Molecular Interactions of Salmonella with the Host Epithelium in Presence of Commensals

Prerak T. Desai
Utah State University

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MOLECULAR INTERACTIONS OF \textit{SALMONELLA} WITH THE HOST EPITHELIUM
IN THE PRESENCE OF COMMENSALS

by

Prerak T. Desai

A dissertation submitted in partial fulfillment
of the requirements for the degree
of
DOCTOR OF PHILOSOPHY
in
Nutrition, Dietetics and Food Sciences

Approved by:

\hspace{1cm}
Bart C. Weimer \hspace{1cm} Marie K. Walsh
Major Advisor \hspace{1cm} Co Advisor

\hspace{1cm}
Dong C. Chen \hspace{1cm} John R. Stevens
Committee Member \hspace{1cm} Committee Member

\hspace{1cm}
Gregory J. Podgorski \hspace{1cm} Byron Burnham
Committee Member \hspace{1cm} Dean of Graduate Studies

\hspace{1cm}
UTAH STATE UNIVERSITY
Logan, Utah

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ABSTRACT

Molecular Interactions of *Salmonella* with the Host Epithelium in Presence of Commensals

by

Prerak T. Desai

Utah State University, 2010

Co-Major Professors: Dr. Bart C. Weimer and Dr. Marie K. Walsh

Department: Nutrition, Dietetics, and Food Sciences

Food-borne infections are a major source of mortality and morbidity. *Salmonella* causes the highest number of Food-borne bacterial infections in the US. This work contributes towards developing strategies to control *Salmonella* by (a) defining receptors used by *Salmonella* to adhere to and invade the host epithelium; (b) developing a host receptor based rapid detection method for the pathogen in food matrix; (C) and defining mechanisms of how probiotics can help alleviate *Salmonella*-induced cell death in the host epithelium.

We developed a cell-cell crosslinking method to discover host-microbe receptors, and discovered three new receptor-ligand interactions. Interaction of *Salmonella* Ef-Tu with Hsp90 from epithelial cells mediated adhesion, while interaction of *Salmonella* Ef-Tu with two host proteins that negatively regulate membrane ruffling (myosin...
phosphatase and alpha catenin) mediated adhesion and invasion. We also showed the role of host ganglioside GM1 in mediating invasion of epithelial cells by *Salmonella*.

Further we exploited pathogen affinity for immobilized gangliosides to concentrate them out of solution and from complex food matrices for detection by qPCR. A sensitivity of \(\sim 4\) CFU/ml (3 hours) in samples without competing microflora was achieved. Samples with competing microflora had a sensitivity of \(40,000\) CFU/ml.

Next we screened several probiotic strains for pathogen exclusion potential and found that *Bifidobacterium longum* subsp. *infantis* showed the highest potential for *Salmonella enterica* subsp. *enterica* ser. Typhimurium exclusion in a caco-2 cell culture model. *B. infantis* shared its binding specificity to ganglioside GM1 with *S. ser.* Typhimurium. Further, *B. infantis* completely inhibited *Salmonella*-induced caspase 8 and caspase 9 activity in intestinal epithelial cells. *B. infantis* also reduced the basal caspase 9 and caspase 3/7 activity in epithelial cells in absence of the pathogen. Western blots and gene expression profiling of epithelial cells revealed that the decreased caspase activation was concomitant with increased phosphorylation of pro-survival protein kinase Akt, increased expression of caspase inhibiting protein cIAP, and decreased expression of genes involved in mitochondrion organization, biogenesis and reactive oxygen species metabolic processes. Hence, *B. infantis* exerted its protective effects by repression of mitochondrial cell death pathway which was induced in the presence of *S. ser.* Typhimurium.
ACKNOWLEDGMENTS

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I am also thankful to my invaluable network of supportive, forgiving, generous, and loving friends. Special thanks to Harshil, Bhavik, Shounak, and Sweta. Many others are not listed here for the lack of space but I truly acknowledge their support.

Above all I dedicate the realization of this endeavor to my parents, Mr. Tushar Desai and Mrs. Priti Desai.

Prerak T Desai
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<td>DMEM</td>
<td>Dubelco Modified Eagle Minimum Essential Media</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>DTT</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<td>HGT-DB</td>
<td>Horizontal Gene Transfer Database</td>
</tr>
<tr>
<td>HMO</td>
<td>Human Milk Oligosaccharides</td>
</tr>
<tr>
<td>IBS</td>
<td>Inflammatory Bowel Syndrome</td>
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<tr>
<td>IEF</td>
<td>Iso-Electric Focusing</td>
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<td>Integrated Microbial Genomes</td>
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<td>LC</td>
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<td>Nicotinamide Adenine Dinucleotide (Oxidized)</td>
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<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide (Reduced)</td>
</tr>
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<td>NARMS</td>
<td>National Antimicrobial Resistance Monitoring System</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroysuccinimide</td>
</tr>
<tr>
<td>NTS</td>
<td>Non-Typhoidal <em>Salmonella</em></td>
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<td>PANP</td>
<td>Presence Absence Calls With Negative Probesets</td>
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<td>Relative Fluorescence Unit</td>
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<td>Relative Luminescence Unit</td>
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<td>SCV</td>
<td><em>Salmonella</em> Containing Vacuole</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<tr>
<td>SEM</td>
<td>Standard Error Of Mean</td>
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<td>SPI</td>
<td><em>Salmonella</em> Pathogenicity Island</td>
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<td>Type 4 Secretion System</td>
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<td>TCA</td>
<td>Tri Carboxylic Acid</td>
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<td>TER</td>
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<td>TES</td>
<td>N-(Tris(Hydroxymethyl)Methyl)-2-Aminoethanesulfonic Acid</td>
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<td>Viable But Non Culturable</td>
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<td>VFDB</td>
<td>Virulence Factors Of Pathogenic Bacteria Database</td>
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CHAPTER I

INTRODUCTION

Enteric bacterial infections cause an estimated 2 million deaths worldwide every year (9). In the U.S. infectious food-borne illnesses cause approximately 76 million cases, 325,000 hospitalizations, and 5,000 deaths annually (8). The total economic burden related to hospitalizations is ~$3 billion and the cost due to lost productivity is estimated to be between $20 billion and $40 billion annually (8). The economic burden due to Salmonella alone in the U.S. is an estimated $2.8 billion (3). Unfortunately, implementation of regulations aimed at reduction and prevention has made little impact in the overall occurrence of Food-borne diseases (1). This places renewed importance on understanding host-microbe interactions to define new methods of control to reduce and prevent the ever-increasing number of Food-borne illnesses.

In the last 12 years, due to efforts undertaken broadly to reduce the incidence of Food-borne illnesses, the relative rates for shiga-toxin producing Escherichia coli (H7:O157), Listeria, and Campylobacter infections declined but those due to Salmonella remained constant (1). In combination, Salmonella, Shigella and Campylobacter cause the vast majority of food associated infectious diseases with these three genera accounting for ~90% of Food-borne infections in 2008 (1). In 2008 Salmonella caused the highest morbidity for all reported bacterial Food-borne illnesses in the U.S. (1). In fact, the health risk related to Salmonella has worsened because of the emergence of antibiotic resistant strains in animals and humans (2, 6). Researchers are aggressively seeking new strategies that circumvent the problem of drug resistance in bacteria with
rejuvenation of programs to discover novel antimicrobials or by using strategies to improve the efficacy of the antibiotics in use. Unfortunately, continued emergence of drug resistant strains and an alarming increase in multi-drug resistant strains of infectious Food-borne disease-causing bacteria warrants increasing effort to search for alternatives to antimicrobial chemicals. Examples of this strategy include anti-virulence therapies (5), anti-adhesive therapy (7, 10), and the use of probiotic bacteria as dietary supplements (4). These approaches result in additional therapeutic options for use of multiple target molecules to intervene at key steps of host/microbe association. An emerging concept is blocking adhesion or providing alternate surfaces that mimic the host cell as a decoy to block disease initiation, thereby blocking disease progression. The success of this approach requires understanding the molecular events at the host microbe interface and how to modulate the initial interaction between pathogens and the host. Devising alternative therapies requires a detailed understanding of the infection process beginning with bacterial-gut association (adhesion) to invasion (host signal transduction routes) to spread (shedding). This study seeks to discover new adhesin molecules used by *Salmonella* for adherence to human gut epithelial cells to begin development of alternate strategies that block disease initiation.

**References**


Salmonella causes food- and water-borne disease around the world. In the U.S. it is transmitted by many types of food, including animal and vegetable sources and is the highest cause of morbidity and mortality in human due to Food-borne infections (Figure 2.1). One reason for host diversity is the ability of Salmonella to adapt uniquely to specific environments and hosts causing an invasive disease. Additionally, Salmonella adapts to a host without causing disease so that an asymptomatic and persistent carrier state develops. This effectively creates a mechanism to consistently spread among populations. Shedding occurs in chickens, pigs, and humans. Consequently, much of the food supply is contaminated with an organism that is quickly transmitted between humans and animals, rapidly becomes multi-drug resistant, readily adapts to new environments, mutates quickly to form new serotypes, forms an asymptomatic carrier state, and is shed after disease symptoms disappear. Therefore, we selected Salmonella to determine the effect of specific macromolecules and other bacteria in the food supply that can mitigate specific host/microbe and microbe/microbe interactions to reduce infection rates. Understanding the molecular interactions during host adherence of Salmonella will provide new insights into an important Food-borne disease and provide many target molecules for use in prevention and intervention of disease.
Salmonella diversity and infection

*Salmonella* is a pathogen of humans and animals, but the pathogenicity of different serotypes with different hosts is not defined well or predictable (Table 2.1). Taxonomic classification of *Salmonella* has evolved continuously over the years. As new molecular technologies are used to differentiate microbes they are being applied to *Salmonella* with the goal of developing consistent and correlative predictors of host/microbe interactions and infections. Unfortunately, even within the most commonly isolated serotypes, many questions remain unanswered that define specific serotype diversity and interactions with the host. Using molecular taxonomic methods, *Salmonella* is divided into only two species - *Salmonella enterica* and *Salmonella bongori*. Foodborne illness is almost entirely caused by serotypes within the *S. enterica* species (1). This fact motivates extensive characterization of the large number of food and human isolates to define specific serotypes to define predictive associations. It is especially important in outbreak investigations, which led to multiple systems of identification beyond 16s DNA typing that include bacteriophage typing, serotyping, metabolic fingerprints, and genetic fingerprints.

Unfortunately, strain differentiation with serotyping is limited in predictive value in non-typhoidal serovars. Hence, there is a need to develop a more refined strategy to characterize and classify different *Salmonella* isolates using more specific genomics-based tools to give predictive information about their host range and physiology for disease or persistence. One emerging option is to utilize comparative genomics based on the 16 finished genome sequences, 21 draft sequences, and the 42 more genome sequences in progress. Comparative genomic hybridizations (CGH) using *S. enterica* ser.
Typhimurium LT2 and another 22 Salmonella members, including all six subspecies of S. enterica and S. bongori, found broad concordance with phylogenomic studies using 16S DNA (68). This research also noted specific genes that changed during the evolution of the groups. With so much sequence information we have the opportunity to harness the power of phylogenomics to unequivocally address the question of Salmonella phylogenetic diversity, host adaptation, and mechanisms of survival in many different environments. This approach promises to link the specific genes or gene clusters to specific Salmonella serotypes and host parameters that enable establishment of a mutualistic partnership that leads to shedding and disease spread.

**Salmonella and antibiotics**

In humans, Salmonella-induced gastroenteritis is usually a self-limiting disease, but in immunocompromised individuals and infants some of the non-typhoidal serovars can cause bacterimia, which requires use of antibiotics. Resistance to antibiotics remains a significant problem in many of the Salmonella isolates. This can be attributed to persistence of Salmonella under constant antibiotic pressure due to indiscriminate use of antibiotics in animal feed coupled to a highly mutable genome (58). According to the National Antimicrobial Resistance Monitoring System (NARMS), in 2005, S. ser. Typhimurium was the most frequently encountered serotype, accounting for 21.3% of Salmonella isolates in the NARMS surveillance program (16). Of all the isolates tested, 34.8% were resistance to at least one of the antibiotics used for clinical salmonellosis. A greater cause of concern though is the prevalence of multidrug resistant isolates; 22.2% of the isolates were resistant to five different antibiotics (ampicillin, chloramphenicol,
streptomycin, sulfamethoxazole, and tetracycline) (16). This problem demands investment in research to define alternative strategies for management of *Salmonella* and the use antibiotics as a last resort.

**Salmonella host adaptation**

Out of the nearly 2,600 known serovars, ~50 serovars of *S. enterica* subsp. *enterica* account for >99.5% of all clinical isolates of *Salmonella* from humans and animals (66). In spite of the predominance of *S. enterica* subsp. *enterica* serotypes, the correlation between serotype, host, and disease is not well established. It is common to find that the same serovar causes different disease symptoms in different hosts. Based on their host specificity *Salmonella* serovars can be loosely categorized into two groups (44). Members of the first group colonize a narrow range of hosts (commonly known as host-adapted serovars) to cause an acute typhoid-like disease (44). Cross-infection of these serovars into other non-host adapted species is possible, but the disease is usually not as invasive as in its primary host (44). Examples of host-adapted serotypes include *S. enterica* ser. Dublin in cattle, *S. enterica* ser. Typhi in humans, *S. enterica* ser. Gallinarum in chickens, and *S. enterica* ser. Choleraesuis in pigs (44). The second group is comprised of *S. enterica* subsp. *enterica* that infect and persist in a broad range of hosts (commonly known as non-host adapted serovars). These cause non-typhoidal gastroenteritis. The most common food-associated examples are *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium, which are found in many food types and host species. *S. enterica* ser. Enteritidis is commonly associated with chickens while *S. enterica* ser. Typhimurium infects humans, cattle, swine, sheep, horses, rodents, chicken, turkeys,
ducks, pigeons, and finches. They are commonly transmitted to humans via direct interaction with the animal or consumption of the meat. Cross contamination of food animal products to water or vegetables is also a source of animal-associated *Salmonella*.

An increasing problem for food safety and public health is the zoonotic transmission of non-host adapted serovars between an animal that is an asymptomatic carrier to food and subsequently to humans that results in an outbreak of gastroenteritis. The carrier state may occur in humans as well as animals. In a *Salmonella*-infected host, after acute salmonellosis is resolved, the organism may persist asymptotically to be shed in the feces for months after the disease symptoms have ceased. In humans ~3% of the people infected with typhoid-causing serovars and ~0.1% of people infected with gastroenteritis-causing serovars become chronic carriers (51). The existence of carrier states in food animals is of particular concern, as *Salmonella* can directly enter the food chain and cause further cross contamination. The exact molecular mechanisms of how *Salmonella* persists in the carrier state are not known for animals or humans, but in some animal models fecal shedding is positively correlated with the prophylactic use of antibiotics (56). It may be due in part to an altered gut microflora composition caused by antibiotic treatment.

**Salmonellosis in humans**

*S. enterica* causes two distinct diseases in humans - typhoid-fever (enteric fever) and non-typhoidal gastroenteritis (Figure 2.2)
Enteric fever

The disease in humans is characterized by invasion of Salmonella through the small intestine before further spread into the liver, spleen, gall bladder, and bloodstream. There are four host-adapted serovars in S. enterica subsp. enterica (Typhi, Paratyphi A, Paratyphi B, Paratyphi C) that cause enteric fever in humans. They account for ~2% of the total Salmonella isolates from human salmonellosis (17). There are no other known hosts, except humans and higher primates, for these four serovars (61).

Untreated enteric fever has a mortality rate of 12-30% while antibiotic-treated cases have ~1% mortality. The organism is usually transmitted through the fecal/oral route and through poor sanitary practices. There are two commercially available vaccines for typhoid fever in humans. The first vaccine is a live attenuated strain of serovar Typhi, while the other is a preparation of the capsular Vi antigen. Both the vaccines have a reported ~55% efficacy (35) at the 3-year interval. Clinical trials of a third, but unlicensed vaccine (rEPA-Vi conjugate), have shown a comparative increase in efficiency (~89%) and it confers immunity for a longer period (35). Since the only source of infection is an infected person, spread of the organism is controlled with antibiotics and good hygiene to prevent cross contamination. Enteric fever is not common in industrialized regions like the US, but travelers to Asia, Africa, and Latin America are at risk.

Non-typhoidal gastroenteritis

Salmonella-associated gastroenteritis is a common food safety concern. It is characterized by nausea, cramping abdominal pain followed by diarrhea, fever, and
sometimes vomiting. The infection is usually restricted to inflammation of the intestine, but complications can arise in at risk populations, such as infants, the elderly and immunocompromised patients. The infective dose for gastroenteritis causing serovars varies from $10^4$-10$^6$ cells (12). Various factors influencing infective dose are serovar, the delivery matrix of the organism and various host specific factors (e.g., age and immune status) (12). The infective dose of *Salmonella* in a food matrix containing high fat or protein (e.g., cheese, meat) is significantly lower than those containing low or no fat or protein (e.g., water, vegetables) (12, 23). Although people from all age groups are susceptible to infection with *Salmonella*, the incidence of salmonellosis in children < 4 years of age is seven times higher compared to healthy adults (1) (Figure 2.1).

The overwhelming majority (roughly 98%) of human clinical isolates of *Salmonella* cause non-typhoidal gastroenteritis. The range of hosts for gastroenteritis-causing *Salmonella* varies from warm-blooded (swine, human, cattle, poultry, rodents) to cold-blooded (reptiles, amphibians) animals. *S. ser.* Typhimurium is the most dominant serovar causing illness in humans and animals, accounting for approximately 17% of the human clinical isolates and 20% of non-human isolates (17). One reason for the high prevalence of *S. ser.* Typhimurium is that it colonizes a broad range of hosts - humans, cattle, poultry, sheep, pigs, horses, and wild rodents (7). The second most isolated serovar is *S. ser.* Enteritidis, which accounts for 16% of human clinical isolates and 3% of non-human isolates (17). No effective vaccine exists for humans against *Salmonella*-causing gastroenteritis.
Molecular mechanisms of Salmonella-induced gastroenteritis

Non-typhoidal salmonellosis in humans is characterized by rapid loss of fluid and electrolytes, abdominal cramps and in few cases vomiting and fever. The main stages of Salmonella infections are adhesion to the host mucosa, invasion of the host mucosa, intracellular replication, and dissemination back into the environment via fecal shedding (Figure 2.3).

The primary symptom of non-typhoidal gastroenteritis is dehydration due to passive loss of protein-rich fluid, and a decreased ability to absorb the lost fluids because of injury to the mucosal lining caused by acute inflammation (63). In some cases that are untreated or in at risk populations death results from dehydration, not the outright infection. Since adhesion and invasion of the host mucosa are the required steps for progression of the disease, intervention at these stages is an attractive therapeutic strategy, especially since antibiotic resistance is increasing.

Adhesion to the host

Adhesion of Salmonella, like other microbial pathogens, to a specific structure on the epithelial surface is the first and necessary step of host colonization (78). Several in vivo studies have defined the importance of adhesion in the infection process of various pathogens. For Salmonella in particular, Sugita-Konishi et al. (80) used a mouse model to demonstrate that preventing Salmonella from adhering to the host mucosa significantly reduced the amount of Salmonella recovered from the spleen. Clinical studies with cranberry juice, an inhibitor of adhesion of uropathogenic E. coli to human epithelial cells showed that the juice significantly decreased the rate of urinary tract infections in
women (49). These and many other in vivo studies (2, 77, 78) have unequivocally established the importance of bacterial adherence in disease progression. A detailed understanding of bacterial adhesins and the cognate host receptors will lead to new therapies that prevent bacterial adhesion, thereby averting disease. Since a single bacterium utilizes many different molecules to bind the host, a thorough characterization of all the bacterial adhesins is required to realize the full potential of anti-adhesive therapies.

Salmonella expresses a variety of adhesins that can be categorized into fimbrial and afimbrial adhesins. Five major types of fimbrial adhesins that mediate adhesion to epithelial cells are defined: 1) thin aggregative fimbriae, 2) long polar fimbriae, 3) plasmid encoded fimbriae, 4) Type I fimbriae, and 5) π-fimbriae. Each fimbrial type uses a slightly different host molecule for adherence to the host cell (Table 2.2)

Thin aggregative fimbriae are encoded by the csg operon and are essential for virulence of Salmonella in the mouse model (81). In vitro, thin aggregative fimbriae of S. ser. Enteritidis bind fibronectin, but the in vivo relevance of this interaction is unknown (21). In mice, csgA is a pathogen-associated molecular pattern (PAMP) that binds TLR-2 (82), which subsequently leads to an inflammatory response by the host.

Long polar fimbriae in Salmonella are encoded by the lpfABCDE operon and mediate attachment of Salmonella to murine Paeyer’s patches, but not to villous enterocytes (8). Deletion mutants of lpfC alone had minimal effect on virulence in mice, as did single gene deletion mutants of invA (necessary for invasion). However, double deletions led to a 150-fold increase in oral LD\textsubscript{50} as compared to the wild type strain or the single deletion mutants (8, 64).
Another fimbrial operon in *Salmonella*’s repertoire is the *pefBACD* operon that is contained on the virulence plasmid. This operon mediates adhesion to the mouse small intestine and is also important to induce the inflammatory response in the host (6).

Plasmid-encoded fimbriae specifically bind the Lewis X blood group antigen in vitro (18). The in vivo significance of this observation remains to be determined; however, it suggests that human hosts are susceptible based on their blood-type, much like found in *Helicobacter pylori* infections (72). Type I fimbriae modulate the adherence of *Salmonella* to human colon carcinoma cells (HT-29) through mannosylated oligosaccharides on the cell surface (48), which provides further evidence for the role of the host glycan status for *Salmonella* infection. Type I fimbriae are encoded by the *fimAICDHF* operon with the *fimH* subunit on the tip of the fimbrial shaft providing binding specificity to the host glycan.

The π-fimbriae are encoded by the *stdABC* operon. The *stdA* gene mediates attachment of *Salmonella* to α-1-2 fucosylated receptor(s) localized in the mucus layer of the murine cecum (19). It is speculated that in vivo *stdA*-mediated binding to the mucus layer gives a competitive advantage during colonization of the mouse cecum. Hence, *Salmonella* fimbrial adhesins act like lectins that bind various terminal sugar residues on the host cell surface which serves two purposes: 1) to help the organism colonize host’s gut and 2) to bring the organism close enough to the host cell surface so that it can invade via other proteins on the host and the microbe.

Recently a few non-fimbrial adhesins and components of the Type III secretion system (T3SS) were implicated to mediate adhesion of *Salmonella* to the host mucosa (Table 2.2). *Salmonella* contains two T3SS loci; T3SS-1 is associated with invasion of
the host mucosa, while T3SS-2 is associated with survival inside host cells. Surprisingly, three translocon components of the T3SS-SP1 (sipB, sipC, sipD) mediate intimate attachment of the organism to epithelial cells in vitro (55). The cognate host receptors for these T3SS molecules are unknown.

Two auto transporters (shdA and misL) bind fibronectin. Human intestinal epithelial cell expression of both these proteins in vitro correlated with increased adhesion and increased invasion of Salmonella (27, 47). Binding to extracellular matrix (ECM) components by enteric pathogens is proposed to serve two functions for pathogens. One advantage is to colonize damaged host tissue where the ECM components are freely accessible. The second function is that binding ECM components, like fibronectin, allows the pathogen to modulate the host cytoskeleton via integrins. This leads to gap junction disruption that opens direct access to the basement membrane and the circulatory system (50, 75). Salmonella uses several receptors to colonize the host via different classes of molecules, which again demonstrates the significance of adhesion in disease. Table 2.2 summaries the known adhesins that mediate attachment of Salmonella to the host mucosal epithelial molecules. The list of molecules implicated as adhesins contains Salmonella adhesins that bind sugar moieties on the host cell surface. These are all associated with fimbriae. Another series of molecules uses other receptors. Location of the sugar moieties it is not entirely clear and no assessment of adhesion with sugars that are part of glycans, glycoproteins, or glycolipids is available.

While a short list of adhesins is known for Salmonella, in many cases the host receptor is unclear. This limits our understanding of the molecular events following adhesion. For example, the plasmid-encoded fimbrial protein (PefC) is important for
adhesion, but the cognate host receptor is unknown. This lack of information is crucial for deciphering the signal transduction events initiated at the host-microbe interface that lead to the disease symptoms via membrane protein signaling. One approach is to delete the genes needed for invasion. In this light, incubation of human epithelial cells with adherent but non-invasive mutants of *Salmonella* leads to an increase in NFkB activation via a signal transduction route initiated from an undescribed receptor (30). Hence, there are yet unidentified T3SS-independent mechanisms that lead to modulation of inflammatory pathways that are likely therapeutic targets. Additional research to characterize the receptors of host *Salmonella* interactions is definitely warranted and will lead to new therapeutic strategies as well as defining new mechanisms of disease initiation.

*Invasion of the host mucosa*

The ability of *Salmonella* to induce its uptake within non-phagocytic epithelial cells is central to its pathogenicity (60). The second step in the infection process is the invasion of enterocytes. Internalization of *Salmonella* is rapid, typically occurring within 15-30 minutes of bacterial contact with the host cell (60). The whole process resembles macropinocytosis (34, 60) and progresses with direct injection of bacterially produced effector molecules through the T3SS into the host cytosol. This manipulates the cytoskeleton via actin filaments causing formation of membrane ruffles among other effects (Figure 2.4). The internalized bacteria remain enclosed within a special membrane-bound vacuole (*Salmonella*-containing vacuole (SCV)). *Salmonella* also interferes with the host’s intravessicular trafficking machinery via a different set of T3SS
effectors to prevent fusion of SCVs to lysosomal compartments. This bacterially directed maturation of SCV leads to the formation of a protected intracellular niche permissive of bacterial replication and persistence (28).

**T3SS Effectors and modulation of the host actin cytoskeleton.** T3SS effectors are eukaryotic-like bacterial proteins that interfere with a wide range of cellular processes. These effectors are injected directly into the host cytosol by the T3SS that resembles a molecular syringe. Table 2.3 lists the characterized T3SS effectors in *Salmonella* involved in invasion and their role in the infection process. Figure 2.5 (adapted from (60)) shows the interplay of the effectors with the host molecules.

*Salmonella* modulates the host actin cytoskeleton by direct stimulation of actin nucleation and polymerization, activation of small GTPases that eventually leads to actin polymerization, and via indirect activation of GTPases via phosphoinositide metabolism (60). Two of the injected effectors proteins, SipA and SipC, bind actin and directly manipulate the host actin cytoskeleton. SipC mimics the actin nucleation activity of the endogenous Arp2/3 complex and stimulates actin assembly by bundling and cross-linking existing actin filaments (60). SipA promotes actin filament polymerization by reducing the monomer concentration necessary for filament assembly, enhancing the filament bundling activity of the host protein fimbrin. SipA also binds to the assembled filaments and prevents ADF/cofilin mediated depolymerization. Thus the synergistic activity of SipC and SipA promotes the formation of actin filaments in proximity to the attached bacteria (60).

The protrusive force required to drive the formation of the phagocytic vesicle requires formation of highly branched actin filaments. The formation of such a network is
mediated by small GTPases of the Rho family, primarily Rac and Cdc42. Two of the
Salmonella effectors, SopE and SopE2, act as guanine nucleotide exchange factors
(GEFs) that are specifically targeted towards Rac and Cdc42 to facilitate the exchange of
GDP to GTP in the GTPases stimulating Rac and Cdc42 activity. Once activated Rac and
Cdc42 stimulate downstream effectors leading to actin assembly (60).

The injected effector protein SopB (also annotated as SopD in some publications)
indirectly stimulates RhoG activity that leads to hydrolysis of phosphoinositides (28).
SopB also exerts inositol phosphatase activity on the intermediates in the phosphoinositol
conversion pathway changing the conversion of PI(3,4)P₂, PI(3,4,5)P₃, PI(3,5)P₂, and
PI(4,5)P₂ (28). The exact mechanism of how RhoG is activated is under investigation, but
it is proposed that modulation of the phosphoinositide concentration recruits endogenous
RhoG GEF, SGEF, which leads to activation of RhoG (28). RhoG activates Rac through
Rac’s GEF which eventually leads to actin polymerization (60).

The next step in invasion is phagosome closure. The PI(4,5)P₂ hydrolyzing
activity of SopB leads to reduction of PI(4,5)P₂ in the vicinity of the attached bacteria. It
is hypotized that the local reduction in the PI(4,5)P₂ levels leads to dissociation of cortical
actin from the nascent phagosome resulting in phagosome closure (60).

Shortly after bacterial entry the host cytoskeleton is remodeled to its initial state.
This phenomenon is pronounced in polarized epithelial cells where apical microvilli are
completely reassembled after invasion. This is achieved by another injected effector SptP
that acts like an endogenous Rho GAP inactivating the previously activated Rac and
Cdc42 (by SopE and SopE2) (60).
Once inside the SCV the bacterium prevents fusion of the SCV membrane with the lysosomal compartment to avoid intracellular clearance by the host lytic enzymes and the immune system. The exact mechanisms of this process are not known, but this phenomenon is mediated by T3SS effectors from *Salmonella* pathogenicity island-2 (SPI-2) that are secreted in the host cytoplasm across the SCV membrane.

Invasion of the host mucosa by *Salmonella* results in increased expression of chemotactic factors by the host. This results in infiltration of neutrophils into the lamina propria (74). As the inflammatory reaction progresses, neutrophils migrate through the epithelial layer with the accumulation of inflammatory cells. Protein-rich fluid is secreted into the intestinal lumen which manifests itself as diarrhea (74).

**Novel therapeutic and prophylactic approaches for control of *Salmonella***

Since adhesion of bacteria to the host initiates the infection process, intervening at that step represents an ideal strategy for disease prevention. *Salmonella* is capable of binding via multiple adhesins (Table 2.2); hence, it may be difficult to develop a universal class of anti-adherence compounds unless common interacting molecules can be found. To fully harness the potential of this approach, extensive characterization of adhesin/receptor affinity and specificity are needed. Various sugar moieties (mannose, fucose, sialic acid, galactose) are important for fimbrial adhesion. Consequently, they are immediate targets for discovery and development. The efficacy of anti-adhesive therapy is demonstrated in animal models where administration of specific oligosaccharides protects the animal from various enteric infections (Table 2.4). The efficacy of various sugar moieties for pathogen blocking in animal models is summarized in Table 2.4.
Interestingly, human breast milk contains oligosaccharides that are similar to cell surface carbohydrates, and some in vitro studies demonstrate their efficacy in disease prevention for some populations (52). To fully realize the potential of this approach, a more thorough characterization of adhesins is required.

The concept of anti-adhesive compounds can be extended to non-carbohydrate adhesion-inhibitory agents like antibodies. The efficacy of this approach is demonstrated in an *E. coli*-mouse infection model to block specific receptors (54). The central concept is that microbial adhesins make ideal vaccine candidates. Langemann et al. (54) showed that immunization of mice with the *E. coli* FimH adhesin reduced in vivo colonization by more than 99%. Furthermore, passive systemic administration of immune sera to FimH also resulted in reduced bladder colonization by uropathogenic *E. coli* (54). Hence, characterization of *Salmonella* adhesins could also provide viable targets for the elusive vaccine against gastroenteritis causing serovars.

The normal gut flora forms the first line of defense against enteric infections (79). The composition of this protective flora can be altered by dietary and environmental influences making the host susceptible to disease. Disturbance in the normal microflora due to pre-treatment of mice with antibiotics makes them more susceptible to *Salmonella* infection (22). The same study also showed that the total number of bacteria in the gut was not altered after pre-treatment with antibiotics, but rather the altered composition of the gut flora was responsible for the increased susceptibility to *Salmonella* (22). The use of probiotics centers on the idea that probiotic treatments re-establish the natural condition which excised in the host before being disrupted by various stresses (36). Many studies have linked prophylactic treatment with probiotics to reduced rate of enteric
infections. A study in the pig model showed that feeding a five-strain probiotic mixture that contained two strains of *Lactobacillus murinus*, and one strain each of *Lactobacillus salivarius*, *Lactobacillus pentosus* and *Pediococcus pentosaceous* reduced pathogen shedding and alleviated disease symptoms when animals were infected with *S. ser.* Typhimurium (15). Although the specific mechanism of the pathogen reduction was not elucidated, this study suggests that probiotics can inhibit infection and reduce symptoms. Broadly, the mechanisms of probiotics to directly influence the pathogen include competing for nutrients, production of antimicrobials, and pathogen exclusion at the host surface (57).

The pathogen exclusion potential of various probiotics is dependent on the probiotic as well as the pathogen strain. Jin et al. (45) showed that in vitro *L. acidophilus* inhibited adhesion of *S. sv* Pullorum, but failed to inhibit adhesion of *S. sv* Enteritidis and *S. ser.* Typhimurium. A recent study in humans correlated the decreased number of *Bifidobacterium* in the gut to acute salmonellosis (14). A study in the cell culture model has also demonstrated the anti-adhesive effect of *Bifidobacterium* on *S. ser.* Typhimurium (10). The ability of *Bifidobacteria* to inhibit pathogen attachment is strain dependent and independent of *Bifidobacteria*’s adhesion properties; an adherent bifidobacterial strain does not guarantee pathogen exclusion (11). The mechanisms of adhesion of *Bifidobacteria* to the host are undefined. There is only one study that implicates a solute binding lipoprotein (BopA) (41, 42) in adhesion of *Bifidobacterium* to caco-2 epithelial cells in vitro (Table 2.5). The cognate receptor on the host is unknown. The exact mechanisms of pathogen exclusion by *Bifidobacteria* are not yet characterized.
Another well documented mode of probiotic action against enteric pathogens is the production of various antimicrobial compounds such as H₂O₂, lactic acid and bacteriocins (57). For example, the strong antimicrobial activity of *L. rhamnosus* GG against *S. ser. Typhimurium* was due to the accumulation of lactic acid (24). Pridmore et al. (70) described an in vitro role of H₂O₂ in the anti-*Salmonella* activity of *L. johnsonni*. Lievin et al. showed that two strains of *Bifidobacterium* isolated from infant gut produced a yet unidentified antimicrobial substance that exerted antimicrobial effect against *S. ser. Typhimurium* in vitro, inhibited cell entry, and killed intracellular *S. ser. Typhimurium* using Caco-2 cells (59).

At the host mucosa there is a complex multi-way interaction between host, pathogen and the resident gut flora that dictates the outcome of the infection. Most studies until now have been dedicated to unravel the effect of probiotics/commensals/pathogen on the host while the literature on the microbe-microbe interaction is sparse at best. Recently there has been a steady increase in reports of intraspecies communication that affect the virulence gene expression of pathogens. For example, short chain fatty acids like acetate, propionate and butyrate induce the expression of hilA (invasion protein transcription activator), which positively regulates virulence gene expression in *Salmonella* (29). Even et al. (33) demonstrated that mixed cultures of *Lactococcus lactis* negatively regulated the virulence genes of *Staphylococcus aureus*. They did not investigate the in vivo relevance of the observation, but the in vitro results open a new dimension of looking at the microbe-microbe interactions.

Among all age groups the incidence of *Salmonella*-induced diarrhea is highest in infants (17). *Bifidobacterium* forms the most abundant genera in breast fed infants (53),
and since they show anti-infective activity towards *Salmonella*, the molecular mechanism by which they inhibit pathogen adhesion and invasion needs to be investigated. The effect of *Bifidobacterium* on gene expression of *Salmonella* and how this influences infectivity needs to be investigated.
Hypothesis and objectives

*B. longum* subspecies. *infantis* reduces *S. ser.* Typhimurium adhesion to gut epithelial cells by competing for the same receptors and also improves cell survival by modulation of specific pathways in the host. These hypotheses will be tested via the following objectives:

**Objective 1.** Define the pathogen exclusion potential of selected probiotic bacteria to gut epithelium in vitro.

**Objective 2.** Define the binding partners of the host-bacteria interaction

**Objective 3.** Identify putative signal transduction and metabolic pathways modulated in the host and the microbes using caco-2 cells in vitro with adhesion of *B. infantis* and *S. ser.* Typhimurium.

References


83. **Yoshio Ishibashi, and Toshihiko Arai.** 2006. Roles of the complement receptor type 1 (CR1) and type 3 (CR3) on phagocytosis and subsequent phagosome-lysosome fusion in *Salmonella*-infected murine macrophages. FEMS Microbiology Letters 64:89-96.
Table 2.1 Number of serovars present in each species of *Salmonella* (66).

<table>
<thead>
<tr>
<th><em>Salmonella</em> species</th>
<th>Number of serovars</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. enterica</em></td>
<td>2,557</td>
</tr>
<tr>
<td><em>S. enterica</em> subsp. <em>enterica</em></td>
<td>1,531</td>
</tr>
<tr>
<td><em>S. enterica</em> subsp. <em>salamae</em></td>
<td>505</td>
</tr>
<tr>
<td><em>S. enterica</em> subsp. <em>arizonae</em></td>
<td>99</td>
</tr>
<tr>
<td><em>S. enterica</em> subsp. <em>diarizonae</em></td>
<td>336</td>
</tr>
<tr>
<td><em>S. enterica</em> subsp. <em>houtenae</em></td>
<td>73</td>
</tr>
<tr>
<td><em>S. enterica</em> subsp. <em>indica</em></td>
<td>13</td>
</tr>
<tr>
<td><em>S. bongori</em></td>
<td>22</td>
</tr>
<tr>
<td>Total (genus <em>Salmonella</em>)</td>
<td>2,579</td>
</tr>
</tbody>
</table>
Table 2.2 Adhesins used by *Salmonella* and their cognate partners on the host for adherence to the host mucosal epithelium.

<table>
<thead>
<tr>
<th><em>Salmonella</em> adhesin molecule</th>
<th>Cognate Host receptor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fimbrial adhesins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid encoded fimbriae (<em>pefC</em>)</td>
<td>Unknown</td>
<td>(6)</td>
</tr>
<tr>
<td>Plasmid encoded fimbriae (<em>pefA</em>)</td>
<td>Galβ1–4(Fucα1–3)GlcNAc</td>
<td>(18)</td>
</tr>
<tr>
<td>Fimbrial Adhesin (<em>stdA</em>)</td>
<td>Fucα1-2Galβ1-4GlcNAc</td>
<td>(19)</td>
</tr>
<tr>
<td>Long Polar fimbriae (<em>lpf</em> operon)</td>
<td>Unknown</td>
<td>(8)</td>
</tr>
<tr>
<td>Thin aggregative fimbriae (<em>agfA</em>)</td>
<td>Unknown</td>
<td>(81)</td>
</tr>
<tr>
<td>Type-1 Fimbriae (<em>fimH</em>)</td>
<td>Mannose moieties on glycoconjugates</td>
<td>(43)</td>
</tr>
<tr>
<td>Thin curled fimbriae (<em>csgA</em>)</td>
<td>Toll-like receptor 2 (TLR2)</td>
<td>(82)</td>
</tr>
<tr>
<td><strong>Non-fimbrial adhesins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-fimbrial adhesin (<em>siiE</em>)</td>
<td>Unknown</td>
<td>(37)</td>
</tr>
<tr>
<td>Auto-transporter like protein (<em>shdA</em>)</td>
<td>Fibronecin</td>
<td>(47)</td>
</tr>
<tr>
<td>Autotransporter (<em>misL</em>)</td>
<td>Fibronecin</td>
<td>(27)</td>
</tr>
<tr>
<td>66-kDA Heat Shock Protein</td>
<td>Mucin</td>
<td>(32)</td>
</tr>
<tr>
<td>Murien Lipoprotein (<em>lppA, lppB</em>)</td>
<td>Unknown</td>
<td>(76)</td>
</tr>
<tr>
<td>Outer Membrane Protein (<em>ratB</em>)</td>
<td>Unknown</td>
<td>(46)</td>
</tr>
<tr>
<td>Intimin-like protein (<em>sinH</em>)</td>
<td>Unknown</td>
<td>(46)</td>
</tr>
<tr>
<td><strong>Type III secretion system</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein Translocase (<em>sipB</em>)</td>
<td>Unknown</td>
<td>(55)</td>
</tr>
<tr>
<td>Protein Translocase (<em>sipC</em>)</td>
<td>Unknown</td>
<td>(55)</td>
</tr>
<tr>
<td>Protein Translocase (<em>sipD</em>)</td>
<td>Unknown</td>
<td>(55)</td>
</tr>
<tr>
<td><strong>Uncharacterized</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer membrane lipoprotein (<em>invH</em>)</td>
<td>Unknown</td>
<td>(4)</td>
</tr>
<tr>
<td>Unknown</td>
<td>Mixed Gangliosides</td>
<td>(26)</td>
</tr>
<tr>
<td>Unknown</td>
<td>Cystic fibrosis transmembrane conductance regulator(^1)</td>
<td>(67)</td>
</tr>
<tr>
<td>Unknown</td>
<td>Galb(1-3)GalNAc</td>
<td>(38)</td>
</tr>
<tr>
<td>Unknown</td>
<td>Integins CR3, CR1</td>
<td>(83)</td>
</tr>
</tbody>
</table>

\(^1\) receptor for ser. Typhi, but not for ser. Typhimurium.
Table 2.3 *Salmonella* T3SS effectors that play a role in invasion of non-phagocytic cells.

<table>
<thead>
<tr>
<th>Effector protein</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SipA</td>
<td>Enhances actin filament assembly and inhibits actin disassembly</td>
<td>(60)</td>
</tr>
<tr>
<td>SipB</td>
<td>T3SS translocon component, acts like an adhesin</td>
<td>(55, 60)</td>
</tr>
<tr>
<td>SipC</td>
<td>T3SS translocon component, acts like an adhesin, promotes actin polymerization</td>
<td>(55, 60)</td>
</tr>
<tr>
<td>SipD</td>
<td>T3SS translocon component, acts like an adhesin</td>
<td>(55, 60)</td>
</tr>
<tr>
<td>SopE</td>
<td>Guanine nucleotide exchange factor (GEF) for Cdc42 and Rac; promotes assembly of branched actin filaments</td>
<td>(60)</td>
</tr>
<tr>
<td>SopE2</td>
<td>Guanine nucleotide exchange factor (GEF) for Cdc42</td>
<td>(60)</td>
</tr>
<tr>
<td>SopB/SigD</td>
<td>Phosphoinositide phosphatase; activates RhoG; Other roles suspected but not characterized</td>
<td>(60)</td>
</tr>
<tr>
<td>SptP</td>
<td>Rho GAP, functions in repression of host membrane ruffling post entry.</td>
<td>(60)</td>
</tr>
</tbody>
</table>
Table 2.4 Anti-adhesive compounds that prevent bacterial infection in vivo. (GIT-Gastrointestinal tract, UT-Urinary tract)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Site of infection</th>
<th>Anti-adhesive molecule</th>
<th>Bacterial Receptor</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. jejuni</em></td>
<td>Mice GIT</td>
<td>HMO</td>
<td>Unknown</td>
<td>(73)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Mice GIT</td>
<td>methyl alpha-D-mannopyranoside</td>
<td>Type 1 Fimbriae</td>
<td>(5)</td>
</tr>
<tr>
<td></td>
<td>Mice UT</td>
<td>Globotetraose</td>
<td>Type 1 Fimbriae</td>
<td>(31)</td>
</tr>
<tr>
<td></td>
<td>Mice GIT</td>
<td>Mannose</td>
<td>P-fimbriae</td>
<td>(39)</td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td>Pig GIT</td>
<td>Sialyl-3'-LacNAc</td>
<td>Unknown</td>
<td>(62)</td>
</tr>
<tr>
<td></td>
<td>Monkey GIT</td>
<td>Sialyl-3'-Lac</td>
<td>Unknown</td>
<td>(62)</td>
</tr>
</tbody>
</table>
Table 2.5 Known adhesins of selected probiotics

<table>
<thead>
<tr>
<th>Organism</th>
<th>Microbe Protein</th>
<th>Host receptor</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactobacillus acidophilus NCFM</strong></td>
<td>Mub</td>
<td>Mucus</td>
<td>(13)</td>
</tr>
<tr>
<td></td>
<td>SlpA</td>
<td>Unknown</td>
<td>(13)</td>
</tr>
<tr>
<td></td>
<td>Fbp</td>
<td>Fibronectin</td>
<td>(13)</td>
</tr>
<tr>
<td></td>
<td>CdpA</td>
<td>Unknown</td>
<td>(3)</td>
</tr>
<tr>
<td><strong>Lactobacillus brevis ATCC 8287</strong></td>
<td>SlpA</td>
<td>Laminin, Collagen, Fibronectin</td>
<td>(25)</td>
</tr>
<tr>
<td><strong>Lactobacillus johnsonni LA1</strong></td>
<td>EF-tu</td>
<td>Mucus</td>
<td>(40)</td>
</tr>
<tr>
<td><strong>Lactobacillus plantarum WCFS1</strong></td>
<td>GroEL</td>
<td>Mucus</td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td>Msa</td>
<td>Mucus via mannose binding</td>
<td>(69)</td>
</tr>
<tr>
<td><strong>Lactobacillus reuteri NCIB 11951</strong></td>
<td>CnBP</td>
<td>Collagen</td>
<td>(71)</td>
</tr>
<tr>
<td><strong>Lactobacillus salivarius UCC 118</strong></td>
<td>LspA</td>
<td>Collagen, Mucin</td>
<td>(20)</td>
</tr>
<tr>
<td><strong>Bifibacterium. bifidum</strong></td>
<td>BopA</td>
<td>Unknown</td>
<td>(42)</td>
</tr>
</tbody>
</table>
Figure 2.1 Reported cases of Food-borne illnesses by age (1)
Figure 2. The two types of diseases caused by Salmonella. Both are initiated at the intestine but enteric fever is characterized by spread of Salmonella to deeper tissues while gastroenteritis is limited to inflammation of the intestine (Adapted from (65)).
Figure 2. 3 Infective cycle of gastroenteritis caused by *Salmonella*. Adapted from Navaneethan et al. (63).
Figure 2. TEM showing invasion of bovine enterocytes by S. ser. Typhimurium. Arrows 1 indicates membrane-bound bacteria after engulfment. Arrow 2 highlights engulfment at the apical membrane while it is still open to the lumen (Adapted from (74)).
Figure 2. Interaction of *Salmonella* with the host through its T3SS effectors (Adapted from (60)).
CHAPTER III
IDENTIFICATION AND CHARACTERIZATION OF HOST MICROBE
RECEPTORS BY WHOLE CELL CROSSLINKING

Abstract

A cell-cell crosslinking method to discover host-microbe receptors was developed. The protocol was first applied to discover extracellular matrix (ECM) binding proteins in *Lactobacillus acidophilus*, *E. coli* and *Salmonella* ser. Typhimurium. I found new bacterial proteins crosslinked to ECM components, in addition to proteins already reported in literature. Further, I applied the protocol to crosslink *S. ser.* Typhimurium to epithelial cells, and discovered three new receptor-ligand interactions. Interaction of *Salmonella* Ef-Tu with Hsp90 from epithelial cells mediated adhesion, while interaction of *Salmonella* Ef-Tu with two host proteins that negatively regulate membrane ruffling (myosin phosphatase and alpha catenin), mediated adhesion and invasion. I also showed the role of host ganglioside GM1 in mediating invasion of epithelial cells by *Salmonella*.

Introduction

Bacterial association with humans is a complex evolutionary partnership the impacts health (34) and disease (51). This co-evolution reflects complex host receptor and bacterial adhesin partnerships (30). Unfortunately, relatively few receptor/adhesin associations are defined (30) and many more exist than can be associated with host response to disease. Host-microbe receptor/adhesin pairings are one of the most critical determinants that control bacterial host range (38), targets bacteria to specific tissues...
(17). Adhesion is the first step in infection. It initiates the host response by triggering signal transduction routes, and prompts the immune system to mount a response to clear the infection. In case of invasive bacteria, such as *Listeria monocytogenes* (45) and *Salmonella enterica* (23), adhesion is the prelude to host cell invasion to cause gastrointestinal infection and tissue migration that leads to systemic disease and secondary infections or long term carrier states. Likewise, recognition of host cell surface molecules by commensal and probiotic bacteria is also important for the observed health benefits of bacterial association with humans (34). Probiotic bacteria initiate a pro-inflammatory or an anti-inflammatory response by the host based on the type of microbial ligands bound by host receptors (58). Hence, identification and characterization of host-microbe receptor pairs is one of the most important areas in cellular microbiology for the discovery of mechanisms initiated by bacterial ligands during adhesion with host cell receptors.

Control of bacterial infection is becoming increasingly challenging with rising antimicrobial resistance (20). This situation calls for alternative compounds and strategies to control infectious disease. Disrupting adhesion is a possible strategy to reduce bacterial adhesion and invasion, and hence slow or stop disease progression (47); however, to fully harness the potential of this approach discovery and extensive characterization of receptor/adhesin specificity is needed. Alternatively, microbial adhesins can be used as vaccine candidates that have increased immunogenicity if they consistently bind to specific host receptors. Langermann et al. (31) successfully demonstrated this approach using an adhesin-based vaccine to reduce *in vivo* colonization of *E. coli* by more than 99% in a murine cystitis model. Identifying the cognate
receptor/adhesin partnerships used by pathogenic bacteria to bind and invade host cells is the first step in development of a series of alternative therapies that will reduce pathogen infection, systemic disease, and bacterial shedding.

Identification of receptor/adhesin pairs is difficult. No protocols or methods are established that define the ligand-receptor relationships. Even though reports of bacterial adhesin are common, they host receptor is usually unknown, or at best inferred from the possible set of host receptors. One approach to define the partnership is to screen libraries of tagged mutants for defects in adhesion/colonization phenotype (42). Unfortunately, this approach does not reveal the identity of cognate host receptor. Considering the usually high redundancy of adhesin molecules (genes) in microbial genomes, screening mutant libraries where only a single loci is disrupted, the expected false negative rate will be very high leading to an excruciatingly slow approach to find cognate receptors. While the affinity pull down approach will define the cognate partnership membership, such as the report by Cabanes et al. (9), identity of one at least one host binding partners must be known before beginning this type of experiment. This report describes an a priori high throughput method to discover cognate receptor/adhesin partners in bacterial associations.

To characterize host-microbe receptor partners, a whole cell cross-linking method was developed using a cell impermeable cross-linking reagent, Sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azido benzamido)-hexanoamido) ethyl-1,3’-dithioproprionate (sulfo-SBED) (Appendix Figure A1) to covalently link cellular proteins during host-bacterial association. Sulfo-SBED is a heterobifunctional reagent that can be used to cross-link proteins (25). The NHS-ester moiety of sulfo-SBED
covalently reacts preferentially, with primary amines of one protein that is followed by photoactivation of the aryl azide moiety to nonspecifically bind interacting molecules within 9-12 Å, thereby covalently cross-linking two protein molecules that are intimately interacting. Additionally, the Sulfo-SBED molecule contains biotin that was used to isolate the covalently bound complex and a reducible disulfide linkage to release both proteins with individual pieces of Sulfo-SBED that remain on the respective proteins. Sulfo-SBED is membrane impermeable cross-linking reagent, hence in intact cells, only the cell surface proteins would be labeled (25) and presence of proteins from lysed cells was prevented by repeated washing of labeled cells in osmotically balanced buffer. By exploiting the molecular properties of Sulfo-SBED to cross-link membrane proteins within whole cells during adhesion a new method was designed to identify cognate receptor/adhesin partners with two-dimension gels and mass spectrometry.

I initially used the method to determine ECM binding protein in L. acidophilus, S. ser. Typhimurium and E. coli. Later, I identified several new putative receptor ligand interactions between S. ser. Typhimurium and epithelial cells. I functionally showed the role of 3 of those partnerships in adhesion and invasion of S. ser. Typhimurium.

Materials and methods

Cell culture and bacterial strains

Caco-2 cells were obtained from ATCC (HTB-37, Manassas, VA) and cultured as per ATCC’s recommendation. All the cells used in the assay were between passage numbers 22-30. In brief, cells were plated at a density of $10^5$ / cm$^2$ in either a T25 or a 96 well plate. Cells were maintained in DMEM/High Modified (Thermo Scientific,
Rockford, IL) with 16.6% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), non-essential amino acids (Thermo Scientific, Rockford, IL), 10mM MOPS (Sigma, St. Louis, MO), 10 mM TES (Sigma), 15 mM HEPES (Sigma) and 2 mM NaH2PO4(Sigma). Cells were considered to be differentiated 14 days post confluence (49), and used for the adhesion and crosslinking assay. Bacterial cells were grown as described in Table 3.1.

*Labeling bacterial cells and purified proteins with Sulfo-SBED*

The bacterial cells were labeled with Sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azido benzamido)-hexanoamido) ethyl, 3’-dithiopropionate (Sulfo-SBED) (Thermo Fisher Scientific, IL, USA). The total cell surface protein content on ~10^9 bacteria was measured using the BCA protein assay (Thermo Fisher Scientific, IL, USA). Assuming an average molecular weight of 60,000 Da for proteins, 10 fold molar excess Sulfo-SBED (dissolved in DMSO at 50 µg/µl) of the determined protein concentration, was added to 10^9 bacteria suspended in 1 ml of tyrode’s (140 mM NaCl, 5mM KCl, 1mM CaCl2, 1mM MgCl2, 10 mM glucose, 10mM sodium pyruvate, 10 mM HEPES, pH 7.4) The labeling reaction was continued in dark on ice for 45 minutes with intermittent shaking. After 45 minutes the reaction was quenched by adding two fold molar excess glycine as compared to sulfo-SBED. The bacteria were washed twice with tyrode’s by centrifugation at 6000 X g for 2 minutes and resuspended in 1 ml tyrode’s buffer.

Fibronectin (Sigma-Aldrich Corp, St. Louis, MO) and fibrinogen Sigma-Aldrich Corp, St. Louis, MO) were dissolved in tyrode’s at 1 mg/ml and were labeled with 10 fold molar excess of sulfo SBED as described earlier. After quenching the labeling
reaction, the proteins were desalted against tyrode’s using microcon YM-30 filter (Millipore, Billerica, MA) exactly as per manufacturer's instructions and resuspended at a concentration of ~1 mg/ml.

Identification of microbial adhesins by label transfer from purified proteins

About ~10⁹ microbial cells were incubated with 1 ml of sulfo-SBED labeled fibronectin (1mg/ml; approximately 1.4 X 10⁵ molecules/bacterial cell) or fibrinogen (1mg/ml; approximately 1.8 X 10⁵ molecules/bacterial cell) for 30 minutes at 37° C. At the end of 30 minutes the suspension was placed under a 15 watt UV lamp (302 nm) at a distance of 5 cm for 10 minutes. The cells were washed twice with 1 ml tyrode’s by centrifugation at 6000 X g for 2 minutes and resuspended in 500 µl lysis buffer (0.1% triton, 150mM DTT) and 250 µl of glass beads (0.1 mm) (BioSpec Products, Inc., Bartlesville, OK). The samples were homogenized in a Mini-Beadbeater (BioSpec Products, Inc., Bartlesville, OK) by giving 3 pluses at full speed for 30 s with intermittent 1 minutes incubation on ice. The free biotin in the sample from the reduced non-crosslinked proteins was removed by passing the lysate through a YM3 microcon filter (Millipore, Billerica, MA) as per manufacturer's instructions. The retentate (volume brought up to 800 µl with tyrode’s) from YM3 filter was incubated with ~30, 3 mm avidin-coated glass beads (Xenopore Corp., Hawthorne, NJ) for 30 minutes on a shaking platform to capture the biotinylated proteins. The glass beads were subsequently washed 3 times with 5 ml of wash buffer (50mM Tris, 1.5 M NaCl, pH adjusted to 7.2) to remove nonspecifically bound proteins. The proteins on the washed beads were digested overnight using 300 ng of proteomics grade trypsin (Sigma-Aldrich Corp, St. Louis, MO)
in 1 ml of 100mM ammonium bicarbonate buffer. The digested proteins were concentrated using a speedvac (Thermo Fisher Scientific, IL, USA) to final volume of 50 µl and submitted to the Center for Integrated Biosystems, Utah State University (Logan, UT) for protein identification by LC-MS/MS. The whole label transfer protocol was repeated using unlabeled ECM components as negative controls.

Identification of microbial adhesins by crosslinking with pure proteins

Microbial cells were interacted, crosslinked with labeled fibronectin/fibrinogen and lysed exactly as described earlier. Fifty µl of the cell lysate was diluted with 50 µl of 2X Laemmli sample buffer (52.5 mM Tri-HCL, pH 6.8, 25% glycerol, 2% SDS, 0.02% bromophenol blue) with or without the addition of 150 mM DTT and heated at 95°C for 10 minutes. The lysate was then centrifuged at 12000 x g in a micro centrifuge and the proteins (50 µl) in the supernatant were separated by SDS-PAGE using the Mini-PROTEAN electrophoresis system (Bio-Rad Laboratories, Hercules, CA) exactly as per manufacturer’s recommendation at a constant current of 30 mAmp per gel, using 4-20 % precast Tris-HCL Gels (Bio-Rad Laboratories, Hercules, CA). The gels were stained overnight with Imperial protein stain (Thermo Fisher Scientific, IL, USA) exactly as per manufacturer’s recommendations. The gels resolved under reducing and non-reducing conditions were imaged using the Kodak Image Station 2000R (Carestream Health, Rochester, NY). The images for gels resolved under reducing and non-reducing conditions were compared and bands missing in the reduced gels were identified. The gel bands were picked, in-gel digested and the proteins identified using LC-MS/MS at the Center for Integrated Biosystems, Utah State University (Logan, UT). The protein
Identification of host microbe receptors by cell-cell crosslinking

About $10^9$ sulfo-SBED labeled bacteria were interacted with ~$10^6$ Caco-2 cells grown in a T25 in a final volume of 3.5 ml tyrode’s buffer for 60 minutes at 37°C. At the end of 60 minutes the bacterial suspension was aspirated from the flask. The flask was then placed under a 15 watt UV lamp (302 nm) at a distance of 5 cm for 10 minutes. The cross linked Caco-2 cells and bacteria were resuspended in 500 µl of lysis buffer (8 M urea, 6.0% ampholytes pH Range (3-10) (Bio-Rad Laboratories, Hercules, CA), 0.4 % CHAPS, 0.25 % Triton 100, 0.15 % n-Dodecyl-B-β-D maltoside, 0.002% bromophenol blue) and 250 µl of glass beads (0.1 mm, BioSpec Products, Inc., Bartlesville, OK). The samples were homogenized in a Mini-Beadbeater (BioSpec Products, Inc., Bartlesville, OK) by giving 3 pluses at full speed for 30 s with intermittent 1 minutes incubation on ice. The samples were stored at -70° C until further use.

The cross linked samples were thawed and centrifuged at 12000 x g in a micro centrifuge and the supernatant was used for 2D gel analysis. Isoelectric focusing (IEF) was performed using 50 µl of sample in tube gels using the Model 175 tube cell (Bio-Rad Laboratories, Hercules, CA) as per the manufacturer’s recommendation. In brief 4% polyacrylamide gels were cast in 1 mm diameter tubes to a height of 11 cm. Fifty µl samples in the lysis buffer was loaded directly on to the tubes and run at 200 V for 2 hours, 400 V for 4 hours, and finally at 800 V for 8 hours. After the IEF run, gels were extruded from the tubes and equilibrated in transfer buffer (3 % SDS, 0.07 M Tris-HCL) with or without 150 mM DTT for 15 minutes. The gels equilibrated in the presence of DTT were subsequently alkylated in transfer buffer using 150 mM iodoacetamide for 15
min. The tube gels were then resolved in the second dimension using the Criterion electrophoresis system (Bio-Rad Laboratories, Hercules, CA) exactly as per manufacturer’s recommendation at a constant current of 45 mAmp per gel using 4-20% precast Tris-HCL Gels (# 345-0104, Bio-Rad Laboratories, Hercules, CA). The gels were stained overnight with Imperial protein stain (Thermo Fisher Scientific, IL, USA) exactly as per manufacturer’s recommendations.

The gels resolved under reducing and non-reducing conditions were imaged using the Kodak Image Station 2000R (Carestream Health, Rochester, NY). The images were compared and spots missing in the reducing gels but present in the non-reduced gel were identified. The gel spots were picked, in-gel digested and the proteins identified using LC-MS/MS at the Center for Integrated Biosystems, Utah State University (Logan, UT).

Purification of Ef-Tu from Salmonella ser. Typhimurium

Elongation factor Tu (Tuf) was affinity purified from S. ser. Typhimurium lysate using a GDP-Sepharose column as described by Jacobson et al. (28) and Meide et al. (60) with a few modifications. S. ser. Typhimurium was grown in 2 liters of brain heart infusion broth (Thermo Fisher Scientific, IL, USA) to early stationary phase (OD600 = ~2.0). The cells were washed once with equal volume of saline and resuspended in 100 ml of TE buffer (10mM Tris, 1 mM EDTA, 0.1mM phenylmethylsulfonyl fluoride, pH 8) containing lysozyme (Sigma-Aldrich Corp, St. Louis, MO) at 10 mg/ml. The cell suspension was incubated at 37 °C for 30 minutes. At the end of 30 minutes sodium deoxycholate was added to the suspension at a final concentration of 1 mg/ml and incubated for 15 more minutes with gentle stirring. At the end of incubation period 100
units of DNase 1 (Promega Corp., Madison, WI) and MgCl₂ at a final concentration of 10 mM were added and incubated for 30 more minutes. The suspension was then centrifuged at 25,000 X g for 30 minutes and the supernatant was used for protein fractionation. Proteins precipitated from the 25,000 X g supernatant by (NH₄)₂ SO₄ between saturations of 37% and 64% were dissolved in binding buffer (50 mM Tris-HCl, 1 mM DTT, 10 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, pH 8) at approximately a concentration of 5 mg/ml.

Periodate oxidation and coupling of the oxidized GDP to the Sepharose were done as follows: 0.1 mmol of GDP was dissolved in 1.3 ml of 0.1 M citrate phosphate buffer (pH 5.0) and to this solution was added 0.11 mmol of sodium periodate dissolved in 0.2 ml of the same buffer. The reaction mixture was incubated in the dark at room temperature for 30 minutes. Excess periodate was eliminated by addition of 10 μl of a 40% (wt/vol) solution of ethylene glycol and incubation for an additional 10 min. One ml of this mixture was added to 3 gm. of EAH Sepharose 4B (#17-0569-01, GE Healthcare, Piscataway, NJ) that had been swollen in a total volume of 24 ml of 0.1 M Tricine-NaOH (pH 8.2) containing 0.5 M NaCl. This suspension was gently rotated for 2 hour at 4°C and was subsequently treated with 1.0 ml of 0.3 M sodium borohydride, freshly dissolved in 0.2 M Tricine-NaOH (pH 8.2). After 1 hour, 0.66 ml of the latter solution (again freshly prepared) was added and incubation was continued for another hour. At the end of incubation GDP-EAH-Sepharose was washed four times with 40 ml of 4 M NaCl.

Previously fractionated Salmonella typhimurium lysate was adsorbed onto the washed GDP-EAH-Sepharose by mixing equal volumes of packed resin and of (NH₄)₂ SO₄ fractionated cell extract. The mixture was dialyzed (12 hours, 22°C) against the binding
buffer using a 3.5K MWCO dialysis tubing (Thermo Fisher Scientific, IL, USA). The mixture was then packed into a column (0.75 X 20 cm) and washed with the same buffer until the absorbance at 280 nm reached baseline. One column volume of the binding buffer containing 100µM GDP was then allowed to flow into the column and elution was interrupted for 1 hour. Subsequent elution with GDP-containing buffer yielded Ef-Tu in homogenous form. The purity of the eluted protein was confirmed by LC-MS/MS.

_Determination of adherent and invasive bacteria_

The role of specific receptors in adhesion and invasion of the host by salmonella was assayed by determining the changes in the amount of total host (intestinal epithelial cells, Caco-2) associated salmonella due to receptor blocking by specific antibodies. The amount of total host associated salmonella was determined by either total viable plate count or by qPCR as described below. The amounts of total invasive salmonella in the host were determined by the gentamicin protection assay with a few modifications as described below (16).

Total host associated bacteria were determined as follows. Caco-2 cells were cultured as described earlier in a 96-well plate. The bacteria were used after two transfers for the adhesion assays. Bacterial cells were collected from 2 ml of media after growth for 14 hours, washed twice with an equal volume of PBS, and re-suspended at ~10^8 CFU/ml, in DMEM/High modified with 1X non-essential amino acids, 10mM MOPS, 10 mM TES, 15 mM HEPES and 2 mM NaH2PO4 but without the FBS. Caco-2 cells were incubated with antibodies or specific proteins as mentioned in Table 3.2 for 60 minutes in a final volume of 50 µl at 37°C in 5% CO₂. At the end of 60 minutes, the Caco-2 cells
were infected with 50 µl of bacterial cell suspension (MOI 1:100) and incubated for 60 minutes at 37°C in 5% CO₂. The bacterial cell suspension was aspirated and the Caco2 monolayer was washed thrice with 200 µl of tyrode’s to remove non-adhered bacterial cells from the monolayer. DNA extraction buffer (AEX Chemunex, France) (50 µl) was used to lyse the monolayer and the bacteria associated with the host, and incubated at 37°C for 15 minutes followed by 95°C for 15 min. Resulting cell lysate was used to determine the number of bacteria associated with the Caco-2 cells (14). Quantitative analysis was done using qPCR with a CFX 96 Real Time System (Bio-Rad, Hercules, CA). Reactions were performed in a final volume of 25 µl including 1 µl of cell lysate, 100 nM of PCR primers and iQ SYBR Green Supermix (Bio-Rad, Hercules CA) as per manufacturer’s instructions. The primers used for the amplification are listed in Table 3.1. The reaction parameters consisted of denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation, annealing and extension at 95°C for 15 s, 56°C for 30 s, 72°C for 30 s, respectively and a final extension at 72°C for 1 min. The product was verified using a melt curve analysis from 50°C to 95°C with a transition rate of 0.2°C/s. The amount of bacterial cells and Caco-2 cells present in each well were estimated using a standard curve of Cₜ vs. Log₁₀ CFU and amount of bacteria per Caco-2 was calculated. The data were normalized relative to control wells which were not given any antibody treatment. The experiment was done in four replicates.

The amount of total invaded and adhered bacteria was determined using the gentamicin protection assay as follows. Caco-2 cells were infected exactly as described earlier. The infected cells were incubated for 60 minutes at 37°C with 5% CO₂. Upon incubation, media was aspirated and cells were washed three times with tyrode’s buffer.
Invaded bacteria were estimated by incubating the cells with 100 µg/ml gentamicin for 2 hours at 37°C with 5% CO₂ to kill bacteria adhered or outside the Caco-2 cells. To determine total host associated bacteria, cells were incubated with cell culture media without any antibiotic. Cells were again washed three times with tyrode’s buffer and lysed with 0.01% triton. The amount of bacteria in the Caco-2 lysate was determined by plating serial dilutions on LB agar. The experiment was performed in four replicates. The number of adhered bacteria were enumerated by subtracting mean of invaded bacteria (B) from mean of total host associated bacteria (A) and error (ΔZ) was calculated as $\Delta Z^2 = (\Delta A)^2 + (\Delta B)^2$ where, ΔA is SEM associated with the A and ΔB is SEM associated with B. The data were normalized relative to control wells which were not given any antibody treatment. Differences in the mean due to treatment were tested by one way ANOVA. Post ANOVA, the means were compared to each other by Tukey-Kramer's method.

**NFkB reporter assay**

A reporter cell line, monitoring the activity of NFkB was generated by transducing Caco-2 cells with lentiviral particles expressing GFP under the control of a minimal CMV promoter and tandem repeats of NFkB transcriptional response element. Caco-2 cells were seeded at a density of 5 X 10⁴ / cm² in a 96 well plate and cultured as described before. After 2 days Caco-2 cells were transfected with ready to transduce lentiviral particles (# CLS-013G, SABiosciences, Fredric, MD) exactly as per manufacturer's recommendations. In brief, cells in each well were transfected with the viral particles at an MOI of 1:10, 0.3 µl of SureFECT transfection reagent (SABiosciences, Fredric, MD) in a final volume of 50 µl. Two days after transfection
the supernatant was aspirated and the cells were maintained in the cell culture media with 12µg/ml puromycin for 18 more days until the cells differentiated, and then they were used for the reporter assays. The cells were serum starved for 24 hours prior to infection with bacteria. The transfected cells were infected with different bacteria as mentioned earlier but at an MOI of 1:1000. At the end of eight hours the amount of fluorescence in each well was measured at an Ex/Em of 485/535 using a DTX 880 Multimode Detector (Beckman Coulter, Brea, CA). Blank reading from non-transfected cells were subtracted from the reported values. Differences in the mean due to treatment were tested by one way ANOVA. Post ANOVA, the means were compared to each other by Tukey-Kramer's method.

**Western Blots**

The relative abundance of target proteins due to different treatments was quantified by densitometric analysis of western blots. Caco-2 cells were cultured as mentioned before in a 48 well plate. The cells were serum starved for 24 hours prior to infection with bacteria. Bacterial cells were grown as mentioned earlier, except that they were grown in cell culture media. Caco-2 cells were incubated with either serum free cell culture media or, Ef-Tu (2 µg/ml) dissolved in serum free cell culture media with or without the presence of S. ser. Typhimurium LT2 ΔinvA at an MOI of 1:1000. The experiment was repeated in 4 replicates. The cells were harvested at the end of 60 minutes by lysis in 30 µl of 1X SDS sample buffer with protease and phosphatase inhibitors (52.5 mM Tri-HCL, pH 6.8, 25% glycerol, 2% SDS, 0.02% bromophenol blue, 50mM DTT, 50 mM Sodium pyrophosphate, 100 mM sodium fluoride, 10 mM
orthovanadate, 1 Roche protease inhibitor cocktail tablet/50ml solvent). The samples were heated at 95°C for 10 minutes and centrifuged at 12,000 X g for 10 minutes. 20 µl of the sample was resolved on precast 10% Tris-HCl polyacrylimide gels (Bio-Rad Laboratories, Hercules, CA) at a constant current of 45 mAmp per gel using the Criterion electrophoresis system (Bio-Rad Laboratories, Hercules, CA). The resolved proteins were then transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA) using the Trans-Blot semi-dry electrophorectic cell (Bio-Rad Laboratories, Hercules, CA) as per manufacturer's recommendation. Upon completion of the transfer, the blots were probed for the presence of p-Akt 473 (#9271), p-AKT 308 (#9275) and p-myosin 19 (#3671), by Pierce® Fast Western Blot Kit (Thermo Fisher Scientific, IL, USA), according to the manufactures recommendations. Primary antibodies for all the proteins were purchased from Cell Signaling Tech. (Boston, MA). The blots were imaged using the Kodak Image Station 2000R (Carestream Health, Rochester, NY). Densitometric analysis of the blot was done using Image J (1). Differences in the means due to treatment were tested by one way ANOVA. Post ANOVA, the means were compared to each other by Tukey-Kramer's method.
Results

*Identification of fibronectin binding proteins in*  
*Lactobacillus acidophilus NCFM*

*L. acidophilus* binds many different extracellular matrix components (ECM) (13), but the identity of most bacterial proteins that mediate this interaction is not known. I used a label transfer strategy to identify bacterial proteins that mediate interactions of *L. acidophilus* NCFM with fibronectin. I also wanted to check the feasibility of ultimately using this reagent to be able to crosslink whole cells. Fibronectin was labeled with sulfo-SBED and interacted with *L. acidophilus* NCFM, crosslinked and subsequently reduced, which transferred the biotin label from fibronectin to any proteins within ~13 Å distance of fibronectin. The biotinylated proteins in *L. acidophilus* NCFM were immobilized onto avidin coated beads and identified by LC-MS/MS. The identified proteins are summarized in Table 3.3. A negative control in which unlabeled fibronectin was interacted with the bacterial cells, identified no proteins as being biotinylated. Of the six proteins identified, functional analysis of a *cdpA* deletion mutant showed ~80% reduction in adhesion to Caco-2 cells (3). InterProScan sequence analysis of *cdpA* predicted the presence of two protein domains, IPR004903- bacterial surface layer protein, and IPR011490 extracellular matrix-binding protein (41). One of the other identified protein; LBA0222 was predicted to contain a signal peptide (41) and a transmembrane domain (11) at its N-terminal. Since there were no annotated protein families in LBA0222, I used Phylobuilder (21) to build neighbor joining trees based on multiple sequence alignments of homologs of LBA0222 (Appendix Figure A2). This analysis revealed that the closest homolog of LBA0222 in *L. acidophilus* NCFM was *cdpA* (E-Value 3.0e-22). Hence this
method functionally identified two homologous proteins in *L. acidophilus* as fibronectin binding proteins, one of which was bioinformatically predicted to contain a domain that binds ECM components. I also identified four ribosomal proteins crosslinked to fibronectin. Although typically found intracellularly, they have also been found expressed on the surface of *Streptococcus* (55) and *E. coli* (54).

**Identification of fibrinogen binding proteins in *E. coli* and *Salmonella* ser. Typhimurium by crosslinking and SDS-PAGE**

The label transfer protocol is useful to identify protein interactions, if the identity of the at least one of the interacting proteins (e.g. fibronectin in the previous case) is known. In a cell-cell interaction model, where the identity of none of the binding partners is known, it is possible to get a list of interacting proteins using the label transfer approach, but it is not possible establish binding partnerships. To be able to establish binding partnerships, I used a slightly different approach with the same crosslinking reagent. I initially verified the feasibility of the approach by crosslinking fibrinogen to *S. ser. Typhimurium* and *E. coli* to identify the fibrinogen interacting proteins, and later used the approach for whole cell crosslinking. After crosslinking the protein to the bacterial cell surface I lysed the bacteria and separated the crosslinked complex by SDS-PAGE under reducing and non-reducing condition. The reducing conditions break the disulfide bond in the crosslinker, separating the two proteins. This was visualized on a gel due to a shift in the molecular weight of the crosslinked complex between the reducing and non-reducing gels. Bands which showed such a shift in molecular weight were cut
out from the gel resolved under non-reducing conditions, and the proteins were identified using LC-MS/MS.

I identified three bands that were present in the gel resolved under non-reducing conditions (Fig 3.1), but absent in the gels resolved under reducing conditions. Of the three bands, two bands contained only fibrinogen, while the third band contained fibrinogen as well as murein lipoprotein (Lpp), for S ser. Typhimurium and universal stress protein B (UspB) for E. coli (Table 3.4). Lpp is a murein lipoprotein expressed on the cell surface and contributes towards the virulence of the organism in the mice model (56). Specifically, Lpp is a murein lipoprotein expressed on the cell surface and is an established virulence factor in S. sv Typhimurium that mediates invasion (56) Persson et al (50) reported csgA fimbriae in Salmonella to be a fibrinogen binding protein, but since csgA is not usually expressed under normal culture conditions (26) it was not found cross-linked to fibrinogen. Fibrinogen binding is reported in E. coli (57), but no known fibrinogen binding proteins have been discovered. We found UspB, a membrane protein in E. coli that is required for ethanol tolerance cross-linked with fibrinogen. Hence using a crosslinking approach I identified a murein lipoprotein that mediates adhesion and invasion in S. ser. Typhimurium and a cell membrane attached stress tolerance protein in E. coli as a fibrinogen interacting protein. Given the success in the initial phase using individual host proteins to find established bacterial adhesins, experiments moved to cross-link whole cells.

*Identification of host microbe receptors by whole cell crosslinking*
After verifying that the crosslinking protocol worked with microbes and pure ECM components, I used the same protocol to crosslink whole cells. I labeled the bacteria with sulfo-SBED, crosslinked it with Caco-2 cells, lysed the cells and resolved the samples on 2D gels (instead of SDS-PAGE) under either reducing conditions or non-reducing conditions. Protein spots that were present in the gel resolved under non-reducing condition, but absent in gels resolved under reducing conditions were picked from the gel, and proteins identified using LC-MS/MS. Figure 3.2 shows a representative gel resolved under non-reducing conditions The gels were resolved in two replicates and only the spots that showed similar migration patterns in both the replicates were picked. Forty four such spots were picked, of which 33 spots yielded protein IDs. Out of the 33 spots with protein ID, nine spots had protein IDs from the host as well as microbe in the same spot. Of those nine spots, five spots had proteins whose sum of the molecular weights matched the observed molecular weight of the spot on the gel. Hence those five spots had proteins from host as well as microbes, which were crosslinked. The identified proteins are listed in Table 3.5. Images of both the replicates resolved under reducing and non-reducing conditions are shown in Appendix Figure A3. Based on the protein identification results (Table 3.5), it can be concluded unambiguously, that STM2699 was crosslinked with SPTAN1 and, STM1956 was crosslinked with HSP90AB1. STM4088 might have been crosslinked to either HSP90B1 or ACTN4, or both the proteins. Ef-Tu from salmonella might have been crosslinked to PPP1R12A, HSP90AB1, CTNNA1, PTPRE, PPP1R9B or MATR3. STM3286 from salmonella might have been crosslinked with ACTB from the host, or it could have also crosslinked with Ef-Tu from salmonella itself.
Characterization of the protein-protein interactions observed from crosslinking

Adhesion and invasion of the host mucosa is the first step in the pathogenesis of salmonella. To verify the functional significance of the crosslinked proteins, I determined their role in adhesion and invasion of *S*. *ser*. Typhimurium. Out of the five bacterial target proteins identified from crosslinking, I investigated the role of one of those proteins and found that, Ef-Tu was involved in adhesion as well as invasion. Out of the 11 host proteins identified, I looked at the role of five of those proteins in adhesion and invasion. I found that two of those proteins were involved in adhesion, one protein was involved in invasion, while two proteins had no role in adhesion or invasion.

Since *Salmonella* binding with host gangliosides has been reported in the literature (14, 37, 48), I also investigated the role of 2 gangliosides, GM1 and GD3 in adhesion and invasion. I narrowed down on these two gangliosides as GM1 has been reported to be a receptor for many bacterial toxins (40) and has also been shown as a coreceptor important in toll like receptor signaling (44). I investigated GD3 as it is the most abundant ganglioside present in differentiated Caco-2 cell membranes (53). Figure 3.3 shows a dose dependent effect of blocking each receptor with antibodies, on the amount of total host associated bacteria. Based on these data, the antibody dilution that showed optimum pathogen blocking was subsequently used to dissect the role of individual molecules in either adhesion or invasion. As evident from the Figure 3.3, PPP1R12A, CTNN1A, HSP90AB1 and ganglioside GM1 were involved in association of *S*. *ser*. Typhimurium to the epithelial cells, as blocking those molecules with antibodies significantly reduced the amount of host associated bacteria. PPP1R9B, PTPRE and
ganglioside GD3 on the other hand had no effect. Also blocking Ef-Tu on the bacteria significantly reduced the amount of host associated bacteria. I further assessed the contributions of PPP1R12A, CTNN1A, HSP90AB1, ganglioside GM1 from the host and Ef-Tu from the bacteria on adhesion and invasion of S. ser. Typhimurium.

I investigated the role of Ef-Tu in adhesion and invasion of S. ser. Typhimurium (Fig 3.4) by either blocking Ef-Tu with an antibody, or by adding excess native purified Ef-Tu to compete with cell surface associated Ef-Tu, from the same bacteria. In a wild type background (WT), blocking Ef-Tu on the bacteria with an antibody caused a significant reduction in adhesion as well as invasion (p<0.05). The fraction of intracellular host associated bacteria, dropped from 5.8% in unblocked control to 2.5 % in Ef-Tu blocked cells. Adding excess Ef-Tu significantly decreased the amount of adhered bacteria, but increased the amount of the invaded bacteria. The fraction of intracellular host associated bacteria increased from 5.8% in untreated control to 16.6 % in cells spiked with excess Ef-Tu, and this effect was dose dependent. The effect of receptor blocking on a non-invasive S. ser. Typhimurium ∆invA strain was also assessed. InvA is an inner membrane protein that is necessary for the secretion of T3SS-1 effectors, which are required for invasion (19). Adding excess Ef-Tu to ∆invA cells caused a decrease in adhered bacteria, while there was no change in amount of invaded bacteria. Based on these results it can be concluded that Ef-Tu was directly involved in adhesion and invasion of S. ser. Typhimurium.

I also investigated the role of the crosslinked host molecules, in adhesion and invasion by blocking specific receptors on the epithelial cells with antibodies. Initial screening (Fig 3.3) revealed that of the seven host molecules tested, four had a role in
either adhesion or invasion. I further tested the role of those molecules in adhesion and invasion. Blocking HSP90AB1, CTNNA1, PPP1R12A and GM1 on the host with antibodies significantly reduced (p<0.05) the total number of adhered wild type bacteria to 26%, 45%, 31%, and 56%, respectively, relative to unblocked control. Concurrently the amount of invaded bacteria was reduced to 78%, 20%, 10%, and 7%, respectively, relative to control. The fraction of intracellular host associated bacteria changed from 5.8% for the control to 15.5%, 1.4%, 3.7%, and 0.68% for HSP90AB1, CTNNA1, PPP1R12A, and GM1 blocking respectively. The effect of host receptor blocking on the amount of host associated bacteria was also tested on a non-invasive S. ser. Typhimurium. Blocking HSP90AB1, PPP1R12A and GM1 on the host reduced the adherence of the noninvasive strain to 38%, 71%, and 49% respectively, relative to the non-blocked control, while blocking CTNNA1 had no effect. Total host associated WT bacteria reduced to 22%, 20%, 19%, and 59% relative to non-blocked control after blocking HSP90AB1, PPP1R12A, CTNNA1 and GM1 respectively. Hence it can be concluded that HSP90AB1 was involved in adhesion of salmonella, while rest of the tested molecules were involved in adhesion and invasion. Also based on the differences in susceptibility to antibody blocking between WT and ΔinvA strain, the role of PPPR12A and CTNNA1 were downstream of the effects induced in the host due to injection of T3SS-1 effectors.

It has been speculated that, since Ef-Tu is a well conserved protein in all bacteria, it is a MAMP (microbe associated molecular pattern) that is recognized by the host cells. Agrobacterium Ef-Tu is recognized by EFR, a receptor kinase in Arabidopsis thaliana, which leads to induction of genes in Arabidopsis necessary for protection from
Agrobacterium mediated DNA transformation (65). It has also been shown that Ef-Tu from Lactobacillus johnsonni elicits a proinflammatory response in cultured epithelial cells (22). Since NFkB is a central regulator of inflammatory response, to test if Ef-Tu from S ser. Typhimurium induced a proinflammatory response in cultured epithelial cells, I quantified NFkB mediated transcription in a reporter Caco-2 cell line in response to excess Ef-Tu. The results are summarized in Figure 3.6. S. ser. Typhimurium (WT and ΔinvA) both induced activity in Caco-2 cells. However there was no effect of adding excess Ef-Tu or blocking Ef-Tu with an antibody on the activity of NFkB.

Of the seven host receptors I tested for an adhesion/invasion related phenotype, three were protein phosphatases (PPP1R12A, PTPRE, and PPP1R9B) and one was a chaperone protein (HSP90AB1). Two of the three phosphatases (PPP1R12A, PTPRE) and HSP90AB1 have been shown to modulate the phosphorylation status of Akt (2, 64) at threonine 308 and serine 473. Hence I hypothesized that Ef-Tu modulates the phosphorylation status of Akt in Caco-2 cells. Since sigD, one of the T3SS-1 effector has been shown to induce phosphorylation of Akt; I tested the hypothesis by relative quantification of P-AKT 473 and P-AKT 308 by incubating Caco-2 cells with ΔinvA strain of Salmonella ser. Typhimurium in presence or absence of excess Ef-Tu (2 µg/ml). Levels of P-Akt3 08 were below detection in all the treatments. Levels of P-AKT 473 due to different treatments are summarized in Figure 3.7. Presence of S. ser. Typhimurium ΔinvA had no effect on levels of P-AKT 473. Presence of excess Ef-Tu with or without the presence of the organisms significantly increased the levels of P-AKT 473. Hence Ef-Tu from S. ser. Typhimurium induced phosphorylation of Akt in epithelial cells
Discussion

I developed a crosslinking method to discover host microbe receptors which doesn't require any apriori knowledge of interacting protein targets on the host or the microbe. I used the method to discover protein-protein interactions between *Salmonella* and cultured intestinal epithelial cells (Caco-2). I discovered five salmonella protein that were crosslinked to 11 proteins from epithelial cells. One of the discovered *Salmonella* proteins was translation elongation factor Tu (Ef-Tu). Ef-Tu is typically found intracellularly and is one of the most abundant proteins in bacterial cells (18). It is a monomeric G protein, which binds and mediates the entry of aminoacyl tRNA into the A site of ribosomes, so as to facilitate the elongation of a protein being translated. Correct binding of an aminoacyl tRNA into the A site of ribosomes triggers the GTPase activity of Ef-Tu. Ef-Ts acts as a guanine nucleotide exchange factor (GEF) for Ef-Tu, and exchanges the hydrolyzed GDP for GTP. Hence for translation, a stoichiometric ratio of 1:1:1 of ribosomes: Ef-Tu: Ef-Ts is sufficient (35). Yet, in a dividing cell, ribosome, Ef-Tu and Ef-Ts exists at a stoichiometric ratio of ~ 1:10:1 (18). Hence in a dividing cell Ef-Tu exists at ~ 10 X concentrations then what is usually required for translation, indicating that perhaps Ef-Tu is also required in the cell for functions other than its role in protein translation. The role of Ef-Tu as a chaperone has also been proposed as it was shown that Ef-Tu promoted functional folding of proteins after urea denaturation (10). Based on the fact that Ef-Tu can form filamentous structures in vitro (7, 8, 62), it has also been proposed that Ef-Tu may be a cytoskeletal element in prokaryotes. Soufo et al. recently showed that in Bacillus subtilis, Ef-Tu co-localizes
with MreB, an actin like cytoskeletal element and is a major determinant of cell shape (12).

I characterized the interaction of *Salmonella* Ef-Tu with host epithelial cells and showed that, it mediates adhesion and invasion of *Salmonella*. Cell surface associated Ef-Tu from *Salmonella* has also been shown to interact with the host tumor necrosis factor (TNF-alpha) (52). The implications of this association in the infection process are not clearly understood. Cell surface expression of Ef-Tu has also been confirmed in phylogenetically diverse group of bacteria including *Lactobacillus johnsonii* (22), *Agrobacterium tumefaciens* (65), *Mycobacterium tuberculosis* (63), *Mycobacterium leprae* (43), *Francisella tularensis* (6), *Helicobacter pylori* (39), *Mycoplasma pneumoniae* (4), *Mycoplasma genetelium* (5), *Streptococcus pyogenes* (55), *Streptococcus gordonii* (29), and *Streptococcus oralis* (61). Table 3.5 lists the interacting partners of Ef-Tu known in different bacterial species and Figure 3.8 shows a neighbor joining tree made by aligning Ef-Tu sequences from bacteria whose Ef-Tu is known to interact with specific host proteins. Figure 3.8 demonstrates that there is casual correlation between taxonomic distance of the Ef-Tu and similarity of binding partners i.e. Ef-Tu from both the *Mycoplasma* tends to bind fibronectin, while Ef-Tu from both the *Lactobacillales* tend to bind mucins. Data from more organisms is needed to have more significant correlations between the phylogenetic distance and similarity of binding partners. One to three Ef-Tu gene copies are present in the bacterial genome depending upon the species (33), but there was no correlation between number of Ef-Tu copies present and surface localization of Ef-Tu. I did not find any correlations between bacteria expressing Ef-Tu on the surface and its phylogenetic position either.
One of the host proteins found crosslinked to Ef-Tu was Hsp90. Hsp90 is a highly conserved molecular chaperone that has a variety of roles in signal transduction, protein folding and protein degradation. A homolog of Hsp90 is also expressed on the cell surface and act as microbial receptors for *Listeria monocytogenes* (9) and *Clostridium difficile* Toxic A (46). Here I show that Hsp90 can specifically mediate adherence of *Salmonella* with epithelial cells.

Surprisingly two of host receptors that I found crosslinked to *Salmonella*, which had a role in invasion (PPP1R12A, CTNNA1), interact with the actin cytoskeleton. PPP1R12A is a cell surface expressed (27, 36) myosin light chain phosphatase that regulates the interaction of actin and myosin downstream of small G protein Rho. Increased phosphatase activity of PPP1R12A, negatively regulates formation of membrane ruffles (59). Since binding of Ef-Tu with PPP1R12A enhanced invasion of *Salmonella*, it can be speculated that Ef-Tu by a yet unknown mechanism decreased the phosphatase activity of PPP1R12A, leading to lesser negative regulation of membrane ruffling, which is a downstream effect of T3SS effectors (24).

Alpha catenin (CTNNA1) has recently been shown to be a molecular switch, that either binds E-cadherin/beta catenin complex as a monomer or, it binds F-actin as a homodimer (15). When bound to F-actin, alpha catenin negatively regulates actin filament branching by suppressing Arp2/3 mediated actin polymerization, possibly by competing with Arp2/3 for actin binding (15). Two of the T3SS effectors (SopE, SopB) stimulate Arp2/3 mediated actin polymerization, which is necessary for the formation of membrane ruffles and invasion (24). Since interaction of Ef-Tu with CTNNA1 promoted invasion only in *Salmonella* that possess T3SS, it can be speculated that binding of Ef-Tu
with alpha catenin decreased its binding with actin by an unknown mechanism, leading to increased Arp 2/3 mediated polymerization and hence increased invasion. Hence interaction of Ef-Tu from *Salmonella* with host PPP1R12A and CTNNA1 most likely promoted invasion by inhibiting the negative regulation of membrane ruffling. This mechanism probably acted in concert with membrane ruffling induced by the T3SS effectors.

The role of gangliosides as receptors for bacteria, viruses and toxins is well studied (14). For *Salmonella* it has been reported that gangliosides act as co receptors with TLR5 for flagellar protein FliC, which leads to induction of NfκB activity in epithelial cells (48). Here I have reported that ganglioside GM1 is involved in the invasion of epithelial cells by *Salmonella*. Blocking host GM1 with antibody reduced adherence ~2-fold, but reduced invasion ~15-fold, hence GM1 probably interacts with a *Salmonella* factor which is very important for mediating invasion. Since a T3SS-1 deletion mutant and GM1 blocking showed similar phenotypes, it can be speculated that GM1 interacts with components of the T3SS. Tejero et al. (32) recently showed that three translocon components of the T3SS-1 (SipB, SipC, SipD) mediate intimate attachment of *Salmonella* with epithelial cells before injection of the effectors (SopE, SopB) that cause membrane ruffling, and this interaction is important for the successful delivery of the SopE and SopB. The identity of the host interacting partners for SipB, SipC and SipD is not known. It can be speculated that since GM1 plays such a crucial role in invasion, perhaps it is the binding partner for the translocon components for the T3SS.
In conclusion, we developed a cell-cell crosslinking method to discover host and microbe receptors that mediate interaction between bacterial and mammalian cells. We initially verified the method by crosslinking pathogens and commensals with pure ECM components and went on to use the method to crosslink *Salmonella* with epithelial cells. New protein-protein interactions were discovered. We showed that interaction of Ef-Tu on the Salmonella cell surface with Hsp90 on Caco-2 cells mediates adhesion while interaction of Ef-Tu with PPP1R12A and CTNNA1 mediates adhesion as well invasion. We also showed that host ganglioside GM1 mediates adhesion and invasion.

**References**


### Table 3.1. Bacteria growth conditions and primers for their quantitation by qPCR.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Growth condition</th>
<th>qPCR primers</th>
<th>Standard curve equation</th>
</tr>
</thead>
</table>
| *Salmonella enterica* ser. Typhimurium LT2 (ATCC 700720) | 37°C / 220 rpm in LB (BD Difco, France) | Forward: TGT TGT GGT TAA TAA CCG CA  
Reverse: CAC AAA TCC ATC TCT GGA | Log$_{10}$ CFU = $Ct$-46.52/-5.034  
$R^2 = 0.99$ |
| Caco2 (ATCC HTB-37) | See cell culture for growth description | Forward: ACC ACA GTC CAT GCC ATC AC  
Reverse: TCC ACC ACC CTG TTG CTG TA | Log$_{10}$ CFU = $Ct$-33.69/-4.13  
$R^2 = 0.99$ |
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Protein Description</th>
<th>Host Species</th>
<th>Dilutions Used</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP90AB1</td>
<td>Heat shock protein 90kDa alpha (cytosolic), class B member 1</td>
<td>Rabbit</td>
<td>1:500, 1:1000, 1:2000, 1:4000</td>
<td>#NB110-96885, Novus Biologicals</td>
</tr>
<tr>
<td>PPP1R12A</td>
<td>Protein phosphatase 1, regulatory (inhibitor) subunit 12A</td>
<td>Rabbit</td>
<td>1:50, 1:100, 1:200, 1:400</td>
<td>#NB110-38890, Novus Biologicals</td>
</tr>
<tr>
<td>PPP1R9A</td>
<td>Protein phosphatase 1, regulatory (inhibitor) subunit 9A</td>
<td>Rabbit</td>
<td>1:250, 1:500, 1:1000, 1:2000</td>
<td>#NB120-3483, Novus Biologicals</td>
</tr>
<tr>
<td>CTNN1A</td>
<td>Catenin (cadherin-associated protein), alpha 1</td>
<td>Rabbit</td>
<td>1:50, 1:100, 1:200, 1:400</td>
<td>#NB120-2364, Novus Biologicals</td>
</tr>
<tr>
<td>PTPRE</td>
<td>Protein tyrosine phosphatase receptor type E (PTPRE)</td>
<td>Mouse</td>
<td>1:100, 1:200, 1:400, 1:800</td>
<td>#H00005791-A01, Novus Biologicals</td>
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<td>Ganglioside GD-3</td>
<td>Ganglioside GD-3</td>
<td>Mouse</td>
<td>1:50, 1:100, 1:200, 1:400</td>
<td>#NB120-6217, Novus Biologicals</td>
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<td>Ganglioside GM-1</td>
<td>Ganglioside GM-1/ Asialo GM1</td>
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<td>1:250, 1:500, 1:1000, 1:2000</td>
<td>#AB31119, Abcam</td>
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<td>Ef-Tu</td>
<td>Elongation factor Tu</td>
<td>Mouse</td>
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Table 3. 3 Fibronectin binding proteins identified in *L. acidophilus* using a label transfer approach.

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<th>Locus Tag</th>
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<th>MASCOT Score</th>
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<td>LBA0222</td>
<td>Hypothetical Protein</td>
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<td>141</td>
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<td>LBA0324</td>
<td>30S ribosomal protein S9</td>
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<td>126</td>
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<td>LBA0223</td>
<td>Cell separation protein, CdpA</td>
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<tr>
<td>LBA0315</td>
<td>30S ribosomal protein S13</td>
<td>2</td>
<td>146</td>
</tr>
<tr>
<td>LBA0291</td>
<td>50S ribosomal protein L3</td>
<td>2</td>
<td>137</td>
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<tr>
<td>LBA0786</td>
<td>30S ribosomal protein S4</td>
<td>2</td>
<td>104</td>
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</tbody>
</table>
Table 3. 4 Fibrinogen binding protein identified in *S. ser. Typhimurium* using a crosslinking approach.

<table>
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<tr>
<th>Band</th>
<th>Locus Tag</th>
<th>Organism</th>
<th>Protein</th>
<th>Unique Peptides</th>
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<th>PLGS Score</th>
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<tr>
<td>K1</td>
<td>B3494</td>
<td>E. coli K12</td>
<td>Universal stress protein B</td>
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<td>103</td>
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<td></td>
<td>FGA</td>
<td>H. sapiens</td>
<td>Fibrinogen alpha chain</td>
<td>22</td>
<td>69713</td>
<td>424</td>
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<td></td>
<td>FGB</td>
<td>H. sapiens</td>
<td>Fibrinogen beta chain</td>
<td>22</td>
<td>55892</td>
<td>320</td>
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<td></td>
<td>FGG</td>
<td>H. sapiens</td>
<td>Fibrinogen gamma chain</td>
<td>20</td>
<td>49464</td>
<td>170</td>
</tr>
<tr>
<td>S1</td>
<td>STM1377</td>
<td>S. Typhimurium lpp</td>
<td>Murien lipoprotein lpp</td>
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<tr>
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<td>FGA</td>
<td>H. sapiens</td>
<td>Fibrinogen alpha chain</td>
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<td>69713</td>
<td>2408</td>
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<td>FGB</td>
<td>H. sapiens</td>
<td>Fibrinogen beta chain</td>
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<td>1596</td>
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<td>H. sapiens</td>
<td>Fibrinogen gamma chain</td>
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<td>1239</td>
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Table 3. 5 Host Microbe proteins identified as binding partners by whole cell crosslinking.

<table>
<thead>
<tr>
<th>Mw of Spot on Gel (Da)</th>
<th>S. Typhimurium Protein</th>
<th>Peptides</th>
<th>PLGS Score</th>
<th>Bacterial protein Mw (Da)</th>
<th>Caco-2 Protein</th>
<th>Peptides</th>
<th>PLGS Score</th>
<th>Host Protein Mw (Da)</th>
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<tr>
<td>276000</td>
<td>Putative Phage Tail like protein (STM2699)</td>
<td>3</td>
<td>98.2</td>
<td>10804</td>
<td>Spectrin, alpha, non-erythrocytic 1 (SPTAN1)</td>
<td>7</td>
<td>446.5</td>
<td>284105</td>
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<td>103000</td>
<td>Flagellar biosynthesis factor FliA (STM1956)</td>
<td>3</td>
<td>87.4</td>
<td>27456</td>
<td>Heat shock 90kDa protein 1 beta (HSP90AB1)</td>
<td>13</td>
<td>651.7</td>
<td>83212</td>
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<td>94000</td>
<td>Putative cytoplasmic protein (STM4088)</td>
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<td>80.1</td>
<td>9306</td>
<td>Tumor rejection antigen gp96 (HSP90B1)</td>
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<td></td>
<td>Actinin alpha 4 (ACTN4)</td>
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<td>131000</td>
<td>Elongation Factor Ef-Tu (STM4146, STM3445)</td>
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<td>368</td>
<td>43256</td>
<td>Protein phosphatase 1 regulatory subunit 12A (PPP1R12A)</td>
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<td>842.1</td>
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<td></td>
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<td></td>
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<td>124.8</td>
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<td>120000</td>
<td>Translation initiation factor IF 2 (STM3286)</td>
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<td>97342</td>
<td>Protein phosphatase 1 regulatory subunit 9B (PPP1R9B)</td>
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<td>611.6</td>
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<td>Elongation Factor Ef-Tu (STM4146, STM3445)</td>
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<td>220.8</td>
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<td>195.5</td>
<td>94564</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Beta actin (ACTB)</td>
<td>4</td>
<td>177.7</td>
<td>41709</td>
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</table>
Table 3. 6 Known interacting host partners of Ef-Tu from other published studies

<table>
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<tr>
<th>Order</th>
<th>Species</th>
<th>Host Binding Partner</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycetales</td>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Plasminogen</td>
<td>(63)</td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td><em>Agrobacterium tumefaciens</em></td>
<td>Receptor kinase-EFR</td>
<td>(65)</td>
</tr>
<tr>
<td>Gamma Proteobacteria</td>
<td><em>Francisella tularensis</em></td>
<td>Nucleolin</td>
<td>(6)</td>
</tr>
<tr>
<td>Gamma Proteobacteria</td>
<td><em>Salmonella ser. Typhimurium</em></td>
<td>TNF-Alpha</td>
<td>(52)</td>
</tr>
<tr>
<td>Bacilli</td>
<td><em>Lactobacillus johnsonii</em></td>
<td>Mucins</td>
<td>(22)</td>
</tr>
<tr>
<td>Bacilli</td>
<td><em>Streptococcus gordonii</em></td>
<td>Mucins</td>
<td>(29)</td>
</tr>
<tr>
<td>Mollicutes</td>
<td><em>Mycoplasma genitalium</em></td>
<td>Fibronectin</td>
<td>(5)</td>
</tr>
<tr>
<td>Mollicutes</td>
<td><em>Mycoplasma pneumoniae</em></td>
<td>Fibronectin</td>
<td>(4)</td>
</tr>
</tbody>
</table>
Figure 3. 1 Fibrinogen binding proteins identified from *E. coli* and *S. ser. Typhimurium* LT2 by crosslinking labeled fibrinogen with the microbes and then separating the proteins under reducing (A4, B3) and non-reducing conditions (A2, A3, B1, B2). Proteins identified from bands S1 and K1 are shown in Table 3.4. Bands K2, K3, S2 and S3 contained fibrinogen subunits (FGA, FGB and FGG) but no bacterial proteins.
Figure 3.2 Crosslinked proteins resolved by 2D gel electrophoresis under non-reducing conditions. The circled spots were picked and identified using LC-MS/MS.
Figure 3.3 Effect of blocking host and microbe receptors on total host associated bacteria as determined by qPCR. Treatments which were significantly different from cells that were given no antibody treatment (Control) (p<0.05) are marked with "**". The actual antibody dilutions of each antibody used are in Table 3.2. Error bars indicate standard error from four replicates.
Figure 3.4 Effect of blocking Ef-Tu with an antibody, and adding excess Ef-Tu, on adhesion and invasion of *S. ser.* Typhimurium in a wildtype (WT) background and non-invasive (ΔinvA) background. Treatments that do not share a letter are significantly different (p<0.05). The % numbers of the x axis represents the fraction of total host associated bacteria that were intracellular (invaded). Error bars indicate standard error from four replicates.
Figure 3.5 (A). Effect of blocking specific host proteins with antibodies on adhesion and invasion of *S. ser.* Typhimurium. (B) Effect of blocking specific host molecules with antibodies on total host associated *S* ser. Typhimurium in a wild type (WT) and a non-invasive (ΔinvA) background. Treatments that do not share a letter are significantly different (p<0.05). The % numbers of the x axis represents the fraction of total host associated bacteria that were intracellular (invaded). Error bars indicate standard error from four replicates.
Figure 3.6 (A) Effect of adding excess Ef-Tu from S. ser. Typhimurium and anti-Ef-Tu on NFkB activity of Caco-2 cells (B) Effect of adding excess Ef-Tu from S. ser. Typhimurium and anti-Ef-Tu on NFkB activity of Caco-2 cells in presence of S. ser. Typhimurium (WT) or S. ser. Typhimurium ΔinvA. Treatments that do not share a letter are significantly different (p<0.05). Cell free lysate from S. ser. Typhimurium was used as a positive control for induction of NFkB mediated transcription. Error bars indicate standard error from four replicates.
Figure 3.7

Relative densitometric quantification of p-Akt 473 by western blots. Treatments not sharing a letter are significantly different (p<0.05). Error bars indicate standard error from four replicates.
Figure 3. Neighbor joining tree based on alignment of amino acid sequences of Ef-Tu from bacteria that have known Ef-Tu binding partners in host cells. The Ef-Tu interacting proteins for each organism are mentioned after the species name.
CHAPTER IV

SOLID-PHASE CAPTURE OF PATHOGENIC BACTERIA BY USING GANGLIOSIDES AND DETECTION WITH REAL-TIME PCR

Abstract

We developed a method for concentrating pathogens from samples without enrichment. Immobilized gangliosides concentrated bacteria for detection with real-time PCR. A sensitivity of ~4 CFU/ml (3 h) in samples without competing microflora was achieved. Samples with competing microflora had a sensitivity of 40,000 CFU/ml. The variance was less than one cycle.

Introduction

The Centers for Disease Control and Prevention (CDC) estimates that food-borne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year, which in 2000 led to an estimated $6.9 billion burden to the U.S. economy for just the top five food-borne pathogens (http://www.ers.usda.gov/briefing/FoodSafety/). This avoidable burden can be reduced if the disease-causing organism is detected and remedial action taken quickly to control the spread of the disease. As such, many efforts to create rapid-detection tests (i.e., <24 h) have been undertaken in the last 10 years. The limitation in most methods is the need for a pre-enrichment step before detection to increase the population of interest with semi-selective or selective media. Methods that eliminate the need for enrichment are needed to break this time barrier so that results are provided earlier in the disease process. This
challenge can be addressed by creating assays that concentrate bacteria or by using detection methods that are very sensitive. More recently, use of genetic detection methods is widely available due to the large number of microbial genome sequences that are available.

The AOAC-approved rapid-detection technologies rely on either antibody-based tests or nucleic acid-based tests that claim detection limits as low as one organism, but these assays still require ≥24 to 48 h to provide a result. This time is somewhat shortened by affinity capture of the infectious agent by antibodies onto a solid matrix and then subsequent detection by PCR or real-time PCR (RT-PCR), but these tests still require enrichment, are still based on antibody reactions that can have false-positive results, and require extensive development time to ensure antibody quality. The approach presented in this work avoids the two most time-limiting steps in antibody-based assays, development and pre-enrichment.

An alternative approach to antibodies is the use of host receptors to capture the pathogen, which changes the paradigm of detection to finding those organisms that can bind host cells. Gangliosides are a large class of glycolipids that are common components of eukaryotic cells. Often, the specific classes are expressed in specific tissues that can be exploited to find organisms that will bind cells from specific tissues. In addition, the binding of bacteria and toxins to gangliosides has been documented in the literature (7, 13, 16, 22). To exploit this paradigm, we used host receptors (isolated mixed gangliosides) to mimic pathogen attachment onto the host cell to capture and concentrate pathogens from various samples onto a solid surface. With these molecules, a variety of pathogens can be captured onto a solid phase and subsequently be detected in a
variety of different formats, depending on the amount of information needed during the testing regime. Here, we demonstrated that most common food-borne pathogens bind immobilized gangliosides onto 3-mm glass beads that are easily removed from the sample with physical separation. We determined the lower detection limit of the assay by using *Escherichia coli* O157:H7 as the model organism. After 5 minutes of binding, we detected up to 10 cells by RT-PCR within 3 h of sample collection. Spiked *E. coli* O157:H7 was detected in frozen spinach, pasteurized apple juice, and bottled water with a detection limit of 4 CFU/g of sample.

**Materials and methods**

*Ganglioside immobilization*

Gangliosides were purified from bovine buttermilk as described by Walsh and Nam (26). Briefly, the purification of gangliosides included ultrafiltration of fresh buttermilk (Gosners, Logan, UT) with a 1-kDa membrane to remove the lactose, followed by an organic extraction with chloroform-methanol-water (40:80:30). The purified gangliosides (6 µg) were used for immobilization with 100 g of 3-mm solid glass beads (catalog no. 11-312A; Fisher Scientific, St. Louis, MO), which produced bioactive beads (gangliobeads) with a mixture of gangliosides on the surface.

*Screening of bacteria for ganglioside binding*
In brief, all the organisms were grown to exponential phase in media and under conditions specific for the organism (Table 4.1). The organisms were washed twice in saline and diluted to an optical density at 600 nm of 0.2 in saline. This suspension (2 ml) was exposed to 10 gangliobeads for 10 minutes at 25°C with agitation and washed thrice with 50 mM Tris-Cl (pH 7.2) for 5 minutes each time. The presence or absence of the particular organism on the beads was confirmed either with specific antibody detection via a tagged tertiary antibody or by RT-PCR using universal bacterial 16S primers (IDT, Coralville, IA). Rabbit anti-Salmonella antibody (OEM Concepts, Toms River, NJ), goat anti-E. coli antibody (BioDesign International, Saco, ME), rabbit anti-Listeria monocytogenes antibody (BioDesign International), rabbit anti-Erwinia antibody (Abcam, Cambridge, MA), goat anti-Bacillus globigii spores (Dugway Proving Grounds, Dugway, UT), rabbit anti-Lactococcus antibody (Center for Integrated BioSystems, Utah State University, Logan, UT), and rabbit anti-Lactobacillus antibody (Biotechnology Center, Utah State University, Logan, UT) were all immunoglobulin G-type polyclonal antibodies. They were diluted as recommended by the manufacturer for an enzyme-linked immunosorbent assay or to a micromolar concentration if they were custom products. Tertiary anti-rabbit and anti-goat conjugated to alkaline phosphatase (Sigma, Saint Louis, MO) were diluted as recommended by the supplier. The substrate, p-nitrophenyl phosphate (Sigma, St. Louis, MO) in 0.1 M glycine buffer, 1 mM MgCl₂, 1 mM ZnCl₂, pH 10.4 (1 mg/ml), was added to each sample, and the plates were incubated for 20 minutes at 37°C. Color development was measured using a BioAssay 7000 plate reader (Perkin-Elmer, Norwalk, CT) at 405 nm. Each plate contained controls consisting of aliquots of each concentration of the specific antibody, with the appropriate tertiary
antibody without bacteria (negative control), the tertiary antibodies alone, and the substrate alone. The signal-to-noise value was calculated by dividing the absorbance readings from each sample by the negative-control value. The entire experiment was done with three biological replicates.

Detection with RT-PCR used washed gangliobeads that were added to 100 µl distilled water and boiled for 10 minutes to release the DNA. An aliquot of the boiled sample (12.5 µl) was used for RT-PCR with universal bacterial 16S primers (IDT, Coralville, IA), using a DyNAmo HS Sybr green quantitative PCR kit (MJ Research, Waltham, MA) per the manufacturer's recommendations and a DNA Engine Opticon 2 thermal cycler (MJ Research). The thermal cycler was run for 50 cycles of 15 s at 95°C (melting), 30 s at 60°C (annealing), and 45 s at 72°C (extension and plate reading). The threshold cycle (C_T) number was reported using Opticon Monitor analysis software (version 2.02; MJ Research). Appropriate negative controls for probing for nonspecific binding (using 3-mm glass beads instead of gangliobeads) and sterility in the beads and buffers (using gangliobeads with saline instead of bacterial suspension) were also used along with the test samples. The experiment was done with three biological replicates, using duplicate RT-PCRs.

Capture and detection of E. coli 0157:H7 from buffers and food. To check the lower detection limit of this approach, we exposed 2-ml 10-fold serial dilutions of log-phase E. coli O157:H7 cells (10^8 to 10^0 CFU/ml; cell population was determined using plate counts with nutrient agar) to 10 gangliobeads. The beads were either unwashed or washed before testing with RT-PCR. The organisms on the beads were detected using the commercially available Genevision rapid pathogen detection system for E.
coli O157:H7 (Warnex Diagnostics, Quebec, Canada) per the manufacturer's instructions. Pasteurized apple juice, commercial bottled water, sterile ultrahigh-temperature milk, salami, hamburger patties, and frozen spinach were purchased from the local grocery store, plated on nutrient agar (as described in the Bacteriological Analytical Manual (17)), and spiked with log-phase E. coli O157:H7 cells at the levels of $10^6$, $10^4$, $10^2$, $10^1$, $10^0$, and 0 CFU/25-g sample after the pure-culture population was determined by plating on nutrient agar. Spinach, hamburger patties, and salami were diluted 1:1 (weight to volume) with saline in a filtered stomacher bag and stomached at high speed using the Seward stomacher laboratory system (Lab3; Bristol, England) for 15 min, and the sample rinse was spiked with the above-mentioned E. coli O157:H7 population. Food samples (25 g) were exposed to 30 gangliobeads on a rocking platform for 30 min. After 30 min, the beads were removed by decanting the liquid from the sample and added to 400 µl of lysis buffer supplied with the Genevision rapid pathogen detection kit, and PCR was performed per the manufacturer’s instructions. The aerobic plate counts of all the foods were estimated prior to spiking with E. coli O157:H7 by using nutrient agar at 32°C as described in the Bacteriological Analytical Manual (17). The average of three replicate plate counts was reported. The experiment was done with three biological replicates, using RT-PCR in duplicate.

Results and Discussion
We screened 20 different bacterial strains for their abilities to specifically bind immobilized gangliosides (Table 4.1). Ten strains of *Salmonella*, two strains of *Escherichia coli*, *Lactococcus lactis*, and *Bacillus globigii* spores bound to the beads. However, *Lactobacillus acidophilus*, *Lactobacillus gasseri*, *Lactobacillus helveticus*, *Listeria monocytogenes*, and *Erwinia herbicola* did not bind to the immobilized ganglioside mixture. All of the tested enterobacteriaceae bound the gangliobeads in this study. While variability among *Salmonella* strains with respect to binding gangliosides is known, we focused on *Salmonella enterica serovar Enteritidis*, where all the strains tested bound. Of the firmicutes tested, only *L. lactis* IL-1403 bound the beads, indicating that organisms used routinely in fermented foods will not interfere with this assay. None of the *Listeria* strains tested bound the beads.

Subsequently, we focused on testing the detection limit by using *E. coli* O157:H7 with RT-PCR (Genevision) as a proof of concept for this system (Figure 4.1). The lower detection limit of the Genevision assay (i.e., without addition of gangliobeads for concentration) using direct extraction was 1,000 CFU/ml. This detection limit was improved with addition of the gangliobead capture step to 10 CFU/ml. Washing beads prior to detection via RT-PCR caused a loss in detection for *E. coli* populations with \( \geq 10^4 \) CFU/ml, but at lower populations, no significant difference (P > 0.05) was observed. Washing the beads led to loss of organisms that were not tightly bound to the bead surface, just as described by Blake and Weimer (3), who used a solid-phase enzyme-linked immunosorbent assay for *Bacillus* spores from water. To further increase the binding capacity, more beads may be added to increase the number bacteria that will be
bound (3). Hence, gangliobeads efficiently concentrated *E. coli* O157:H7 from solution, even when the organisms were present at 10 CFU/ml.

The inability of the Genevision system alone (without the use of a solid-phase concentration) to detect organisms at <1,000 CFU/ml is due to the need for at least 1 CFU/10 µl (the volume of cell lysate used in each PCR), which equates to 1,000 CFU/ml. This limitation was overcome by the addition of gangliobeads to the sample to concentrate low cell numbers from a large volume. This resulted in a volume reduction that is more compatible with sample processing requirements for PCR (i.e., microliter volumes rather than milliliter volumes).

After ascertaining the ability of gangliobeads to concentrate *E. coli* O157:H7 from solution, we tested the ability of this approach to detect spiked organisms directly from food (Table 4.2). We detected spiked *E. coli* O157:H7 in bottled water, apple juice, and frozen spinach with a lower detection limit of 4 CFU/g. The lower detection limit for hamburger was 400 CFU/g, while for salami and milk the limit was 40,000 CFU/g. Except with milk, a significant correlation ($r^2 = 1; P < 0.0001$) between the level of background microflora and the minimum detection limit for each food was observed (Table 4.2). Hence, it is likely that the background microorganisms out-competed the spiked *E. coli* O157:H7 for binding to gangliosides, which explains the higher detection limits for hamburger and salami. The higher detection limit for milk with no competing flora is likely due to the presence of milk fat globule membranes that contain gangliosides and lactoferrin, which binds gangliosides. In either case, this would lead to competitive binding with *E. coli* O157:H7 for the gangliosides (26). Weimer et al. (27) did not observe competition for solid-phase capture when using antibodies immobilized
onto glass beads. In light of these data, the efficiency of binding with gangliobeads is affected by the levels of background microflora and other molecules in the foods that bind gangliosides. Hence, ganglioside-immobilized beads would be suitable for food systems containing low initial microbial populations and foods of non-animal origin (e.g., bread, fruit juices, ready-to-eat salad, water, fruits, and vegetables).

The variability of the assay was measured for each approach with buffer and foods. The standard errors of the means (SEMs) for the decreasing concentrations of *E. coli* ranged from 0.03 to 0.81 cycles in unwashed samples, while washed samples had a range of 0.36 to 0.92 cycles (Figure 4.1, error bars). This translated into coefficients of variation (CVs) of between 1.9 and 3.9% over the concentrations in the wash treatments. For the food samples, the variance and % CV differed by sample, but all SEMs were less than 1.32 and all CVs less than 6.8%. The average CV for concentrations in all foods was 3.1%.

The specificity and sensitivity of PCR-based rapid-detection systems have driven their increased popularity in the last 2 decades (6). There are several PCR-based pathogen detection systems available commercially, such as BAX (DuPont Qualicon, Wilmington, DE), Probelia (Sanofi Diagnostic Pasteur, Marnes La Coquette, France), and Genevision (Warnex Diagnostics, Quebec, Canada), but all are hindered by a detection limit ranging from $10^3$ to $10^6$ CFU/ml tied to the presence of PCR inhibitors in the sample, sample dilution prior to cell lysis, or large sample volumes that do not allow a representative sample for PCR. To overcome these limitations, the sample is pre-enriched in laboratory media for 6 to 48 h prior to detection in at least 2 liters of media. The enrichment procedures are labor-intensive and do not allow quantitative or high-throughput
automated screening procedures. In addition, all the commercially available PCR-based systems require at least 24 to 48 h to report a result. To lower the detection times, various researchers have used antibody-coated immunomagnetic beads (4, 8-10, 15, 20, 25, 28) to capture a variety of pathogens, such as *Salmonella*, *E. coli*, *Campylobacter*, *Listeria*, *Yersinia*, and *Clostridia*, for subsequent detection of them with PCR or RT-PCR. These studies achieved sensitivities of 5 to 1,000 CFU/ml within 3 to 10 h in food systems. The advantage of using gangliosides over using antibody-coated beads is that one can capture a broad repertoire of pathogens by using a single reagent. The specificity of detection can be adjusted by using PCR for each pathogen of interest with various primer designs and combinations. Simultaneous detection of various pathogens in one PCR is also possible by employing multiplex PCR (19). Additionally, coupling gangliobead capture with RT-PCR without pre-enrichment for detection makes the assay quantitative even at a low population of 4 CFU/g. Furthermore, the specificities of ganglioside binding to polyomavirus, simian virus 40 (12), Newcastle disease virus (11), human immunodeficiency virus (21), adenovirus (2), and human influenza virus (23) suggest that this technology has potential use for viral detection when coupled with real-time reverse transcriptase PCR (14). Ganglioside-immobilized beads could also be used for capturing specific toxins, such as botulinum toxin (1, 24), cholera toxin (5), and *E. coli* heat-labile enterotoxin (18), with subsequent detection via a solid-phase immunoassay, such as that reported by Weimer et al. (27). Mason et al. (16) successfully used liposomes with encapsulated DNA reporters and ganglioside receptors embedded in the bilayer to detect botulinum toxin with a lower detection limit of 0.02 fg/ml. Coupling of ganglioside-immobilized beads with the ImmunoFlow system (27), which uses a
fluidized bead bed for capture, may increase the sensitivity to as low as 1 CFU independent of the sample size, as demonstrated for *E coli* O157:H7 and *Bacillus globigii* spores by using antibody-coated beads.

In conclusion, we demonstrated the ability of gangliosides immobilized on 3-mm glass beads to concentrate pathogenic organisms and Bacillus spores directly from samples without pre-enrichment. Use of gangliobeads prior to a commercial RT-PCR kit for food pathogen detection improved the sensitivity 100-fold in samples that did not contain competing bacteria or molecules that bind gangliosides. However, with competing microflora and biomolecules the detection limit was 40,000 CFU/g. This approach concentrated *E. coli* O157:H7 directly from foods containing low initial bacterial counts and foods of non-animal origin with a detection limit of 4 CFU/g in 3 h.

References


Table 4. 1 Growth conditions and ganglioside binding specificity of selected bacteria. (NB = Nutrient broth, NA = not done, MRS = MRS broth, BHI = BHI broth, 1 = organism detected via specific antibody, 2 = organism detected by RT-PCR). The *Bacillus* spores were provided as a powder and no growth was needed.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Strain</th>
<th>Medium</th>
<th>Temp. (°C)</th>
<th>Growth Condition</th>
<th>Ganglioside Binding</th>
<th>Detection Method</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>K12</td>
<td>NB</td>
<td>37</td>
<td>Shaking</td>
<td>+</td>
<td>1, 2</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>O157:H7</td>
<td>NB</td>
<td>37</td>
<td>Shaking</td>
<td>+</td>
<td>1, 2</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em></td>
<td>8326</td>
<td>NB</td>
<td>37</td>
<td>Shaking</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em></td>
<td>13076</td>
<td>NB</td>
<td>37</td>
<td>Shaking</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em></td>
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<td>NB</td>
<td>37</td>
<td>Shaking</td>
<td>+</td>
<td>1</td>
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<tr>
<td><em>Salmonella enteritidis</em></td>
<td>31194</td>
<td>NB</td>
<td>37</td>
<td>Shaking</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em></td>
<td>49214</td>
<td>NB</td>
<td>37</td>
<td>Shaking</td>
<td>+</td>
<td>1</td>
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<tr>
<td><em>Salmonella enteritidis</em></td>
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<td>1</td>
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<tr>
<td><em>Salmonella enteritidis</em></td>
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<td>Shaking</td>
<td>+</td>
<td>1</td>
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<tr>
<td><em>Salmonella enteritidis</em></td>
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<td>37</td>
<td>Shaking</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
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<td>700720</td>
<td>NB</td>
<td>37</td>
<td>Shaking</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td><em>Bacillus globigii spores</em></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>IL1403</td>
<td>MRS</td>
<td>37</td>
<td>Static</td>
<td>+</td>
<td>1, 2</td>
</tr>
<tr>
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<td>4355</td>
<td>MRS</td>
<td>37</td>
<td>Static</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
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<td>MRS</td>
<td>37</td>
<td>Static</td>
<td>-</td>
<td>2</td>
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<td><em>Lactobacillus gasseri</em></td>
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<td>Shaking</td>
<td>-</td>
<td>2</td>
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<td>-</td>
<td>1</td>
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<tr>
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<td>37</td>
<td>Shaking</td>
<td>-</td>
<td>1</td>
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<tr>
<td><em>Listeria monocytogenes</em></td>
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<td>BHI</td>
<td>37</td>
<td>Shaking</td>
<td>-</td>
<td>2</td>
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<tr>
<td><em>Erwinia herbicola</em></td>
<td>33243</td>
<td>NB</td>
<td>37</td>
<td>Shaking</td>
<td>-</td>
<td>1</td>
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</table>
Table 4.2 Minimum detection limit of spiked *E. coli* O157:H7 in the food types used in this study and the levels of food associated background microflora.

<table>
<thead>
<tr>
<th>Food Type</th>
<th>Aerobic Plate Count before <em>E. coli</em> O157:H7 spike (CFU/gm.)</th>
<th>Lower Detection Limit (CFU/gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Apple juice</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Spinach</td>
<td>577</td>
<td>4</td>
</tr>
<tr>
<td>Hamburger</td>
<td>24856</td>
<td>400</td>
</tr>
<tr>
<td>Salami</td>
<td>9350000</td>
<td>40000</td>
</tr>
<tr>
<td>Milk</td>
<td>0</td>
<td>40000</td>
</tr>
</tbody>
</table>
Figure 4. Threshold cycle numbers (CT) when gangliobeads were exposed to different cell populations of *E. coli* O157:H7 with or without washing the beads. The error bar represents the SEM of CT values from six observations. A CT of >45 was considered no detection. Shorter CT is equal to a faster detection time, which indicates a higher population.
CHAPTER V
SYSTEMS LEVEL ANALYSIS OF CROSS TALK BETWEEN THE HOST COMMENSAL AND PATHOGEN

Abstract

*Bifidobacterium longum* subspp. *infantis* showed the highest potential for *Salmonella enterica* subspp. *enterica* ser. Typhimurium exclusion in a Caco-2 cell culture model among the five screened probiotic strains. *B. infantis* and *S. ser. Typhimurium* shared binding specificity to host ganglioside GM1 among the tested receptors. Further, *B. infantis* completely inhibited, *Salmonella*-induced caspase 8 and caspase 9 activity in intestinal epithelial cells. *B. infantis* also reduced the basal caspase 9 and caspase 3/7 activity in the epithelial cells in absence of pathogen. Western blots and gene expression profiling of epithelial cells revealed that the decreased caspase activation was concomitant with increased phosphorylation of the pro-survival protein kinase Akt, increased expression of caspase inhibiting protein cIAP and decreased expression of genes involved in mitochondrion organization and biogenesis and reactive oxygen species metabolic processes. Hence *B. infantis* exerted it’s protective effect by repression of mitochondrial cell death pathway which was induced in presence of *S. ser.* Typhimurium. Gene expression analysis of epithelial cells also revealed that *B. infantis* strongly induced expression of genes downstream of cAMP signaling, MAPK signaling and NFkB signaling. Using both gene expression and small metabolite profiling of bacterial and epithelial cells, I observed a three way crosstalk between the epithelial cells, *Salmonella* and *Bifidobacteria*. In these interactions, *B. infantis* prevented catabolism of
arginine (a substrate for nitric oxide (NO) production) by S. ser. Typhimurium, making it available for increased NO production by the host. This was evidenced by the induction of genes necessary for nitrate and nitrite respiration in the pathogen (nitrate and nitrite are breakdown products of NO). Using a comparative and functional genomics approach I also discovered a cluster of 50 proteins that have been acquired in the B. infantis genome through lateral gene transfer, are found only in subspp. infantis (and absent in subspp. longum) and are induced only in the presence of the host. Therefore these genes likely have been maintained in the genome through selective pressure from the host.

**Introduction**

The normal gut flora forms the first line of defense against enteric infections (77). The composition of this protective flora can be altered by dietary and environmental influences making the host susceptible to disease. The use of probiotics centers on the idea that probiotic treatments re-establishes the natural condition that exists in the host which is disrupted by various stresses (31). Recently, there has been a renewal of interest in the use of probiotics, due to the rise in number of multidrug resistant pathogens; and the increased knowledge of the roles played by human microbioata in health and disease. Renewed interest in probiotics is also reflected by the rapid increase in the numbers of “probiotic” products being marketed.

Efficacy of probiotics in the prevention of gastrointestinal diseases has been observed in several in vivo and in vitro models. A study in the pig model showed that feeding a five-strain probiotic mixture (that contained two strains of Lactobacillus murinus, and one strain each of Lactobacillus salivarius, Lactobacillus pentosus and
*Pediococcus pentosaceous* reduced pathogen shedding and alleviated disease symptoms when infected with *S. ser. Typhimurium* (11). Although the specific mechanism of pathogen reduction was not elucidated, this suggests that probiotics can inhibit infection and reduce symptoms. The pathogen exclusion potential of various probiotics is dependent on the probiotic as well as the pathogen strain. Jin et al. (38) showed that in vitro *L. acidophilus* inhibited adhesion of *S. sv Pullorum*, but failed to inhibit adhesion of *S. sv Enteritidis* and *S. sv Typhimurium*. A cell culture study has also demonstrated the anti-adhesive effect of *Bifidobacterium* on *S. ser. Typhimurium*. The ability of *Bifidobacteria* to inhibit pathogen attachment is strain-dependent and independent of strain specific adhesion properties (6).

Le Blanc et al. (17) recently showed the preventative and therapeutic efficacy of *L. casei* in a mouse model of *S. ser. Typhimurium* infection. Administration of *L. casei* not only decreased neutrophil infiltration and inflammation, but also increased the release of *Salmonella* specific IgA in the intestinal lumen. Hence the protective effect of probiotics goes beyond the commonly reported modes of action that include competing for nutrients, production of antimicrobials, and pathogen exclusion at the host surface (48).

Silva et al. reported the protective effect of *B. longum* in a mouse model of salmonellosis, where mice fed *B. longum* and infected with *S. ser. Typhimurium* showed higher survival than those infected without the probiotic intervention (76). Surprisingly, pathogen levels in the animal feces were similar with or without *B. longum* even though survival rate was higher in the animals supplemented with *B. longum*. Hence additional mechanisms other than competitive exclusion accounted for higher survival of
Salmonella infected animals. Among all age groups the incidence of Salmonella-induced diarrhea is highest in the infant population (13). Bifidobacteria are one of the most numerically abundant and among the first colonizers of the human gut (3). It is estimated that in breast fed infants ~10% of the gut microbiota is composed of Bifidobacteria (43). Since Bifidobacteria show anti-infective activity towards Salmonella, the molecular mechanism by which they inhibit pathogen adhesion and protect the host needs to be investigated.

At the host mucosa there is a complex multi-way interaction between host, pathogen and the resident gut flora that dictates the outcome of the infection. Most studies have been dedicated to unraveling the effect of probiotics/commensals/pathogen on the host while the literature on the microbe-microbe interaction is sparse at best. Recently there is a steady increase in literature that reports interspecies communication that would affect the virulence gene expression of pathogens. For example, short chain fatty acids like acetate, propionate and butyrate induce the expression of hilA (invasion protein transcription activator), which positively regulates the virulence gene expression in Salmonella (21). Communication between Salmonella and Yersinia through acyl homoserine lactones (AHLs) has also been reported, which enhances Salmonella colonization in the murine intestine (22). Hence interspecies crosstalk can lead to different outcome of the disease

I hypothesize that B. longum spp. infantis reduces S sv. Typhimurium adhesion to gut epithelial cells by competing for the same receptors and also improves cell survival by modulation of specific pathways in the host as well as the pathogen. Modulation in cellular networks was probed by determining gene-expression profile and small
metabolite profiles of the epithelial cells, *B. infantis*, and *S. ser.* Typhimurium during co-infection.

**Materials and method**

*Cell culture and bacterial strains*

Caco-2 cells were obtained from ATCC (HTB-37, Manassas, VA) and cultured as per ATCC’s recommendation. All the cells used in the assay were between passage numbers 22-30. In brief, cells were plated at a density of $10^5$ / cm$^2$ in either a T75 or a 96 well plate. Cells were maintained in DMEM/High Modified (Thermo Scientific, Rockford, IL) with 16.6% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), non-essential amino acids (Thermo Scientific, Rockford, IL), 10mM MOPS (Sigma, St. Louis, MO), 10 mM TES (Sigma), 15 mM HEPES (Sigma) and 2 mM NaH$_2$PO$_4$(Sigma). Cells were considered to be differentiated 14 days post confluence (65), and used for the adhesion assays, caspase assays and gene expression. Bacterial cells were grown as described in Table 5.9.

*Determination of adhered and invaded bacteria*

The ability of specific bacteria to block *Salmonella* binding to epithelial cells was tested by determining the changes in the amount of total host (intestinal epithelial cells, Caco-2) associated salmonella in presence of specific bacteria. The amount of total host associated salmonella was determined by qPCR as described below. The amounts of
intracellular salmonella in the host were determined by the gentamicin protection assay with a few modifications as described below (24).

Total host associated bacteria were determined as follows. Caco-2 cells were cultured as described earlier in a 96-well plate. The bacteria were used after two transfers for the adhesion assays. Bacterial cells were collected from 2 ml of media after growth for 14 hours, washed twice with an equal volume of PBS, and resuspended at ~10^8 CFU/ml, in DMEM/High modified with 1X non-essential amino acids, 10mM MOPS, 10 mM TES, 15 mM HEPES and 2 mM NaH2PO4 but without the FBS. Caco-2 cells were infected with either *Salmonella* alone or *Salmonella* in conjunction with other bacteria (Fig 5.1) in a final volume of 50 µl at an MOI (multiplicity of infection) of 1:1000. The ratio of *Salmonella* with other bacteria was 1:1. Caco-2 cells were incubated with bacteria for 60 minutes. The bacterial cell suspension was aspirated and the Caco-2 monolayer was washed thrice with 200 µl of tyrode’s (140 mM NaCl, 5mM KCl, 1mM CaCl2, 1mM MgCl2, 10 mM glucose, 10mM sodium pyruvate, 10 mM HEPES, pH 7.4) to remove non-adhered bacterial cells from the monolayer. DNA extraction buffer (AEX Chemunex, France) (50 µl) was used to lyse the monolayer and the bacteria associated with the host, and incubated at 37°C for 15 minutes followed by 95°C for 15 min. Resulting cell lysate was used to determine the number of bacteria associated with the Caco-2 cells (19). Quantitative analysis was done using qPCR with a CFX 96 Real Time System (Bio-Rad, Hercules, CA). Reactions were performed in a final volume of 25 µl including 1 µl of cell lysate, 100 nM of PCR primers and iQ SYBR Green Supermix (Bio-Rad, Hercules CA) as per manufacturer’s instructions. The primers used for the amplification are listed in Table 5.9. The reaction parameters consisted of denaturation
step at 95°C for 5 min, followed by 40 cycles of denaturation, annealing and extension at 95°C for 15 s, 56°C for 30 s, 72°C for 30 s respectively and a final extension at 72°C for 1 min. The product was verified using a melt curve analysis from 50°C to 95°C with a transition rate of 0.2°C/s. The amount of bacterial cells and Caco-2 cells present in each well were estimated using a standard curve of $C_T$ vs. $\log_{10}$ CFU and amount of bacteria per Caco-2 was calculated. The data were normalized relative to control wells which were incubated with *Salmonella* alone. The experiment was done in four replicates. Differences in the mean due to treatment were tested by one way ANOVA. Post ANOVA, the means were compared to control using Dennett’s multiple comparison test.

The amount of intracellular and adhered bacteria was determined using the gentamicin protection assay as follows. Caco-2 cells were infected exactly as described earlier. The infected cells were incubated for 60 minutes at 37°C with 5% CO$_2$. Upon incubation, media was aspirated and cells were washed three times with tyrode’s buffer. Invaded bacteria were estimated by incubating the cells with 100 µg/ml gentamicin for two hours at 37°C with 5% CO$_2$ to kill bacteria adhered or outside the Caco-2 cells. To determine total host associated bacteria, cells were incubated with cell culture media without any antibiotic. Cells were again washed three times with tyrode’s buffer and lysed with 0.01% triton. The amounts of bacteria in the Caco-2 lysate were determined by plating serial dilutions on LB agar. The experiment was performed in four replicates. The number of adhered bacteria were enumerated by subtracting mean of invaded bacteria (B) from mean of total host associated bacteria (A) and error ($\Delta Z$) was calculated as $(\Delta Z)^2 = (\Delta A)^2 + (\Delta B)^2$ where, $\Delta A$ is SEM associated with the A and $\Delta B$ is SEM associated with B. The data were reported as $\log_{10}$ colony forming units (CFU)/well. Differences in the
mean due to treatment were tested by one-way ANOVA. Post ANOVA, the means were compared to each other by Tukey-Kramer's method.

Caspase assays

Caco-2 cells were cultured in a 96 well plate as mentioned earlier and were washed with phosphate buffered saline (PBS) before infection with bacteria. Caco-2 cells were infected with either *Bifidobacterium longum* subsp. *infantis* ATCC 15697, *Salmonella ser.* Typhimurium LT2 ATCC 700720, or both the organisms simultaneously. Upon adding bacterial treatments (MOI of 1:1000) in a volume of 50 µl, cells were incubated at 37°C with 5% CO₂. After 8 hours, caspase 8, 9 and 3/7 activities was measured using Caspase-Glo assay kits (Promega, Madison, WI) exactly as per manufacturer’s instructions. Bioluminescence due to caspase activity was measured using the DTX 880 Multimode Detector (Beckman Coulter, Brea, CA). Differences in the mean due to treatments were tested by one way ANOVA. Post ANOVA, the means were compared to each other by Tukey-Kramer's method.

Infection of epithelial cells for gene expression

Caco-2 cells were cultured in T75 as mentioned earlier, and were serum starved for 24 hours prior to infection with bacteria. Caco-2 cells were infected with either *Bifidobacterium longum* subsp. *infantis* ATCC 15697, *Salmonella ser.* Typhimurium LT2 ATCC 700720, or both the organisms simultaneously at an MOI of 1:1000 in a final volume of 10 ml. All the three bacterial treatments were also incubated in absence of the Caco-2 cells in the exact same conditions. All the infected cells were incubated at 37°C
with 5% CO₂. At 30, 60, and 120 minutes after infection, media with non-adherent bacteria was aspirated and 10 ml TRIzol LS reagent (Invitrogen, Carlsbad, CA) was added to the cells. This was gently mixed with pipette followed by centrifugation at 7200 \( \times g \) for 5 minutes to pellet the bacteria. TRIzol LS supernatant was stored in a clean tube and further processed for RNA extraction from Caco-2 cells. The bacterial pellet was re-suspended in 2 ml of fresh TRIzol LS, gently mixed and further processed for RNA extraction from host associated bacteria

_Bacterial RNA extraction and gene expression_

The TRIzol LS suspension containing host associated bacteria was centrifuged at 7200 \( \times g \) for 5 minutes. The TRIzol LS supernatant was stored in a clean tube for later use. 1 ml of lysis enzyme cocktail containing 50 mg/ml of lysozyme (Sigma) and 200 U/ml mutanolysin (Sigma) in TE buffer (10mM Tris and 1mM EDTA, pH 8) was added to bacterial pellet obtained by centrifugation. The solution was mixed gently and incubated at 37°C for 1 hour followed by centrifugation at 7200 \( \times g \) for 5 minutes. The supernatant was discarded and pellet was re-suspended in 250 μl of proteinase K buffer (100 mM Tris-HCl, 5 mM EDTA, 200 mM NaCl, and 0.2 % SDS, pH 8) containing 8 U/ml of proteinase K (Fermentas, Glen Burnie, MD). This was incubated at 55°C for 1 hour with intermittent mixing. To this, previously stored TRIzol LS was added and gently mixed. RNA was isolated from TRIzol LS exactly as per manufacturer's recommendations. RNA concentration, A260/280 and A260/230 were measured on NanoDrop (Thermo scientific, Waltham, MA) and samples were processed further only if the RNA concentration was at least 0.5 μg/μl and ratios were ≥ 1.8. The RNA samples
were analyzed for integrity on 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Total RNA (10 µg in 20 µl) was reverse transcribed into cDNA with random hexamers and Superscriptase II (Invitrogen, Carlsbad, CA) exactly as per manufacturer’s recommendations by using 400 U of Superscriptase II/ 10 µg RNA. Upon cDNA synthesis, the enzyme was heat inactivated and the RNA templates were degraded with 80 U of RNaseH (Epicentre, Madison, WI) at 37°C for 20 minutes. The reaction mixture was cleaned using the Qiaquick-PCR purification kit (Qiagen, Valencia, CA) exactly as per manufacturer’s instructions. Purified cDNA was eluted from the column twice with a total of 100 µl of nuclease free water (Ambion, Austin, TX).

cDNA was fragmented using DNaseI (Promega, Madison, WI) according to manufacturer's recommendations by using 0.6 U of DNaseI/ µg cDNA at 37 °C for 20 minutes. The fragmented cDNA was labeled using GeneChip DNA Labeling reagent (Affymetrix, Santa Clara, CA) and Terminal Transferase enzyme (TdT) (New England Biolabs, Ipswich, MA) as per manufacturers' recommendation by using 2µl GeneChip labeling reagent and 3µl TdT / µg cDNA at 37 °C for 60 minutes. The samples were denatured prior to hybridization, at 98°C for 10 minutes followed by snap cooling at 4°C for 5 minutes.

Labeled cDNA was hybridized onto two different custom made Affymetrix GeneChip designed against all the annotated coding sequences of *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (50) and *Salmonella enterica* subsp. *enterica ser. Typhimurium* LT2 ATCC 700720. Briefly the *Salmonella* array contained 9852 probe sets, of which 4735 probe sets were designed against S. *ser. Typhimurium*. Each probe
set contained 11 probes, each 25 nucleotides long. The *Bifidobacteria* chip has been described by Locascio et al (50). 500 ng of labeled cDNA for samples extracted from pure culture of *B. infantis* and *S. ser*. Typhimurium; 1000 ng labeled cDNA for samples extracted from co-culture of *B. infantis* and *S. ser* Typhimurium; 2000 ng of labeled cDNA for samples extracted from co-culture of *B. infantis*/*S. ser*. Typhimurium and Caco-2; and 2500 ng of labeled cDNA for samples extracted from co-culture of *B. infantis*, *S. ser*. Typhimurium and Caco-2 was hybridized onto the respective chips. In total, 24 chips *Salmonella* chips were processed (2 replicates X 3 Time points (30 minutes, 60 minutes, 120 minutes) X 4 Treatments ) and 8 *Bifidobacteria* chips were processed (2 reps X 1 Time point (120 mins) X 4 Treatments). 

*Microarray data normalization and statistical analysis of microbial chips*

Raw data (.cel files) was back ground corrected, quantile normalized and summarized using RMA-MS (78).The resultant normalized log₂ transformed intensity matrix was used for further statistical analysis. To detect differentially regulated genes, the *Salmonella* chip data was analyzed as unpaired time course analysis with “signed area” as the time summary method, while for *Bifidobacteria* chips the data was analyzed as two class unpaired data, with T statistic, using Significance Analysis of Microarrays (SAM) (84). All the genes were ranked based on the score(d) from SAM output. This pre ordered ranked gene list was then used in Gene Set Enrichment Analysis software (GSEA) (60, 79) to detect the coordinate changes in the expression of groups of functionally related genes, upon respective treatments. Gene sets for *Bifidobacteria* were defined based on annotations from KEGG (41), Cluster of Orthologous Groups of
proteins (COGs) (81), Carbohydrate Active Enzyme Database CAZY (66) and genes identified from Locasio et al (50). Putative horizontally transferred genes in *B. infantis* were identified using Integrated microbial genomes system (IMG) (55). Genes sets based on protein localization were created based on annotations from CoBaltDB (34). Gene sets for *Salmonella* were defined based the annotations from Comprehensive Microbial Resource (CMR) (68), Cluster of Orthologous Groups of proteins (COGs) (81), Virulence Factors of pathogenic bacteria Database (VFDB) (14, 89), Carbohydrate Active Enzyme Database (CAZY) (66), and Biocyc (12). Gene sets based on putative horizontally transferred genes in *Salmonella* were defined using predictions from the Horizontal Gene Transfer Database (HGT-DB) (32). Genes sets based on protein localization were created using predictions from CoBaltDB (34).

*Caco-2 RNA extraction and gene expression*

The TRIZol LS supernatant obtained after pelleting the bacteria was freeze thawed twice in liquid nitrogen. Two hundred fifty μl water was added to 750 μl of TRIZol LS sample. This was further processed for RNA extraction exactly as per manufacturer’s instructions. RNA concentration, A260/280 and A260/230 were measured on NanoDrop (Thermo Scientific, Waltham, MA) and samples were processed further only if the RNA concentration was at least 0.5 μg/μl and ratios were ≥ 1.8. The RNA samples were analyzed for integrity on 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Synthesis of cDNA, biotin labeled cRNA, fragmentation and purification of cRNA were carried out using one-cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA) exactly as per manufacturer's instructions. 10 μg of labeled and fragmented cRNA was hybridized
microarray data normalization and statistical analysis of the HGU133 Plus2 chips

Raw data (.cel files) was back ground corrected, quantile normalized and summarized using RMA (37). RMA normalized data was then filtered through the PANP algorithm (86) to make presence-absence calls for each probe set. Probe sets that were called present in at least one of the samples were included in further statistical analysis while rests were excluded. The resultant normalized, filtered, log₂ transformed intensity matrix was analyzed as two class unpaired time course data with “signed-area” as the time summary method, using Significance Analysis of Microarrays (SAM) (84). All the genes were ranked based on the score(d) obtained from SAM. This pre ordered ranked gene list was used as an input for Gene Set Enrichment Analysis software (GSEA) (79) to detect the coordinate changes in the expression of groups of functionally related genes, upon respective treatments. The gene sets were based on Gene Ontology annotations, KEGG annotations, Biocarta annotations and TRANSFAC annotations and were downloaded from the molecular signatures database (79). Ingenuity pathway analysis (IPA) was used to map expression data onto pathways. Hierarchical clustering with multiscale bootstrapping (1000 times) of all the samples based on normalized data was performed using PVCLUST (80).
Western Blots

Relative abundance of target proteins due to different treatments was quantified by densitometric analysis of western blots. Caco-2 cells were cultured and infected exactly as those infected for the gene expression. The cells from the T75 were removed from the flask by scraping; suspended in 1 ml protease/phosphatase inhibitor cocktail (30 mM HEPES, 1 mM EDTA, 50 mM Sodium pyrophosphate, 100 mM sodium fluoride, 10 mM orthovanadate, 1 Roche protease inhibitor cocktail tablet/50ml solvent) and lysed using a bead beater at full speed with three pulses of 30 s each with intermittent incubation on ice for 1 min. The cell lysates were stored at -70°C for further analysis. 50µg protein was diluted in 1X SDS sample buffer and the samples were heated at 95°C for 10 minutes and centrifuged at 12,000 X g for 10 minutes. The sample was resolved on precast 10% Tris-HCl polyacrylimide gels (Bio-Rad Laboratories, Hercules, CA) at a constant current of 45 mAmp per gel using the Criterion electrophoresis system (Bio-Rad Laboratories, Hercules, CA). The resolved proteins were then transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA) using the Trans-Blot semi-dry electrophoretic cell (Bio-Rad Laboratories, Hercules, CA) as per manufacturer's recommendation. Upon completion of the transfer, the blots were probed for the presence of p-Akt 473 (#9271) and p-AKT 308 (#9275) by Pierce® Fast Western Blot Kit (Thermo Fisher Scientific, IL, USA ), according to the manufactures recommendations. Primary antibodies for all the proteins were purchased from Cell Signaling Tech. (Boston, MA). The blots were imaged using the Kodak Image Station 2000R (Carestream Health, Rochester, NY). Densitometric analysis of the blot was done using Image J (2).
Differences in the means due to treatment were tested by two way ANOVA with repeated measures.

**Small metabolite profiling**

Fifty µl of extracellular supernatant and intracellular cell lysate collected from the infection experiments were extracted with equal volume of ice cold methanol and incubated at -20°C for 60 minutes. The resultant precipitates were separated from the sample by centrifugation at 20,000 X g for 10 minutes. The supernatant was evaporated to dryness using a speed-vac at room temperature. Metabolite profiles for all the samples were determined using GC-MS at the Genome Center, UC Davis (Davis, CA) exactly as described by Fiehn et al (28). In brief, the dried sample was derivatized using N-methyl-N-(trimethylsilyl)-trifluoroacetamide (Sigma, St. Louis, MO) and spiked with retention index markers (RI) exactly as described by Fiehn et al (28). The samples were analyzed on Agilent 6890 gas chromatograph controlled using Leco ChromaTOF software version 2.32, as described by Fiehn et al (28). GC/MS peaks were annotated based on the mass spectra and retention index using the BinBase database (27). Peak areas were normalized by calculating the sum area of all identified compounds for each and subsequently dividing all data associated with a sample by the corresponding metabolite sum. The resulting data were multiplied by a constant factor in order to obtain values without decimal places and, the peak intensities were log2 transformed to remove the heteroscedasticity (28). Hierarchical clustering with multiscale bootstrapping (1000 times) of all the samples based on normalized data was performed using PVCLUST (80).
Results

The pathogen exclusion potential of different probiotics is strain dependent. Since intestinal epithelial cells are the primary host target during the initial phase of Salmonella infection, I screened five different probiotic strains for their ability to block host association of Salmonella sv. Typhimurium (Figure 5.1) in cultured gut epithelial cells (Caco-2). Of the 5 strains tested B. infantis showed the highest potential for Salmonella exclusion. Hence I further characterized the effect of B. infantis on adhesion and invasion of S. ser. Typhimurium of intestinal epithelial cells.

Effect of B. infantis on adhesion and invasion of S. ser. Typhimurium

I first tested if B. infantis could displace already adhered S. sv. Typhimurium from the epithelial cells, by incubating Caco-2 cells with Salmonella for 30 minutes prior to adding B. infantis into the co-culture. As evident from Figure 5.2, adding B. infantis had no effect on adhesion and invasion of S. ser. Typhimurium, if the pathogen was allowed to interact with the epithelial cells first. However if B. infantis was added to the Caco-2 cells 30 minutes prior to Salmonella, it significantly reduced adhesion and invasion of Salmonella. A similar effect was observed when both the organisms were added together. The degree of reduction of adhered and invaded Salmonella was similar to when the Caco-2 cells were incubated with B. infantis first. Interestingly, incubation of Salmonella with B. infantis for 120 minutes prior to infection of Caco-2 cells significantly caused more adhesion and invasion as compared to when Caco-2 cells were infected without the interaction (Figure 5.2). However it should be noted that when compared to the control (i.e. adhesion of S. ser. Typhimurium alone) there was still
significantly less adhesion and invasion. Hence pre-incubation of *S.* ser. Typhimurium with *B. infantis* primed *Salmonella* to be more invasive, and this effect was most likely due to gene regulation in *Salmonella*. To the test the hypothesis, gene expression of *S.* ser. Typhimurium in co-culture with *B. infantis* (30, 60, and 120 minutes) was determined and compared to gene expression profile of *S.* ser. Typhimurium cultured alone.

Differentially expressed genes between the two treatments were determined using SAM as an unpaired time course analysis using “signed area” as the time summary method (84). All the genes were ranked based on the (d) score obtained from SAM (84), and the pre-ranked gene list was analyzed using GSEA (79) to make inferences about regulation of pre-defined functionally related gene-sets. Table B1 (Appendix B) lists all the enriched gene sets in *Salmonella* when they were incubated with *B. infantis* for up to 120 minutes. Genes coding for both the type 3 secretion systems (SPI1 T3SS (q≤0) and SPI2 T3SS (q≤0.04) as well the effectors for both the T3SS (q<0.05) were significantly induced (Figure 5.3). Hence the increased adhesion and invasion of *S.* ser. Typhimurium due to incubation with *B. infantis* was concomitant with increased expression of genes coding for the T3SS structural proteins and effectors required for adhesion (47) and invasion (52).

Next I tested if *B. infantis* excluded the pathogen from the epithelial cells by competing for similar receptors. I blocked the same receptors which I had previously shown to be important for *Salmonella* adhesion and invasion, and looked at the effect of receptor blocking on adhesion of *B. infantis* to the host. Blocking HSP90, PPP1R12A, CTNN1A and ganglioside GD3 on the host and blocking Ef-Tu on the bacteria had no
effect on *B. infantis* binding (Appendix Figure B1). Blocking ganglioside GM1 on the host reduced the adhesion of *B. infantis* to 75% as compared to unblocked control (p=0.054). Hence Salmonella shared its binding specificity for GM1 with *B. infantis*.

*Effect of *B. infantis* on caspase activation and AKT phosphorylation in epithelial cells*

Since *Salmonella* mediated cell death of host cells is important for disease progression (15), I probed the potential of *B. infantis* to block *Salmonella* mediated cell death by measuring the activity of caspase 8, caspase 9 and caspase 3/7. Hence I measured the activity of the effector caspases (caspase 3/7), and I measured the activity of the initiator caspases of the extrinsic cell death pathways (caspase 8) and the intrinsic cell death pathway (caspase 9). Figure 5.4 shows the level of caspase activity after 8 hours in epithelial cells alone or in presence of different bacteria. Epithelial cells incubated with *B. infantis* showed a significant reduction in activity of caspase 9 and caspase 3/7 (p<0.05), but not caspase 8 as compared to control. Epithelial cells incubated with *S. ser.* Typhimurium showed a significant increase in activity of caspase 8 and caspase 9 (p<0.05) but not caspase 3/7. Epithelial cells incubated with *S. ser.* typhimurium and *B. infantis* both showed a caspase activity profile similar to that of cells incubated with *B. infantis* alone, where activity of caspase 9 and caspase 3/7 was significantly lower than that of control cells (p<0.05) while there was no effect on the activity of caspase 8. Hence *B. infantis* not only blocked the increase in *Salmonella* mediated caspase activity, but also lowered the basal caspase activity in epithelial cells.

Since *B. infantis* lowered the basal caspase activity, I probed the activation (phosphorylation) status of pro survival kinase Akt in epithelial cells. Akt is protein
kinase which regulates cell survival through two separate pathways. Akt inhibits apoptosis by phosphorylating the Bcl-2 family member Bad, which then interacts with 14-3-3 and dissociates from Bcl-xL, which prevents downstream activation of caspase 9 and caspase 3/7 (16). Alternatively, Akt activates IKKα that ultimately leads to NFκB activation. This leads to transcription and translation of proteins that directly inhibit caspase 9 and caspase 3/7 activity (25). Figure 5.5 shows the phosphorylation status of Akt at ser-473 and thr-308 at 30, 60, and 120 minutes after infection with different microbes. Incubation of B. infantis with the epithelial cells increased the phosphorylation of Akt at ser-473 and thr-308 in a time dependent manner (Figure 5.5) as compared to the control, while incubation with Salmonella decreased the phosphorylation at ser 473 and thr-308 as compared to the control. Phosphorylation levels of Akt at ser-473 and thr-308 when both the microbes were present together was similar to those of cells treated with B. infantis at 30 and 60 minutes. However by 120 minutes, Akt ser-473 and thr-308 phosphorylation levels were similar to cells infected with S. ser. Typhimurium. Hence, the decrease in caspase activity in epithelial cells due to B. infantis was concomitant with increased Akt phosphorylation at ser-473 and thr-308. To further characterize the interaction of B. infantis with epithelial cells I determined gene expression profile of Caco-2 cells alone, Caco-2 cells with B. infantis, Caco-2 cells with S. ser. Typhimurium and Caco-2 cells with both the bacteria after 30, 60, and 120 minutes.

Gene expression profile and small metabolite profile of Caco-2

I determined the global gene expression profiles and small metabolite profiles (intracellular and extracellular) of Caco-2 cells alone, and in presence of different
bacteria (*B. infantis* alone, *S. ser. Typhimurium* alone and *B. infantis* and *S. ser. Typhimurium* together) at 30, 60, and 120 minutes in two biological replicates. Figure 5.6 shows the dendogram based on hierarchical clustering of averaged global gene expression profile for each treatment at different time points. Hierarchical clustering of all samples indicated that the global transcriptome of epithelial cells infected with *S. ser. Typhimurium* and *B. infantis* simultaneously was more similar to that of epithelial cells infected with *B. infantis* than to the transcriptome of epithelial cells infected with *S. ser. Typhimurium* alone (p<0.01). Figure 5.7 shows the dendogram based on hierarchical clustering of intracellular and extracellular small metabolite profiles under different infection conditions. Metabolite profiling using GC/MS identified 294 compound peaks using the BinBase database (27), out of which 110 GC/MS peaks could be assigned positive identification. Hierarchical clustering based on log2 peak areas of all compound peaks, revealed that the small metabolite profile formed two big clusters, i.e. a cluster of samples with extracellular metabolites and the other with intracellular metabolites (p<0.01) (Figure 5.7). Within the extracellular metabolite cluster, Caco-2 cells co-infected with *Salmonella* and *Bifidobacteria* together clustered with Caco-2 cells treated with *Bifidobacteria* alone (p<0.12), while Caco-2 cells infected with *Salmonella* (60 minute and 120 minute samples) formed their own cluster (p<0.01). Hence the gene expression profile as well as the extracellular metabolite profile of cells co-infected with *Salmonella* and *Bifidobacteria* together was more similar to those of cells infected with *Bifidobacteria* alone.

To gain insight into the functional changes in the epithelial cells due to presence of the microbes, I compared the gene expression profile of epithelial cells when they
were incubated alone (control) to when they were incubated with either *B. infantis* (treatment) or *B. infantis* and *S. ser.* Typhimurium co-culture (treatment). Gene expression profile of epithelial cells with microbes was compared to gene expression profile of cells without any microbes using SAM (84) as an unpaired time course analysis using “signed area” as the time summary method. All the genes were ranked based on the (d) score obtained from SAM, and this pre-ranked gene list was analyzed using GSEA (79) to make inferences about pre-defined functionally related gene-sets. The gene sets consisted of genes annotated by the same Gene Ontology (GO) terms, KEGG pathways, Biocarta Pathways and genes that share same transcription factor binding sites defined in the TRANSFAC and were downloaded from the molecular signatures database (79).

Table 5.1 and Table 5.2 show enriched gene sets (based on GO annotations) that were differentially regulated in epithelial cells when incubated with either *B. infantis* alone or with *B. infantis* and *S. ser.* Typhimurium co-culture, as compared to epithelial cells without any microbial treatment. At an FDR≤0.21; 82 gene sets were enriched in epithelial cells treated with *B. infantis* (12 were induced while 70 were repressed) alone (Table 5.1) while 61 gene sets were enriched (all repressed) in epithelial cells treated with *B. infantis* and *S. ser.* Typhimurium co-culture. There were 36 gene sets (all repressed) that were commonly regulated between both the microbial treatments.

Incubation of *B. infantis* with epithelial cells significantly induced expression of proteins localized at cell junctions (q≤0.05), particularly adherens junctions (q≤0.11), cell matrix junction (q≤0.13) and intercellular junction (q≤0.11). Although tight junctions as a gene set was not induced significantly, two proteins important for maintenance of the tight junctions; zona occludens 2 (TJP2) (q≤0.01) and occludin (OCLN) (q≤0.14) were
induced. None of the above mentioned gene sets were differentially regulated in presence
of both *B. infantis* and *S. ser. Typhimurium*. Hence incubation of epithelial cells with *B.
infantis* induced expression of proteins important for maintenance of cellular junctions,
but the effect was negated in presence of the pathogen.

Incubation of *B. infantis* with the epithelial cells significantly repressed genes
required for the functioning of the proteasome (*q*≤0.01) and the peroxisome (*q*≤0.02) and
they remained repressed even in presence of the pathogen.

Incubation of *B. infantis* with the epithelial cells caused repression of the genes
involved in biogenesis of the mitochondrial matrix (*q*≤0), mitochondrial membrane
(*q*≤0), mitochondrial respiratory chain (*q*≤0) as well as mitochondrial ribosomes (*q*≤0).
All these gene sets were also repressed (*q*≤0.01) when the epithelial cells were incubated
with *B. infantis* and *S. ser. Typhimurium* co-culture. Subsequently genes involved in
aerobic respiration (*q*≤0.21), electron transport (*q*≤0.20) and oxygen and reactive oxygen
species metabolic processes (*q*<0.18) were also repressed. Hence irrespective of the
presence of pathogen, *B. infantis* repressed genes related to the biogenesis of
mitochondria and genes necessary for respiration and reactive oxygen species metabolic
processes.

Figure 5.8 represents expression of genes related to apoptosis signaling in
epithelial cells after 120 minutes of exposure to *B. infantis*. Caspase activity data (Figure
5.4) and phosphorylation of Akt (Figure 5.5) have also been overlaid on the same
pathway. Multiple anti-apoptotic pathway signaling leading to BAD phosphorylation was
induced (*q*≤0.04) (Table 5.3). Hence it is evident from Figure 5.4, that repression of
caspase 3/7 and caspase 9 activity in epithelial cells by *B. infantis* was concomitant with
increase in expression of cIAP (cIAP directly represses the activity of the effector caspases (71)), as well as increase in phosphorylation of Akt and repression of pro-apoptotic proteins BAD (20) and BID (51). The expression profile of the pathway was similar in presence of the pathogen (Appendix Figure B2). Also, since \textit{B. infantis} repressed the basal caspase 9 and 3/7 activity, but not that of caspase 8, it can be concluded that \textit{B. infantis} down regulated the intrinsic death pathway which is initiated by non-receptor-mediated intracellular signals that cause changes in the inner mitochondrial membrane.

To gain further insights into signaling pathways and transcriptional networks regulated in the epithelial cells due to \textit{B. infantis}, enrichment analysis was performed based on gene-sets from pathway databases (KEGG and Biocarta), and gene sets that contained genes which share similar transcription factor binding sites as defined in the TRANSFAC database (Table 5.3, Table 5.4, Table 5.5). Some of the enriched pathways (Oxidative phosphorylation, mitochondrial biogenesis, proteasome, peroxisome, cell junctions) were redundant with GO gene sets because of overlapping annotations.

Presence of \textit{B. infantis} significantly induced genes necessary for functioning of four receptor tyrosine kinase (RTK) mediated signaling pathways (i.e. hepatocyte growth factor signaling (q≤0.05), platelet derived growth factor signaling (q≤0.04), epidermal growth factor signaling (q≤0.05) and insulin signaling pathway (p≤0.05)). Expression of epiregulin (EREG), which is a ligand for epidermal growth factor receptor (44) was also strongly induced (q≤0)(~12-fold) after 120 minutes of incubation with \textit{B. infantis}.

Presence of \textit{B. infantis} significantly induced expression of genes in the canonical G-protein coupled receptor pathway (GPCR) (q≤ 0.04). Expression of four GCPR ligands
((CXCL1, CXCL2, CXCL3 and CCL20) was also significantly induced (q≤0).

Presence/absence calls based on PANP (86) revealed that, of the four induced GPCR ligands, receptor for CXCL3 (CXCR4 (82)) was expressed in the epithelial cells, while receptors for the other three ligands were not expressed. Expression of CXCR4 in Caco-2 cells has also been reported by other groups (30). Subsequently, genes under regulation of transcriptional factors, whose activity is modulated downstream of RTK and GPCR pathways by phosphorylation through the MAPK pathway i.e. CEBPA, CREB1, NFkB, and SRF (Table 5.6) were also significantly induced (q≤0). IL6 along with four other chemokines (CXCL1, CXCL2, CXCL3 and CCL20) which are under the transcriptional regulation of NFkB (83) were strongly induced (q≤0) (16-32-fold) after 120 minutes in presence of *B. infantis*. Eight more genes associated with IL6 signaling pathway were also induced (q≤0.04). Furthermore genes downstream of the IL6 signaling, which are under the positive transcriptional control of phosphorylated STAT3 were also induced (Table 5.6) (q≤0). Hence *B. infantis* induced expression of genes downstream of RTK, GPCR and JAK-STAT signaling pathways. Also expression of agonists of the three signaling pathways (IL6 for JAK-STAT (35), EREG for RTKs (36) and CXCL3 for GPCR (88)) were also significantly induced, which likely induced an autocrine signaling loop in the epithelial cells.

Expression profile of the epithelial cells exposed to *B. infantis* alone and those exposed to *B. infantis* and *S. ser.* Typhimurium were directly compared, to look at the effect of *S. ser.* Typhimurium on the epithelial cells in presence of *B. infantis*. Addition of *S. ser.* Typhimurium blocked *B. infantis* mediated induction of the IL6 pathway (q≤0.02) (Appendix Table B2) in the epithelial cells. Genes annotated under GO category cell
junctions induced by *B. infantis* were also significantly repressed (q≤0.13) in presence of the pathogen as compared to when only *B. infantis* was present. Transcription of genes having a cAMP responsive element (CRE) in their promoter which is mediated through CREB1 binding was also significantly repressed (q≤0) (Appendix Table B2). CREB1 is activated by a variety of stimuli leading to phosphorylation of CREB1 (39). Repression of genes under the transcriptional control of CREB1 was concomitant with repression of four chemokines (CXCL1, CXCL2, CXCL3 and CCL20) (q≤0.01) which are GPCR ligands (88). Induction of epiregulin (EREG), which is a ligand for epidermal growth factor receptor (44) was also blocked in presence of the pathogen. Hence presence of the pathogen blocked *B. infantis* induced CREB1 mediated gene expression, which was likely a downstream effect of repression of ligands for the GCPR (CXCL3), RTK (EREG) and JAK-STAT (IL6) pathways.

*Gene expression profile of S. ser. Typhimurium*

To gain insights into how *S. ser. Typhimurium* adapts to the presence of *B. infantis* in the host environment, I determined the gene expression profile of *S. ser. Typhimurium* interacting with Caco-2 cells with or without the presence of *B. infantis* at 30 minutes, 60 minutes, and 120 minutes after interaction. Gene expression profile of *Salmonella* between the two treatments was compared using SAM (84) as an unpaired time course experiment with “signed area” as the time summary method. All the genes were ranked based on the score(d) from SAM output and this pre-ranked gene list was used with Gene Set Enrichment Analysis software (GSEA) (79) to detect the coordinate changes in the expression of groups of functionally related genes, upon respective
treatments. Table 5.6 lists differentially regulated gene sets in *Salmonella* interacting with the host, with or without the presence of *B. infantis*. Presence of *B. infantis* induced genes down-stream of the Qse two component system in Salmonella (57) irrespective of the presence of host (q≤0.09) (Table 5.6 and Appendix Table B1). Hence most likely the cross-talk between *B. infantis* and *S. sv. Typhimurium* was mediated through soluble factors sensed by the Qse two component system. The known ligands that trigger Qse two component system are epinephrine/norepinephrine (4) and autoinducer 3 (AI-3) (61). Epinephrine/norepinephrine are host stress hormones and have been shown to induce increased colonization and systemic spread of Salmonella (58). The exact biochemical pathway or the genetic basis for production of AI-3 is not characterized, however AI-3 production has been shown to occur in variety of Enterobacteriaceae (85) including *Salmonella*, while AI-3 production in *Bifidobacteria* has not been tested yet.

Interestingly addition of *B. infantis* to the host/ *Salmonella* co-culture repressed genes related to arginine catabolism in *Salmonella* (Figure 5.9). This was reflected in the small metabolite profile, where addition of the *B. infantis* lead to accumulation of arginine and ornithine (the substrates), and depletion of putrescine (product). Catabolism of arginine is important from a virulence perspective for two reasons. First, catabolism of arginine is important for resistance to acid stress (29) and is required for full virulence. Secondly, arginine is a source of production of nitric oxide (NO) via nitric oxide synthase (iNOS) which is an important inflammatory mediator and necessary for protection of the host cell against the invading pathogen (46). Though the exact source of arginine, for NO production is not known, it is clear that in addition to intracellular sources of arginine, luminal sources of arginine are also required for optimal production of NO by the host.
(67). *Giardia lamblia* uses depletion of arginine as a strategy to prevent NO production in the intestinal epithelium as shown by Eckmann et al. (23). I observed a depletion of arginine in absence of *B. infantis* (from *Salmonella*/Caco-2 co-culture), while arginine accumulated in *B. infantis* presence. Hence in presence of *B. infantis* there was an increased pool of arginine available for NO production. The major pathway for NO metabolism is the stepwise oxidation to nitrite (NO$_2^-$) and nitrate (NO$_3^-$) (91). In presence of *B. infantis*, 9 genes related to nitrate and nitrite reduction were strongly induced (q≤0) in *Salmonella* (Figure 5.9, Anaerobic Metabolism). Genes required for synthesis of cofactors necessary for functioning of nitrate and nitrite reductases were also significantly induced (q≤0) (Table 5.9, Biosynthesis of cofactors- Prosthetic Groups- and Carriers- Molybdopterin). This *Bifidobacteria* mediated induction of nitrate and nitrite reductases in *Salmonella* did not happen in absence of the epithelial cells. Hence induction of nitrate and nitrite reductases in *Salmonella* was most likely a down-stream effect production of NO from the host. Hence our data suggests an elegant cross talk between the host, commensal and the pathogen, where the commensal prevented catabolism of arginine by the pathogen, which probably lead to increased NO production by the host as evidenced by induction of genes necessary form nitrate and nitrite in the pathogen.

Addition of *B. infantis* to the host/ *Salmonella* co-culture also repressed genes in *Salmonella* necessary for the TCA cycle (q≤0.09) and induced genes necessary for anaerobic metabolism (q≤0) (Table 5.6, Figure 5.9). Surprisingly genes necessary for functioning of the mitochondrial respiratory chain were also repressed in the epithelial cells as mentioned earlier by the addition of *B. infantis* into the co-culture (Table 5.1,
Table 5.2). Hence addition of *B. infantis* repressed genes necessary for respiration in Caco-2 as well as *Salmonella*. This was reflected in the small metabolite profile of the co-culture where (Figure 5.9), addition of *B. infantis* in the Caco-2/*Salmonella* co-culture caused a significant accumulation of glucose, fructose, trehalose and maltose (q<0.05) extracellularly as well as intracellularly. Glucose 6P, pyruvate and citrate also accumulated significantly while rest of the detected TCA metabolites (*α* ketoglutarate, malate, fumarate and succinate) though not statistically significant, showed a depleting trend. Hence based on the gene expression and small metabolite profile it can be concluded that addition of *B. infantis* to the co-culture of the host and pathogen, reduced the carbon flux through glycolysis and the TCA cycle, while presence of *Salmonella* alone with the host induced genes related to glycolysis (q≤0.01). Induction of glucose metabolism in the host epithelium due to *Salmonella* infection was also reported in vivo by Rodenburg et al. (70). The increased glucose catabolism perhaps leads to increased mitochondrial ETC function to regenerate the spent reducing equivalent (NAD⁺), which is the main source of reactive oxygen species (ROS) generation in the cell (33). Uncontrolled production of ROS directly activates caspase 9 (42) (intrinsic pathway), and indirectly activates caspase 8 (extrinsic pathway) via induction of Fas and Fas ligand expression (18), which further amplifies caspase 9 pathway. Hence, *Bifidobacteria* mediated blocking of *Salmonella*-induced caspase 8 and caspase 9 was concomitant with decreased carbohydrate metabolism in the host, as evidenced by gene expression in the host as well the small metabolite profile.
Gene expression profile of B. infantis

To gain insights into how B. infantis responds to the presence of the epithelial cells and Salmonella, gene expression profiles of B. infantis were determined in cell culture media alone; in presence of Caco-2 cells and in presence of Caco-2 /S. ser. Typhimurium co-culture after 120 minutes of exposure. Expression profile of the treated B. infantis was compared to those of B. infantis incubated alone using SAM (84) as two class unpaired data, with t statistic. All the genes were ranked based on the score (d) from SAM output. This pre ordered ranked gene list was used in Gene Set Enrichment Analysis software (GSEA) (79) to detect the coordinate changes in the expression of groups of functionally related genes, upon respective treatments. Table 5.7 lists differentially regulated gene sets (q≤0.15) in B. infantis when they were incubated with epithelial cells (treatment) as compared to when they were incubated in the same conditions without the epithelial cells (control) for 120 minutes. Table 5.8 lists differentially regulated gene sets (q≤0.15) in B. infantis when they were incubated with Caco-2 /S. ser. Typhimurium co-culture (treatment) as compared to when they were incubated in the same conditions alone (control) for 120 minutes.

Presence of epithelial cells significantly induced transcription of genes with %GC content greater than 70 (q≤0) and less than 40(q≤0), irrespective of the presence of Salmonella. Hence these genes were important for the interaction of B. infantis with the host. Also since average %GC content of B. infantis is 60% (74) those genes were putative horizontally transferred genes (55). Protein translation as a gene set was induced (q≤0.01) when B. infantis was incubated with epithelial cells alone while the same gene set was significantly repressed (q≤0) when B. infantis was incubated in presence of
epithelial cells and S. ser. Typhimurium. Along with translation, oxidative phosphorylation was also significantly repressed ($q\leq 0$) in presence of epithelial cells and S. ser. Typhimurium. Hence presence of Salmonella along with epithelial cells reduced translation and energy metabolism in B. infantis. This effect was not observed when B. infantis was in presence of Salmonella alone (Appendix Table B4). Hence interaction of the pathogen and epithelial cells reduced the fitness of B. infantis. This was surprising as presence of B. infantis and Salmonella did not lead to increased expression of enzymes needed for reactive species generation (ROS) or increased expression of defensins in the epithelial cells which could reduce the fitness of Bifidobacteria. On the contrary in presence of both the bacteria, mitochondrial biogenesis and genes needed for generation of ROS were repressed in epithelial cells. Hence the fitness of B. infantis in presence of the pathogen and epithelial cells was reduced by a yet uncharacterized mechanism.

Recently, a comparative genomic hybridization (CGH) study with 15 different Bifidobacteria strains revealed that B. longum spp. has at least two distinct subspecies, subsp. infantis, specialized to utilize human milk oligosaccharides, and subsp. longum, specialized for plant-derived oligosaccharides (50). I reanalyzed the CGH data using GSEA and determined what functionally related gene sets were conserved in the infantis lineage but divergent in the longum lineage. The results are summarized in Appendix Table B5. As reported by Locascio et al. gene enrichment analysis revealed that the genes divergent between infantis and longum strains were enriched in functions related to human milk oligosaccharide (HMO) metabolism, urease metabolism and solute binding proteins of ABC transporters. Furthermore I also discovered clusters of putative horizontally transferred genes that were only present in the infantis lineage and absent or
highly divergent in the longum lineage. Surprisingly three of the gene sets related to horizontally transferred genes (genes with % GC >70, genes with % GC<40 and horizontally transferred genes from Firmicutes) were induced in *B.infantis* in presence of the host and pathogen. Hence these genes were most likely acquired through horizontal gene transfer in the infantis lineage and conserved in the genome under selective pressure from the host. Of the cluster of 50 genes that were putatively acquired through lateral gene transfer and also induced in presence of the host (Appendix Figure B3) 28 were hypothetical proteins with no known functions.

**Discussion**

Many studies have linked prophylactic use of probiotics to reduced rate of enteric infections (6, 8, 11, 48). The pathogen exclusion potential of various probiotics is dependent on the probiotic as well as the pathogen strain (38). Hence I screened 5 different probiotic strains and found that *B. infantis* excluded *S. ser.* Typhimurium most effectively among the tested strains. *B. infantis* blocked pathogen adhesion and invasion only if it was added before or with the pathogen. This indicates that it was not able to displace already adhered pathogen from the host cells. Among the tested host receptors *B. infantis* shared its binding specificity for ganglioside GM1 with *Salmonella*. Further I also showed that *B. infantis* blocked *Salmonella* mediated caspase 8 and caspase 9 activation. It also reduced the basal caspase 9 and 3/7 activity in the epithelial cells. The reduction in caspase activity was concomitant with increased Akt phosphorylation and increased expression of the caspase-inhibiting cIAP protein family. Hence, *B. infantis* prevented the activation of the mitochondria-mediated intrinsic cell death pathway.
Most pathogens have evolved mechanisms to subvert host signal transduction cascades to influence host cell death decisions (72). Mitochondria integrates a variety of signals and mitochondrial membrane permeabilization is the decisive event between cell survival and death (72). A variety of pathogen induce cell death by causing mitochondrial dysfunction (10, 53, 62) which is mediated through cytochrome c release in the cytoplasm leading to caspase 9 activation. During *Salmonella* infection the antioxidant defense system in the host is impaired due to excessive production of reactive oxygen species (ROS) (56). This has been shown to increase caspase 9 and caspase 8 activity and also observed in this study. A recent study (Shah et al, unpublished data) correlated the induction of genes involved in production of ROS, respiration, and mitochondrial biogenesis to increased caspase 9 and caspase 3/7 activation during *Salmonella* infection. Surprisingly, *B. infantis* repressed the same gene categories irrespective of presence of *Salmonella* and also repressed caspase 9 activity irrespective of the presence of pathogen. Hence apart from competitive exclusion, *B. infantis* also mitigated *Salmonella* mediated mitochondrial dysfunction in epithelial cells. It was recently shown that production of ROS in the gut gives *Salmonella* a selective advantage, as the ROS can react with luminal sulfur compounds to form tetrathionate which can be used by *Salmonella* to respire, giving it a competitive edge over fermenting gut microbes (87). Our data suggests that *B. infantis* could possibly block ROS production in the gut by down regulation of genes needed for aerobic respiration and functioning of the electron transport chain. This mechanism could contribute to the *Bifidobacteria* mediated resistance to *Salmonella* as observed in vivo in mice (75).
*B. infantis* also induced NFkB mediated transcription of four cytokines IL6, CXCL1, CXCL2, CXCL3, and CCL20. Significantly all these genes were repressed in presence of *Salmonella*. *Salmonella* interferes with the host signal transduction by secreting a vast array of effector molecules directly into the host cytosol. One of the secreted effectors AvrA blocks NFkB activation by deubiquitination of IkBα (90) which leads to down regulation of target genes in the NFkB pathway s IL6, as observed in this study as well as by Ye et al (90). Hence presence of *Salmonella* blocked *B. infantis* mediated induction of NFkB, most likely through AvrA activity, which was induced in presence of *B. infantis* (q≤0). Immunostimulatory effects of *Bifidobacteria* have also been reported by other groups. Ruiz et al. (73) reported that in a mouse model, *B. lactis* triggers IL6 secretion from intestinal epithelial cells which was concomitant with activation of MAPK and NFkB signaling (73). In mouse embryogenic fibroblasts, *B. lactis* mediated induction of IL6 was completely dependent on TLR2 signaling (73). Li et al. have also reported the immunostimulatory properties of TLR9 dependent CpG motifs in *Bifidobacteria* DNA in macrophages (49).

Patients suffering from inflammatory bowel syndrome (IBS) typically report increased gut permeability and increased apoptotic rates of intestinal epithelial cells (40). In this study, I observed that *B. infantis* treated epithelial cells showed increased expression of tight junction and adherens junction proteins. This was also observed by Ewaschuk et al. where soluble factors in *B. infantis* conditioned media lead to increased transepithelial resistance (TER) in epithelial cells and concomitant increased expression of tight junction proteins. Hence *B. infantis* was functionally shown to improve the barrier function of epithelial cells. I also observed a *B. infantis* mediated decrease in
activation of caspase 9 and caspase 3/7 concomitant with increased phosphorylation of Akt in colonic epithelial cells. Two recent clinical trials showed that *B. infantis* intervention significantly reduced IBD related symptoms (7). The probiotic mixture VSL#3 has also been shown effective in management of IBS symptoms. Incidentally *B. infantis* is one of the eight strains present in the mixture (5). These observations suggest that *B. infantis* may be involved in maintaining the barrier functions of the gut epithelium in vivo by increased expression of tight junction proteins and by modulating the cell turnover rate.

To summarize, *B. infantis* competitively excluded *Salmonella* from binding to epithelial cells. It prevented *Salmonella* mediated caspase 8 and caspase 9 activation in epithelial cells and in absence of the pathogen *B. infantis* lowered the basal caspase activity of epithelial cells. The lowered caspase activity was concomitant with increased Akt phosphorylation, increased expression of caspase inhibiting protein (cIAP) and repression of genes involved in mitochondrion organization and biogenesis and reactive oxygen species metabolic processes. Using gene expression and small metabolite profile I also observed a three way cross talk between the epithelial cells, *Salmonella* and *Bifidobacteria*; where *B. infantis* prevented catabolism of arginine (a substrate for nitric oxide (NO) production) by *S. ser. Typhimurium*, making it available for increased NO production by the host as evidenced by induction of genes necessary for nitrate and nitrite (break down products of NO) respiration in the pathogen. Using comparative and functional genomics approach, I also discovered clusters of mostly hypothetical proteins which have been acquired in the genome of *B. infantis* through lateral gene transfer and are important for the interaction of *B. infantis* with epithelial cells.
References

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Table 5.1 GO gene sets that were differentially regulated \((q \leq 0.21)\) in Caco-2 cells when exposed to \(B.\ infantis\). NES= Normalized enrichment score. A positive score indicates that the gene set was induced in Caco-2 cells when exposed to \(B.\ infantis\), while a negative score indicates that the gene set was repressed.

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**Molecular function**

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**Molecular function**

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Table 5.3 Differentially regulated pathways \((q \leq 0.05)\) in Caco-2 cells when exposed to \(B.\) \(infantis\). NES= Normalized enrichment score. A positive score indicates that the gene set was induced in Caco-2 cells when exposed to \(B.\) \(infantis\), while a negative score indicates that the gene set was repressed.

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Table 5.4 Differentially regulated Pathways (q≤0.05) in Caco-2 cells when they were exposed to *B. infantis* and *S. ser.* Typhimurium. NES= Normalized enrichment score. A positive score indicates that the gene set was induced in Caco-2 cells when exposed to *B. infantis*, while a negative score indicates that the gene set was repressed.

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<td>0.00</td>
</tr>
<tr>
<td>Oxidative Phosphorylation</td>
<td>96</td>
<td>56</td>
<td>-2.22</td>
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</tr>
<tr>
<td>Antigen Processing And Presentation</td>
<td>37</td>
<td>24</td>
<td>-1.91</td>
<td>0.03</td>
</tr>
<tr>
<td>Complement And Coagulation Cascades</td>
<td>32</td>
<td>17</td>
<td>-1.86</td>
<td>0.05</td>
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<tr>
<td>Melanogenesis</td>
<td>64</td>
<td>25</td>
<td>1.92</td>
<td>0.03</td>
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<tr>
<td>Basal Cell Carcinoma</td>
<td>30</td>
<td>16</td>
<td>2.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Notch Signaling Pathway</td>
<td>35</td>
<td>20</td>
<td>2.04</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Biocarta Pathway</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteasome Complex</td>
<td>18</td>
<td>11</td>
<td>-1.91</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Table 5.5 Top ten differentially regulated (q≤0) transcriptional regulons in Caco-2 cells when exposed to *B. infantis*. NES= Normalized enrichment score. A positive score indicates that the gene set was induced in Caco-2 cells when exposed to *B. infantis*, while a negative score indicates that the gene set was repressed.

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Binding Motif</th>
<th>Transcription Factor</th>
<th>Remarks</th>
<th>Size of Gene Set</th>
<th>Genes Regulated</th>
<th>NES</th>
<th>FDR q-val</th>
</tr>
</thead>
<tbody>
<tr>
<td>V$CEBP_Q$ 2 _01</td>
<td>NTTRCNNA ANNN</td>
<td>CEBPA: CCAAT/enhancer binding protein (C/EBP), alpha</td>
<td>Positive regulation by activated STAT3 (63), positive regulation by activity of Erk 1/2 (69)</td>
<td>128</td>
<td>59</td>
<td>2.17</td>
<td>0.00</td>
</tr>
<tr>
<td>V$CDPCR1_01$</td>
<td>NATCGATC GS</td>
<td>CUTL1: cut-like 1, CCAAT displacement protein (Drosophila)</td>
<td>Negatively regulates transcription of genes; repression is relieved by phosphorylation of CUTL1 by PKA (59) and PKC</td>
<td>53</td>
<td>19</td>
<td>2.04</td>
<td>0.00</td>
</tr>
<tr>
<td>V$CREB_Q$ 2</td>
<td>NSTGACGT AANN</td>
<td>CREB1: cAMP responsive element binding protein 1</td>
<td>Activated by a variety of stimuli leading to phosphorylation of CREB1 mediated by PKA principally through GPCR signaling (39)</td>
<td>140</td>
<td>69</td>
<td>1.96</td>
<td>0.00</td>
</tr>
<tr>
<td>V$CRX_Q4$</td>
<td>YNNNTAAT CYCMN</td>
<td>CRX: cone-rod homeobox</td>
<td>SNPs in CRX are correlated to Crohn's disease (1)</td>
<td>104</td>
<td>50</td>
<td>2.05</td>
<td>0.00</td>
</tr>
<tr>
<td>V$NFKAPP_AB_01$</td>
<td>GGGAMTT YCC</td>
<td>NFKB RELA: v-rel reticuloendotheliosis viral oncogene homolog A</td>
<td>Activated by a diverse group of receptors which include cytokine receptors, T cell receptors, growth factor receptors and Toll like receptors (54)</td>
<td>95</td>
<td>46</td>
<td>1.97</td>
<td>0.00</td>
</tr>
<tr>
<td>V$PAX8_01$</td>
<td>NNNNTNNNG NGTGANN</td>
<td>PAX8: paired box gene 8</td>
<td>Activity is negatively regulated by glutathionylation of PAX8 (9)</td>
<td>16</td>
<td>11</td>
<td>1.94</td>
<td>0.00</td>
</tr>
<tr>
<td>V$SOPC1_01$</td>
<td>NNNNWTA TGCAAATN TNNN</td>
<td>POU2F1: POU domain, class 2, transcription factor 1</td>
<td>Activity positively regulated by BRCA (26) and negatively regulated by Glucocorticoid-GCR complex (45)</td>
<td>103</td>
<td>54</td>
<td>2.27</td>
<td>0.00</td>
</tr>
<tr>
<td>V$OCT1_03$</td>
<td>NNNRTAAT NANNN</td>
<td>POU2F1: POU domain, class 2, transcription factor 1</td>
<td>Activity positively regulated by BRCA (26) and negatively regulated by Glucocorticoid-GCR complex (45)</td>
<td>93</td>
<td>40</td>
<td>2.05</td>
<td>0.00</td>
</tr>
<tr>
<td>V$SRF_Q4$</td>
<td>SCCAWATA WGGMNMMN NNN</td>
<td>SRF: serum response factor (c-fos serum response element-binding transcription factor)</td>
<td>This gene is the downstream target of many pathways; for example, the mitogen-activated protein kinase pathway (MAPK)</td>
<td>124</td>
<td>45</td>
<td>2.10</td>
<td>0.00</td>
</tr>
<tr>
<td>V$STAT3_0</td>
<td>2</td>
<td>NNNTCCN</td>
<td>STAT3: signal transducer and activator of transcription 3 (acute-phase response factor)</td>
<td>This protein is activated through phosphorylation in response to various cytokines and growth factors including IFNs, EGF, IL5, IL6, HGF, LIF and BMP2 (64)</td>
<td>75</td>
<td>25</td>
<td>2.08</td>
</tr>
</tbody>
</table>
Table 5.6 Differentially regulated gene sets (q≤0.09) in *S. ser.* Typhimurium when incubated with epithelial cells as compared to when incubated with epithelial cells and *B. infantis* for 120 minutes. NES= Normalized enrichment score. A positive score indicates that the gene set was induced in *S. ser.* Typhimurium when incubated with Caco-2 cells and *B. infantis* together, while a negative score indicates that the gene set was repressed.

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Size of Gene Set</th>
<th>Genes Regulated</th>
<th>NES</th>
<th>FDR q-val</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biosynthesis Of Cofactors- Prosthetic Groups- And Carriers- Molybdopterin</td>
<td>9</td>
<td>6</td>
<td>1.60</td>
<td>0.08</td>
</tr>
<tr>
<td>Cog J-Translation</td>
<td>170</td>
<td>107</td>
<td>2.51</td>
<td>0.00</td>
</tr>
<tr>
<td>Cog K-Transcription</td>
<td>246</td>
<td>96</td>
<td>1.76</td>
<td>0.04</td>
</tr>
<tr>
<td>DNA Metabolism- DNA Replication- Recombination-And Repair</td>
<td>119</td>
<td>54</td>
<td>1.61</td>
<td>0.09</td>
</tr>
<tr>
<td>Energy Metabolism- Anaerobic</td>
<td>66</td>
<td>26</td>
<td>2.21</td>
<td>0.00</td>
</tr>
<tr>
<td>Genes Induced By QSE Two Component System</td>
<td>44</td>
<td>29</td>
<td>1.63</td>
<td>0.09</td>
</tr>
<tr>
<td>Glycan Biosynthesis-Lps Biosynthesis</td>
<td>27</td>
<td>13</td>
<td>1.74</td>
<td>0.05</td>
</tr>
<tr>
<td>Membrane Transport-Protein Export</td>
<td>16</td>
<td>11</td>
<td>1.80</td>
<td>0.03</td>
</tr>
<tr>
<td>Protein Fate- Protein Folding And Stabilization</td>
<td>34</td>
<td>11</td>
<td>1.60</td>
<td>0.09</td>
</tr>
<tr>
<td>Protein Fate- Protein Modification And Repair</td>
<td>20</td>
<td>11</td>
<td>1.64</td>
<td>0.09</td>
</tr>
<tr>
<td>Protein Synthesis- Ribosomal Proteins- Synthesis And Modification</td>
<td>61</td>
<td>46</td>
<td>1.94</td>
<td>0.01</td>
</tr>
<tr>
<td>Protein Synthesis- Translation Factors</td>
<td>28</td>
<td>19</td>
<td>1.88</td>
<td>0.01</td>
</tr>
<tr>
<td>Protein Synthesis- tRNA And rRNA Base Modification</td>
<td>28</td>
<td>17</td>
<td>2.27</td>
<td>0.00</td>
</tr>
<tr>
<td>Purines- Pyrimidines- Nucleosides- And Nucleotides-2-Deoxyribonucleotide Metabolism</td>
<td>10</td>
<td>4</td>
<td>1.58</td>
<td>0.09</td>
</tr>
<tr>
<td>SPI-3</td>
<td>9</td>
<td>4</td>
<td>1.60</td>
<td>0.09</td>
</tr>
<tr>
<td>Transcription-RNAProcessing</td>
<td>7</td>
<td>6</td>
<td>1.60</td>
<td>0.08</td>
</tr>
<tr>
<td>Mobile And Extrachromosomal Element Functions-Prophage Functions</td>
<td>143</td>
<td>64</td>
<td>-2.41</td>
<td>0.00</td>
</tr>
<tr>
<td>Mobile And Extrachromosomal Element Functions-Plasmid Functions</td>
<td>28</td>
<td>24</td>
<td>-2.01</td>
<td>0.00</td>
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<tr>
<td>Membrane Transport-ABC Transporters</td>
<td>174</td>
<td>57</td>
<td>-1.99</td>
<td>0.00</td>
</tr>
<tr>
<td>HGT-High %GC</td>
<td>70</td>
<td>46</td>
<td>-2.60</td>
<td>0.00</td>
</tr>
<tr>
<td>Genes With %GC Greater Than 60</td>
<td>196</td>
<td>100</td>
<td>-2.54</td>
<td>0.00</td>
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<tr>
<td>Energy Metabolism- TCA Cycle</td>
<td>35</td>
<td>8</td>
<td>-1.65</td>
<td>0.09</td>
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<td>Cellular Processes- DNA Transformation</td>
<td>30</td>
<td>26</td>
<td>-1.99</td>
<td>0.00</td>
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<tr>
<td>Biosynthesis Of Cofactors- Prosthetic Groups- And Carriers- Biotin</td>
<td>8</td>
<td>4</td>
<td>-1.89</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Table 5.7 Differentially regulated gene sets (q≤0.15) in *B. infantis* when incubated with epithelial cells as compared to when incubated in the same conditions without the epithelial cells for 120 minutes. NES= Normalized enrichment score. A positive score indicates that the gene set was induced in *B. infantis* when incubated with Caco-2 cells, while a negative score indicates that the gene set was repressed.

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Size of Gene Set</th>
<th>Genes Regulated</th>
<th>NES</th>
<th>FDR q-val</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes with %GC &gt;70</td>
<td>48</td>
<td>26</td>
<td>2.53</td>
<td>0.00</td>
</tr>
<tr>
<td>Genes with %GC&lt; 40</td>
<td>34</td>
<td>17</td>
<td>2.05</td>
<td>0.00</td>
</tr>
<tr>
<td>Translation</td>
<td>57</td>
<td>24</td>
<td>1.93</td>
<td>0.01</td>
</tr>
<tr>
<td>Pyrimidine Metabolism</td>
<td>35</td>
<td>15</td>
<td>1.93</td>
<td>0.01</td>
</tr>
<tr>
<td>Purine Metabolism</td>
<td>43</td>
<td>17</td>
<td>1.60</td>
<td>0.11</td>
</tr>
<tr>
<td>Cog J Translation</td>
<td>137</td>
<td>41</td>
<td>1.89</td>
<td>0.01</td>
</tr>
<tr>
<td>Cog F Nucleotide Transport And Metabolism</td>
<td>61</td>
<td>29</td>
<td>1.82</td>
<td>0.01</td>
</tr>
<tr>
<td>CHO-Binding Module Family (Cazy)</td>
<td>5</td>
<td>2</td>
<td>-1.79</td>
<td>0.13</td>
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<tr>
<td>Glycosyl Hydrolases (Cazy)</td>
<td>41</td>
<td>14</td>
<td>-1.78</td>
<td>0.07</td>
</tr>
</tbody>
</table>
Table 5.8 Differentially regulated gene sets (q≤0.15) in *B. infantis* when incubated with epithelial cells and *S. ser. Typhimurium* as compared to when incubated alone for 120 minutes. NES= Normalized enrichment score. A positive score indicates that the gene set is induced in *B. infantis* when incubated with Caco-2 cells, while a negative score indicates that the gene set was repressed.

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Size of Gene Set</th>
<th>Genes Regulated</th>
<th>NES</th>
<th>FDR q-val</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes with %GC &gt;70</td>
<td>48</td>
<td>24</td>
<td>2.44</td>
<td>0.00</td>
</tr>
<tr>
<td>Genes with %GC&lt; 40</td>
<td>34</td>
<td>17</td>
<td>2.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Unclassified</td>
<td>683</td>
<td>232</td>
<td>1.85</td>
<td>0.02</td>
</tr>
<tr>
<td>Genes with %GC Content Between 40 and 50</td>
<td>108</td>
<td>24</td>
<td>1.60</td>
<td>0.14</td>
</tr>
<tr>
<td>HGT from Firmicutes</td>
<td>114</td>
<td>36</td>
<td>1.54</td>
<td>0.15</td>
</tr>
<tr>
<td>Translation</td>
<td>57</td>
<td>30</td>
<td>-2.31</td>
<td>0.00</td>
</tr>
<tr>
<td>Oxidative Phosphorylation</td>
<td>11</td>
<td>8</td>
<td>-2.12</td>
<td>0.00</td>
</tr>
<tr>
<td>Cog C Energy Production and Conversion</td>
<td>42</td>
<td>17</td>
<td>-2.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Cog J Translation</td>
<td>137</td>
<td>40</td>
<td>-1.81</td>
<td>0.02</td>
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</tbody>
</table>
Table 5.9 Bacteria with their growth conditions and primers for their quantitation by qPCR

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Growth condition</th>
<th>qPCR primers</th>
<th>Standard curve equation</th>
</tr>
</thead>
</table>
| *Salmonella enterica* ser. Typhimurium LT2 (ATCC 700720) | 37°C / 220 rpm in serum free cell culture media. Cells were washed and suspended in fresh serum free cell culture media before interaction with Caco-2 cells | Forward: TGT TGT GGT TAA TAA CCG CA  
Reverse: CAC AAA TCC ATC TCT GGA | Log$_{10}$ CFU = Ct-46.52/-5.034  
$R^2 = 0.99$ |
| Caco2 (ATCC HTB-37) Gene: G3PDH      | See cell culture for growth description                                         | Forward: ACC ACA GTC CAT GCC ATC AC  
Reverse: TCC ACC ACC CTG TTG CTG TA | Log$_{10}$ CFU = Ct-33.69/-4.13  
$R^2 = 0.99$ |
| *Bifidobacterium longum* subsp. *infantis* (ATCC 15697) | 37°C/ Anaerobic in MRS broth + 0.05% Cys. Cells were washed and suspended in fresh serum free cell culture media before interaction with Caco-2 cells | Forward: ACT ACC CCT GGC CTG AAC TT  
Reverse: GAC AGA GCG TAA CCC AGC TC | Log$_{10}$ CFU = Ct-36.31/-2.624  
$R^2 = 0.97$ |
Figure 5.1 Efficacy of different strains to block *Salmonella* host association. Treatments with "*" are significantly different (p≤0.5) as compared to control (Caco-2 cells incubated with *Salmonella* alone). Error bars indicate standard error from 4 replicates.
Figure 5.2 Effect of presence of *B. infantis* on the relative adhesion and invasion of *S. ser.* Typhimurium to Caco-2 cells. Control (Caco-2 cells incubated with *S. ser.* Typhimurium); Sal-Pre + Bif (Caco-2 cells pre-incubated for 30 minutes with *S. ser.* Typhimurium and then with *B. infantis* for 30 more minutes); Bif-Pre+ Sal (Caco-2 cells pre-incubated with *B. infantis* for 30 minutes and then with *S. ser.* Typhimurium for 60 more minutes); Sal+Bif (Caco-2 cells incubated with *S. ser.* Typhimurium and *B. infantis* together for 60 minutes); Sal+Bif Incubated (S. ser. Typhimurium and *B. infantis* were first incubated together for 60 minutes and then incubated with Caco-2 cells for 120 minutes). Treatments that do not share an alphabet are significantly different (p<0.05). The % numbers of the x axis represents the fraction of total host associated bacteria that were intracellular (invaded). Error bars indicate standard error from three replicates.
Figure 5.3 Log₂ ratios of genes involved in biogenesis of T3SS and its effectors in *S. ser.* Typhimurium grown in co culture with *B. infantis* or grown alone. A positive log₂ ratio represent induction of genes in presence of *B. infantis* while a negative log₂ ratio represents repression of genes. The q value was obtained from SAM.
Figure 5.4 Activity of caspase 8, caspase 9 and caspase 3/7 after 8 hours of incubation with either no microbe (control), or with *B. infantis* alone, or *S. ser. Typhimurium* alone, or *B. infantis* and *S. ser. Typhimurium* together. Means significantly different from control (p≤0.05) for each caspases are marked with "*". Error bars indicate standard error from 3 replicates.
Figure 5.5 Relative levels of phosphorylated Akt at ser-473 and thr-308 in epithelial cells upon infection with different microbes. Error bars indicate standard error from 2 replicates.
Figure 5.6 Dendogram visualizing similarity between global gene expression profiles of Caco-2 cells. The number at each cluster edge represents Approximately Unbiased % p value estimated by multiscale bootstrap resampling 1000 times. (Host=Caco-2 cells incubated with no microbes, Host+Bif= Caco-2 cells incubated with *B. infantis*, Host+Sal= Caco-2 cells incubated with *S. ser. Typhimurium*, Host+Bif+Sal= Caco-2 cells simultaneously incubated with 1:1 ratio of *B. infantis* and *S. ser. Typhimurium*. The time in minutes represent the time after infection at which the gene expression was determined.
Figure 5.7 Dendogram constructed by hierarchical clustering of small metabolite profile of all the host-microbe interaction samples. The number at each cluster edge represents Approximately Unbiased % p value estimated by multiscale bootstrap resampling 1000 times (Host= Caco-2, Bif= B. infantis, Sal= S. ser. Typhimurium. The time in minutes represent the time after infection at which the metabolite profile was determined. "E" represents the extracellular metabolite profile from culture supernatant, while "I" represent the intracellular metabolite profile of all the cells in co-culture.
Figure 5.8 Log2 ratios of differentially expressed genes (q≤0.1); caspase 8,9, 3/7 activity and phosphorylation status of Akt in epithelial cells when they are exposed to \textit{B. infantis}. Data for Akt and caspase activity are same as Fig 5.2 and 5.3. For gene expression data, red represents induction while blue represents repression of genes when epithelial cells were exposed to \textit{B. infantis}. 
Figure 5.9 Log_2 ratios of gene expression intensities of S. ser. Typhimurium and selected small metabolite peak areas when Caco-2 cells were treated with either S. ser. Typhimurium alone or with a co-culture of S. ser. Typhimurium and B. infantis.

Induction of genes represent that the gene was induced in Salmonella in presence of B. infantis as compared to its absence.
CHAPTER VI

SUMMARY

Food-borne bacterial infections are a substantial source of morbidity and mortality in humans (3). In 2008 *Salmonella* caused the highest morbidity for all reported bacterial Food-borne illnesses in the U.S. (1). The economic burden due to *Salmonella* alone in the U.S. is an estimated $2.8 billion (2). *Salmonella* is a big problem for food safety and public health. Broadly, this is because of zoonotic transmission of non-host adapted serovars between an animal, that is an asymptomatic carrier to food and subsequently to humans, that results in food related out breaks. While it is not possible to completely get rid of *Salmonella* from the livestock, efforts can be still be undertaken to reduce the load of the organism from the food chain. The number of pathogen outbreaks can also be reduced if the disease causing organism is detected earlier, so that remedial action can be taken quickly to control the spread of the disease. This work focused on developing strategies to block *Salmonella* infection and allow for rapid pathogen detection.

I hypothesized that *B. longum* subspecies. *infantis* reduces *S* ser. Typhimurium adhesion to gut epithelial cells by competing for the same receptors and also improves cell survival by modulation of specific pathways in the host. These hypotheses were tested via the following objectives.

**Objective 1.** Define the pathogen exclusion potential of selected probiotic bacteria *in vitro* to gut epithelium.

**Objective 2.** Define the binding partners of the host-bacteria interaction.
Objective 3. Identify signal transduction and metabolic pathways modulated in the host (Caco-2 cells) and the microbes in vitro due to adhesion of B. infantis and S. ser. Typhimurium over time.

Efficacy of probiotics in the prevention of gastrointestinal diseases has been observed in several in vivo and in vitro models, but this effect is strain dependent and there is limited knowledge about the mode of action of probiotics against enteric pathogens. I screened several probiotic strains for pathogen exclusion potential and found that Bifidobacterium longum subsp. infantis showed the highest potential for Salmonella exclusion in a cell culture model.

To identify new protein binding partnerships, I developed a cell-cell cross-linking protocol, in which bacterial cells were chemically cross-linked to host epithelial cells during infection using a cleavable crosslinker. The crosslinked proteins were identified by resolving the cross-linked complexes on 2D-gels. This approach identified several new receptor pairs in Salmonella and epithelial cells. Interaction of Salmonella Ef-Tu with Hsp90 from epithelial cells mediated adhesion, while interaction of Salmonella Ef-Tu with myosin phosphatase and alpha catenin; two host proteins that negatively regulate membrane ruffling, mediated adhesion and invasion. Ef-Tu from Salmonella also induced phosphorylation of pro-survival kinase Akt. Ef-Tu is one of the most abundant protein on the cell, and this work proved that a sub population of the protein is membrane associated. Also Ef-Tu is a highly conserved protein across all Salmonella serovars, hence maybe an ideal vaccine candidate effective against all Salmonella serotypes.

I also identified host ganglioside GM1 as a receptor that mediated adhesion and invasion of Salmonella. Based on the fact that host glycosphigolipids can mediate
pathogen attachment, I screened a variety of enteric pathogens, commensals and probiotics for ganglioside binding. All the screened *Salmonella, E. coli, Bifidobacteria* and *Bacillus* bound a mixed population of gangliosides isolated from bovine buttermilk. Armed with this knowledge I developed a method for concentrating pathogens from samples without pre-enrichment. Immobilized gangliosides concentrated bacteria for detection with real-time PCR. A sensitivity of ~4 CFU/ml (3 h) in samples without competing microflora was achieved. Samples with competing microflora had a sensitivity of 40,000 CFU/ml. In conclusion, I demonstrated the ability of gangliosides immobilized on 3-mm glass beads to concentrate pathogenic organisms and *Bacillus* spores directly from samples without pre-enrichment. Use of gangliobeads prior to a commercial RT-PCR kit for food pathogen detection improved the sensitivity 100-fold.

Next I tested, if *B. infantis* blocked *Salmonella* adhesion and invasion by competing for the same epithelial cell receptors. I found that *B. infantis* shared its binding specificity to ganglioside GM1 with *S. ser. Typhimurium*, while it did not use 90KD heat shock protein, alpha catenin or myosin phosphatase for binding to the host. Furthermore, *B. infantis* completely inhibited, *Salmonella*-induced caspase 8 and caspase 9 activity in intestinal epithelial cells. *B. infantis* also reduced the basal caspase 9 and caspase 3/7 activity in epithelial cells in absence of the pathogen. Western blots and gene expression profiling of epithelial cells revealed that the decreased caspase activation was concomitant with increased phosphorylation of the pro-survival protein kinase Akt, increased expression of caspase inhibiting protein cIAP and decreased expression of genes involved in mitochondrion organization and biogenesis and reactive oxygen
specie metabolic processes. Hence *B. infantis* exerted it protective effects by repression of mitochondrial cell death pathways which was induced in presence of *S. ser.* Typhimurium. Gene expression analysis also revealed that *B. infantis* strongly induced expression of genes downstream of cAMP signaling, MAPK signaling and NFkB signaling. Using gene expression and small metabolite profiles I also observed a three way cross talk between the epithelial cells, *Salmonella* and *Bifidobacteria*. In these interactions *B. infantis* prevented catabolism of arginine (a substrate for nitric oxide (NO) production) by *S. sv.* Typhimurium, making it available for increased NO production by the host (Figure 6. 1). This was evidenced by induction of genes necessary for nitrate and nitrite (break down products of NO) respiration in the pathogen. Using a comparative and functional genomics approach I also discovered a cluster of 50 proteins that have been acquired in the *B. infantis* genome through lateral gene transfer. These gene clusters are highly conserved in only subspp. *infantis* and are induced only in presence of the host. Hence they have likely been conserved in the genome through selective pressure from the host.

In conclusion this work identified novel receptors in *Salmonella* that mediate adhesion and invasion, and therefore are candidates for vaccine development against *Salmonella*. Further, using immobilized host receptors I developed a method for concentrating pathogens from samples without pre-enrichment. Mechanisms for pathogen blocking by *B. infantis* included competition for GM1 binding, and inhibition of mitochondrial cell death pathway in the host as well as the catabolism of arginine in *Salmonella*. Therefore, I accept the hypothesis that *B. infantis* reduces *S sv.* Typhimurium
adhesion to gut epithelial cells by competing for the same receptors and improves cell survival by modulation of specific pathways in the host.

References


Figure 6.1 Proposed three way interactions between host epithelial cells, *S. ser. Typhimurium* and *B. infantis*
APPENDICES
Appendix A - Supplementary Data for Chapter 3
Figure A 1 Molecular structure of Sulfo-SBED as provided by the manufacturer.
Figure A 2 Neighbor joining tree made by using Phylobuilder based on multiple sequence alignments of homologs of LBA0222. The blue box indicates the homologous proteins in *L. acidophilus* NCFM.
Figure A 3 Cross-linked proteins resolved under non reducing conditions (1,2) and under reducing conditions (3,4)
Appendix B-Supplementary Data for Chapter 5
Table B1 Two way ANOVA with repeated measures of relative levels of phosphorylated AKT at ser-473 and thr-308 in epithelial cells upon infection with different microbes at 30, 60 and 120 minutes post infection.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Df</th>
<th>Sum-of-squares</th>
<th>Mean square</th>
<th>F</th>
<th>% of total variation</th>
<th>P value</th>
</tr>
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<td>pAKT-473</td>
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<td>1213</td>
<td>202.2</td>
<td>4.514</td>
<td>18.69</td>
<td>0.0272</td>
</tr>
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<td>304</td>
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<td>7.69</td>
<td>0.0002</td>
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<td>5419</td>
<td>1806</td>
<td>26</td>
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<td>Residual</td>
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<tr>
<td>pAKT-308</td>
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<td>202.2</td>
<td>4.514</td>
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Table B 2 Differentially regulated pathways (q≤0.05) in *S. sv. Typhimurium* when exposed to *B. infantis* as compared to normal growth. NES= Normalized enrichment score. A positive score indicates that the gene set was induced when exposed to *B. infantis*, while a negative score indicated that the gene set was repressed.

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Size of Gene Set</th>
<th>Genes Regulated</th>
<th>NES</th>
<th>FDR q-val</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino Acid Biosynthesis- Glutamate Family</td>
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<td>1.57</td>
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<td>1.55</td>
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<td>Cell Envelope- Biosynthesis And Degradation Of Surface Polysaccharides And Lipopolysaccharides</td>
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<td>Cellular Processes- Detoxification</td>
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<td>Cog J-Translation</td>
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<td>94</td>
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<td>Cog M-Cell Wall Membrane Biogenesis</td>
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<td>Mobile And Extra chromosomal Element Functions- Plasmid Functions</td>
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</table>
Table B 3 Differentially regulated pathways (q≤0.20) in caco-2 cells when exposed to *B. infantis* and *S. sv. Typhimurium* as compared to when exposed to only *B. infantis*. NES= Normalized enrichment score. A positive score indicates that the gene set was induced in Caco-2 cells when exposed to *B. infantis* and *S. sv. Typhimurium*, while a negative score indicated that the gene set was repressed.

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Size of Gene Set</th>
<th>Genes Regulated</th>
<th>NES</th>
<th>FDR q-val</th>
</tr>
</thead>
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<tr>
<td><strong>Kegg Pathway</strong></td>
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<tr>
<td>Nod Like Receptor Signaling Pathway</td>
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<td>Steroid Hormone Biosynthesis</td>
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<td><strong>Biocarta Pathway</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Ataxia Telangiectasia-mutated gene Pathway</td>
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<tr>
<td>Il6 Pathway</td>
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<td>9</td>
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<td>0.02</td>
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<tr>
<td><strong>GO Annotations</strong></td>
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<td>Cell Junction</td>
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<td>Basolateral Plasma Membrane</td>
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<td>Chemokine Activity</td>
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<td>6</td>
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<td>0.20</td>
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Table B 4 Transcriptional regulons that are differentially regulated (q≤0) in Caco-2 cells when they were exposed to *B. infantis* and *S. sv.* Typhimurium as compared to when they were exposed to only *B. infantis*. NES= Normalized enrichment score. A positive score indicates that the gene set was induced in Caco-2 cells when exposed to *B. infantis* and *S. sv.* Typhimurium, while a negative score indicated that the gene set was repressed.

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Binding Motif</th>
<th>Transcription Factor</th>
<th>Remarks</th>
<th>Size</th>
<th>Genes Regulated</th>
<th>NES</th>
<th>FDR q-val</th>
</tr>
</thead>
<tbody>
<tr>
<td>V$CREB__Q2$</td>
<td>NSTGAC GTAANNN</td>
<td>CREB1: cAMP responsive element binding protein 1</td>
<td>Activated by a variety of stimuli leading to phosphorylation of CREB1 mediated by PKA</td>
<td>140</td>
<td>29</td>
<td>-2.17</td>
<td>0</td>
</tr>
<tr>
<td>V$CREB__Q4_01$</td>
<td>CNNTGA CGTMA</td>
<td>CREB1: cAMP responsive element binding protein 1</td>
<td>Activated by a variety of stimuli leading to phosphorylation of CREB1 mediated by PKA</td>
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<td>40</td>
<td>-2.25</td>
<td>0</td>
</tr>
<tr>
<td>V$CREB__Q4$</td>
<td>NSTGAC GTMAN N</td>
<td>CREB1: cAMP responsive element binding protein 1</td>
<td>Activated by a variety of stimuli leading to phosphorylation of CREB1 mediated by PKA</td>
<td>109</td>
<td>25</td>
<td>-1.99</td>
<td>0</td>
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<tr>
<td>V$CREBP_1__Q2$</td>
<td>VGTGAC GTMAC N</td>
<td>ATF2: activating transcription factor 2</td>
<td>This protein binds to the cAMP-responsive element (CRE),The protein forms a homodimer or heterodimer with c-Jun and stimulates CRE-dependent transcription.</td>
<td>134</td>
<td>31</td>
<td>-1.99</td>
<td>0</td>
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</table>
Table B 5 Differentially regulated genesets in *B. infantis* after 120 minutes of incubation with *S. sv.* Typhimurium as compared to *B. infantis* incubated alone. NES= Normalized enrichment score. A positive score indicates that the gene set was induced in *B. infantis* when exposed to *S. sv.* Typhimurium, while a negative score indicated that the gene set was repressed.

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Size of Gene Set</th>
<th>Genes Regulated</th>
<th>NES</th>
<th>FDR q-val</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes with%GC &gt;70</td>
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<td>Cog J Translation</td>
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<td>66</td>
<td>1.97</td>
<td>0.01</td>
</tr>
<tr>
<td>Sec System</td>
<td>9</td>
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</tr>
<tr>
<td>Translation</td>
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<td>Other Transcription Related Proteins</td>
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<td>Drug Metabolism</td>
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<td>4</td>
<td>1.65</td>
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<td>Histidine Metabolism</td>
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<td>8</td>
<td>1.62</td>
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<tr>
<td>Multi Transmembrane</td>
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<td>198</td>
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<tr>
<td>Cog G Carbohydrate Transport and Metabolism</td>
<td>191</td>
<td>84</td>
<td>-1.90</td>
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<td>Glycosyl Hydrolases</td>
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<td>Cog V Defense Mechanism</td>
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Table B 6 Gene sets that are conserved in *B. infantis* but divergent in *B. longum* based on comparative genomic hybridization of 15 *Bifidobacteria* strains (q≤0.07).

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Size of Gene Set</th>
<th>Divergent Genes</th>
<th>NES</th>
<th>FDR q-val</th>
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<tbody>
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<td>Cog V Defense Mechanism</td>
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<tr>
<td>Genes%GC Less Than 40</td>
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<td>Genes with%GC Between 40 And 50</td>
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<td>HGT from Actinomycetales</td>
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<tr>
<td>HGT from Firmicutes</td>
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<td>31</td>
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</tbody>
</table>
Figure B 1 Effect of blocking selected *Salmonella* receptors with antibodies in Caco-2 cells on the adhesion of *B. infantis*. Control represents the adhesion of *B. infantis* to unblocked caco-2 cells. Error bars represent standard error from 4 replicates.
Figure B.2 Log2 ratios of differentially expressed genes (q≤0.1) in epithelial cells when exposed to *B. infantis* and *S. sv.* Typhimurium. For gene expression data, red represents induction while blue represents repression of genes when epithelial cells were exposed to *B. infantis* and *S. sv.* Typhimurium.
Figure B 3 Genes in *B. infantis* that were putatively acquired by lateral gene transfer; were only conserved in the subspp. *infantis* and divergent in subspp. *longum*; and were induced in presence of epithelial cells and *S. sv. Typhimurium* (q<0.15). The heat maps represent averaged relative expression level of the genes under specific conditions from two replicates.
CURRICULUM VITAE

Prerak T. Desai
10835 Road to the Cure, Suite 150, San Diego CA 92122, prerak.desai@usu.edu

PhD, Food Science (Microbiology,) May 2011
Dissertation: Molecular interactions of Salmonella with the host in presence of commensals.
Department of Nutrition Dietetics and Food Sciences, Utah State University, Logan

M.S, Food Science (Microbiology), May 2006
Thesis: Antimicrobial properties of syringopeptin 25A and rhamnolipids
Department of Nutrition Dietetics and Food Sciences, Utah State University, Logan

B. Tech, Dairy Science, May 2003
SMC College of Dairy Science, Anand, Gujarat, India

Competencies

Statistics and Bioinformatics
- Analysis and visualization of high throughput “omics” datasets including gene expression microarrays, high throughput protein expression (LC-MS/MS), metabolite profiles (LC-MS, GC-MS and NMR) and high throughput DNA sequencing
- Use of R and Bioconductor for statistical analysis (analysis of variance and multivariate analysis) of high throughput data sets
- Use of enrichment analysis tools like Gene Set Enrichment Analysis (GSEA) for enrichment analysis (based on GOs, COGs, signaling and metabolic pathways or phylogenetic distance) of high throughput datasets
- Primer/probe design for qPCR and microarrays for gene expression and phylogenomic analysis
- Comparative genomic analysis of sequenced microbes using many different genome databases and visualization tools including but not limited to JCVI-CMR, JGI-IMG, CAZY, Pathway tools, Kegg, Prodiric, Ingenuity pathway analysis
- Identification of genus or species specific gene clusters by subtractive genome analysis of sequenced strains
- Genome annotation and curation using various sequence analysis tools (For eg. Blast2GO, ClustalW, Blast, BioEdit, Phylobuilder) and databases (For eg. PFAM, eggNOG, PROSITE, GenBank, Swiss-Prot, COG)

Genomics/Genetics
- Design, validation and use of microarrays for gene expression profiling in eukaryotic and prokaryotic systems. I have worked on many different microarray platforms which include in house hand spotted glass chips/nylon membranes, Nimblegen and Affymetrix GeneChips.
- Comparative genomics and phylogenomics using sequence analysis, comparative genomic hybridization (CGH) and multilocus sequence typing (MLST)
- Microbial community analysis from environmental, clinical and food samples using qPCR, microarrays (Phylochip) and amplicon sequencing
- Heterologous gene expression/gene knock downs in bacteria (stable isogenic knock out mutants) and mammalian cell culture (shRNA, lentiviral vector)

**Proteomics**
- Identification of binding partners in host-microbe interaction studies by chemical cross-linking of whole cells
- Identification of vaccine targets in bacteria by employing a combination of label transfer approach and antigen screening using serum from infected animals
- Protein and small molecule purification using column chromatography and HPLC
- Protein separation using 2D and 1D gels
- Protein detection and quantitation using western blots and ELISA

**Cell based assays**
- High throughput qPCR based adhesion and invasion assays to quantify adhered/invaded pathogens and commensals in cell culture models
- High throughput screening of growth inhibitors (antimicrobials) and growth promoters (prebiotics) in microbial systems
- Construction of pathway reporters in mammalian cells using lentiviral vectors
- Rapid and quantitative detection of microbes by solid phase capture and qPCR
- Manufacture of biomimetic liposomes for functional immobilization of membrane proteins
- Immobilization of protein, DNA and small molecules on solid surfaces

Summary of Experience

**Junior Specialist**
January 2009 to August 2010
Dept. of Population Health and Reproduction, UC Davis, Davis, CA
- Use of modified aptamers as a therapy against multi drug resistant *S. aureus*
- Screening of prebiotics and probiotics for pathogen blocking
- Development of a peptide vaccine against *Salmonella*
- Metabolomic profile during infection of host with *Salmonella* in presence of commensals
- Comparative genomics of bifidobacteria
- Microbial community analysis of plants infected with *E. coli*
- Effect of transgenic goat milk expressing human lysozyme on the diversity of gut microbial community

**Graduate Research Assistant**
January 2004 to December 2008
Center For Integrated Biosystems, Utah State University, Logan, UT
- Antimicrobial discovery from natural products
- Host microbe interactions using functional genomics
- Identification of host-microbe receptors
- Rapid detection of pathogens from food and environment
- Microbial community analysis of the Great Salt Lake (Utah)
- Microbial community analysis of cheese supplemented with probiotics

**Graduate Research Assistant**
August 2003 to December 2003
Agricultural Research Station, Fort Valley State University, Fort Valley, GA
- Fatty acid profile of goat milk cheese.

**Quality Control Officer**
March 2003 to August 2003
Vasudhara Dairy, Alipore, Gujarat, India

Awards and Scholarships
- Best Oral Presentation, American Society for Microbiology, 2006 - Received 1st prize at the 2006 American Society for Microbiology Meeting Intermountain Section, Provo, UT
- Gandhi Scholarship, Utah State University, 2004 - Recipient of the Gandhi Scholarship

Patents

Manuscripts
Published

In Review

In Preparation
- Desai P., Dong Chen, B.C. Weimer. A whole-cell crosslinking approach to identify host microbe receptors.
- Desai P., Dong Chen, B.C. Weimer. Identification of Novel Receptors for *Salmonella* that mediates host-microbe adhesion and invasion.
Conference Posters


Oral Presentations

- Ganesan, Balasubramanian, Bart C. Weimer, Giovanni Rompato P. Desai, Janneth Pinzon, Carl Brothersen, and Donald J. Mcmahon. 2010 Addition of probiotic bacteria modifies the
biodiversity of other lactic acid bacteria in Cheddar cheese. American Dairy Science Association annual meeting, Denver, CO. (Invited Talk)
