenotype, enabling positive selection of excision recombinant mutants. Described by Goh and Klaenhammer, 2009).

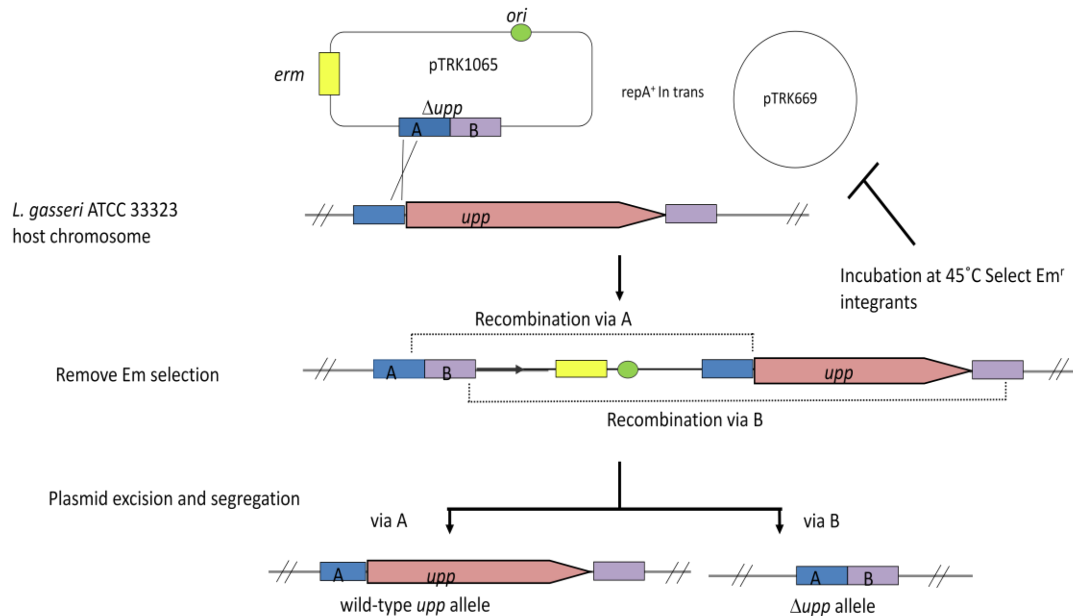


Figure 2. Gene replacement strategy employed in lactobacilli. Elimination of the temperature sensitive plasmid pTRK669 by incubation at non-permissive temperatures facilitates single crossover site-directed homologous recombination and integration of the non-replicative pORI plasmid via host recombinant machinery. Subsequent to the integration of the targeting plasmid, removal of selection allows for a second recombination event, resulting in retention of the wild-type allele or successful gene replacement exist depending on whether recombination via A or via B occurs, respectively. Modified from Goh and Klaenhammer, 20009)

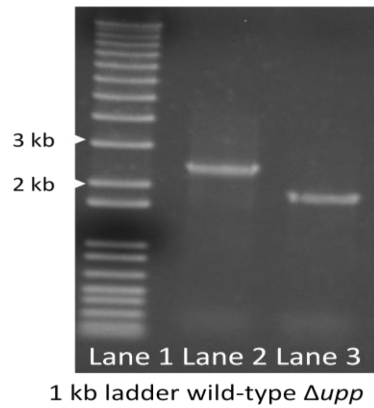


Figure 3. PCR confirmation of *upp* deletion. Agarose gel electrophoresis image of PCR amplicons from gDNA with primers flanking *upp* in *L. gasseri* ATCC 33323 strains. Lane 1 is a 1 kb ladder (Invitrogen). Lane 2 is the amplicon from gDNA of the wild-type control and lane 3 is the amplicon from gDNA of the putative Δ*upp* clone. The loss of 571 bp in the deletion clone (lane 3) was confirmed.

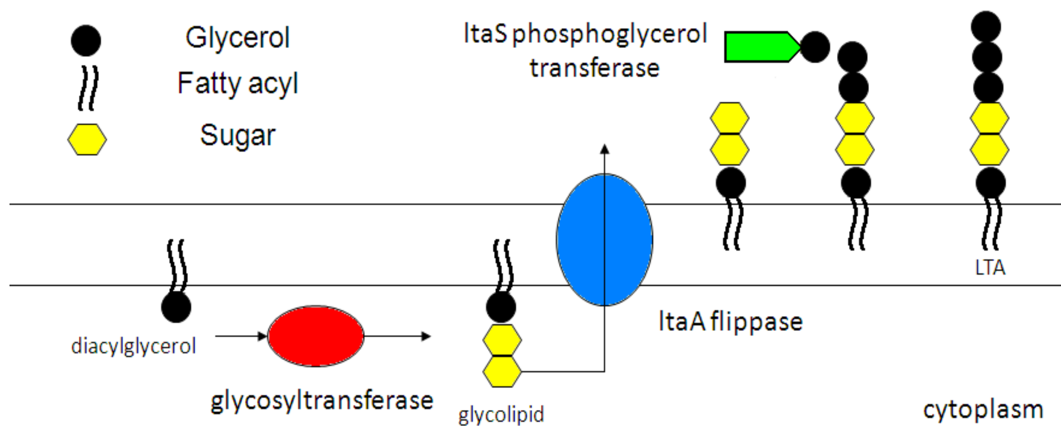


Figure 4. Putative biosynthetic pathway of LTA in lactobacilli. Synthesis begins with diacylglycerol on the cytoplasmic side of the cell membrane, where glycosyltransferases

conjugate it with variable sugars including n-acetylglucosamine and glucose. The putative membrane integrated protein ltaA flips the glycolipid to the outer face of the cell membrane where phosphoglycerol transferase catalyzes polymerization of polyglycerol phosphate chains to variable lengths to the form the complete LTA molecule. Deletion of the *ltaS* gene prevents formation of the polyglycerol phosphate backbone, reducing LTA to a glycolipid moiety in the cell membrane, abolishing activity of the canonical LTA. Modified from Rahman et al, 2009.

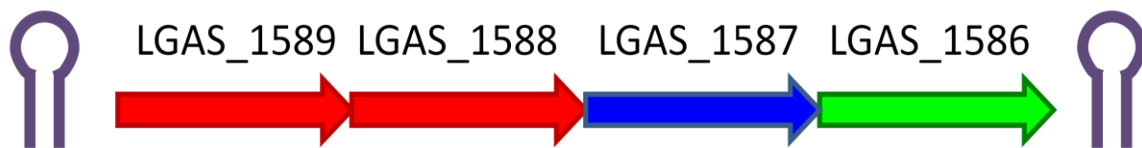


Figure 5. Lipoteichoic acid biosynthetic operon in *L. gasseri*. Organization of the putative LTA biosynthetic genes in *L. gasseri* ATCC 33323 indicates they are co-transcribed as a potential operon, consisting of four genes flanked by two putative rho-independent terminators. The operon structure is also conserved in *L. acidophilus* NCFM and consists of two glycosyltransferases (LGAS_1589, LGAS_1588), an integral membrane protein (LGAS_1587) and a phosphoglycerol transferase (LGAS_1586). The target for knockout mutagenesis is the phosphoglycerol transferase gene LGAS_1586.

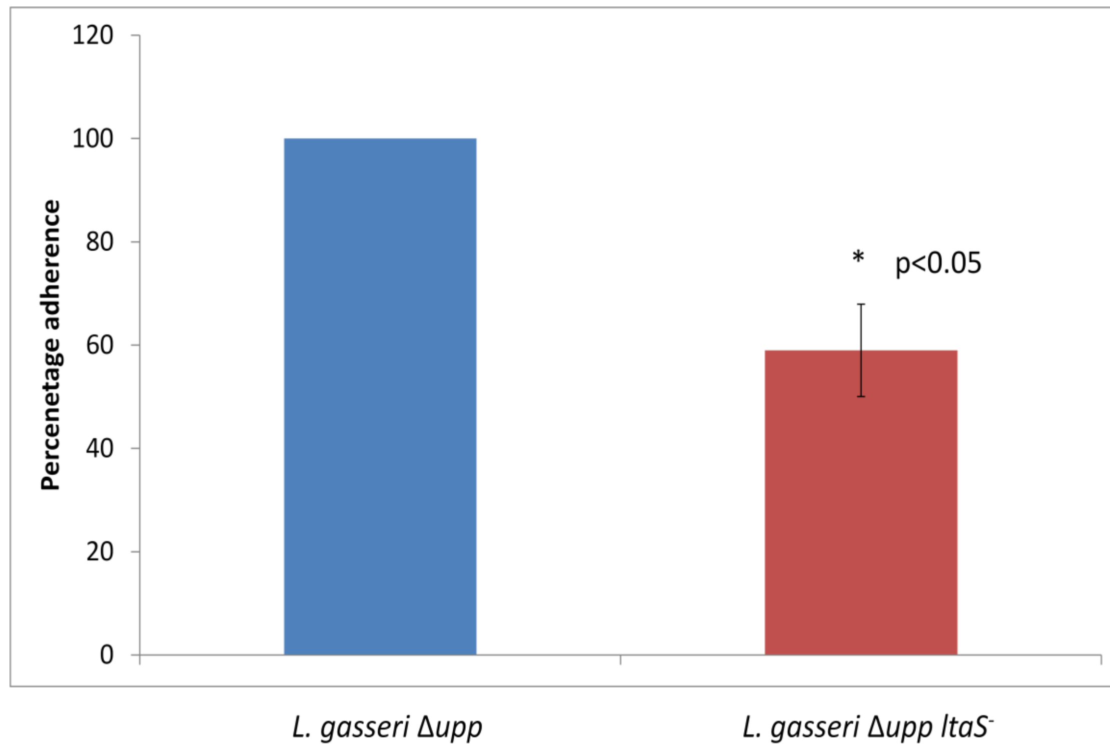
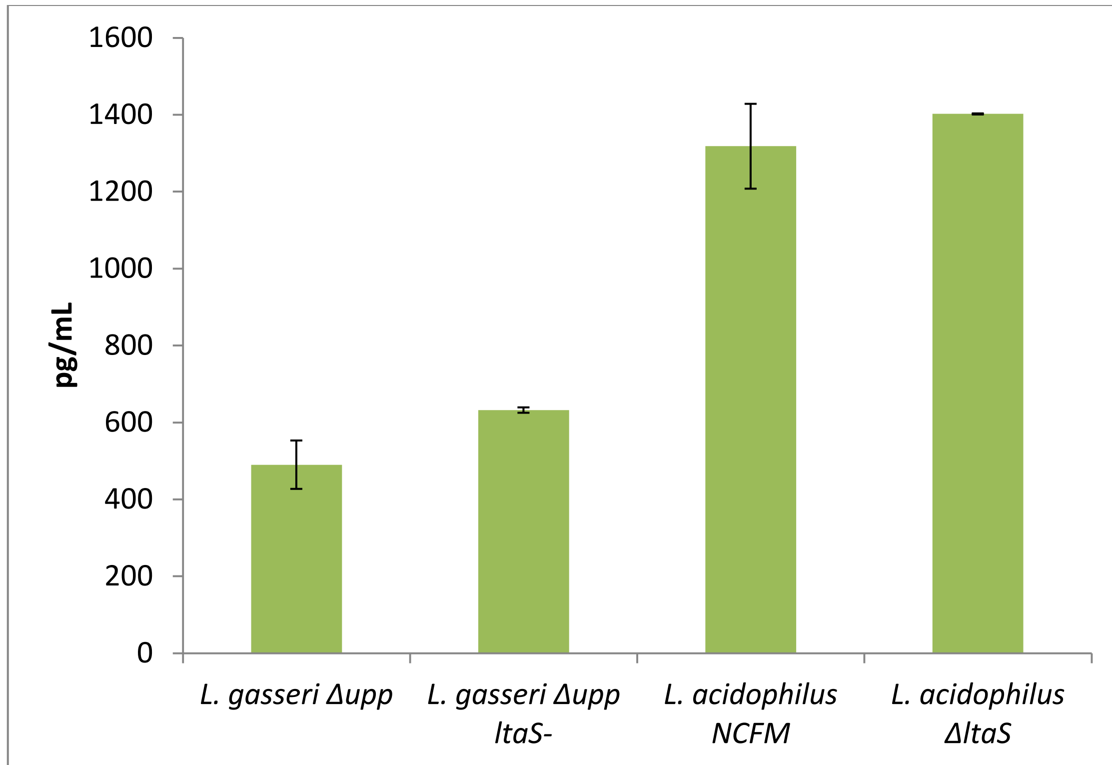


Figure 6. Adhesion of *ltaS* deficient *L. gasseri* to Caco-2 cell line.

Adherence capacity of *L. gasseri* Δ*upp ltaS*$^-$ to the Caco-2 intestinal epithelial cell line monolayer *in vitro*. Results presented as percentage of adhesion when compared to the wild-type parent strain. The mean percentage of adhesion was calculated based on three replicates from three independent experiments. Student's t-test was used to determine statistical significance. (α=.05)



Figures 7. Quantification of IL-10 elicited from Balb/c murine iDCs upon incubation with *ltaS* deficient lactobacilli by ELISA. The bacterial cells and iDCs were co-incubated at a ratio of 10 to 1 respectively, in RPMI-1640 media supplemented with 10% FBS and 25 µg/mL streptomycin. Cultures were maintained at 37˚C and 5% CO_2 for 24 hours, after which supernatants were harvested by centrifugation. Data were normalized by subtracting cytokine levels secreted by DCs incubated with the media alone from the levels in the bacterial treated cells. *L. gasseri* Δ*upp ltaS*$^-$ exhibited increased levels of IL-10.

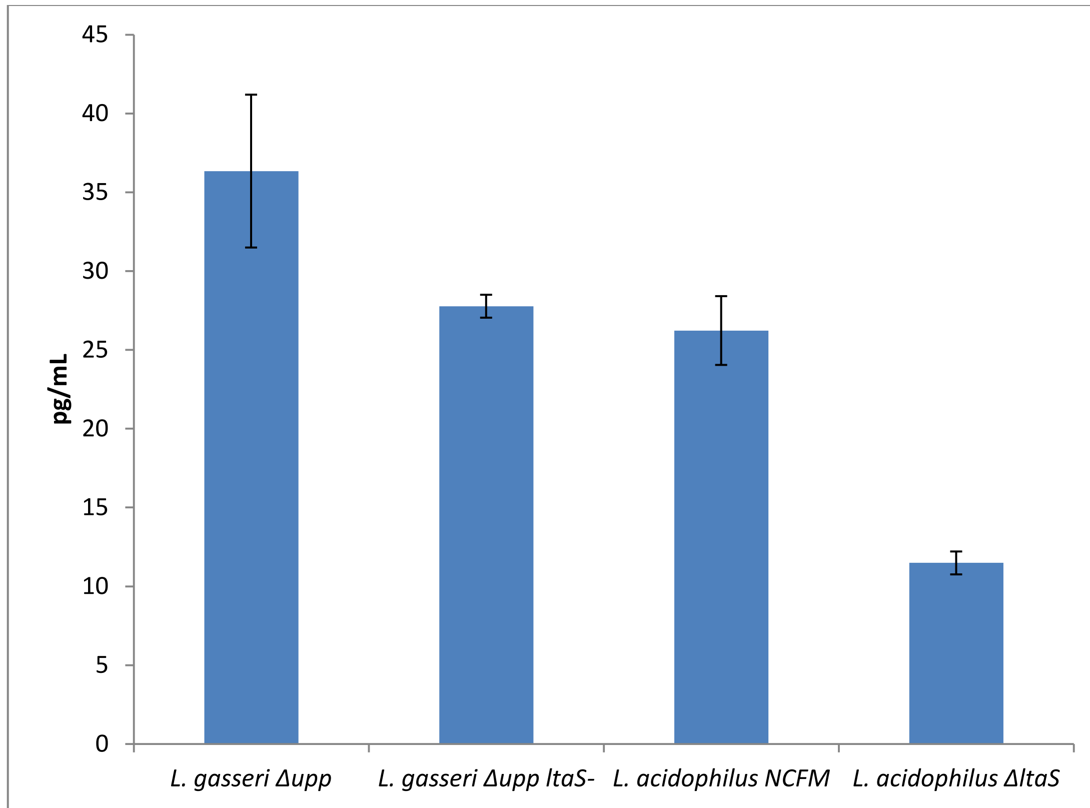


Figure 8. Quantification of IL-12 elicited from Balb/c murine iDCs upon incubation with *ltaS* deficient lactobacilli by ELISA. Data were normalized by subtracting cytokine levels secreted by DCs incubated with the media alone from the levels in the bacterial treated cells. *L. gasseri* Δ*upp ltaS*$^{-}$ elicited lower levels of IL-12 than the *L. gasseri* Δ*upp* control.

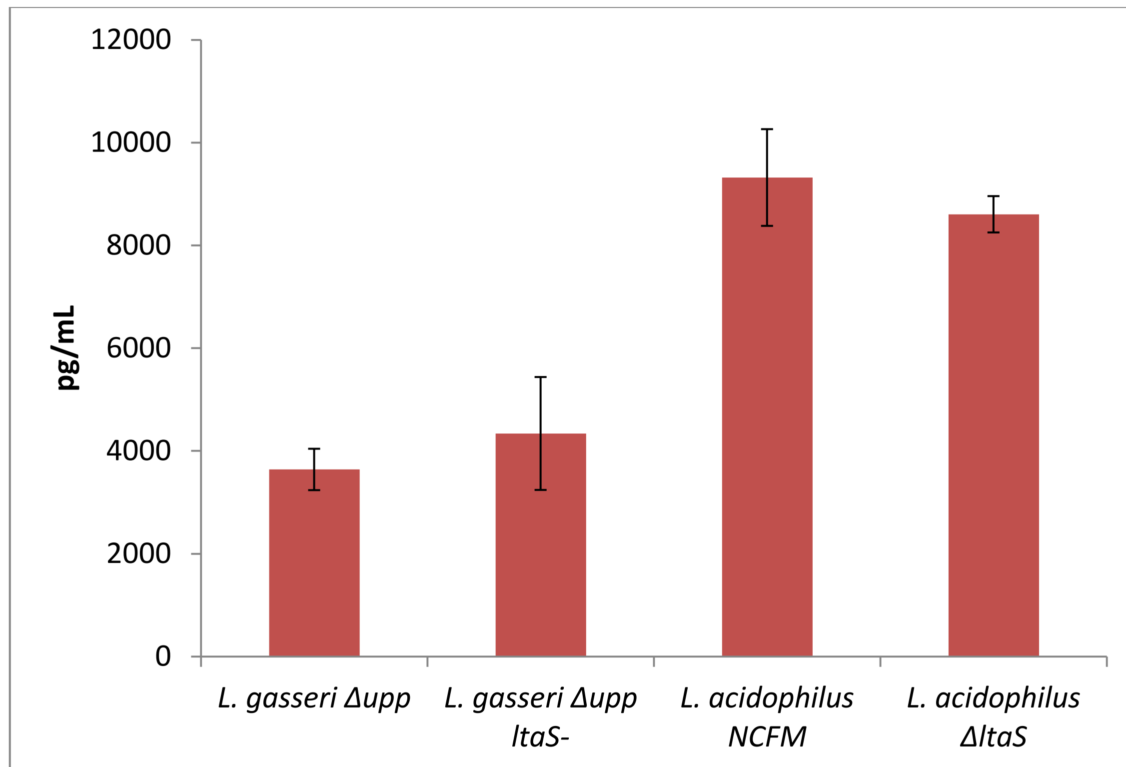


Figure 9. Quantification of IL-6 elicited from Balb/c murine iDCs upon incubation with *ltaS* deficient lactobacilli by ELISA. Data were normalized by subtracting cytokine levels secreted by DCs incubated with the media alone from the levels in the bacterial treated cells.

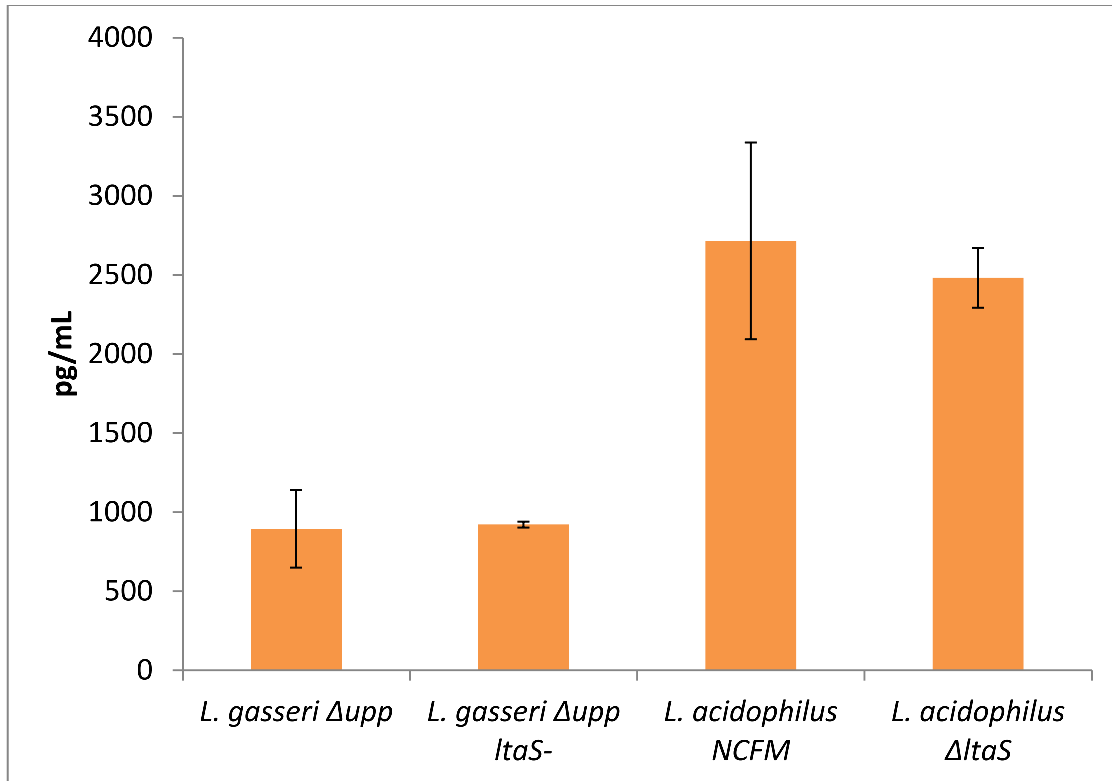


Figure 10. Quantification of TNF-α elicited from Balb/c murine iDCs upon incubation with *ltaS* deficient lactobacilli by ELISA. Data were normalized by subtracting cytokine levels secreted by DCs incubated with the media alone from the levels in the bacterial treated cells.

APPENDIX

Table A1. Primers used in this study.

Primer Name	Sequence	Use	Source
M13f	TGTAAAACGACGGCCAGT	General cloning	
M13r	TCACACAGGAAACAGCTATGAC	General cloning	
pORI28f	TTGGTTGATAATGAACTGTGCTG	General cloning	Goh
pORI28r	TTGTTGTTTTTATGATTACAAAGTGA	General cloning	Goh
repAf	TTGGGCGTATCTATGGCTGT	Integration screen	Goh
repAr	CTGATAATTGCCCTCAAACCA	Integration screen	Goh
Erm323	CAAAACGCTCATTGGCATTA	Integration screen	Goh
Erm615	TTCCTGAGCCGATTTCAAAG	Integration screen	Goh
1245-SOE-1	CAGCAGGGATCCGATTTATCAGTAGAAACGCCTACTG	Create deletion construct	This study
1245-SOE-2	AACGGTAAACTTACCCCATAATGG	Create deletion construct	This study
1245-SOE-3	CCATTATGGGTAAGTTTACCGTTGATGCAGGAGATAGATTGTTCG	Create deletion construct	This study
1245-SOE-4	CAGCAGGAGCTCGTAAAAATCCAACTGGTTTTGCAT	Create deletion construct	This study
1245-up	TGTTAATGAAGTACCAGAGCGA	Integration screen	This study
1245-dwn	GTTGTCTTGCGATACCTTCAAT	Integration screen	This study
1245-seqf	ACGCCTACTGTTCTTCGT	Sequencing	This study
1245seqr	GTGATTTGACAAGGATATGGTCAT	Sequencing	This study
1586-SOE-1	CTGCTGGGATCCGGACGCCTAAAAGCATCATAT	Create deletion construct	This study
1586-SOE-2	TCGCATTACTTTGAGCTGTTT	Create deletion construct	This study
1586-SOE-3	AAACAGCTCAAAGTAATGCGACTCTAATCATGCTTTATGGTGATC	Create deletion construct	This study
1586-SOE-4	CAGCAGGAGCTCCAATTCTGGAGCATCCGTT	Create deletion construct	This study
1586-up	CTGTGAAGCCGCAATTATTT	Integration screen	This study
1586-dwn	CATTGTGGAAGATTCCCTAC	Integration screen	This study