THE ROLE OF THE INTESTINAL MICROBIOTA IN HOST SUSCEPTIBILITY TO *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

by

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Abstract

Intestinal microbiota comprise microbial communities that reside in the gastrointestinal tract and are critical to normal host physiology. Understanding the microbiota's role in host response to invading pathogens will further expand our knowledge of host-microbe interactions, as well as foster advances in the design of novel therapeutic and prophylactic methods.

In this dissertation I used clinically relevant doses of antibiotics to disturb the intestinal microbiota balance in a murine infection model. Pre-infection perturbations in the microbiota with two antibiotics resulted in increased mouse susceptibility to *Salmonella enterica* serovar Typhimurium intestinal colonization, greater post-infection alterations in the microbiota, and more severe intestinal pathology. This demonstrates the importance of a balanced microbiota community in host response to an enteric pathogen.

This infection model also allowed further characterization of the host-pathogen-microbiota interactions during enteric salmonellosis. It was shown that in the presence of high numbers of indigenous microbes *S*. Typhimurium deficient in *Salmonella* pathogenicity island 2 (SPI2) is unable to trigger intestinal inflammation, while a SPI1 mutant strain promotes late typhlitis. Additionally, it was demonstrated that pathogen-induced intestinal inflammation does not always translate into extensive alterations to the host microbiota, as inflammation during a SPI1 mutant infection did not promote the same changes in host microbiota composition and numbers as inflammation induced by wild-type *S*. Typhimurium. Differential neutrophil recruitment by the three *S*. Typhimurium strains was implicated as one possible agent of microbiota perturbations.

A thorough understanding of the tripartite host-microbiota-pathogen relationship in the progression of the enteric infections is needed to fully appreciate the disease process, as well as

to suggest new avenues through which to interfere with the infection progression. These studies enhance our understanding of the microbiota's role in the progression of *S*. Typhimurium infection and the effects of inflammation upon the microbiota, thus broadening our knowledge of *S*. Typhimurium pathogenesis and associated host response.

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List of Abbreviations

ANOVA	analysis of variance
CD (e.g. CD18)	cluster of differentiation
CDC	Centres for Disease Control
CFB	Cytophaga-Flavobacteria-Bacteroides
CFU	colony forming units
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DDGE	denaturing detergent gradient gel electrophoresis
EHEC	Enterohemorrhagic *Escherichia coli*
ELISA	enzyme-linked immunosorbant assay
et al	*et alii*
FISH	Fluorescent *in situ* hybridization
GALT	gut-associated lymphoid tissue
Gm(-)/Gm(+)	Gram negative / Gram positive
GF	germ free
IBD	inflammatory bowel disease
IgA	Immunoglobulin A
IL (e.g. IL1)	interleukin
i.v.	intravenous
LB	Luria-Bertani
LPS	lipopolysaccharide
MAPK	MAP kinase
MPO	myeloperoxidase
NFκB	nuclear factor kappa B
NLR	Nod-like receptor
NK	natural killer
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PEEC	pathogen-elicited epithelial chemoattractant
PFA	paraformaldehyde
p.i.	post infection
PMN, neutrophil	polymorphonuclear leukocyte; neutrophil
p.o.	*per oral*
PRR	pattern recognition receptor
RFLP	restriction fragment length polymorphisms
SCV	*Salmonella* containing vacuole
SFB	Segmented filamentous bacteria
Sif	*Salmonella* induced filaments
SPF	specific pathogen free
SPI	*Salmonella* pathogenicity island
TLR	Toll-like receptor
TNFα	tumour necrosis factor alpha
T3SS	type three (III) secretion system
WHO	World Health Organization
WT	wild-type

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CHAPTER 1:
INTRODUCTION

1.1 Intestinal Microbiota

1.1.1 Overview

Humans and other multicellular eukaryotic organisms are made up of more than just their own cells – they exist in a tight relationship with a myriad of assorted microorganisms that inhabit numerous sites on our bodies. This entourage of microorganisms constitutes our normal flora, or microbiota; colonization by the microbiota is a very dynamic process that begins at birth and continues throughout life. Although every surface that is exposed to the environment harbours some number and variety of microbial tenants, the gastrointestinal tract contains by far the most numerous and diverse microbial community (Salzman et al., 2007). The intestinal microbiota are notable for their many roles in the proper development, health and disease of the host, contributing to numerous aspects of the development of the gastrointestinal tract itself, and also affecting many other organ systems.

The intestinal microbiota are comprised of eukaryotes (Scupham et al., 2006), viruses (Furuse et al., 1983) and bacteria. The bacterial microbiota (henceforth refered to as microbiota) constituents are incredibly diverse and numerous, outnumbering the host cells by at least one order of magnitude (Savage, 1977). Microbial colonization varies along the length of the gastrointestinal tract, with a low of 10^1-10^3 bacteria/ml in the stomach and the duodenum, progressing to 10^4-10^7 bacteria/ml in the jejunum and ileum and culminating in 10^{11}-10^{12} bacteria/ml in the colon (O'Hara and Shanahan, 2006). Obligate anaerobes make up most of the colonic microbiota, with facultative anaerobes occurring about ~1000-fold less. Firmicutes and Bacteroidetes phyla dominate both the human and the murine intestinal microbiota. Proteobacteria, Actinobacteria, Verrucomicrobia, Cyanobacteria and Deferribacteres have also

been detected in humans and mice, and Fusobacteria in humans only (Figure 1.1) (Backhed et al., 2005; Eckburg et al., 2005; Stecher and Hardt, 2007).

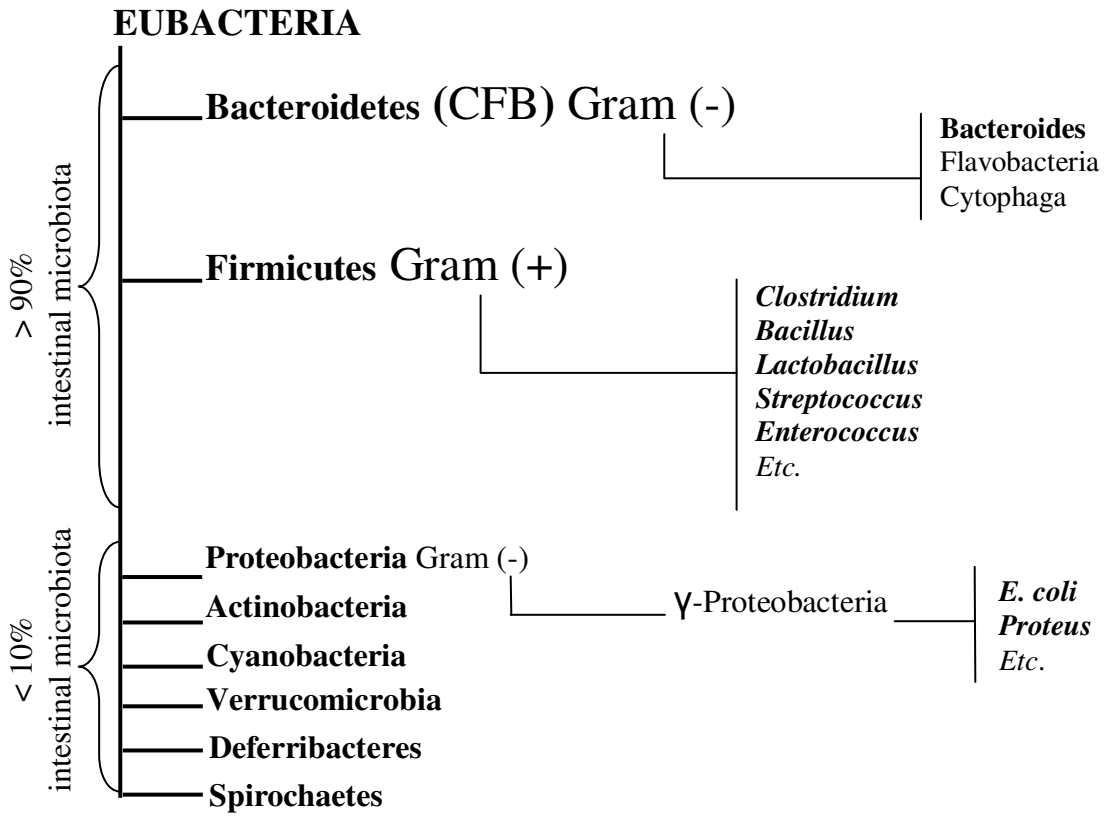


Figure 1.1 Composition of the murine intestinal microbiota
Murine large intestinal microbiota cluster within only a few phyla, with over 90% of the bacteria belonging to Bacteroidetes and Firmicutes and the remaining 10% distributing among Proteobacteria, Actinobacteria, Cyanobacteria, Verrucomicrobia, Deferribacteres, and Spriochaetes. While the composition at phylum level is similar in mice, and even between humans and mice, the composition at finer genus and species levels varies greatly between individual members of the same species.

Bacteroidetes are Gram negative (Gm(-)) obligately anaerobic rods with specific metabolic abilities, GC content and cell wall composition (Shah, 1992). As true anaerobes, they are well positioned to colonize the anaerobic intestinal environment, and therefore it is not surprising that they represent one of the two most abundant phyla of the intestinal microbiota.

This phylum contains such important microbiota members as *Bacteroides thetaiotaomicron*, whose role in the development of the intestinal epithelium is discussed below.

Firmicutes are Gm(+) bacteria of diverse shapes and metabolic abilities. Their name means "strong" in Latin, in reference to their durable Gm (+) wall. They are divided into two large groups according to their G-C ratio – high G-C (>55% G-C in their DNA) and low G-C (<55% G-C in their DNA) (Daubin et al., 2002). Members of the *Clostridia*, *Bacilli* and *Mollicutes* classes (low G-C Firmicutes) are found in the human intestinal microbiota (Eckburg et al., 2005). Bacteria from this phylum contribute to many aspects of proper gastrointestinal tract functioning, as well as to a number of pathological conditions. Details of their contribution are discussed below.

Proteobacteria are Gm(-) bacteria whose name is derived from the Greek sea god Proteus known for his ability to change his shape (Stackebrandt, 1988). This phylum is subdivided into 5 classes – α-, β-, γ-, δ- and ε- Proteobacteria. Proteobacteria harbor many important pathogens and opportunistic pathogens, such as *Rickettsia spp*. (α-Proteobacteria), *Burkholderia* and *Neisseria spp*. (β-Proteobacteria), *E. coli* and *Salmonella spp.* (γ-Proteobacteria) and *Campylobacter and Helicobacter spp.* (ε-Proteobacteria). Members of all 5 classes have been found in the human intestinal microbiota (Eckburg et al., 2005), while imbalances in the proportion of γ-Proteobacteria have been implicated in the etiology of various gastrointestinal diseases (Sartor, 2008).

Actinobacteria are Gm(+) bacteria with high G-C content. Most Actinobacteria are aerobic, with few exceptions. Some members form branching filaments, resembling unrelated fungi, with which they were originally mistakenly classified as Actinomycetes. Many soil bacteria from this phylum are known antibiotic producers.

Cyanobacteria are blue-green photosynthetic bacteria that are important contributors to the oceanic ecosystem as well as many freshwater lakes. 16S rRNA sequencing of human intestinal microbiota by Eckburg and colleagues revealed sequences that are near but not closely related to Cyanobacteria and chloroplast sequences, which appear to represent a novel lineage deeply branching from the Cyanobacteria phylum (Eckburg et al., 2005).

Verrucomicrobia is a recently described phylum (Hedlund et al., 1997) with many potential biotechnological and medical applications (Wagner and Horn, 2006). One member of the Verrucomicrobia, *Akkermansia muciniphila,* has been detected in and cultured from the human intestinal microbiota (Derrien et al., 2004); Eckburg et al., 2005). It is a Gm(-), strictly anaerobic non-motile, non-spore-forming mucin degrading bacterium.

Deferribacteres have been recently demonstrated to be present in the murine cecum (Stecher et al., 2007). This phylum incorporates only 4 genera (Huber, 2001) and has been implicated in the etiology of human periodontal disease (Siqueira et al., 2005).

Spirochaetes is a phylum of Gm(-) bacteria with distinctive long, helically coiled cells. Their presence in the human intestinal tract has been described by Backhed and colleagues (Backhed et al., 2005). Members of this phylum cause a variety of human infections, such as syphilis, lyme disease and leptospirosis. Like Deferribacteres, Spirochaetes have recently been implicated as a causative agent of human periodontal infections (Duncan, 2003).

Fusobacteria are obligately anaerobic Gm(-) bacilli which have only recently been assigned to their proper phylum (Bennett and Eley, 1993). As a component of the digestive tract microbiota, Fusobacteria are most commonly found in the oral cavity, although they also contribute to the intestinal microbiota. Their involvement in opportunistic infections of many organ systems has been long recognized.

Microbiota composition varies along the length of the gastrointestinal tract, as well as between the luminal and epithelium-associated bacteria (Swidsinski et al., 2007). Although the composition at phylum level is fairly similar between individuals and even between members of certain different species, the composition at the genus and species levels varies widely between individuals (Eckburg et al., 2005; Gill et al., 2006; Ley et al., 2005). Detailed knowledge of microbiota composition at both levels can foster our understanding of microbiota's contributions to various aspects of normal host physiology, as well as to an array of pathological conditions in which microbiota play a role.

1.1.2 Intestinal microbiota in health

The host and its indigenous microbiota have evolved together, as it has been shown that transplantation of microbial communities between different host species results in the transplanted community morphing to resemble the native microbiota of the recipient host (Rawls et al., 2006). Moreover, the host has evolved intricate mechanisms that allow local control of the resident microbiota without the induction of concurrent damaging systemic immune responses (Macpherson and Uhr, 2004). This adaptation is not surprising when considering that different bacterial groups and species have been implicated in various aspects of normal intestinal development and function of their host (Figure 1.2), as well as the causative agents of certain intestinal pathologies (which will be discussed in the following section).

Intestinal microbiota play a central role in the proper development of the gastrointestinal structure and function, provides a great contribution to host metabolism and nutrient processing, and impacts on many aspects of the proper development, maturation and functioning of the

intestinal immune system. All of these diverse roles position the intestinal microbiota at the center of the host's health.

Intestinal microbiota contribute to many aspects of host defense against invading pathogens both through direct microbial antagonism and promotion of maturation of the intestinal immune system. Bacterial metabolism results in the production of several by-products with an antimicrobial effect, such as peroxides and various acids. Some of these substances do not just inhibit the growth of pathogenic microorganisms themselves, but might also potentiate the effectiveness of other antimicrobial substances (Alakomi et al., 2000). In addition, production of biosurfactants by the microbiota (Velraeds et al., 1996) and competition for sites of attachment and nutrients (Reid et al., 2001) prevent the pathogens from establishing themselves within the host.

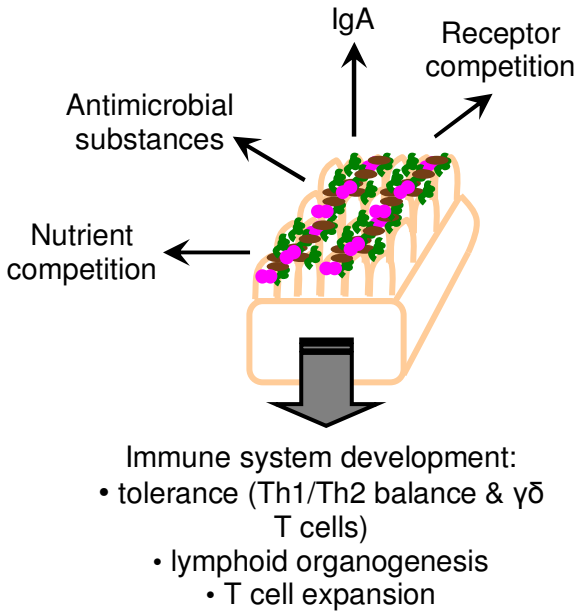
The intestinal microbiota are in constant contact with a variety of host cells of the gastrointestinal tract, such as the surface epithelial cells, M cells and dendritic cells (DCs) (O'Hara and Shanahan, 2006). The commensal bacterial antigens are being constantly sampled by the host pattern recognition receptors (PRRs), namely the extracellular Toll-like receptors (TLRs) and the intracellular NOD-like receptors (NLRs). It is not surprising, therefore, that the immunomodulatory role of the intestinal microbiota is integral to the proper development of the intestinal immune system. Comparisons of germ-free (GF) and conventionally raised laboratory animals confirm a role for the indigenous microbes in cytokine production, serum immunoglobulin levels, development of intraepithelial lymphocytes and Peyer's patches (O'Hara and Shanahan, 2006), as well as a role in systemic lymphoid organogenesis (Macpherson and Harris, 2004). Depletion of intestinal microbiota constituents has demonstrated that microbiota-induced activation of TLR signaling in a healthy host is necessary to maintain proper epithelial

homeostasis and promote repair following intestinal injury (Rakoff-Nahoum et al., 2004). Additional studies have implicated specific bacterial groups and species in various aspects of intestinal immunity.

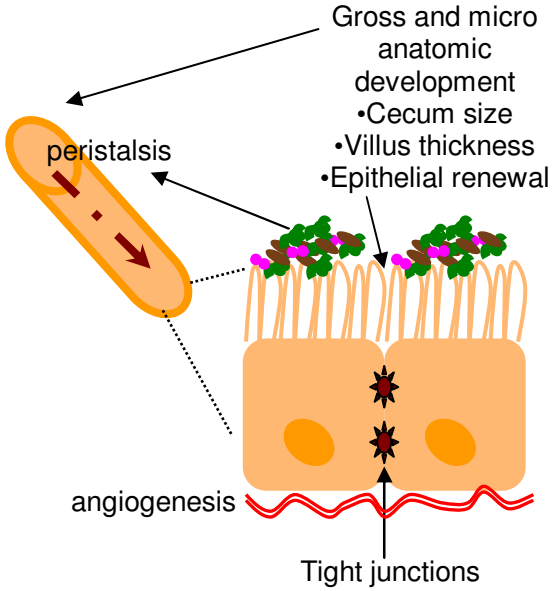
Polysaccharide antigen of *Bacteroides fragilis*, a prominent member of the gut microbiota, promotes the expansion of splenic CD4 T-cells and regulates Th1/Th2 cytokine production, as well as restores the splenic architecture to that of conventionally raised mice (Mazmanian et al., 2005). *Bacteroides thetaiotaomicron*, another ubiquitous microbiota species, affects host gene expression resulting in a plethora of effects in a number of organ systems (Xu and Gordon, 2003); for instance, it induces the expression of an angiogenin with bactericidal activity against intestinal microbes (Hooper et al., 2003). Different *Lactobacillus* species, also important members of the gut microbiota, differentially activate DCs, inducing them to produce different arrays of inflammatory cytokines, and thus playing an important role in the modulation of the Th1, Th2 and Th3 balance (Christensen et al., 2002). Moreover, *Lactobacillus*-stimulated DCs proceed to activate natural killer (NK) cells, thus potentiating gastrointestinal immunity (Fink et al., 2007). Segmented filamentous bacteria (SFB) are implicated in the induction of the intestinal IgA (Suzuki et al., 2004).

The gut architecture is directly influenced by its resident microbiota, with germ-free animals exhibiting gross morphological alterations, such as cecum enlargement (Gustafsson et al., 1970), as well as microscopic changes, such as villi hyperplasia and reduced crypt cell cycling (Alam et al., 1994). The complexity of the intestinal capillary network is also severely reduced in the germ-free mice, and recently *B. thetaiotaomicron* has been shown to regulate the intestinal angiogenesis through its interactions with Paneth cells (Stappenbeck et al., 2002). *Lactobacilli*

A. Protection and immunomodulation



B. Structure and function



C. Metabolism and nutrition

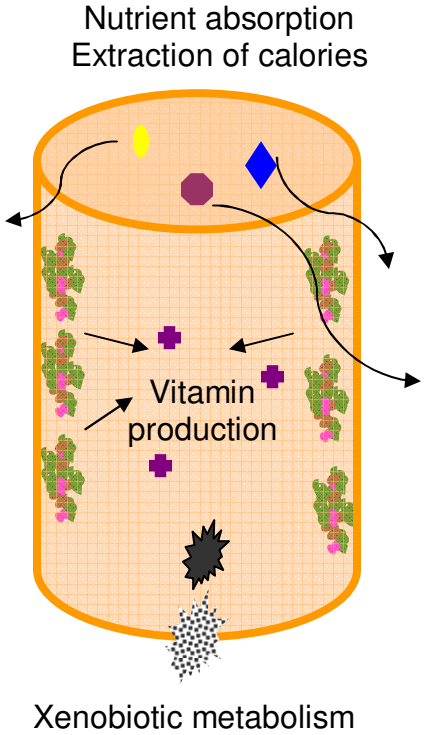


Figure 1.2 Contributions of the intestinal microbiota to proper GI development and function

A. Microbiota constituents contribute to host defenses against invading pathogens through both direct interference and promotion of proper maturation of the intestinal immune system. **B.** Intestinal microbiota help establish proper gross and microscopic architecture of the digestive tract and promotes healthy GI motility. **C.** Intestinal microbiota maximize caloric availability and contribute essential vitamins; some constituents help in xenobiotic degradation

have been shown to contribute to the maintenance of epithelial tight junctions (Lutgendorff et al., 2008). Indigenous bacteria also alter the intestinal function: intestinal motility is restricted in the absence of microbiota, with reduced frequency and propagation of migrating myoelectric complexes in the small intestines of germ-free rats (Husebye et al., 1994).

Nutrient processing and metabolism is also affected by the intestinal microbiota. GF animals require a higher-calorie diet than conventionally raised animals (Wostmann et al., 1983) and are highly susceptible to various vitamin deficiencies (Backhed et al., 2005). *Clostridium* and *Bifidobacterium* species are specifically implicated in metabolism of dietary fiber to short-chain fatty acids, such as butyrate, accounting for a significant part of the human energy source (Sartor, 2008). Production of butyrate is not only important as an energy source for the host, but also prevents the accumulation of potentially toxic metabolic by-products, such as D-lactate (Duncan et al., 2004). In addition to being able to break down indigestible polysaccharides to absorbable monosaccharides, the intestinal microbiota have been recently shown to increase body fat deposition by suppressing the inhibition of lipoprotein lipase (Wolf, 2006). Certain intestinal microbiota constituents have also been implicated in the metabolism of some xenobiotic compounds (Backhed et al., 2005). *Oxalobacter formigenes*, for instance, has the ability to degrade oxalate, thus reducing kidney stone formation (Sidhu et al., 2001).

1.1.3 Intestinal microbiota in disease

While a normal and healthy intestinal microbial community is prerequisite to the normal and healthy development of its host, microbiota imbalances (or dysbiosis) have been linked to various pathological processes (Figure 1.3). Interestingly, dysbiosis in the intestinal tract has the capacity to produce pathological manifestations in remote organ systems, yet again

demonstrating the crucial role of the indigenous intestinal microbial community in host physiology.

Various disorders of the gastrointestinal tract and associated digestive organs are commonly linked to either specific microbial species or general intestinal microbiota dysbiosis. *Helicobacter pylori* was shown to be a causative agent of gastric cancer (Correa and Houghton, 2007), initiating an adverse immune response that ultimately leads to dysplasia and neoplasia. A role for microbiota in the initiation of colorectal cancer has also been suggested (Hope et al., 2005). *Bacteroides* and, surprisingly, *Bifidobacterium* species were shown to be enriched in populations at high risk for colorectal cancer (Moore and Moore, 1995). *Enterococcus faecalis* was shown to induce chromosomal aberrations in colonic epithelial cells (Wang et al., 2008). Conversely, *Lactobacillus* species and *Eubacterium aerofaciens* (also a major lactic acid producer) were shown to be enriched in populations at low risk for colorectal cancer (Moore and Moore, 1995). Several researchers have demonstrated significant dysbiosis in both mucosally associated and fecal microbiota of patients with various inflammatory bowel diseases (IBD), such as Crohn's disease, ulcerative colitis and pouchitis (Sartor, 2008). An increased proportion of γ-Proteobacteria, specifically *Enterobacteriaceae*, is a common finding, mostly due to a decrease in the number of Firmicutes, specifically class Clostridia, rather than an absolute increase in γ-Proteobacteria. Additionally, *Bacteroides vulgatus* was recently shown to cause chronic inflammatory changes in the intestine in a rabbit model of IBD (Shanmugam et al., 2005), and early exposure to antibiotics was shown to be associated with an increased risk for subsequent development of Crohn's disease (Hildebrand et al., 2008). A role for the intestinal microbiota has also been suggested in the instigation of Type I diabetes, mostly due to alterations in disease susceptibility of laboratory animals treated with various antimicrobial agents (Vaarala

et al., 2008). Carcinogenesis, IBD and Type I diabetes all have immune dysregulation as part of their etiology, and it is currently unclear which component initiates the pathogenesis.

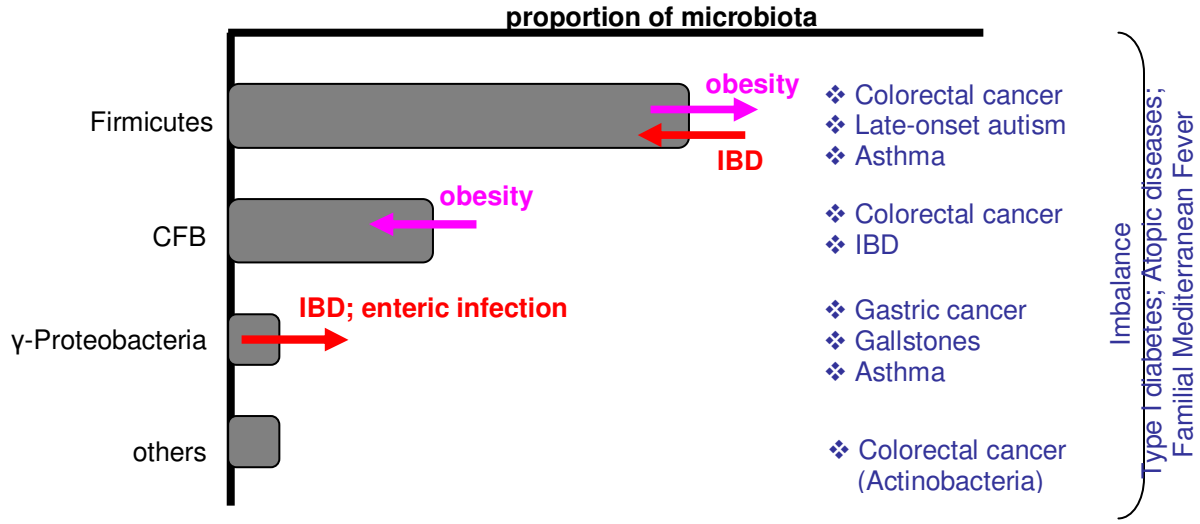


Figure 1.3 Diseases associated with the intestinal microbiota
Multiple diseases of various organ systems have been associated with either imbalances in or certain members of the intestinal microbiota; arrows indicate increase or decrease of microbial proportion in indicated disease; bullets indicate diseases associated with particular microbiota members; bracket indicates diseases associated with overall microbiota imbalance; IBD = inflammatory bowel diseases

Several disorders with complex manifestations and involving multiple organ systems in addition to, or other than the gastrointestinal system, have been linked to intestinal microbiota imbalances. Both genetically- and diet-induced obesity in mice and humans were shown to be associated with a decrease in the proportion of CFB bacteria and a concomitant increase in the proportion of Firmicutes (Ley et al., 2005; Turnbaugh et al., 2008; Turnbaugh et al., 2006). The "obese" microbiota were shown to have an increased capacity to harvest energy from host diet, thus possibly contributing to the pathophysiology of obesity (Turnbaugh et al., 2006).

Clostridium spp. were suggested to play a role in late-onset childhood autism, with fecal microbiota of autistic children containing significantly more of several *Clostridium* members than that of control subjects (Song et al., 2004). Microbial imbalance has been implicated in the etiology of atopic disorders, such as asthma, eczema and allergic rhinoconjunctivitis (Penders et al., 2007), while recently the carriage of *H. pylori* and increased concentrations of *Clostridium spp.* were shown to be inversely associated with asthma (Reibman et al., 2008; Verhulst et al., 2008). Patients suffering from familial Mediterranean fever, a hereditary inflammatory disorder, have been shown to harbour a significantly altered intestinal microbial community, demonstrating a decrease in total bacterial numbers and a loss of diversity (Khachatryan et al., 2008).

It was mentioned previously that the intestinal microbiota play an important role in the establishment of the host immune system and protection against invading pathogens. In recent years it was also shown that gastrointestinal infections exert a dramatic effect on the indigenous intestinal microbiota. Infection with various *Helicobacter* species was shown to reduce the diversity of and decrease colonization by the indigenous microbial community in the lower intestinal tract, while the diversity of the gastric microbiota was increased (Aebischer et al., 2006; Kuehl et al., 2005); Whary et al., 2006). Infection with *Citrobacter rodentium*, a murine enteric pathogen that is used to model enterohemorrhagic *Escherichia coli* (EHEC) infection (Luperchio and Schauer, 2001), was shown to decrease the total microbial population in the colon and drastically alter the microbiota composition (Lupp et al., 2007). *Salmonella enterica* serovar Typhimurium was shown to outcompete the intestinal microbiota during successful infection (Stecher et al., 2007) and also alter the composition of the indigenous microbes (Barman et al., 2008; Stecher et al., 2007). An inflammatory response to the invading pathogen

was implicated in the mechanism of microbiota perturbation during infection in many of these studies (Lupp et al., 2007; Stecher et al., 2007; Whary et al., 2006). As the intestinal microbiota are an important contributor to host resistance to infection, the details of the interplay of the pathogen and commensal biota warrant further investigation.

The presence itself of the indigenous microbiota, either in balance or dysbiosis, is of course insufficient to affect either the normal or abnormal development of the host. It is the reaction of the host to the microbiota that is responsible for the establishment of either a normal or a pathological effect. An assault upon the intestinal microbiota in the form of a pathogen, another foreign agent, an autoimmune reaction or abnormal host metabolism disrupts the microbial balance. This results in a modified host response to the "new" microbial community, a disturbance in the epithelial homeostasis, and a consequent impact upon the disease progression. The host's inflammatory response is often tied to the disease-associated disturbances in the intestinal microbiota. In the case of gastrointestinal infections, the commensal bacteria, the invading pathogen and the intestinal inflammation have been dubbed the "ménage à trois" in the progression of the infection (Pedron and Sansonetti, 2008).

1.1.4 Microbiota manipulation and study models

Antibiotics, probiotics and prebiotics

The ability to manipulate the intestinal microbiota is necessary in order to successfully examine its various contributions to host physiology. A number of techniques can be employed to modify the microbial composition. Antibiotics and similar bactericidal agents with specific mechanisms of action and antimicrobial spectrum can be used to conveniently and selectively modify the composition of the intestinal microbial community. There are some caveats to the use

of antibiotics, however. For instance, their spectrum of activity is mostly defined with respect to the clinical pathogenic isolates, not complex microbial communities. Consequently detailed evaluation of their effects on the indigenous intestinal microbes needs to be undertaken in order to establish with certainty the outcome of administration of various agents on the microbial community. Probiotics are live microorganisms that can efficiently colonize the gastrointestinal tract and consequently interact with the host's established microbial community and mucosal immune system (Pham et al., 2008). Administration of probiotics can, therefore, modify the composition of the host's microbiota and allow evaluation of the contribution of various bacterial species to various health and disease states. There are also some potential limitations to the use of probiotics. The indigenous microbiota are a stable community and might quickly revert to its steady state condition if only a single or small dose of the probiotic bacteria is introduced. Further, the exact effects on microbiota composition upon probiotic administration have not yet been determined. Prebiotics are "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well being and health", and some non-digestible oligosaccharides, such as inulin, its hydrolysis product oligofructose, and galactooligosaccharides, are currently available that fulfill this description (de Vrese and Schrezenmeir, 2008). Their use, by definition, also provides a tool for microbiota modifications, although, as with the use of probiotics, the exact effects of their administration need to be carefully detailed. The concurrent synergistic use of pro- and prebiotics, called synbiotics, is another potential microbiota modification technique.

Microbiota study models

A number of model systems are currently available to study the role of the intestinal microbiota in a variety of conditions, all with certain advantages and limitations. GF animals (such as mice, rats, zebrafish) offer great potential to the study of contribution of single microbiota species to the establishment of both normal host physiology and pathophysiology. A significant limitation to the use of GF animals, however, are gross anatomical and functional abnormalities resulting from compromised development in the absence of the microbiota, as was described above. An interesting alternative to the use of completely axenic animals is the use of animals associated with a defined flora cocktail, such as the altered Schaedler flora (Dewhirst et al., 1999), for instance. The use of these poly-associated animals provides the advantage of studying a well-defined microbial community in a host without gross developmental abnormalities. The draw-back, however, is that the full complexity of the intestinal microbial ecosystem is lacking. An attractive model was described recently by Pang and colleagues, involving transplantation of human fecal flora to GF piglets (Pang et al., 2007). The obvious benefit of this model is the study of a truly humanized microbial population in a host that is physiologically very similar to humans. The weakness, however, is that knock-out pigs are not readily available, especially compared to the multitude of commercially available knock-outs in the murine host. This limits the range of questions that can be successfully answered using this model. Regular specific pathogen free (SPF) mice can also be successfully used to study the contribution of the intestinal microbiota to the progression of various physiological and pathophysiological processes. Human and murine intestinal microbiota are similar at the phylum level (Backhed et al., 2005), making findings in SPF mice relevant to humans. Additionally the whole complexity of the microbial community is preserved, providing an accurate picture of its

contribution to the host. The shortcoming of this model lies in the difficulty of accurate microbiota manipulation; it will improve, however, after further assessments of the effects of anti-, pro- and prebiotics are undertaken.

1.2 *Salmonella enterica* serovar Typhimurium

1.2.1 *Salmonellae* epidemiology

The burden of enteric infections is colossal worldwide, especially in developing countries. *Salmonella enterica* serovars are Gm(-) enteropathogenic bacteria that cause the most common and widely distributed food-born disease in humans, being a significant public health hazard and accruing to a substantial economic burden world-wide (WHO, 2005). *S. enterica*-induced diseases, or salmonelloses, can range from gastrointestinal to systemic infections, depending on the host and the *S. enterica* serovar. More than 2500 *S. enterica* serovars have been described that exhibit different degrees of host adaptation and consequently produce diverse diseases in different host species (Coburn et al., 2007a). The outcome of infection usually falls within one of the following 5 categories: enteric fever (e.g. typhoid), enterocolitis/diarrhea, bacteremia, metastatic infections, or chronic asymptomatic carriage. Human enterocolitis is one of the most common *S. enterica*-associated salmonelloses, with serovars Typhimurium and Enteritidis being the most frequent isolates. Infection usually occurs through consumption of contaminated food or water; after passage through the stomach bacteria colonize the intestine from where they initiate their virulence strategy (Grassl and Finlay, 2008). Although *S.* Typhimurium typically causes uncomplicated gastroenteritis, it can be more critical in the young, the elderly and the immunocompromised, leading to severe and life-threatening outcomes (Fierer and Guiney, 2001). Most cases do not require specific therapy beyond oral rehydration and are self-limiting; very

young infants, elderly and immunocompromized patients that might present with a more severe disease manifestation can benefit from antimicrobial therapy (2004). Administration of antibiotics, however, does not guarantee complete eradication of the pathogen and a chronic carriage state may ensue. Chronic carriers promote further spread of the pathogen and are themselves predisposed to an array of additional medical problems.

1.2.2 *Salmonella* virulence factors

S. Typhimurium carries an impressive arsenal of virulence genes, the majority of which are organized into pathogenicity islands called *Salmonella* pathogenicity islands (SPI). At least 10 SPI have been recognized in *S. enterica*, endowing it with a diverse array of functions, such as secretion, nutrient uptake and expression of adhesins (Hensel, 2004).

The key to the *S.* Typhimurium virulence strategy are two type III secretion systems (T3SS) encoded on SPI1 and SPI2. T3SS are complex multi-protein structures that allow bacteria to directly inject bacterial proteins called effectors into host cells across bacterial and host membranes, where they can manipulate host cell function (Coburn et al., 2007b). SPI1- and SPI2- encoded T3SS secrete bacterial effectors that are encoded either within or outside the pathogenicity island (Tables 1.1 and 1.2) and promote a sequence of events that lead to a successful establishment of *S.* Typhimurium within the host and generation of infection-associated symptoms.

SPI1 is a 40kb island inserted between genes that are consecutive in the *E. coli* K-12 chromosome (Mills et al., 1995). Effectors translocated by SPI1 contribute to two aspects of *S.* Typhimurium-associated disease: invasion of non-phagocytic cells, a process that involves extensive host cell cytoskeleton rearrangements (Schlumberger and Hardt, 2006), and

enteropathogenesis, a process that involves induction of a strong inflammation at the intestinal epithelium (Wallis and Galyov, 2000). Loci related to SPI1 have been detected in *Shigella spp*. (Groisman and Ochman, 1993) and *Burkholderia pseudomallei* (Stevens et al., 2002).

SPI2 is also 40kb in size but is composed of at least two distinct elements (Hensel et al., 1999). A 25kb portion is essential for systemic pathogenesis, enabling Salmonella to modify endosomal trafficking and replicate within macrophages (Hensel et al., 1998) while a 15kb part is involved in anaerobic respiration (Hensel, 2004). Recently, SPI2 was also shown to play a significant role in the intestinal phase of the disease (Coburn et al., 2005; Coombes et al., 2005a; Hapfelmeier et al., 2005).

Table 1.1 SPI1 effectors

Protein	Gene location	Proposed function	Reference
SipA	SPI1	Invasion; PMN recruitment	(Hapfelmeier et al., 2004; Lee et al., 2000; Raffatellu et al., 2005; Zhang et al., 2002; Zhou et al., 1999)
SipB	SPI1	Effector translocation; macrophage apoptosis	(Hayward et al., 2000; Hersh et al., 1999)
SipC	SPI1	Effector translocation; actin rearrangements	(Hayward and Koronakis, 1999)
SopA	Chromosome	Invasion; PMN recruitment	(Raffatellu et al., 2005; Wood et al., 2000; Zhang et al., 2002)
SopB/SigD	SPI5	Invasion	(Wood et al., 2000; Zhang et al., 2002; (Zhou et al., 2001)
SopD	Chromosome	Invasion	(Raffatellu et al., 2005; Zhang et al., 2002)
SopE	Phage	Invasion; nuclear responses	(Hapfelmeier et al., 2004; Patel and Galan, 2006)
SopE2	Phage remnant	Invasion; nuclear responses	(Hapfelmeier et al., 2004; Patel and Galan, 2006)
SptP	SPI1	Actin cytoskeleton disruption	(Zhang et al., 2002)
SteA	Chromosome	?	(Geddes et al., 2005)
AvrA	SPI1	Inhibition of NFκB activation	(Collier-Hyams et al., 2002)
SlrP	Chromosome	?	(Zhang et al., 2002)
SspH1	Gifsy prophage	Down-regulates NFκB-dependent gene expression	(Zhang et al., 2002)

Table 1.2 SPI2 effectors

Protein	Gene location	Proposed function	Reference
SifA	Chromosome	Sif formation; SCV maintenance	(Beuzon et al., 2000); Brumell et al., 2002; Brumell et al., 2001; (Ruiz-Albert et al., 2002)
SifB	Chromosome	Targeted to Sif	(Brumell et al., 2002)
SseE	SPI2	?	(Hensel et al., 1998)
SseF	SPI2	Sif formation; replication in macrophages	(Hensel et al., 1998; (Kuhle and Hensel, 2002)
SseG	SPI2	Sif formation; replication in macrophages	(Hensel et al., 1998; Kuhle and Hensel, 2002)
SseJ	Phage	Deacylase; SCV maintenance	(Ohlson et al., 2005); Ruiz-Albert et al., 2002)
SseI/SrfH	Gifsy prophage	Increases infected phagocyte motility	(Ruiz-Albert et al., 2002; (Worley et al., 2006)
SseK1/2	Chromosome	?	(Kujat Choy et al., 2004)
SopD2	Chromosome	Sif formation	(Jiang et al., 2004; Lawley et al., 2006)
SpiC	SPI2	Effector translocation; modification of vesicular trafficking	(Uchiya et al., 1999; Yu et al., 2002)
PipB	SPI5	Targeted to Sif; function unknown	(Knodler and Steele-Mortimer, 2005); Knodler et al., 2003)
PipB2	Chromosome	Sif formation	(Knodler and Steele-Mortimer, 2005; Knodler et al., 2003)
SspH1	Gifsy prophage	Promotes virulence in calf model together with SspH2	(Miao et al., 1999)
SspH2	Phage	Promotes virulence in calf model together with SspH1	(Miao et al., 1999)
SlrP	Chromosome	?	(Tsolis et al., 1999)
GogB	Gifsy prophage	?	(Coombes et al., 2005b)

Current knowledge of the functions of other *S.* Typhimuruim pathogenicity islands is lacking. They are involved in Mg2+ uptake (SPI3), adhesion (SPI4), invasion (SPI6 or SCI (*Salmonella* chromosomal island)) and antibiotic resistance (*Salmonella* genomic island or SGI1), or are known to encode effectors for SPI1 and SPI2 (SPI5) and fimbriae (SPI10) (Hensel, 2004).

1.2.3 *Salmonella*-host interactions

Successful infection implies numerous interactions between the host and the invading pathogen. Colonization needs to be established, followed by survival in and dissemination from the infected host. Various disease symptoms, such as inflammation, systemic spread and persistence, are all manifestations of host – pathogen interactions. Invasion and intracellular survival, as well as the induction of an inflammatory response at the intestinal mucosa are important hallmarks of *S*. Typhimurium-associated disease.

Invasion and intracellular survival

Salmonella promotes its own uptake into non-phagocytic cells by targeting several signalling pathways that converge to induce formation of transient actin-rich membrane ruffles that engulf the infecting bacterium (Gruenheid and Finlay, 2003). A number of mechanisms are involved, such as direct interactions with the actin and microtubular cytoskeleton (Abrahams and Hensel, 2006; Schlumberger and Hardt, 2006) and manipulation of signalling cascades that manage the actin cytoskeleton architecture (Schlumberger and Hardt, 2006).

SPI1-associated effectors are integral to the *Salmonella* invasion of the intestinal epithelium. SipA and SipC promote invasion of epithelial cells through their interactions with the actin cytoskeleton (Hayward and Koronakis, 1999; Zhou et al., 1999). SopE, SopE2 (Zhou and Galan, 2001) and SopB/SigD (Schlumberger and Hardt, 2006) activate host Rho GTPases that are central to the regulation of cytoskeletal functions (Ridley, 2006). The functions of SopB/SigD, SopE and SopE2 are overlapping, as deficiency in any one of these effectors still allows *Salmonella* to invade extremely efficiently, whereas a lack of all of these effectors simultaneously results in a completely entry-deficient mutant (Zhou et al., 2001). SptP

counteracts the effects of SopE, SopE2 and SopB/SigD by inactivating the Rho GTPases following *Salmonella* engulfment, thus downregulating the changes in the actin cytoskeleton induced by the invasion process, and returning it to a somewhat normal state (Gruenheid and Finlay, 2003). Thus the concerted efforts of SipA, SipC, SopB/SigD, SopE, SopE2 and SptP result in the successful engulfment of *Salmonella* by the host cell.

Following engulfment *Salmonella* needs to survive in the phagocytic vacuole. To this end SPI2 is activated and interferes with phagosome maturation, resulting instead in the formation of the *Salmonella*-containing vacuole (SCV), which is the compartment where *Salmonella* resides during the intracellular stage. SCVs provide *Salmonella* with a convenient, safe niche for intracellular survival and replication. SCV formation proceeds through a number of steps, such as interference with the endocytic pathway, preventing the acquisition of late-endosome markers and fusion with the lysosome, acidification of the SCV to a final pH of 4-5, transport of the SCV to the microtubule organizing center in the perinuclear region and development of *Salmonella*-induced filaments (SIFs). A number of host factors mediating these effects have been identified; however the identity of *Salmonella* effectors that recruit and subvert them is still elusive (Kuhle and Hensel, 2004). A number of SPI2 effectors required for SCV and SIFs biogenesis have been identified, however; specifically SifA (Boucrot et al., 2005), SseF and G (Guy et al., 2000), SseJ (Kuhle and Hensel, 2004), SopD2 (Jiang et al., 2004) and PipB2 (Knodler and Steele-Mortimer, 2005). Intracellular *Salmonella* interferes with cell transport processes in a SPI2-dependent manner (Uchiya et al., 1999), redirecting the Golgi-derived exocytic transport vehicles, which are normally targeted to the cytoplasmic membrane, to the SCV instead (Kuhle et al., 2006). The recruitment of Golgi-derived vesicles was suggested to provide Salmonella in the SCV with nutrient supply or provide membrane material for SCV expansion. And while SPI2 is necessary

to promote successful intracellular survival, a role for SPI1 has also been demonstrated (Steele-Mortimer et al., 2002).

Inflammatory response

Enteric salmonellosis is characterized by profound inflammation of the intestinal mucosa with recruitment of various inflammatory cell populations (PMN, macrophages and DCs), formation of crypt abscesses, epithelial degeneration and pronounced submucosal edema (Coburn et al., 2007a; Grassl and Finlay, 2008). Various interactions between *S*. Typhimurium virulence factors and host cells lead to activation of pro-inflammatory processes and signalling cascades, culminating in inflammation-induced damage to the intestinal mucosa.

Recognition of *Salmonella* by host PRRs is a strong inducer of host response. *S*. Typhimurium LPS and flagellin are two pathogen-associated molecular patterns (PAMPs) of principal importance to *S*. Typhimurium-induced pathophysiology (Coburn et al., 2007a). *Salmonella* LPS induces a strong inflammatory response in macrophages and is required to mount a complete inflammatory response to intravenously administered bacteria (Rosenberger et al., 2000; (Royle et al., 2003). While a role for LPS in systemic inflammatory response does not necessarily imply a similar role during the intestinal phase of the disease, it remains a possibility as macrophages are recruited to the infected intestinal epithelium. Flagellin was shown to be of critical importance to the induction of early potent inflammation at the intestinal mucosa in a high dose streptomycin pre-treatment mouse model of *Salmonella* colitis (Stecher et al., 2004), as well as to lead to the activation of a number of proinflammatory signalling pathways *in vitro* (Miao et al., 2007). Flagellin recognition is mediated by TLR5 and Ipaf, a NLR. TLR5-mediated recognition of flagellin occurs at the basolateral surface of polarized epithelial monolayers

(Gewirtz et al., 2001) and SPI2 is required for flagellin transcytosis (Lyons et al., 2004). In contrast, Ipaf-mediated recognition of flagellin requires a functional SPI1 (Miao et al., 2006). In fact, $TLR5^{-/-}$ mice (that could still recognize flagellin through Ipaf) were not defective in *Salmonella*-induced macrophage killing, suggesting that sufficient amount of flagellin can be imported through SPI1 T3SS to activate caspase-1 (Steiner, 2007). Flagellin detection by TLR5 leads to IL-12 production, while Ipaf recognition leads to caspase-1 activation and secretion of IL-1β and IL-18; the two pathways likely synergize in the activation of IFNγ-producing Th1 cells (Grassl and Finlay, 2008). Lamina propria DCs, one of the key cell populations involved in *S.* Typhimurium infection, constitutively express high levels of TLR5. Evidence from $TLR5^{-/-}$ mice suggests that flagellin-mediated inflammation in the lamina propria promotes *Salmonella* uptake and/or dissemination by the DCs (Steiner, 2007).

SPI1 was shown to be critical for the instigation of early colitis in the high dose streptomycin pre-treatment mouse model (Coburn et al., 2005; Hapfelmeier et al., 2005) and calf intestinal loops (Coombes et al., 2005a); it contributes to the initiation of inflammatory responses in a number of ways. SopE, a SPI1 effector, has been shown to activate Cdc42, resulting in the induction of mitogen-associated protein kinases (MAPK), thus targeting NFκB signalling. SipA, another effector, is responsible for the recruitment of basolateral neutrophils to the apical epithelial membranes through the induction of IL-8 and pathogen elicited epithelial chemoattractant (PEEC) production (Lee et al., 2000; McCormick et al., 1995). PEEC was identified as eicosanoid hepoxilin A_3 (Mrsny et al., 2004). A domain of SipA responsible for neutrophil recruitment has been recently identified (Wall et al., 2007). Additional domains of SipA have been shown to be necessary for the induction of colitis in high dose streptomycin pre-treated mice (Schlumberger et al., 2007). SipA, SopE, SopE2 and SopB were shown to be

responsible for the disruption of tight junctions *in vitro* (Boyle et al., 2006) and in high dose streptomycin pre-treated mice (Boyle et al., unpublished data). SPI1-mediated colitis was shown to progress via MyD88-independent pathways (Hapfelmeier et al., 2005).

A role for SPI2 in the enterocolitic phase of *Salmonella* pathogenesis has emerged only recently, but has already been corroborated in the high dose streptomycin pre-treatment mouse model (Coburn et al., 2005; Coombes et al., 2005a; Hapfelmeier et al., 2005), the bovine host (Bispham et al., 2001); Coombes et al., 2005a) and human patients (Hu et al., 2008). While SPI1 plays a role in the early stages of colitis, in animal models SPI2 was shown to be required during the later stages of the disease. Details of SPI2 contribution to enterocolitis are still sparse. In contrast to SPI1-mediated colitis, it requires signalling via MyD88 (Hapfelmeier et al., 2005) and was shown to be involved in the induction of cyclooxygenase-2 in macrophages (Uchiya and Nikai, 2004) and to regulate cytokine expression (Uchiya et al., 2004; Uchiya and Nikai, 2005).

As described in previous sections, inflammation has been intimately linked with perturbations in the intestinal microbiota, and elucidation of further details regarding the *S.* Typhimurium-induced inflammatory response will undoubtedly help to shed additional light on the host – *S.* Typhimurium – microbiota interactions.

1.2.4 Enteric salmonellosis models

As mentioned previously, many of the *S. enterica* serovars exhibit narrow host adaptations, either being non-infective in all but one host, or eliciting distinct syndromes in different host species. This can complicate the *in vivo* studies of *S. enterica* pathogensis (Kingsley and Baumler, 2000).

S. Typhimurium is adapted to a broad range of hosts, manifesting as primarily intestinal disease in humans and cattle and systemic disease in most unmanipulated susceptible laboratory mouse strains (Santos et al., 2001). A number of animal models exist that imitate *S.* Typhimurium-induced human enterocolitis.

Calf model of enterocolitis

S. Typhimurium infection of calves results in clinical and histopathological features similar to human intestinal disease (Santos et al., 2001). Calves can be infected either orally or by direct inoculation into ileal loops. Ileal loops can be either ligated (Santos et al., 2001), allowing examination of early time points of infection, or patent (Coombes et al., 2005a), providing the possibility for experiments of longer duration. This model provides an opportunity to examine the contribution of bacterial factors to disease progression; however, as the bovine host is not easily amenable to genetic manipulation, the evaluation of the role of host factors during infection is limited. The examination of the role of the intestinal microbiota during *S.* Typhimurium infection would also be difficult in this model system: little information is available about the natural composition of bovine microbiota, especially its similarity or lack thereof to human intestinal microbes, and the logistics of calf husbandry would preclude a convenient control of the microbiota composition.

Mouse high dose streptomycin pre-treatment model of colitis

While unmanipulated susceptible mice only get a systemic disease following *S.* Typhimurium infection, pre-treatment of mice with a high dose of streptomycin was shown to promote *S.* Typhimurium colonization and generate intestinal inflammation in the large intestine

(Barthel et al., 2003). The inflammatory response is characterized by mucosal edema and inflammatory infiltrates rich in polymorphonuclear leukocytes (PMN), similar to human histopathology (Santos et al., 2001). Mice are easily amenable to genetic manipulation, and so this model provides an excellent tool for the study of both the host's and the pathogen's contribution to the infection progression. A significant impediment, however, is posed to the study of the microbiota component during infection, as the massive dose of streptomycin given to mice prior to infection eliminates over 90% of the intestinal microbiota (Stecher et al., 2007).

1.2.5 *S*. Typhimurium and microbiota – current knowledge

As mentioned previously, intestinal microbiota are an important, albeit little characterized, participant during host response to enteric infections. Recent studies have highlighted the importance of *S*. Typhimurium interactions with the host intestinal microbiota during infection.

Normal mouse intestinal microbiota provide the host with colonization resistance to *S*. Typhimurium, while its reduction with high doses of antibiotic treatment allows the pathogen to successfully establish itself in the intestinal environment and generate intestinal disease manifestations (Barthel et al., 2003; Woo et al., 2008). Studies in the high dose streptomycin pre-treatment mouse model have shown that *S*. Typhimurium-induced intestinal inflammation allows it to outcompete the indigenous microbial community, promoting successful host colonization (Stecher et al., 2007). *S*. Typhimurium utilization of the high-energy nutrients released in the inflamed intestine, as part of the host defense, was proposed to contribute to this enhanced pathogen fitness (Stecher et al., 2008). Additionally, host microbiota were shown to contribute to *S*. Typhimurium transmission (Lawley et al., 2008).

Since the studies described in this thesis were initiated, another group has demonstrated differential alterations of the intestinal microbiota during infection of FvB mice (that do not require antibiotic pre-treatment to manifest the intestinal phase of the disease during infection with WT *S.* Typhimurium) with different *S.* Typhimurium strains (Barman et al., 2008).

A myriad of questions regarding the role of the microbiota during *S.* Typhimurium infections still remain, however. The relative contributions of different bacterial groups to host resistance and susceptibility to *S.* Typhimurium infection needs to be addressed, as well as the *S.* Typhimurium and host factors contributing to the microbiota alterations during infection ought to be evaluated.

1.3 Rationale and Hypothesis:

At the time when these studies were initiated, the impact of infectious agents on the intestinal microbiota was little studied. Kuehl and colleagues (Kuehl et al., 2005) have shown that murine cecal microbiota are disturbed during *H. hepaticus* infection, while studies in our laboratory shed light on the effects of *C. rodentium* infection and inflammation on the murine colonic microbiota (Lupp et al., 2007). Since then, additional research has been published on the effects of *S.* Typhimurium on the microbiota (Stecher et al., 2007), highlighting the importance of elucidating the details of host-microbiota-infection interplay.

As a pathogen colonizes and invades the host intestinal mucosa, it interacts with and disrupts the balance of the host microbial community. Therefore, it stands to reason that pre-infection alterations in the composition of the host intestinal microbiota will have an effect on the progression of the enteric infection. Furthermore, as pathogens utilize various virulence factors to interact with and successfully establish themselves within the host, these virulence

factors are bound to also affect the pathogens' ability to disrupt the host microbiota, either through direct interactions or through induction of a suitable host response. Consequently, we hypothesized that pre-infection modifications of the composition of the murine intestinal microbiota will affect the progression of enteric salmonellosis, and that various *S.* Typhimurium virulence factors will play a role in the observed effects of *S*. Typhimurium infection on the host microbiota.

CHAPTER 2:
ANTIBIOTIC-INDUCED ALTERATIONS IN THE MURINE INTESTINAL MICROBIOTA

2.1 Summary

The use of antibiotics is wide-spread in our society, however their effect on the indigenous intestinal microbiota has received little attention beyond studies of induction of resistance. We used a mouse model to investigate the spectrum of activity of orally administered streptomycin, vancomycin and tetracycline on intestinal microbiota. Both streptomycin and tetracycline were found to have a similar effect on microbiota at the phylum level, increasing the proportion of CFB bacteria. Their effects at genus level differed, however, with streptomycin decreasing the levels of *Enterococci*/D-*Streptococci* and *Enterobacteriaceae*, which were unaffected by tetracycline treatment. Vancomycin was found to decrease the proportion of CFB bacteria and increase the proportion of γ-Proteobacteria at the phylum level, while decreasing the amounts of *Enterococci*/D-*Streptococci* and increasing the amounts of *Enterobacteriaceae* and culturable aerobic bacteria. All antibiotics were found to decrease the levels of *Lactobacilli*. While all antibiotics were found to affect the composition of the microbiota, they did not significantly reduce the total numbers of intestinal bacteria, although the higher dose of vancomycin reduced the total bacterial numbers more noticeably than did other antibiotics. Additionally, treatment with vancomycin, unlike treatment with streptomycin and tetracycline, promoted translocation of enteric bacteria to systemic sites three days following treatment.

2.2 Introduction

While the use of antibiotics is vital in the treatment of many ailments, too often they are prescribed unnecessarily and frequently their use is associated with complications such as antibiotic-associated diarrhea. To maximize the benefit of antibiotic treatment and minimize the

potential for associated co-morbidities it is essential to know the full extent of antibiotics' spectrum of activity, including their effects on the intestinal microbiota.

Although antimicrobial agents are known to affect the microbiota, to date the effect of antibiotics on intestinal microbiota has been poorly evaluated beyond studies of induction of resistance (Lofmark et al., 2006; Raum et al., 2008; Zolezzi et al., 2007). Additionally, most previous studies used culture techniques to evaluate the effect of tested antibiotics on microbiota (Ianniello et al., 2005; Sakata et al., 1986; Wynne et al., 2004), which severely limit the scope of assessment; although recently a more thorough analysis has been done to evaluate the effects of some antibiotics on human fecal flora (Jernberg et al., 2007). Our evaluation involved culture-independent techniques, providing a novel glimpse at the extent of antibiotic-induced changes in the intestinal microbiota. Streptomycin and tetracycline were chosen as antibiotics with activity against both Gm(+) and Gm(-) bacteria and vancomycin as an antibiotic with activity against Gm(+) bacteria only. Both streptomycin and tetracycline inhibit protein synthesis by binding to the bacterial ribosome. Tetracycline prevents the binding of tRNA to the ribosome, while streptomycin binds to the 30S ribosomal subunit, inhibiting translation initiation and promoting miscoding. Vancomycin inhibits cell wall synthesis, and consequently is a bacteriocidal antibiotic. It prevents the incorporation of N-acetylmuramic acid and N-acetylglucosamine into the peptidoglycan matrix. These antibiotics are used in clinical practice for a wide variety of indications (Table 2.1).

We hypothesized that treatment of mice with these different antibiotics at different doses would cause different and specific shifts in the composition and/or numbers of the murine intestinal microbiota.

Table 2.1 Antibiotics used in this study

Antibiotic	Dose (mg/L)	Dose (mg/kg mouse/day) / Total dose received (mg)	Clinical dose and administration §		Mechanism of action	Clinical uses (some examples) §
			Adult	Pediatric		
Streptomycin	150	30 / 0.9	15 mg/kg (up to 2 g); 3X/week to daily for 2 weeks to 3 months, depending on indication; i.m. or i.v.; Average 70 kg adult: 2.1 to 4 g over 2 days ¥	20-40 mg/kg (up to 1 g) daily; 10-14 days and up to 3 months, depending on indication; i.m. or i.v.; Average 20 kg child: 0.8 to 1.6 g over 2 days ¥	Inhibits protein synthesis by binding to the 30S ribosomal subunit, causing miscoding or inhibiting initiation.	Tuberculosis Brucellosis Plague Endocarditis Tularemia; Given in combination therapy
	300	60 / 1.8				
	450	90 / 2.7				
Tetracycline	75	15 / 0.45	1-2 g daily in 2-4 divided doses, duration depends on indication; p.o.; Average 70 kg adult: 2 to 4 g over 2 days ¥	25-50 mg/kg daily in 4 divided doses (for children over 8 yrs.), duration depends on indication; p.o.; Average 20 kg child: 1 to 2 g over 2 days ¥	Inhibits protein synthesis by preventing binding of aminoacyl-tRNA to the 30S ribosomal subunit.	Acne Chlamydial and mycoplasmal infections H. pylori infection and duodenal ulcer disease; Given alone or in combination therapy
	150	30 / 0.9				
	250	50 / 1.5				
Vancomycin	50	10 / 0.3	0.5-2 g daily for 7-10 days; p.o. or i.v.; Average 70 kg adult: 1 to 4 g over 2 days ¥	40mg/kg (up to 2 g) daily for 7-10 days; p.o. or i.v.; Average 20 kg child: 1.6 g over 2 days ¥	Inhibits cell wall synthesis by preventing the incorporation of N-acetylmuramic acid and N-acetylglucosamine into the peptidoglycan matrix	Staphylococcal and streptococcal infections Endocarditis *C. difficile*-associated diarrhea and colitis; Given alone or in combination therapy
	100	20 / 0.6				

§ - source: AHFS Drug Information

¥ - over 2 days as that was the duration of antibiotic administration to mice in these studies

i.m. – intramuscular; i.v. – intravenous; p.o. – per oral

2.3 Results

2.3.1 Antibiotic treatment at selected doses does not significantly change the total numbers of murine intestinal microbiota

We used SYBR Green staining to determine the effect of antibiotic treatment on the total numbers of intestinal bacteria. Treatment with selected doses of streptomycin, tetracycline and vancomycin did not significantly change the total numbers of microbiota (Figure 2.1). However, treatment with vancomycin had a greater effect on the total numbers of microbiota than treatment with either streptomycin or tetracycline. To determine if higher vancomycin doses would further reduce the total numbers of bacteria, we treated mice with 200 mg/L of vancomycin for 2 days. No bacteria were detected in the colonic microbiota of these mice using SYBR green staining, indicating that this higher vancomycin dose reduced the total murine microbiota below the limit of SYBR green detection. Some bacterial genera were still detected at 10^4 – 10^8 cfu/g colon using differential plating, indicating that although treatment with 200 mg/L of vancomycin severely reduced the total numbers of murine microbiota, they were not eliminated completely. The 200 mg/L dose of vancomycin was not used in any further studies due to inability to evaluate the microbiota of treated mice using culture-independent methods at this dose.

2.3.2 Antibiotic treatment modifies microbiota composition in an antibiotic- and dose-dependent manner

Once the effect of antibiotic treatment on the total numbers of intestinal bacteria was determined, we sought to evaluate the effects of streptomycin, tetracycline and vancomycin on the composition of murine intestinal microbiota. To this end we employed FISH and differential plating methods. FISH provides a good overview of the distribution of the major phyla in the

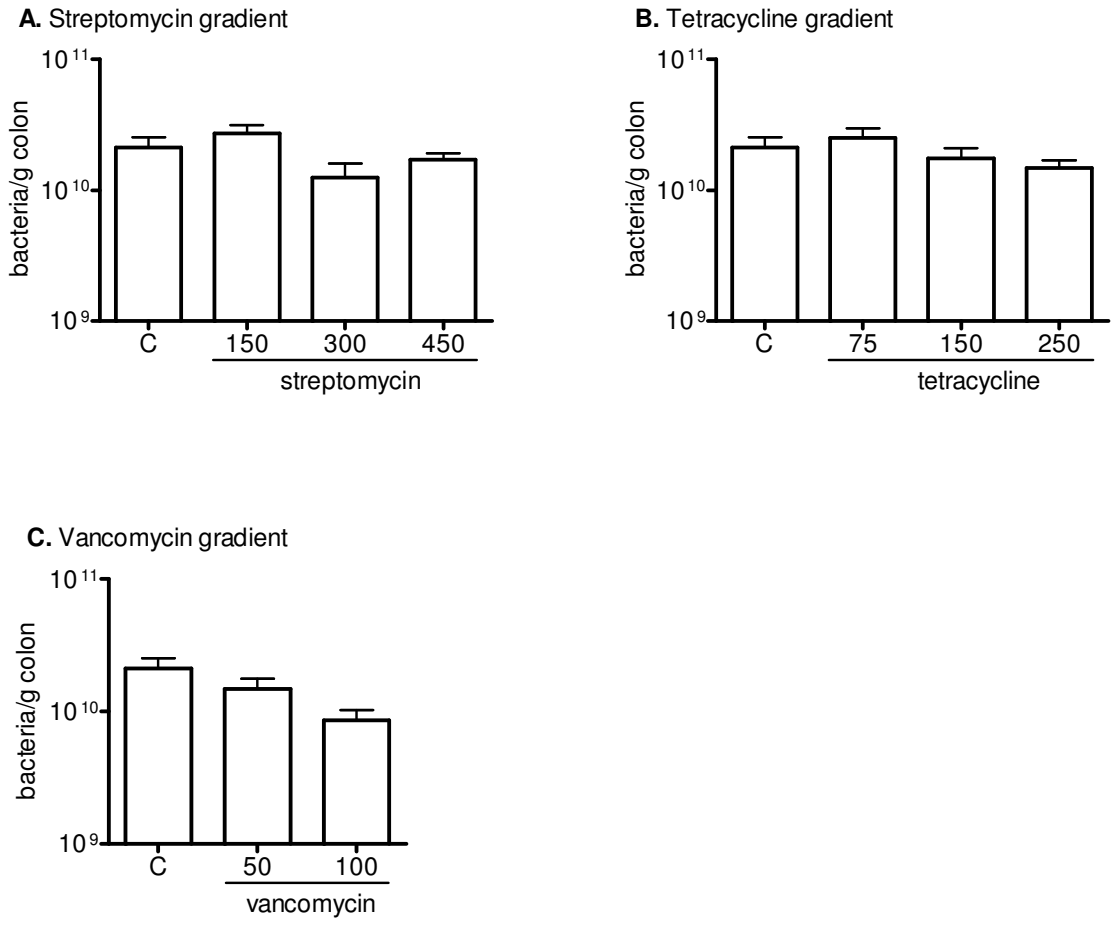


Figure 2.1 SYBR Green assessment of total colonic microbial numbers in C57Bl/6 mice prior to and following antibiotic treatment
Mice were treated with specified antibiotics in drinking water for 2 days; doses are in mg/L; C = control group. p values were calculated using one-way ANOVA with Bonferroni post test with 95% confidence interval; no significant differences between antibiotic-treated and control groups were found.

intestinal microbiota, and was previously shown to correlate well with the results of the more laborious and expensive sequencing method (Lupp et al., 2007). Differential plating provides an evaluation of a selection of bacteria at a finer genus level. Colonic microbiota of antibiotic-treated uninfected mice were evaluated. Mice were treated with increasing doses of streptomycin, tetracycline and vancomycin, administered in drinking water for 2 days. The dosage was either within the range or lower than what is used in clinical practice for several indications (Table 2.1).

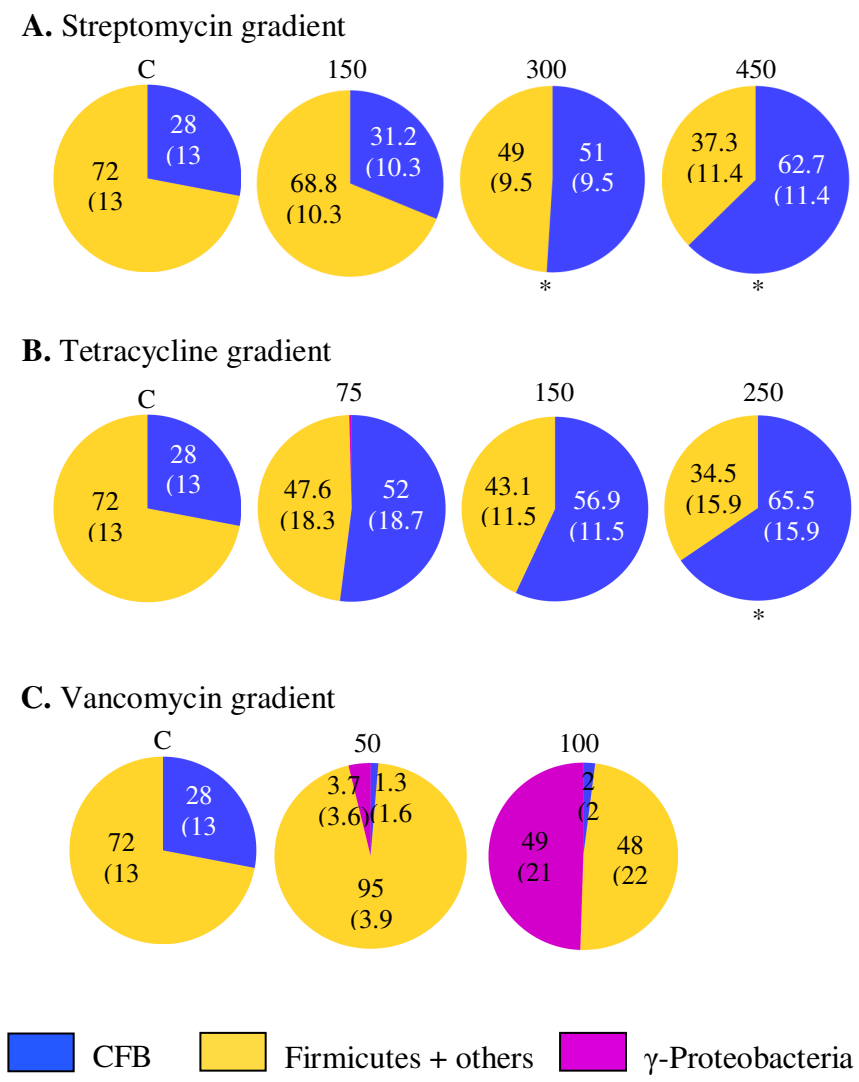


Figure 2.2 FISH assessment of colonic microbiota composition at phylum level in C57Bl/6 mice prior to and following antibiotic treatment

Mice were treated with specified antibiotics in drinking water for 2 days; doses are in mg/L; C = control group.

Values are % of all Eubacteria (SD). Proportions of CFB and γ-Proteobacteria were determined as described in Materials and Methods chapter. Proportion of Firmicutes + other bacteria were estimated as 100% - %CFB - %γ-Proteobacteria. Groups marked with an asterisk have proportion of CFB significantly different from C group (with $p<0.05$ or smaller). p values were calculated using one-way ANOVA with Bonferroni post test with 95% confidence interval.

As demonstrated in Figure 2.2, treatment of mice with increasing doses of streptomycin and tetracycline increased the proportion of CFB bacteria in their colonic microbiota in a dose-dependent manner. The increase in CFB bacteria became statistically significant for streptomycin at 300 mg/L and 450 mg/L and statistically significant for tetracycline at 250 mg/L. At the genus level, the numbers of *Lactobacilli* and *Enterococci* / D-*Streptococci* were significantly decreased with streptomycin treatment (Figure 2.3C and 2.3D), consistent with a gradual decrease in the proportion of Firmicutes and "other" bacteria evidenced by FISH evaluation. Tetracycline, on the

other hand, did not have a significant effect on the numbers of *Enterococci* / D-*Streptococci* in the murine colonic microbiota, but did significantly decrease the numbers of *Lactobacilli*. The decrease in the numbers of *Lactobacilli* following tetracycline treatment was far greater than following streptomycin treatment, and also was consistent with a reduction in Firmicutes and "other" bacteria shown by FISH.

The effect of vancomycin on the microbiota was strikingly different from the effects of streptomycin and tetracycline (Figure 2.2C). The low vancomycin dose caused a dramatic reduction in the proportion of CFB bacteria and a small increase in the proportion of γ-Proteobacteria. With a higher vancomycin dose, the proportion of the CFB bacteria was still reduced, and the γ-Proteobacteria increased to nearly 50% of the microbiota. At the genus level, vancomycin treatment adversely affected both *Lactobacilli* and *Enterococci*/D-*Streptococci*, and promoted the overgrowth of *Enterobacteriaceae* and culturable aerobic bacteria in a dose-dependent manner (Figure 2.3). Overgrowth of *Enterobacteriacea* shown by differential plating was consistent with the increase in the proportion of γ-Proteobacteria demonstrated by FISH.

Overall the evaluation of bacteria at the genus level for all groups of mice showed variability between mice, consistent with the fact that individuals display variation in their microbiota composition at species level (Eckburg et al., 2005; Gill et al., 2006; Ley et al., 2005). But despite this variation, treatment of mice with selected antibiotics consistently modified the murine microbiota in an antibiotic- and dose-dependent manner.

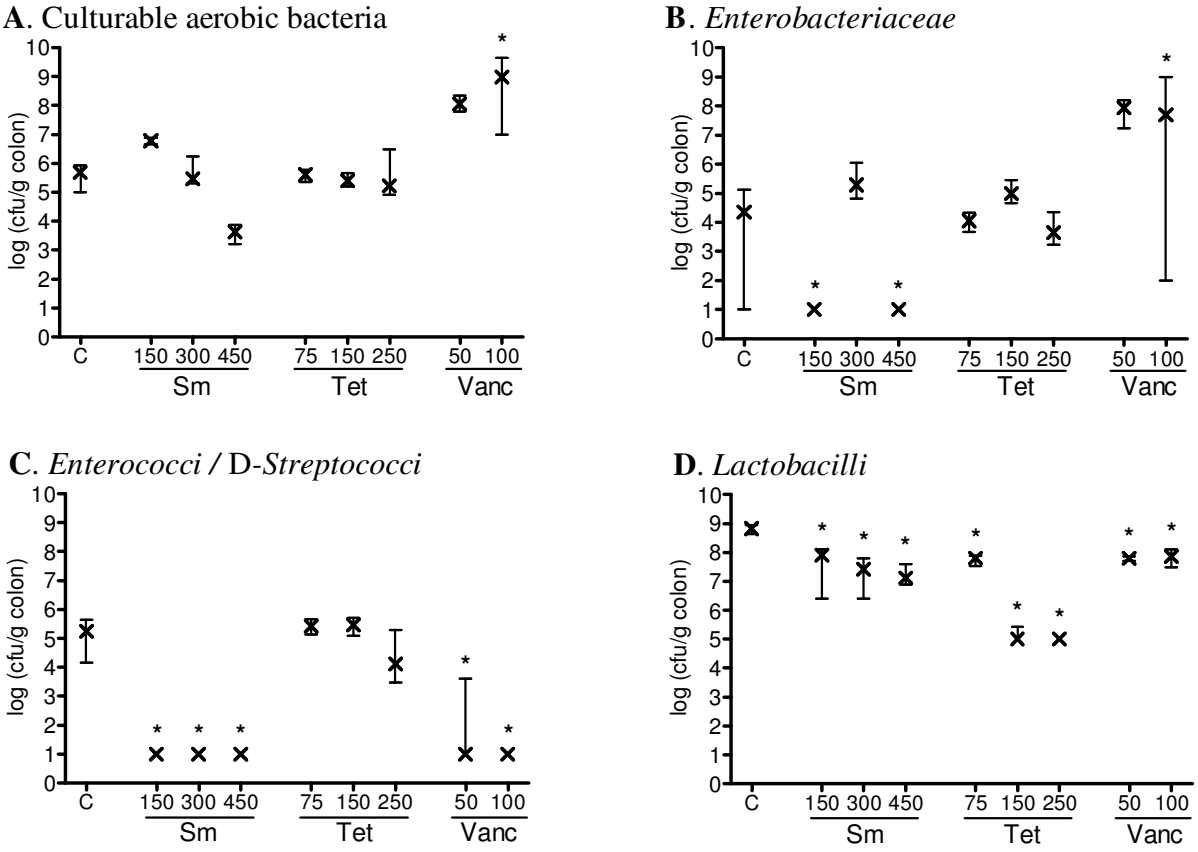


Figure 2.3 Differential plating assessment of colonic microbiota composition at genus level in C57Bl/6 mice prior to and following antibiotic treatment

Mice were treated with specified antibiotics in drinking water for 2 days; doses are in mg/L; C = control group, Sm = streptomycin, Tet = tetracycline, Vanc = vancomycin.

Colonization by each group of bacteria was determined as described in Materials and Methods section. Medians and interquartile ranges are shown. Groups marked with an asterisk are significantly different from C group (with $p<0.05$ or smaller). p values were calculated using one-way ANOVA with Bonferroni post test with 95% confidence interval.

2.3.3 Treatment with vancomycin but not with streptomycin or tetracycline promotes translocation of Enterobacteriacea *to systemic sites*

Translocation of enteric bacteria to systemic sites has been associated with a number of disease states, leading to sepsis, inflammatory reactions and shock. Systemic translocation is most often observed in hospitalized patients undergoing various medical procedures, and

Enterobacteriaceae species are a common culprit (Henry-Stanley et al., 2005; White et al., 2006). As hospitalized patients commonly receive antibiotics, it was of interest to investigate whether treatment with different antibiotics promoted translocation of enteric bacteria to systemic sites. To assess this, homogenized spleens of antibiotic-treated mice were plated on MacConkey media.

When spleens were examined after two days of oral antibiotic administration, no bacteria were found in either of the treated or control groups (data not shown). However, three days after antibiotics were discontinued, mice that received vancomycin were shown to harbor *Enterobacteriaceae* in their spleens (Figure 2.4), while spleens of streptomycin- and tetracycline-treated mice were still sterile (data not shown). Spleens of mice that were pre-treated with 100 mg/L of vancomycin were colonized by *Enterobacteriaceae* more heavily than those of mice pre-treated with 50 mg/L of vancomycin.

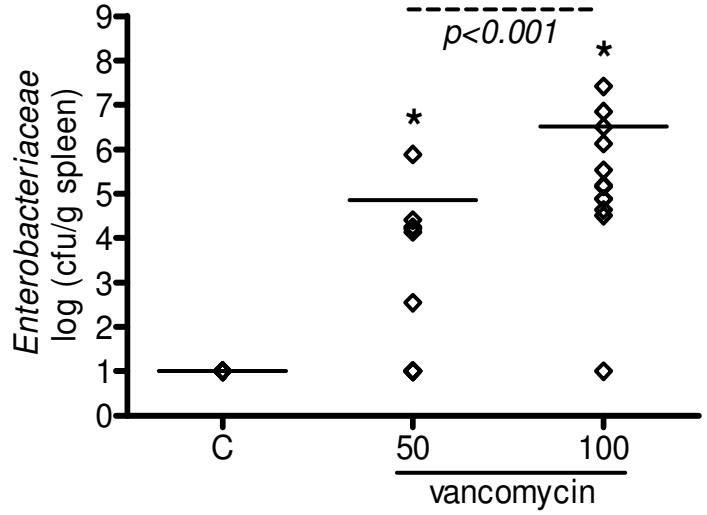


Figure 2.4 Assessment of translocation of enteric bacteria to spleens of C57Bl/6 mice following vancomycin treatment
Mice were treated with vancomycin for two days and then sacrificed three days following discontinuation of treatment; doses are in mg/L; C = control group.
Means are shown. Groups marked with an asterisk are significantly different from C group (with $p<0.05$ or smaller). p values were calculated using one-way ANOVA with Bonferroni post test with 95% confidence interval.

2.4 Discussion

While the use of antibiotics is exceedingly common in our society, their effects on the intestinal microbiota have not been thoroughly evaluated to date. Since the intestinal microbiota make extensive contributions to host physiology in health and disease (Canny and McCormick, 2008); O'Hara and Shanahan, 2006; Xu and Gordon, 2003), it is imperative to assess the impact that commonly used therapeutics have on them. To this end, the effects of streptomycin, tetracycline and vancomycin on microbiota numbers, composition and translocation to systemic sites were investigated.

The main effect of streptomycin and tetracycline on microbiota was in promoting the growth of CFB bacteria at the expense of other phyla. As this effect was dose-dependent it was likely due to a selective inhibition of a microbial population whose vacated niche CFB organisms were then able to colonize. It is possible that CFB bacteria are less sensitive to the bacteriostatic effects of streptomycin and tetracycline, which both work through inhibition of protein synthesis.

However, streptomycin and tetracycline had different effects on the members of the Firmicutes phylum. Streptomycin reduced colonization by *Enterococci* and D-*Streptococci*, which were not significantly affected by tetracycline. Conversely, treatment with tetracycline resulted in a much more pronounced reduction in the numbers of *Lactobacilli* than treatment with streptomycin.

It was surprising to see that although vancomycin's spectrum of activity is exclusively against Gm(+) bacteria (2008), Gm(-) bacteria (CFB phylum) appeared to be adversely affected by vancomycin treatment. It is possible that certain Gram positive bacteria that were resistant to the effects of vancomycin were able to dominate the niches of their more sensitive relatives (as evidenced by an increase in the proportion of Firmicutes + other bacteria following treatment

with 50 mg/L of vancomycin), as a result making the overall colonic environment more hostile to the members of the CFB phylum. However, vancomycin treatment promoted the proliferation of Gram negative bacteria of the γ-Proteobacteria phylum, specifically the *Enterobacteriaceae*. A possible explanation to this discrepancy is that bacteria of the CFB phylum are obligate anaerobes, while *Enterobacteriaceae* and some members of the Firmicutes phylum are facultative anaerobes. Thus, vancomycin treatment could create an environment favourable to the survival and proliferation of oxygen-resistant bacteria and detrimental to oxygen-sensitive ones.

Additionally, it was interesting to see that although *Lactobacilli* (a Gm(+) genus) were sensitive to the effects of vancomycin, and were reduced in numbers compared to the control mice, the magnitude of reduction was not that astounding (from $6.75X10^8$ cfu/g colon in control mice to $6.5X10^7$ cfu/g colon in 50 mg/L and $7.45X10^7$ in 100 mg/L vancomycin-treated groups), especially compared to the reduction in the numbers of *Enterococci* and D-*Streptococci*. This result falls in line with clinical evidence of the ease with which *Lactobacilli* are able to acquire resistance to vancomycin (Chen et al., 2007; Klare et al., 2007). Furthermore it supports the cautionary notes regarding the use of *Lactobacilli* as a probiotic strains, especially in immunocompromised patients and in patients receiving antimicrobial therapy (Mater et al., 2008).

An additional effect of vancomycin treatment was the translocation of enteric *Enterobacteriaceae* to spleens of treated mice following discontinuation of treatment. This translocation could be due to the increase in the proportion of *Enterobacteriaceae* in the colons of vancomycin-treated mice, as well as to possible alterations in intestinal permeability.

As a role for microbiota in determining body weight and predisposition to obesity has received mounting attention in recent years (Ley et al., 2005; Turnbaugh et al., 2008; Turnbaugh et al., 2006), it was of interest to examine whether antibiotic-induced alterations in microbiota composition would translate into changes in mouse weight. The weight of mice in vancomycin-treated groups was examined in one of the experiments, as vancomycin treatment resulted in the establishment of "obesity-promoting" microbiota, i.e. a higher proportion of Firmicutes and a lower proportion of CFB bacteria. Surprisingly, mice treated with vancomycin had a slightly lower body weight than control mice (data not shown), contrary to our expectations. This discrepancy could be due to a number of factors. The duration of antibiotic treatment was short, and consequently there was only a short period of time during which the mice were exposed to "obese" microbiota. Alternatively vancomycin treatment could result in an increase in members of Firmicutes other than those responsible for increased energy harvest. As this aspect of antibiotic treatment was not central to the experiments described in this chapter, it was not pursued further. However, it might be of interest to examine the long-term effects of antibiotic treatment of longer duration on body weight in both animal models and humans, if possible.

The observed different effects on intestinal microbiota of the three evaluated antibiotics underscore the importance of thorough assessment of the antibiotics currently available and the ones that will be later entering clinical practice. Detailed knowledge of the impact antibiotic treatment has on the microbiota, in addition to their impact on the pathogenic bacteria, will help in devising better regimens that might avoid inducing such conditions as antibiotic-associated diarrhea or *C. difficile*-associated diarrhea. It will help in designing optimal combinations of antibiotics and pro/pre-biotics, thus advancing health care.

CHAPTER 3:
ANTIBIIOTIC-INDUCED ALTERATIONS IN THE MURINE SUSCEPTIBILITY TO *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

3.1 Summary

Intestinal microbiota comprise microbial communities that reside in the gastrointestinal tract and are critical to normal host physiology. Understanding the microbiota's role in host response to invading pathogens will further advance our knowledge of host-microbe interactions. *Salmonella enterica* serovar Typhimurium was used as a model enteric pathogen to investigate the effect of intestinal microbiota perturbation on host susceptibility to infection. Antibiotics were used to perturb the intestinal microbiota. C57BL/6 mice were treated with streptomycin, tetracycline and vancomycin for 2 days in drinking water, followed by oral infection with *S.* Typhimurium. Alterations in microbiota composition and numbers were evaluated by fluorescent in situ hybridization (FISH), differential plating, and SYBR green staining. Greater antibiotics-induced pre-infection perturbations in microbiota resulted in increased mouse susceptibility to *S.* Typhimurium intestinal colonization, greater post-infection alterations in the microbiota, and more severe intestinal pathology. These results suggest that alterations in the balance of the microbial community predispose the host to *S.* Typhimurium infection, highlighting the importance of a healthy microbiota in host response to enteric pathogens.

3.2 Introduction

Many diseases have been linked to imbalances in the intestinal microbial community, including obesity (Ley et al., 2005), inflammatory bowel disease (Frank et al., 2007), colorectal cancer (Yang and Pei, 2006), and even atopic diseases (Penders et al., 2007). For most of these associations, however, it is not clear whether the microbial imbalance is a predisposing factor that precedes the onset of the pathology, or is the result of the pathological condition. Diseases such as vaginal candidiasis and *C. difficile* colitis frequently start following a course of antibiotic

therapy that disrupts the microbiota balance (Crogan and Evans, 2007; Sobel, 2007), which favours the hypothesis that the imbalance precedes the onset of pathology. However, whether this conclusion can be extrapolated to all disorders in question is not known. Furthermore, little is known about the effect of perturbations in the intestinal microbiota on host susceptibility to invading pathogens. It has recently been demonstrated that enteric pathogens interact extensively with the intestinal microbiota (Barman et al., 2008; Kuehl et al., 2005; Lupp et al., 2007; Stecher et al., 2007), thereby prompting the hypothesis that different microbiota compositions, as a result of perturbations in the microbial community, would affect the outcome of enteric infections.

To investigate this hypothesis we disrupted the murine intestinal microbial community with various doses of streptomycin, tetracycline and vancomycin, followed by infection with *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) to investigate the effects of the pre-infection perturbation on the outcome of infection. *S.* Typhimurium was chosen as the model pathogen since the normal intestinal microbiota of mice provides a barrier to *S.* Typhimurium-induced intestinal disease (Santos et al., 2001), making the effects of any perturbation in the microbial community more obvious and easier to evaluate. The doses of antibiotics used in this study were considerably lower than those used in the conventional streptomycin *S.* Typhimurium infection model (Barthel et al., 2003; Stecher et al., 2007), and did not significantly reduce the total number of intestinal microbes, providing a better insight into their role in *S.* Typhimurium-induced intestinal disease.

3.3 Results

3.3.1 Streptomycin- and vancomycin-, but not tetracycline-induced modifications in the composition of the murine microbiota promote further infection-induced alterations

The intestinal microbiota of mice following infection with *S.* Typhimurium were examined, using methods described in section 2.3.2. When untreated and antibiotic-treated mice were infected with *S.* Typhimurium, a striking feature was an increase in the proportion of γ-Proteobacteria in all infected groups (Figure 3.1), compared to untreated uninfected mice (Figure 2.2). Examination of both the colonic and the cecal microbiota yielded similar results at both the phylum and the genus levels. The increase in γ-Proteobacteria was higher in mice that were pre-treated with streptomycin and vancomycin prior to infection, compared to untreated and tetracycline pre-treated groups. The increase in γ-Proteobacteria in the streptomycin and vancomycin pre-treated groups depended on the dose of antibiotic administered prior to infection, with higher doses producing a greater increase in the proportion of intestinal γ-Proteobacteria. Treatment of mice with the intermediate and high doses of streptomycin and high dose of vancomycin resulted in a statistically significant increase in the proportion of γ-Proteobacteria, compared to the untreated infected group ($p<0.05$ or smaller).

Examination of infection-induced changes in the microbiota at genus level demonstrated that the numbers of *Enterobacteriaceae* and culturable aerobic bacteria were increased in the streptomycin and vancomycin pre-treated mice, but not in the mice that received tetracycline (Figure 3.2). The numbers of *Enterococci* and D-*Streptococci* were increased in the vancomycin pre-treated groups, compared to the untreated infected mice, and decreased in the ceca of mice pre-treated with 300 and 450 mg/L of streptomycin.

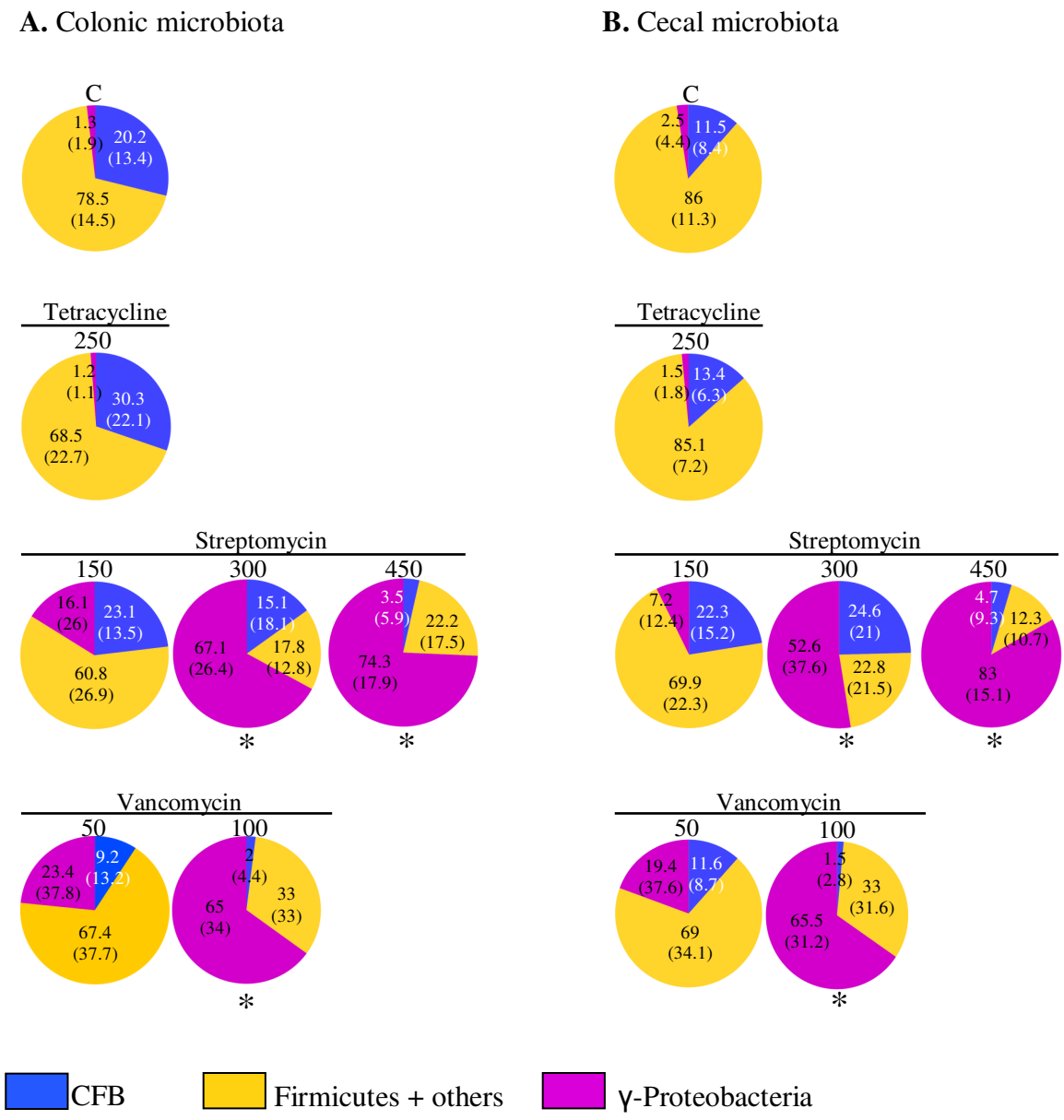


Figure 3.1 FISH assessment of intestinal microbiota composition at phylum level in antibiotic-treated and untreated C57Bl/6 mice following *S.* Typhimurium infection

Mice were treated with specified antibiotics in drinking water for 2 days; doses are in mg/L; After antibiotic withdrawal, mice were infected with $2.7X10^8$ cfu of *S.* Typhimurium for 3 days. C = control group.

Values are % of all Eubacteria (SD) Proportion of CFB and γ-Proteobacteria were determined as described in Materials and Methods section. Proportion of Firmicutes+other bacteria were estimated as 100% - %CFB - %γ-Proteobacteria. Groups marked with an asterisk have proportion of γ-Proteobacteria significantly different from C group (with $p<0.05$ or smaller). p values were calculated using one-way ANOVA with Bonferroni post test with 95% confidence interval.

The numbers of *Lactobacilli* were unaffected in all streptomycin and vancomycin pre-treated infected mice, but were reduced in the ceca of the tetracycline pre-treated group, compared to control infected mice.

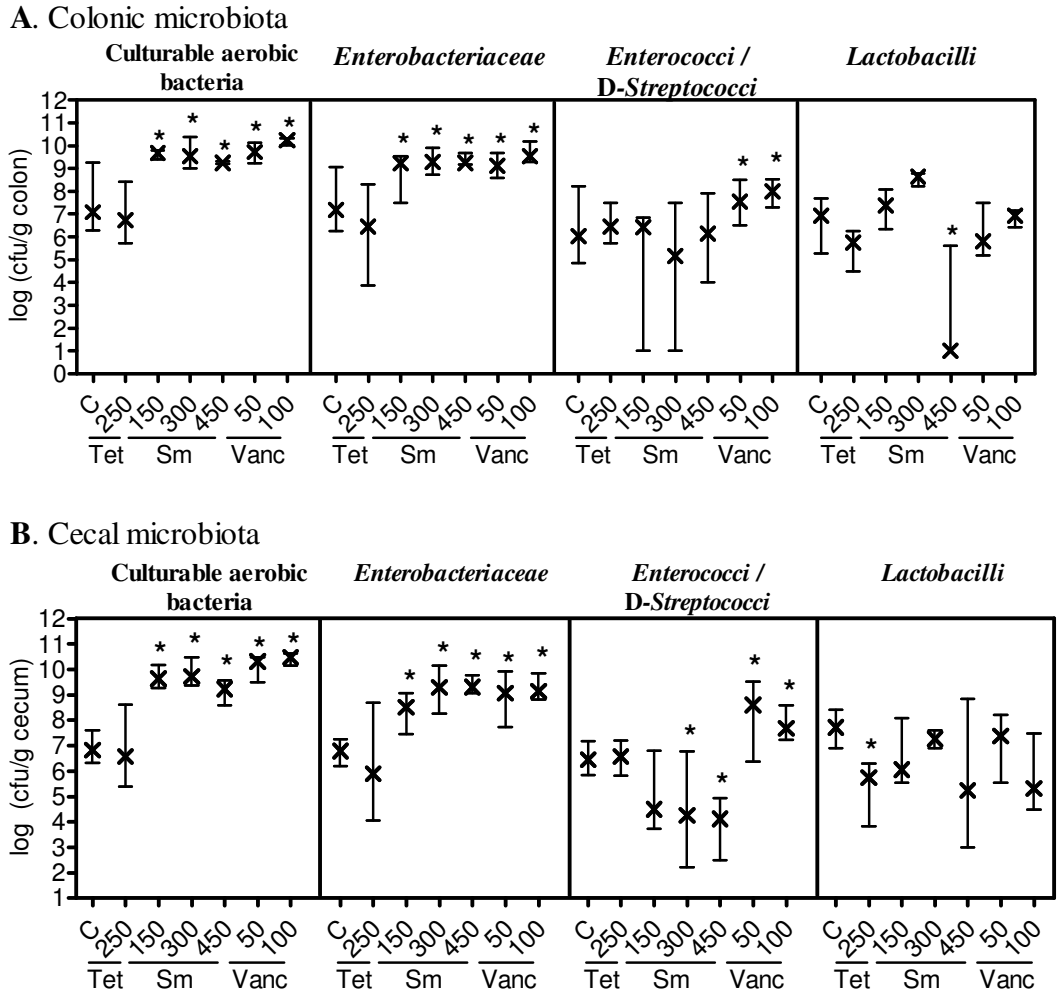


Figure 3.2 Differential plating assessment of colonic microbiota composition at genus level in antibiotic treated and untreated C57Bl/6 mice following infection with *S.* Typhimurium

Mice were treated with specified antibiotics in drinking water for 2 days; doses are in mg/L; After antibiotic withdrawal, mice were infected with $2.7X10^8$ cfu of *S.* Typhimurium for 3 days. C = control group, Tet = tetracycline, Sm = streptomycin, Vanc = vancomycin.

Colonization by each group of bacteria was determined as described in Materials and Methods chapter. Medians and interquartile ranges are shown. Groups marked with an asterisk are significantly different from C group (with $p<0.05$ or smaller). p values were calculated using one-way ANOVA with Bonferroni post test with 95% confidence interval.

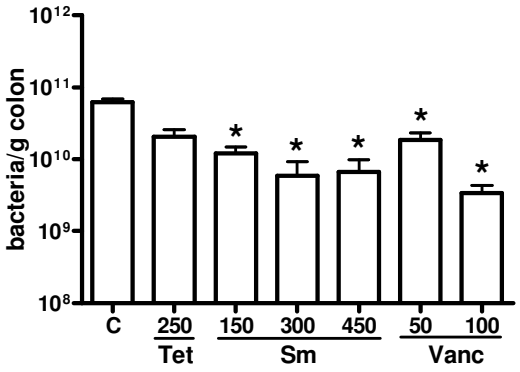
These data clearly indicate that modifications in the pre-infection intestinal microbiota composition impact on the ability of the infectious agent to further interact with and modify the microbiota. Pre-treatment of mice with streptomycin and vancomycin afforded *S.* Typhimurium a chance to cause a more significant shift in the intestinal microbiota than did pre-treatment with tetracycline or no antibiotic treatment.

3.3.2 Streptomycin and vancomycin, but not tetracycline pre-treatment promotes a decrease in the total numbers of the intestinal microbiota post S. *Typhimurium infection, concurrent with an increase in* S. *Typhimurium colonization in the murine large intestine*

To investigate the reasons for alterations in post-infection microbiota composition observed by FISH and differential plating evaluation, we examined the total numbers of the intestinal bacteria and *S.* Typhimurium colonization.

Infection with *S.* Typhimurium caused a dramatic reduction in the total microbiota of streptomycin and vancomycin, but not tetracycline pre-treated mice, compared to the untreated infected mice ($p<0.05$ or smaller), in both the colons and the ceca (Figure 3.3 A,B). The observed reduction was not due to the effect of antibiotic treatment itself, as it did not have a significant effect on total microbial numbers (Figure 2.1). The extent of *S.* Typhimurium-induced reduction in total microbial numbers in streptomycin and vancomycin pre-treated mice was associated with increased *S.* Typhimurium burdens in both the colons and the ceca, while intestinal *S.* Typhimurium colonization in tetracycline pre-treated mice did not differ from that in the control group (Figure 3.4 A, B).

A. Colonic microbiota numbers



B. Cecal microbiota numbers

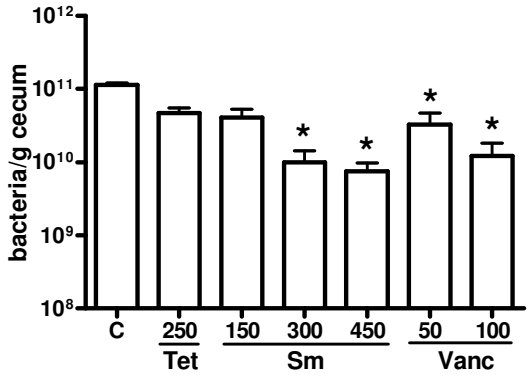
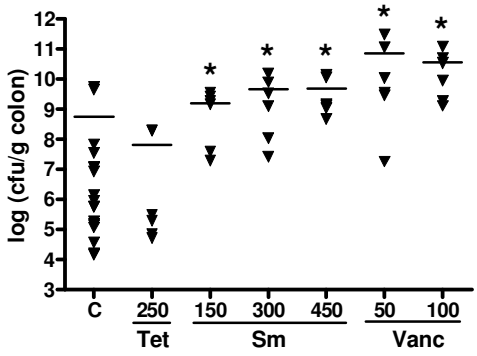


Figure 3.3 SYBR Green assessment of total intestinal microbial numbers in *S.* Typhimurium-infected C57Bl/6 mice with and without antibiotic pre-treatment
Mice were treated with specified antibiotics for 2 days in drinking water; doses are in mg/L. After antibiotic withdrawal, mice were infected with $2.7X10^8$ cfu of *S.* Typhimurium for 3 days. p values were calculated using one-way ANOVA with Bonferroni post test with 95% confidence interval. Groups marked with an asterisk are significantly different from C group (with $p<0.05$ or smaller). C = control; Tet = tetracycline; Sm = streptomycin; Vanc = vancomycin.

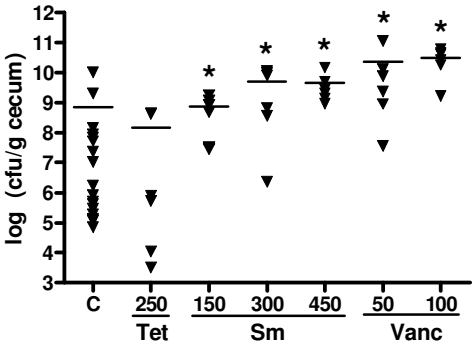
Therefore, the increased proportion of the γ-Proteobacteria in the microbiota of streptomycin- and vancomycin-treated infected mice (Figure 3.1) was due to a combination of a decrease in total numbers of intestinal microbes in these mice (Figure 3.3) and an increased intestinal colonization by *S.* Typhimurium (Figure 3.4), which also belongs to γ-Proteobacteria. In fact, *S.* Typhimurium appeared to account for the vast majority of the intestinal γ-Proteobacteria.

Although intestinal *S.* Typhimurium colonization was affected by antibiotic-induced alterations in the composition of the murine microbiota, systemic infection was not affected. *S.* Typhimurium burdens in the spleens of mice in all antibiotic-treated groups did not differ from those in control mice (Figure 3.4C).

A. *S.* Typhimurium colonization in mouse colons



B. *S.* Typhimurium colonization in mouse ceca



C. *S.* Typhimurium colonization in mouse spleens

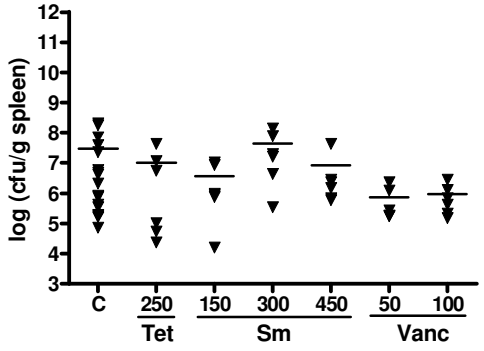


Figure 3.4 *S.* Typhimurium colonization in mouse organs with and without antibiotic treatment

Mice were treated with specified antibiotics for 2 days in drinking water; doses are in mg/L. After antibiotic withdrawal, mice were infected with $2.7X10^8$ cfu of *S.* Typhimurium for 3 days. *S.* Typhimurium colonization was enumerated by plating serial dilutions of organ homogenates on LB or XLD plates with 100 μg/ml streptomycin. p values were calculated using one-way ANOVA with Bonferroni post test with 95% confidence interval. Groups marked with an asterisk are significantly different from C group (with $p<0.05$ or smaller). C = control; Tet = tetracycline; Sm = streptomycin; Vanc = vancomycin.

3.3.3 The degree of S. *Typhimurium-induced intestinal pathology correlates with the extent of pre-infection microbiota perturbation*

To examine how increased *S.* Typhimurium intestinal burdens and greater microbiota perturbations in streptomycin and vancomycin pre-treated mice correlate with *S.* Typhimurium-induced intestinal disease, cecal necropsies were examined. Ceca were chosen for histopathological evaluation, as they are the focal point of *S.* Typhimurium-induced intestinal pathology in mice. Tissues from tetracycline pre-treated infected mice were not included in the

examination as their intestinal microbiota and *S.* Typhimurium numbers did not differ significantly from those in untreated mice and their ceca did not appear sick on gross examination.

Large intestine of infected control mice appeared normal on gross examination, consistent with the fact that normal murine microbiota are protective against *S.* Typhimurium-induced intestinal disease. Conversely, mice that were pre-treated with increasing doses of streptomycin and vancomycin showed progressive shrinkage of ceca and loosening of colonic fecal contents (Figure 3.5A).

Microscopic examination of H&E-stained tissues from uninfected mice, both untreated and antibiotic-treated, showed no signs of intestinal disease, indicating that treatment with antibiotics alone did not induce a pathological response in the mouse intestinal tract (Figure 3.5B). There were no signs of edema, presence of inflammatory infiltrate, or alterations in the epithelial architecture. When infected tissues were examined, signs of intestinal disease were observed in all but the untreated infected group (Figure 3.5B). Mice pre-treated with increasingly higher antibiotic doses prior to infection exhibited increasingly advanced pathology, starting with mild edema and minimal alterations to the epithelial layer, and progressing to copious inflammatory infiltrate, severe edema and complete loss of epithelial architecture. Although all the mice infected with *S.* Typhimurium appeared sick (ruffled fur, sluggish movements) at the time of sacrifice due to *S.* Typhimurim systemic infection, mice with more extensive intestinal pathology were more moribund (seemingly more lethargic) than other infected groups.

Pathological scoring (Coburn et al., 2005) demonstrated that while tissues from uninfected animals were healthy with minimal pathology scores, *S.* Typhimurium-infected animals showed overt signs of intestinal pathology that correlated with the extent of pre-infection

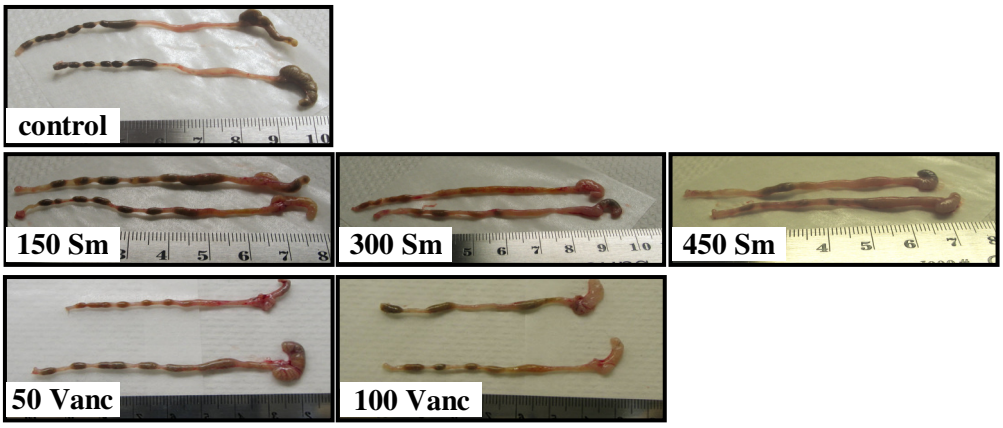
microbiota perturbation (Figure 3.5C). Pathological indices were increased in all of the tissue sections of antibiotic pre-treated infected mice, becoming significantly greater than that of the untreated infected group with higher doses of antibiotics administered prior to infection ($p<0.05$ or smaller).

3.3.4 The severity of S. *Typhimurium-induced typhlitis in streptomycin and vancomycin pre-treated mice correlates with the levels of inflammatory cytokines in the diseased ceca*

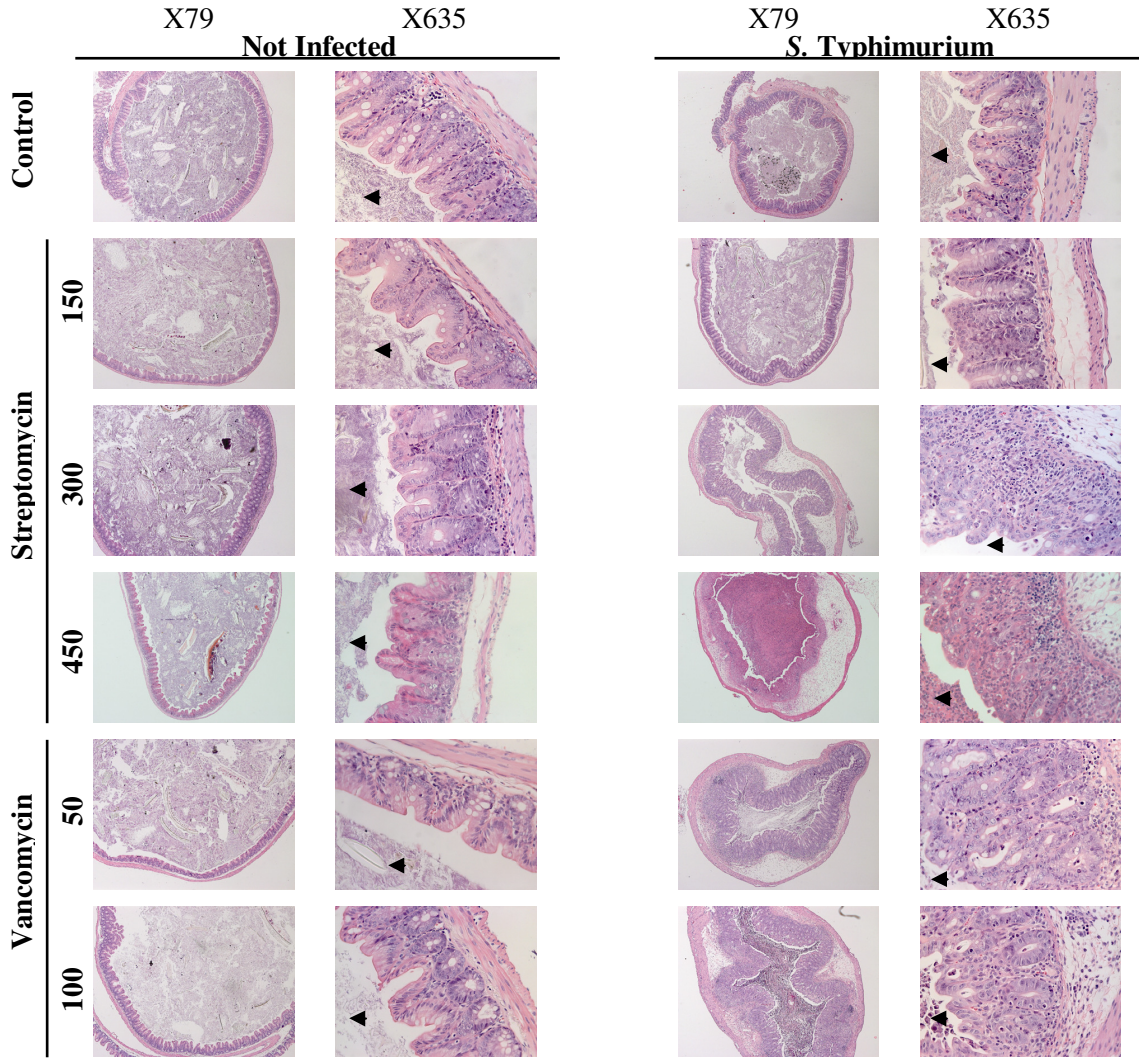
To evaluate the infiltrating inflammatory cells in cecal inflammation (typhlitis), a panel of 4 inflammatory cytokines and chemokines was examined: tumour necrosis factor α (TNFα), monocyte chemotactic protein-1 (MCP-1), keratinocyte chemoattractant (KC) and interleukin-6 (IL-6). Together, these inflammatory mediators function to activate and/or attract a wide array of inflammatory cells to the site of injury or infection, such as macrophages, neutrophils, T and B cells (Akira et al., 1990; Khan et al., 2006; Kobayashi, 2008), and their presence can therefore be used as an indication of the inflammatory infiltrate.

None of the uninfected ceca had extensive levels of any of the examined inflammatory mediators present (Figure 3.6). Thus, antibiotics alone did not induce an inflammatory milieu in the intestinal tract of the treated mice. Similarly, untreated infected ceca had negligible levels of all of the examined inflammatory mediators, consistent with the fact that regular mouse microbiota provide its host with protection from *S.* Typhimurium-induced intestinal disease.

A. Gross intestinal pathology



B. Cecal histopathology



C. Pathology Scores

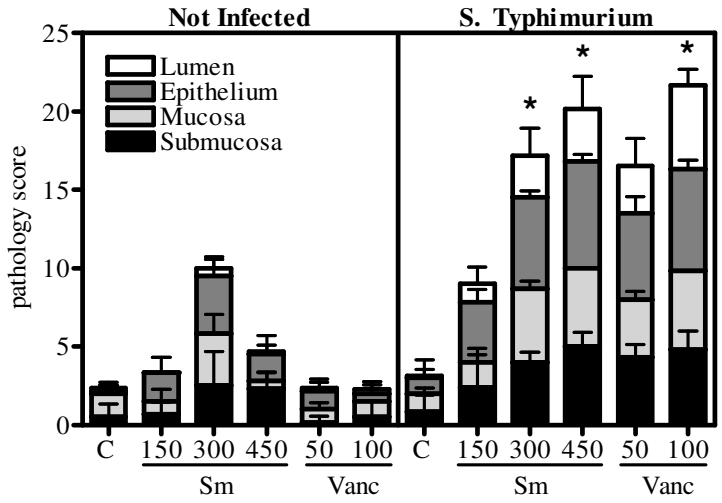


Figure 3.5 *S.* Typhimurium-induced intestinal pathology is aggravated in antibiotic pre-treated infected mice

Mice were treated with specified antibiotics for 2 days in drinking water; doses are in mg/L. Following antibiotic withdrawal, mice were infected with $2.7X10^8$ cfu of *S.* Typhimurium for 3 days. p values were calculated using Kruskal-Wallis with Dunn's post test with 95% confidence interval. C = control; Sm = streptomycin; Vanc = vancomycin.

A. Ceca and colons of *S.* Typhimurium-infected mice are shown. Absence of fecal pellets and cecal shrinkage in antibiotic-pretreated mice are indicative of aggravated pathology.

B. Tissues were harvested, fixed in formalin and stained with H&E. Antibiotic-treated *S.* Typhimurium-infected sections show escalating pathology indicated by rising levels of inflammatory infiltrate starting from submucosa and spreading to the lumen, as well as increasing epithelial disorganization indicated by mucinous plugs in crypts, mounting epithelial regenerative changes, desquamation and presence of dead epithelial cells in the lumen. Arrow-heads indicate lumen.

C. Quantification of indicators of pathology. Groups marked with an asterisk are significantly different from respective C group (with $p<0.05$ or smaller).

Conversely, ceca of infected mice that have been pre-treated with streptomycin or vancomycin had high levels of all the examined inflammatory mediators, indicating that more severe typhlitis observed in cecal necropsies (Figure 3.5) was due to increased inflammatory infiltrate in the infected tissues. The increase in inflammatory mediators was dependent on the dose of streptomycin and vancomycin that mice received prior to infection, and consequently on the extent of pre-infection microbiota perturbation.

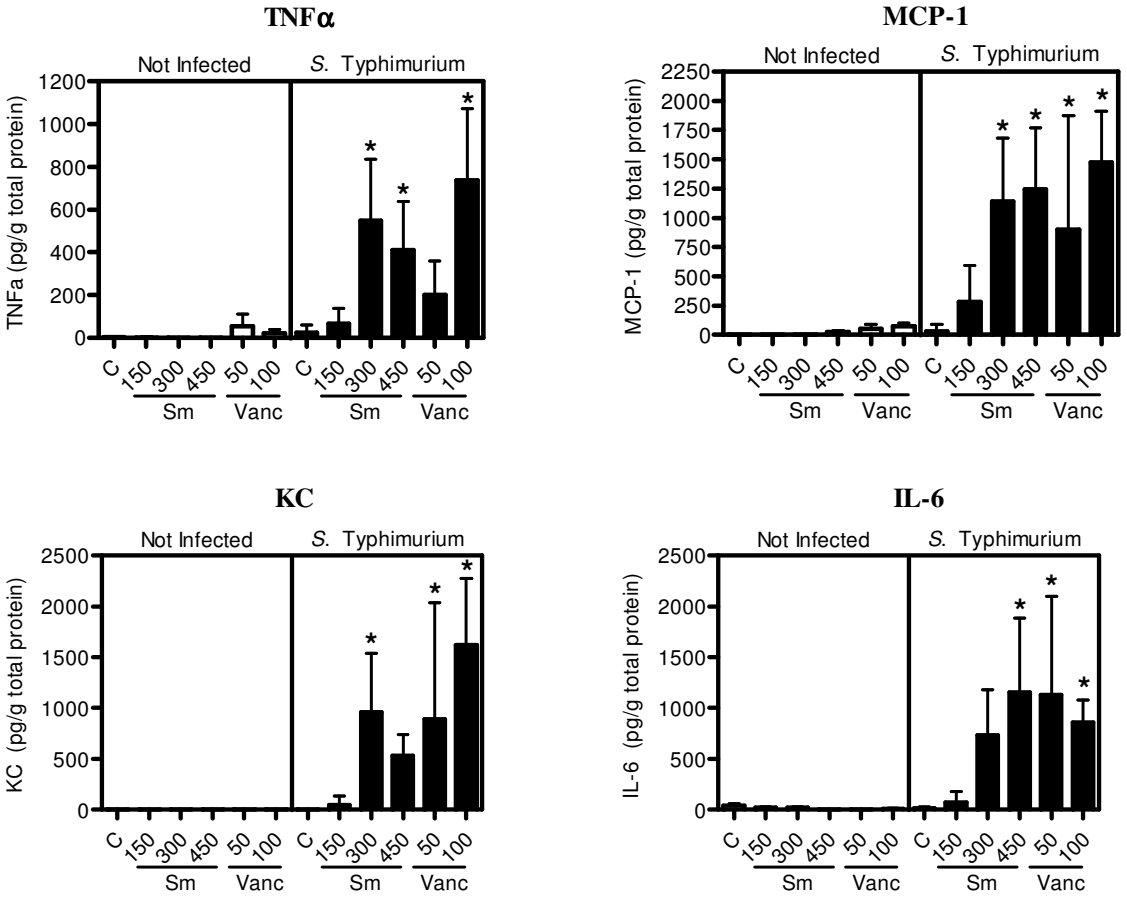


Figure 3.6 Inflammatory mediators in the ceca of infected and uninfected mice
Mice were treated with specified antibiotics for 2 days in drinking water; doses are in mg/L. Following antibiotic withdrawal, mice were infected with $2.7X10^8$ cfu of *S.* Typhimurium for 3 days. Levels of inflammatory mediators were determined by ELISAs in infected (black bars) and uninfected (white bars) animals. Levels are normalized to total protein content in samples. p values were calculated using one-way ANOVA with Bonferroni post test with 95% confidence interval. Groups marked with an asterisk are significantly different from respective C group (with $p<0.05$ or smaller). C = control; Sm = streptomycin; Vanc = vancomycin.

3.4 Discussion

Recently, many diseases have been linked to perturbations in the intestinal microbiota composition and dysbiosis (Ley et al., 2005; Sartor, 2008). Moreover, it was demonstrated that complex interactions exist between an invading pathogen and the host microbiota (Kuehl et al., 2005; Lupp et al., 2007; Stecher et al., 2007). To further characterize these interactions and to shed light on the cause-and-effect sequence in the etiology of enteric infections, we have studied

the progression of enteric salmonellosis in the setting of antibiotics-induced microbiota perturbations.

Mice pre-treated with streptomycin, but not with tetracycline exhibited an increased susceptibility to *S*. Typhimurium infection evidenced by increased intestinal colonization by the pathogen and more profound post-infection perturbations in both the composition and the numbers of intestinal microbiota. Interestingly, both antibiotics had a similar effect on the microbiota at the phylum level, increasing the proportion of the intestinal CFB bacteria (Figure 2.2). The differences in the effects of streptomycin and tetracycline on the microbiota at genus level, however, could account for the observed disparity in host susceptibility to *S.* Typhimurium post-treatment. While streptomycin treatment resulted in a profound reduction in the numbers of *Enterococci* and D-*Streptococci*, treatment with tetracycline did not have a significant effect on these bacteria (Figure 2.3).

Pre-treatment of mice with vancomycin also resulted in an increased susceptibility to *S.* Typhimurium. Similarly to streptomycin, vancomycin acted to decrease the numbers of *Enterococci* and D-*Streptococci*. A higher variability in response to *S.* Typhimurium was observed in infected mice treated with the low vancomycin dose, than in those treated with the higher vancomycin dose. Streptomycin pre-treated mice exhibited a more uniform response to *S.* Typhimurium infection than vancomycin pre-treated mice. This could be due to the fact that streptomycin, unlike vancomycin, did not encourage the growth of commensal *Enterobacteriaceae* (Figure 2.3), thus preventing the potential competition between them and *S.* Typhimurium.

Bacteria of the CFB phylum were more adversely affected during infection with *S.* Typhimurium than bacteria in Firmicutes + other phyla (Figure 3.1), a result that is similar to that

noted by Stecher *et al*. during *S.* Typhimurium infection in a high dose streptomycin pre-treatment model (Stecher et al., 2007). The higher sensitivity of bacteria belonging to the CFB phylum could be due to the inflammatory changes observed during *S*. Typhimurium infection. The presence of reactive oxygen species during inflammation could be detrimental to the strictly anaerobic CFB bacteria.

The increased susceptibility of mice to *S.* Typhimurium infection as a result of treatment with streptomycin and vancomycin could be due to a number of factors, such as alterations in the host immune response due to a disturbance in the microbiota (as it is known that the microbiota contribute to the establishment of intestinal immunity (Mazmanian et al., 2005)), a selective removal of a group of bacteria that usually provide a barrier to *S.* Typhimurium colonization and/or persistence, or a combination of these two options. As *Enterococci*/D-*Streptococci* were selectively inhibited by both streptomycin and vancomycin, they could represent one of the bacterial groups responsible for murine colonization resistance to *S.* Typhimurium. These bacteria belong to the Gram positive Firmicutes phylum, and it has been previously shown in vitro that Gram positive fecal isolates are better able than Gram negative ones to inhibit the growth of *S.* Typhimurium (Gomes et al., 2006). Also consistent with this observation is the fact that bacteria of the Gram negative CFB phylum appear not to play a role in host colonization resistance to *S.* Typhimurium; mouse susceptibility to *S.* Typhimurium was increased following both streptomycin and vancomycin treatment, which had opposing effects on the CFB phylum. Further studies focusing on detailing the effects of antibiotics on different species making up the microbial community could shed more light on which members are specifically needed for protection against various invading pathogens. To date most studies on the contribution of particular members of the bacterial population to inhibition of enteric pathogens focused on

probiotic bacteria in epithelial cell models (Broekaert et al., 2007; Corr et al., 2007); Gomes et al., 2006). Our studies highlight the importance of the microbiota in host response to infection in an animal model and identify potentially important groups of microbiota that could become the focus of future studies.

Higher *S.* Typhimurium burdens in mice treated with increasing doses of streptomycin and vancomycin were also associated with more profound intestinal inflammation and pathology, as well as with greater post-infection alterations in microbiota, as evidenced by both a reduction in total numbers of bacteria and a higher proportion of γ-Proteobacteria making up the intestinal microbiota. The enhanced intestinal inflammation and pathology were likely due to a greater activation of the host immune system by higher numbers of *S.* Typhimurium. The cytokine profile was indicative of infiltration by neutrophils and macrophages, which is consistent with the current knowledge of the innate immune response to *S.* Typhimurium infection (Wick, 2004). Additionally, the cytokine profile was reminiscent of that observed in inflammatory bowel diseases (Banks et al., 2003; Sartor, 2006) (increases in TNFα, MCP-1), which are also characterized by disturbances in intestinal microbiota (Frank et al., 2007), particularly an increase in colonization by γ-Proteobacteria. We have previously shown that a strong inflammatory response acts to reduce the total numbers of intestinal microbiota (Lupp et al., 2007), which is likely the reason for a reduction in total numbers of bacteria observed post-infection. As well, a strong inflammatory response was shown to benefit the growth of enteropathogens (Lupp et al., 2007; Stecher et al., 2007) and non-pathogenic aerobic bacteria (Lupp et al., 2007). *S.* Typhimurium-induced inflammation was previously shown to adversely affect the members of murine cecal microbiota, promoting the overgrowth of the pathogen (Stecher et al., 2007), which is also confirmed by our results.

Most previous studies looking at murine *S.* Typhimurium-induced colitis utilize extremely high doses of antibiotics (streptomycin, 20 mg/mouse) (Barthel et al., 2003; (Bohnhoff et al., 1954), eliminating 90-98% of the intestinal microbiota prior to infection (Stecher et al., 2007). Thus it was ambiguous which component of the antibiotic-induced perturbation of the microbiota was responsible for the disruption of resistance to *S.* Typhimurium colitis: a change in the composition of the normal flora or the reduction in total numbers of bacteria. We have shown that a perturbation in the composition alone is sufficient to increase the susceptibility of the murine host to *S.* Typhimurium colitis. A recently published study (Lawley et al., 2008) has also found that even when bacterial numbers returned to normal levels after antibiotic treatment, mice still remained more susceptible to *S.* Typhimurium infection. This study, however, did not examine the shifts in microbiota composition either pre- or post-infection, and did not conclusively demonstrate the correlation between the perturbations in microbiota composition and increased susceptibility to *S.* Typhimurium.

Our results demonstrate that select imbalances in microbiota predispose the host to more severe enteropathogenic infection. These observations could be part of an explanation to the high rates of nosocomial infections, where antibiotics are abundantly used. In fact, nosocomial *Salmonella enterica* infections, particularly with multi-drug resistant strains, are a concern in developing countries (Kumar et al., 1995; Vaagland et al., 2004), and occasionally even happen in developed countries (Kay et al., 2007; Olsen et al., 2001). Although we cannot indiscriminately extrapolate our findings to all diseases where microbiota imbalance has been implicated in the etiology, they show that at least in infectious colitis, microbial imbalance precedes the onset of pathology, rather than being the result of it. Consequently initiation of pathology could potentially be averted or corrected by maintaining a balanced microbial

community.

CHAPTER 4:
CHARACTERIZATION OF THE INTESTINAL INFLAMMATION AND ASSOCIATED MICROBIOTA CHANGES IN THE MURINE *S.* TYPHIMURIUM INFECTION

4.1 Summary

Gastrointestinal infections involve a tripartite relationship between the invading pathogen, the host and the host's intestinal microbiota. However, the details of these interactions, including the main contributing facets of the host response, the pathogen's virulence factors involved in microbiota alterations, etc., have not yet been fully elucidated. To characterize the inflammatory response and associated microbiota changes in enteric salmonellosis, C57Bl/6 mice were pre-treated with 450 mg/L of streptomycin for 2 days, modifying their intestinal microbiota composition but not reducing the total microbial numbers, and then infected with WT, Δ*invA* and Δ*ssaR S.* Typhimurium strains. *S.* Typhimurium cecal colonization and induced typhlitis were evaluated to assess infection success and progression, while total microbial numbers, microbiota appearance, and the proportion of γ-Proteobacteria were measured as indicators of associated microbiota changes. Successful persistent colonization by *S.* Typhimurium was associated with *S.* Typhimurium-induced intestinal pathology, as both WT and Δ*invA* strains, which were able to colonize the intestine successfully and persistently, were also able to induce significant typhlitis. However, intestinal inflammation and persistently high pathogen burdens were not always associated with similar perturbations in the intestinal microbiota, as mice infected with Δ*invA* strain did not have a consistent reduction in their total intestinal microbes and diversity, or a consistent increase in the proportion of cecal γ-Proteobacteria, as did wt-infected mice. Upon assessment of neutrophil infiltration in the diseased ceca, it was found that while WT-infected mice had large numbers of neutrophils in the lumen starting at day 3 post infection, neither Δ*invA*-, nor Δssa*R*- infected mice had evident intestinal neutrophil invasion. Thus neutrophils were implicated as a potential mediator of perturbations observed in the indigenous microbiota during WT *S.* Typhimurium infection. These studies offer a new model system for the study of *S.*

Typhimurium-induced intestinal disease phase that retains all three participants of the disease process: the host, unaltered numbers of its indigenous microbiota, and the pathogen. The use of this model system can shed additional light upon the role of the host microbiota in *S.* Typhimurium-induced intestinal disease, the virulence strategies of *S.* Typhimurium, and upon the effects of the intestinal inflammation on the host's microbiota.

4.2 Introduction

It is now well appreciated that an infectious process is a tripartite relationship between the invading pathogen, the host and the host's indigenous microbiota (Pedron and Sansonetti, 2008). Furthermore, we now recognize that pathogen-elicited host response can prove detrimental to the intestinal microbiota, as a result promoting pathogen colonization (Lupp et al., 2007; Stecher et al., 2007). However, it is not known which aspects of the inflammatory response are responsible for the observed effects on the microbiota, whether the indigenous microbial population would be adversely affected by all inflammatory changes, and which virulence factors are responsible for the observed effects.

Salmonella enterica serovar Typhimurium is a gastrointestinal pathogen and a frequent cause of food poisoning, accounting for much morbidity and mortality in the developing world and even exerting a significant toll on the healthcare systems of the developed countries. *S.* Typhimurium's virulence strategy is dependent on virulence genes encoded on a number of pathogenicity islands, termed *Salmonella* pathogenicity islands, or SPI (Hensel, 2004). Two type III secretion systems (T3SS) encoded on SPI1 and SPI2 mediate many aspects of both the intestinal and systemic aspects of *S.* Typhimurium infection, such as invasion of the intestinal epithelium (Lostroh and Lee, 2001); Schlumberger and Hardt, 2006), induction of the

inflammatory response (Wallis and Galyov, 2000), and intracellular survival (Abrahams and Hensel, 2006). The SPI1-encoded T3SS is crucial for *S*. Typhimurium invasion of the intestinal epithelium and also plays an important role in the induction of the host inflammatory response. Mutation of *invA*, a part of the SPI1 apparatus, abrogates the ability of *S*. Typhimurium to invade cultured intestinal epithelial cells (Galan and Curtiss, 1989). The SPI2-encoded T3SS is activated during the intracellular stage of *S*. Typhimurium infection (Schlumberger and Hardt, 2006), allowing it to evade killing in the phagocytic vacuole and instead establish a successful niche for intracellular survival. Deletion of *ssaR*, a part of the SPI2 apparatus, renders SPI2 nonfunctional and *S*. Typhimurium susceptible to intracellular killing.

Careful dissection of mechanisms accounting for *S.* Typhimurium-induced intestinal disease can further our understanding of the disease process and facilitate development of better prevention and treatment methods. Until recently, no convenient model system was available to study the intestinal phase of *S.* Typhimurium-induced disease, as the murine host was only susceptible to the systemic illness, while the bovine model was genetically far more heterogeneous and clearly not as amenable to manipulation, complicating data interpretation. In 2003, Hardt's laboratory described a murine *S.* Typhimurium colitis model, which allowed analysis of pathogen and host factors involved in the disease process (Barthel et al., 2003). However, pre-treatment of mice with massive doses of streptomycin (20mg/mouse) in this model severely reduced the total numbers of the murine intestinal microbiota (Stecher et al., 2007). Consequently, while this model offered great advantages to study the host and the pathogen, the role of the third participant in the process – the intestinal microbiota – could not be fairly assessed.

We offer here a low dose streptomycin pre-treatment murine model of *S.* Typhimurium-

induced typhlitis, in which total microbial levels remain unaltered prior to infection (Sekirov et al., 2008), facilitating the examination of microbiota perturbations during infection. Using this model we studied the effects of *S.* Typhimurium infection on the murine intestinal microbiota, focusing on the virulence factors needed to induce the post-infection alterations and the host response components that promote them. We found that while both WT and Δ*invA S.* Typhimurium strains were able to induce significant inflammation in the mouse ceca, only infection with the WT strain was associated with pronounced perturbations to the intestinal microbiota. We hypothesized that this disparity was due to the differences in the inflammatory response elicited during infection with these two strains. When we sought to evaluate the infection-induced typhlitis, it was noted that infection with the WT strain induced a large neutrophil infiltrate into the lumen, unlike infection with Δ*invA S.* Typhimurium. Neutrophils were thus implicated as one of the host agents involved in the inflammation-induced damage to the host microbiota. SPI1-secreted virulence factors were shown to be necessary for *S.* Typhimurium to induce a host response that adversely affects the indigenous microbial population.

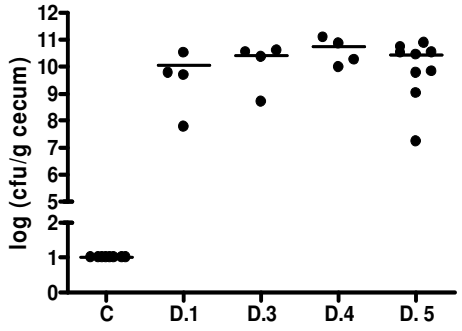
4.3 Results

*4.3.1 Cecal colonization by WT and Δ*invA *S. Typhimurium strains, but not by the Δ*ssaR *strain, does not change over the time course of infection*

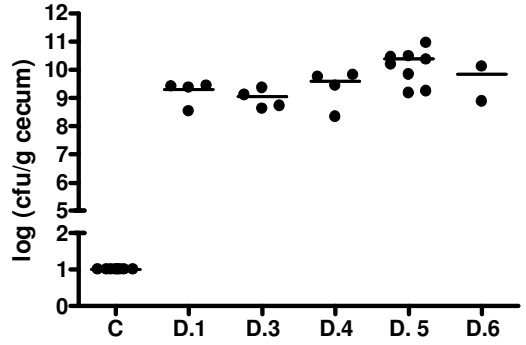
To assess the ability of different *S.* Typhimurium strains to colonize the murine intestine in the presence of adequate numbers of the intestinal microbiota we examined colonization of the infected mouse ceca by WT, Δ*invA* and Δ*ssaR S.* Typhimurium over the duration of infection. Colonization by all three strains reached maximal levels as early as 1 day post infection (p.i.),

peaking at 10^9-10^{10} cfu/g cecum (Figure 4.1). WT and Δ*invA S.* Typhimurium numbers remained

A. WT *S*. Typhimurium colonization



B. Δ*invA S*. Typhimurium colonization



C. Δ*ssaR S*. Typhimurium colonization

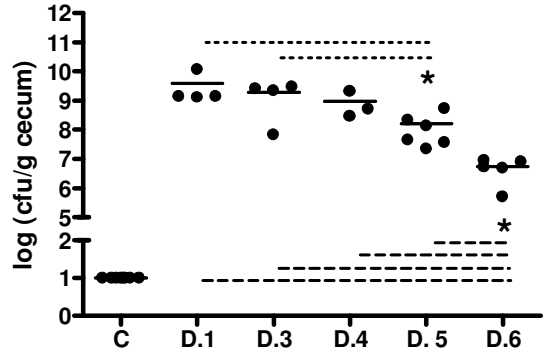


Figure 4.1 Cecal colonization by WT and Δ*invA*, but not Δ*ssaR S.* Typhimurium does not change over the time course of infection

Mice were treated with 450 mg/L of streptomycin for 2 days in drinking water. After antibiotic withdrawal, mice were infected with $2.7X10^8$ cfu of indicated *S.* Typhimurium strain. 2 to 8 mice per group. *S.* Typhimurium colonization was enumerated by plating serial dilutions of cecum homogenates on XLD plates with 100 μg/ml streptomycin. p values were calculated using one-way ANOVA with Bonferroni post test with 95% confidence interval. Time points marked with an asterisk are significantly different from time points indicated by dashed lines with $p<0.05$ or smaller. C = uninfected control; D = days post infection

Table 4.1 Statistical analysis of cecum colonization by WT, Δ*invA* and Δ*ssaR* S. Typhimurium strains over time course of infection

	Day 1		Day 3		Day 4		Day 5		Day 6	
vs.	Δ*invA*	Δ*ssaR*	Δ*invA*	Δ*ssaR*	Δ*invA*	Δ*ssaR*	Δ*invA*	Δ*ssaR*	Δ*invA*	Δ*ssaR*
WT	ns	ns	ns	ns	ns	$p<0.05$	ns	$p<0.01$	NA	NA
Δ*invA*	-	ns	-	ns	-	ns	-	$p<0.001$	-	$p<0.01$

ns = not significant
NA = not available
Colonization by different strains at the same time points was compared. All p-values were calculated using ANOVA, except for day 6 Δ*ssaR* vs. Δ*invA* comparison, which was calculated using unpaired t-test, as only 2 groups of mice survived to this time point.

relatively unaltered for the entire duration of the infection and did not significantly differ from day to day or from one strain to the other (Figure 4.1A, B, Table 4.1). However, cecal colonization by the Δ*ssaR S.* Typhimurium strain declined gradually over the time course of infection with significant reductions in the microbial burdens at days 5 and 6 p.i., compared to the burdens at day 1 (Figure 4.1C). Moreover, Δ*ssaR S.* Typhimurium cecal burdens became significantly lower than those of the WT strain at days 4 and 5 p.i. and lower than those of the Δ*invA* strain at days 5 and 6 p.i. (Table 4.1).

Mice infected with WT and Δ*invA S.* Typhimurium quickly became very moribund with no surviving WT-infected mice past day 5 and only 2 surviving Δ*invA*- infected mice at day 6 p.i.. Mice infected with Δ*ssaR S.* Typhimurium, on the other hand, appeared considerably less sick with all the infected mice surviving at day 6 p.i.. The disparity was most likely due to the inability of the Δ*ssaR* strain to cause systemic illness (Hensel, 2000).

*4.3.2 Infection with WT or Δ*invA*, but not Δ*ssaR S. Typhimurium results in cecal inflammation*

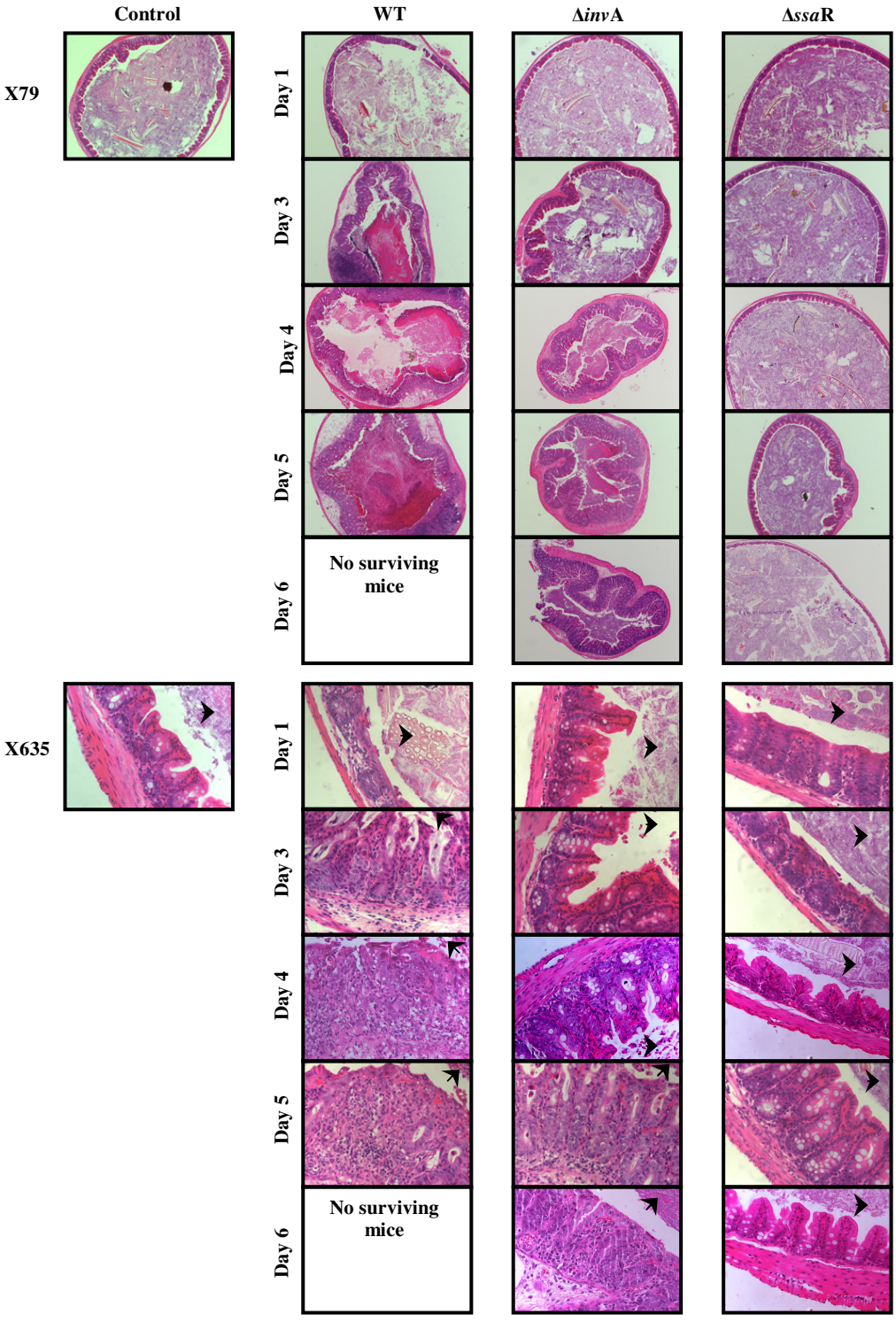
Next we sought to assess the induction of intestinal inflammation by the three selected *S.* Typhimurium strains in this model system. As *S.* Typhimurium induces the most pronounced

inflammatory changes in the ceca of the infected mice (Barthel et al., 2003; Coburn et al., 2005), the presence and extent of typhlitis was evaluated as a marker of intestinal inflammation.

It was noted that infection with WT *S.* Typhimurium induced significant typhlitis in infected mice as early as day 3 p.i. (Figure 4.2B). Inflammation was characterized by increasing amounts of inflammatory infiltrate, starting from submucosa and spreading to the lumen. Increased submucosal and mucosal thickness, mounting epithelial disorganization and presence of mucinous plugs in the crypts and dead epithelial cells in the lumina of the tissues also indicated increased pathology (Figure 4.2A). The inflammatory changes persisted until day 5 p.i., at which time point the mice became too moribund and were sacrificed. In conclusion, WT *S.* Typhimurium strain was able to induce significant typhlitis in the infected host in the presence of normal numbers of the intestinal microbiota. The inflammatory changes persisted until the end of the infection time-course.

Mice infected with Δ*invA* mutant did not have any notable inflammatory changes in their ceca in the early time points post-infection (Figure 4.2), consistent with previous findings in the high dose streptomycin pre-treatment mouse model (Coburn et al., 2005; Hapfelmeier et al., 2004). However, at the later time points of infection the ceca became appreciably inflamed, reaching significant levels at day 5 p.i. (Figure 4.2B), also in agreement with previous findings about the role of SPI2 in intestinal inflammation (Coombes et al., 2005a). Typhlitis at day 6 p.i. was at a similar level to that at day 5 p.i., however did not reach significance. This may be due to only 2 mice surviving at this time point. Typhlitis induced by Δ*invA* mutant was similar to that induced by WT *S.* Typhimurium, with epithelial degeneration, increased submucosal and mucosal thickness, and presence of cellular debris in the lumen, mucinous plugs and crypt abcesses. Although Δ*invA*-induced typhlitis was pronounced and significant, it never reached the

A. Cecal Histopathology



B. Pathology scores

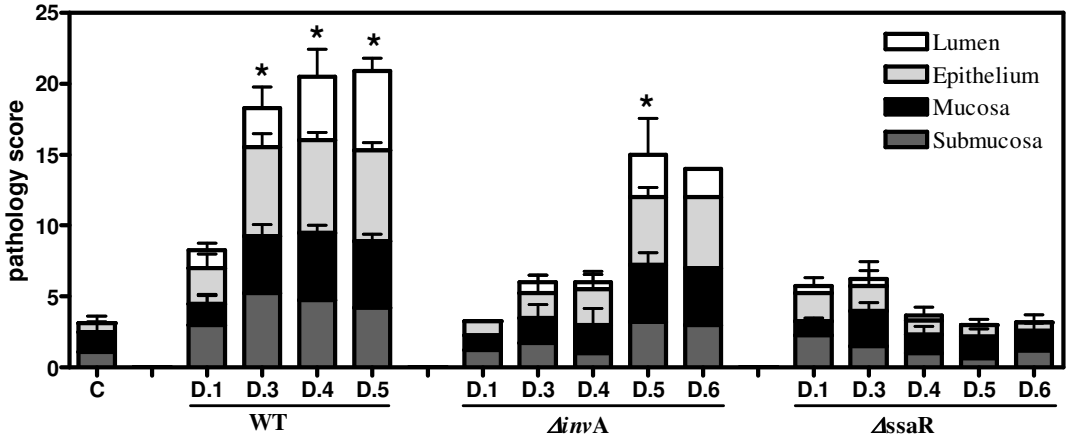


Figure 4.2 Murine infection with WT and Δ*invA*, but not Δ*ssaR S.* Typhimurium results in cecal inflammation

Mice were treated with 450 mg/L streptomycin in drinking water for 2 days. Following antibiotic withdrawal, mice were infected with $2.7X10^8$ cfu of specified *S.* Typhimurium strain. 2 to 8 mice per group were used. p values were calculated using Kruskal-Wallis with Dunn's post test with 95% confidence interval. C = uninfected control, D = days post infection.

A. Tissues were harvested, fixed in formalin and stained with H&E. WT and Δ*invA S.* Typhimurium-infected sections show escalating pathology over time, indicated by rising levels of inflammatory infiltrate starting from submucosa and spreading to the lumen, as well as evident crypt abscesses; increasing epithelial disorganization is indicated by mucinous plugs in crypts, mounting epithelial regenerative changes, desquamation and presence of dead epithelial cells in the lumen. Arrow-heads indicate lumen.

B. Quantification of indicators of pathology. Groups marked with an asterisk are significantly different from control group, $p<0.05$ or smaller.

extent of that induced by the WT strain, remaining significantly lower at the same time points for the duration of the infection time course (p=0.004 at day 5 p.i., Mann-Whitney U-test). This is in contrast to the situation in the high dose streptomycin pre-treatment mouse model (Coombes et al., 2005a).

Mice infected with Δ*ssaR S.* Typhimurium did not exhibit any significant cecal inflammation for the duration of the infection and cecal tissues were morphologically similar to those of the unifected mice (Figure 4.2), indicating that in the presence of adequate numbers of the intestinal microbiota SPI1 pathogenicity island alone is not sufficient to trigger typhlitis. This

is in contrast to the findings in a high dose streptomycin model, in which significant (albeit significantly lower than WT-induced) typhlitis was observed at 2 days p.i. in Δ*ssaR*-infected mice (Coburn et al., 2005; Coombes et al., 2005a).

*4.3.3 Intestinal microbiota are extensively modified during infection with WT, but not Δ***invA** *or Δ***ssaR** ***S. Typhimurium strains***

To determine the effect of infection with the three *S.* Typhimurium strains on the host intestinal microbiota we evaluated the total bacterial numbers, morphology and the proportion of γ-Proteobacteria in the murine ceca over time. We expected to see profound perturbations to the host microbiota during infection with WT and Δ*invA S.* Typhimurium due to the intestinal inflammation observed in response to these two strains (Figure 4.2).

Infection with WT *S.* Typhimurium resulted in a significant decrease in total cecal bacterial numbers, as assessed by SYBR green staining, starting at day 3 p.i. (Figure 4.3A). The observed reduction was consistent among animals and the intestinal microbiota numbers did not recover for the whole duration of the experiment. Surprisingly, total bacterial numbers of mice infected with both Δ*invA* and Δ*ssaR S.* Typhimurium strains were unaffected by the infection, except for a transient reduction observed at day 4 in Δ*invA*-infected mice (Figure 4.3B, C). This reduction, however, did not persist and was appreciably smaller than that observed during WT infection.

Microbiota appearance during infection with the three *S.* Typhimurium strains was evaluated as described in section 6.3.4. The reduction of total microbial numbers during WT *S.* Typhimurium infection (assayed using SYBR green staining) was also evident in the DAPI staining of luminal microbiota. In addition to a decrease in total numbers, it was also apparent

that the diversity of the microbial population was greatly reduced, and there was a lot of cellular debris in the lumen (Figure 4.4 D, E and F). The diversity of the microbiota remaining in the lumen of WT infected mice was strikingly different than that of uninfected mice with mostly small rod-shaped bacteria adhering to the cellular debris. Upon counter-staining for *Salmonella* LPS, it was confirmed that the majority of the remaining microbiota were the infecting *S.* Typhimurium (Figure 4.4 D, E and F).

In contrast to the situation that occurs with WT *S.* Typhimurium infection, infection with Δ*invA* and Δ*ssaR* strains did not result in a prominent reduction of total microbial numbers, or diversity (based on microbial appearance). Additionally, a much smaller proportion of the luminal microbiota were *S.* Typhimurium (Figure 4.4 G-I and J-L). The microbiota appearance was evaluated in mice at day 5 p.i., as the latest time point at which there were surviving mice infected with each of the three *S.* Typhimurium strains.

To assess the perturbations in the composition of the intestinal microbiota as a the result of infection, the proportion of cecal γ-Proteobacteria was determined using FISH. Infection with WT *S.* Typhimurium resulted in a prominent and significant increase in the proportion of cecal γ-Proteobacteria starting at day 3 p.i. (Figure 4.5A). As noted from figure 4.4 D-F, majority of these γ-Proteobacteria were the infecting *S.* Typhimurium. In contrast to that, infection with each of the mutant *S.* Typhimurium strains did not result in a pronounced or consistently significant increase in the proportion of cecal γ-Proteobacteria (Figure 4.5B, D). The observed significant increases did not last throughout the time course of the infection and were far smaller than the increases observed during infection with the WT strain.

All of the above three methods of the microbiota analysis demonstrated that while infection with the WT *S.* Typhimurium strain results in consistently prominent and significant

alterations to both the numbers and composition of the intestinal microbiota, as was expected, infection with each of the *S*. Typhimurium mutant strains did not demonstrate similar profound

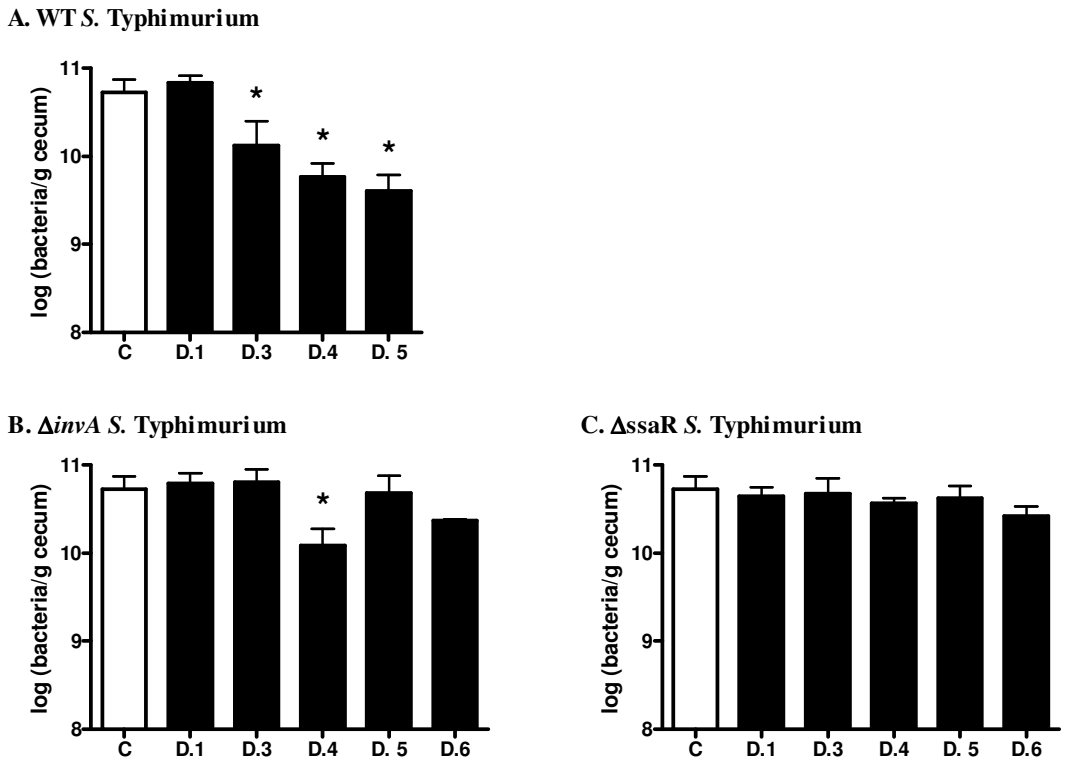


Figure 4.3 Total cecal bacterial numbers are consistently reduced during infection with WT, but not Δ*invA* or Δ*ssaR* *S*. Typhimurium strains
Mice were treated with 450 mg/L of streptomycin for 2 days in drinking water. After antibiotic withdrawal, mice were infected with $2.7X10^8$ cfu of indicated *S*. Typhimurium strain. 2 to 8 mice per group were used. Total bacteria were enumerated by SYBR green staining. p values were calculated using one-way ANOVA with Bonferroni post test with 95% confidence interval. Time points marked with an asterisk are significantly different from control mice. C = uninfected control; D = days post infection

modifications. Threrefore, although Δ*invA S*. Typhimurium was able to provoke an inflammatory response in the infected host, the induced inflammation did not have the same detrimental effect on the host intestinal microbiota as inflammation observed during WT *S.* Typhimurium infection.

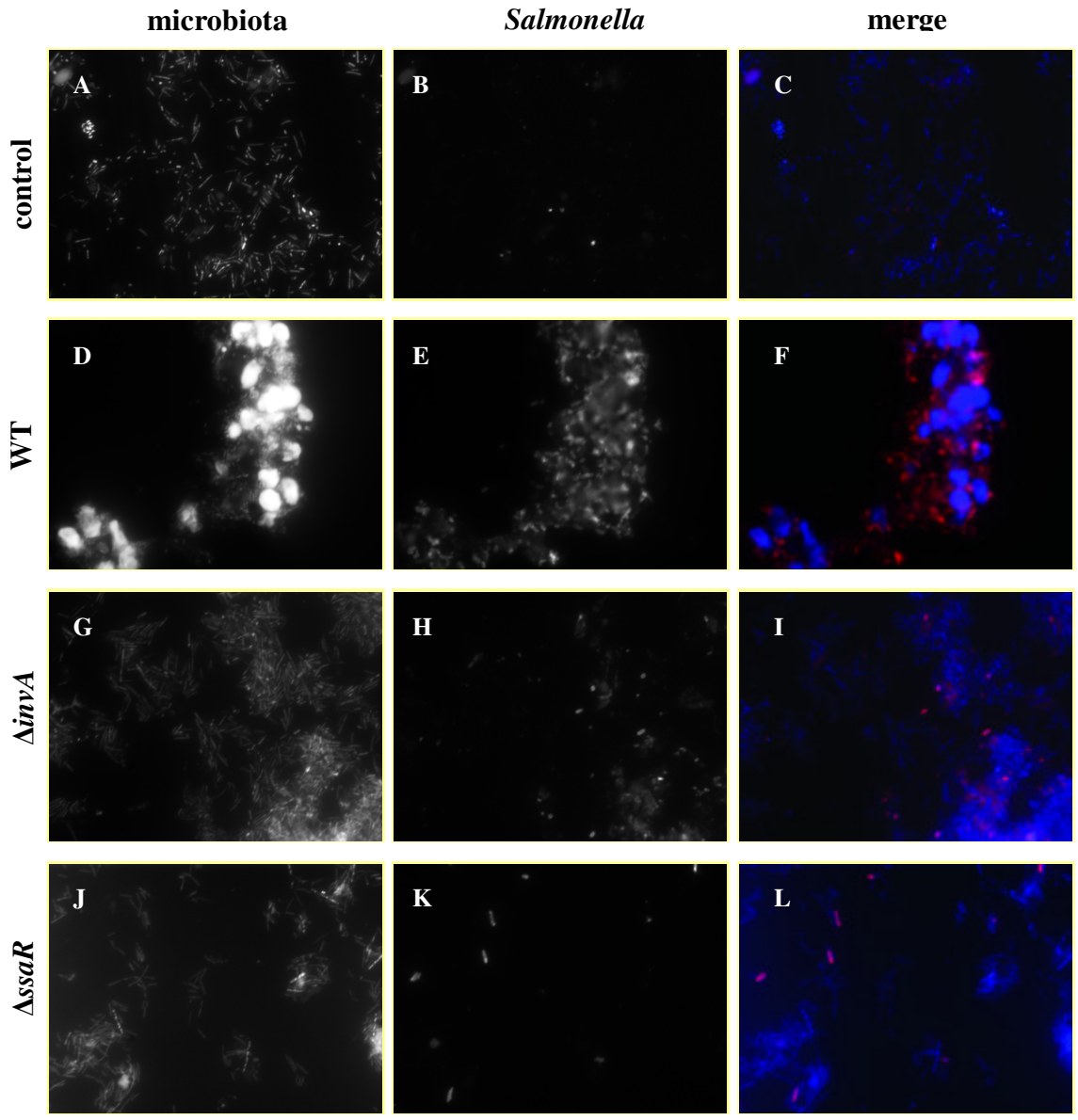


Figure 4.4 Microbiota appearance is extensively modified during infection with WT, but not Δ*invA* or Δ*ssaR S.* Typhimurium

Mice were treated with 450 mg/L of streptomycin for 2 days in drinking water. After antibiotic withdrawal, mice were infected with $2.7X10^8$ cfu of indicated *S.* Typhimurium strain for 5 days. Micrographs are from a representative mouse with 5 mice per group. PFA-fixed cecum cryosections were stained for DNA (with DAPI [4',6'-diamidino-2-phenylindole; blue in merge) and *Salmonella* LPS (red in merge) and lumina visualized. Control mice had a very morphologically diverse and numerous microbiota and did not stain specifically for *Salmonella* (A,B and C). Mice infected with WT *S.* Typhimurium exhibited a profound reduction of both microbial numbers and diversity, as well as a lot of cellular debris in the lumen. Majority of remaining luminal microbiota were the infecting *S.* Typhimurium (D, E and F). Mice infected with both the Δ*invA* and Δ*ssaR* strains had luminal microbial numbers and diversity very similar to the control mice. While *S.* Typhimurium was present in the lumina of mice infected with both mutant strains, it did not constitute the majority of present microbes (G-I and J-L). Images are pseudocolour and are shown at 1000X magnification.

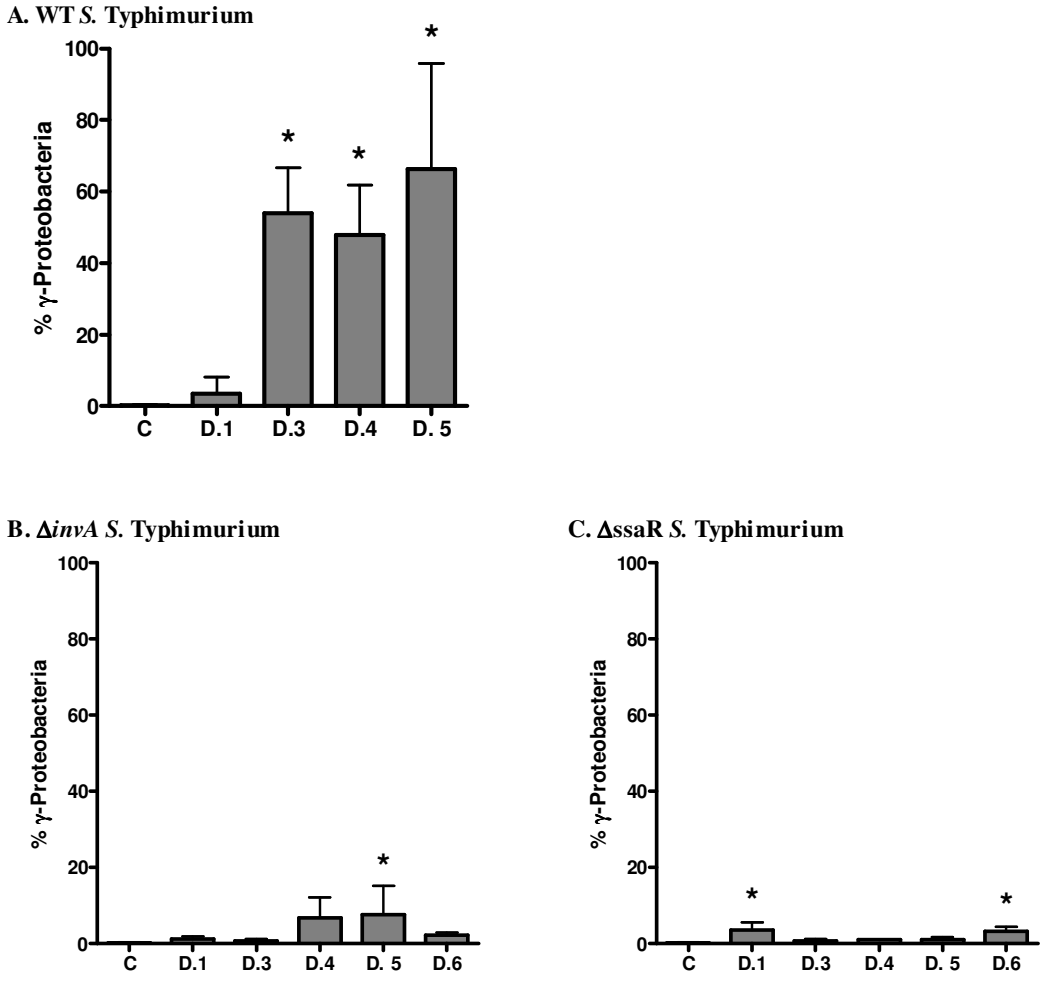


Figure 4.5 Proportion of γ-Proteobacteria is consistently increased during infection with WT, but not Δ*invA* or Δ*ssaR S*. Typhimurium strains

Mice were treated with 450 mg/L of streptomycin in drinking water for 2 days; After antibiotic withdrawal, mice were infected with $2.7X10^8$ cfu of specified *S*. Typhimurium strain. 2 to 8 mice per group were used. Values are % of all Eubacteria; Proportion of γ-Proteobacteria was determined by FISH as described in Materials and Methods chapter. Time points marked with an asterisk are significantly different from control mice. p values were calculated using one-way ANOVA with Bonferroni post test with 95% confidence interval. C = uninfected control; D = days post infection

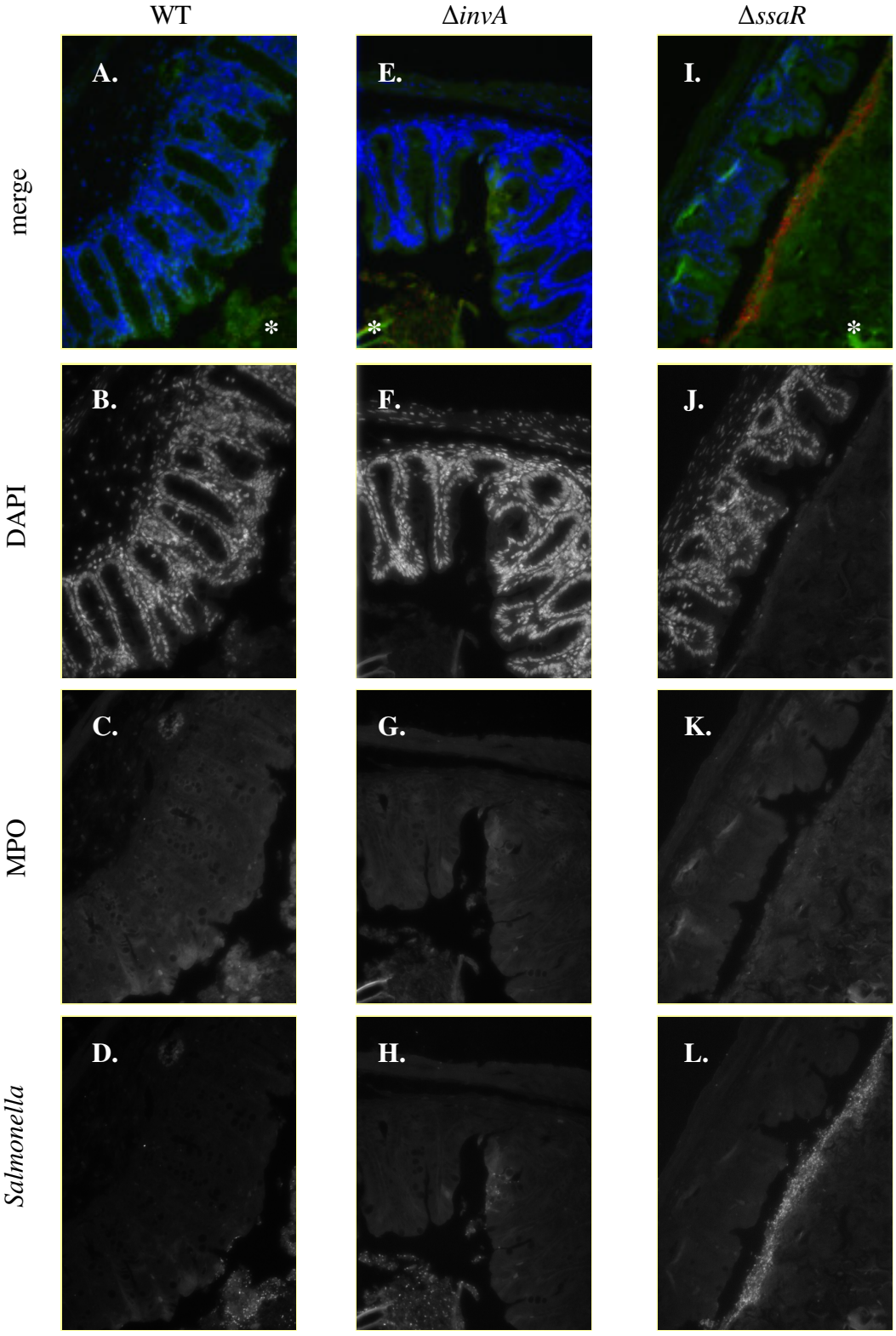
*4.3.4 Infection with WT but not Δ*invA *or Δ*ssaR *S. Typhimurium induces neutrophil infiltration of the infected ceca*

It was surprising to see that although both the WT and the Δ*invA S*. Typhimurium strains were able to cause typhlitis in the infected mice, only the WT strain also induced profound microbiota perturbations. The possible explanations that could account for this disparity are differences in the extent of the induced inflammation, the type of the induced inflammatory response or direct interactions between SPI1 effector(s) and the indigenous microbiota. Due to the complexity of the indigenous microbiota and culturing difficulties, it was not feasible to evaluate the latter option. The first two possibilities, however, could be assessed.

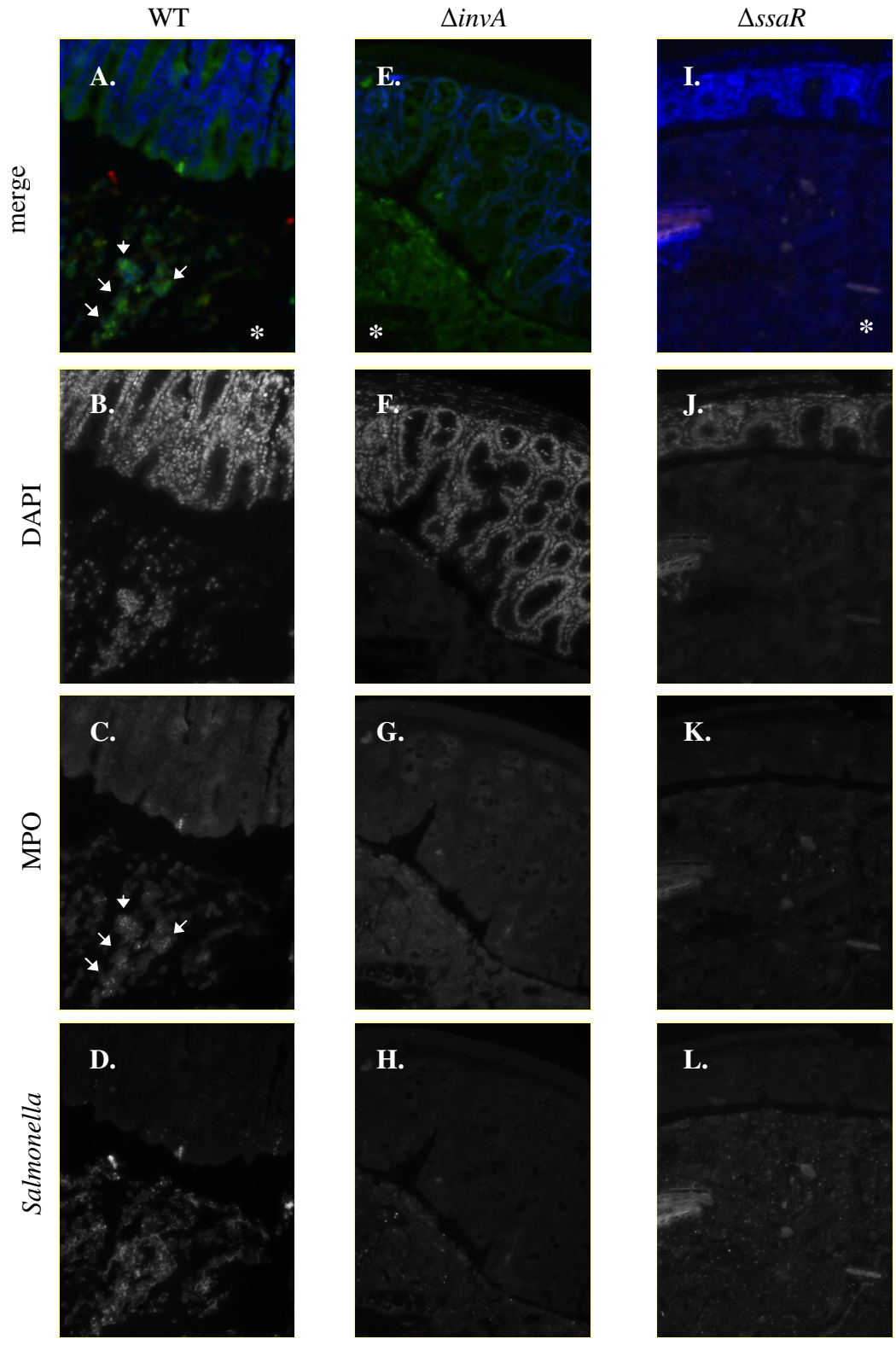
Although the extent of the inflammation induced by the Δ*invA* mutant was less than that induced by the WT strain for each given time point, the pathology score for Δ*invA* at day 5 p.i. was not significantly lower than that of the WT strain at day 3 p.i. (p>0.05, Mann-Whitney U test) (Figure 4.2B). Nonetheless, no profound changes to the microbiota were observed in the *Δinv*A-infected mice at day 5 p.i., unlike the situation in WT-infected mice at day 3 p.i. (Figures 4.3, 4.4, 4.5). Consequently it was more likely that the differences in the type of the inflammatory response induced by the two strains are accountable for the disparity in their affect on the host intestinal microbiota.

WT *S*. Typhimurium has been previously shown to induce a strong neutrophil influx into the ceca of mice pre-treated with a high dose of streptomycin, in contrast to Δ*ssaR S*. Typhimurium (Coburn et al., 2005). We hypothesized that in our model system there might also be a differential neutrophil recruitment by the different *S*. Typhimurium strains. Immunostaining of cecal necropsies for myeloperoxidase (MPO) granules found in neutrophils showed a neutrophil influx into the lumina of the WT-infected mice starting as early as day 3 p.i. and

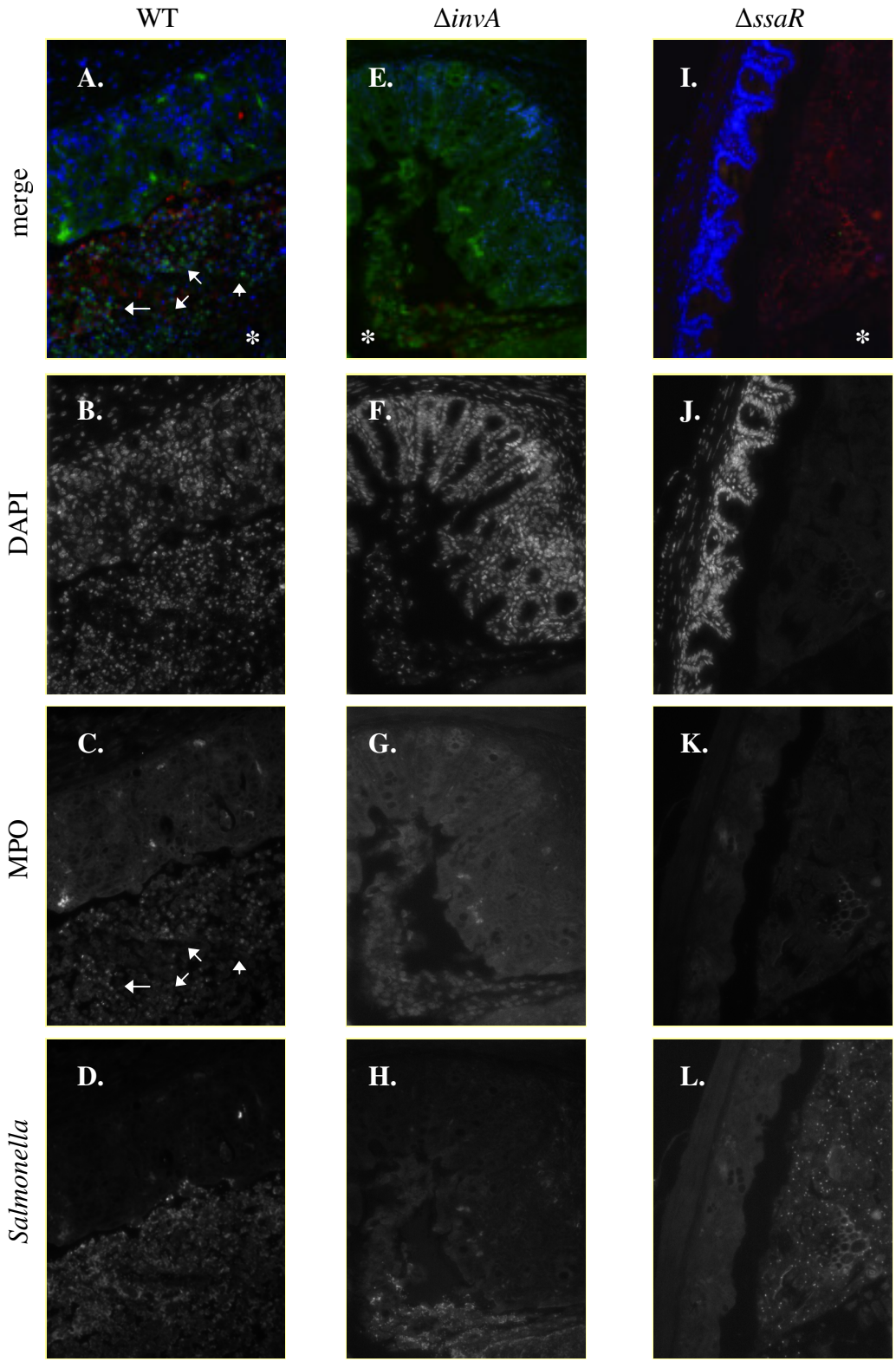
Day 1



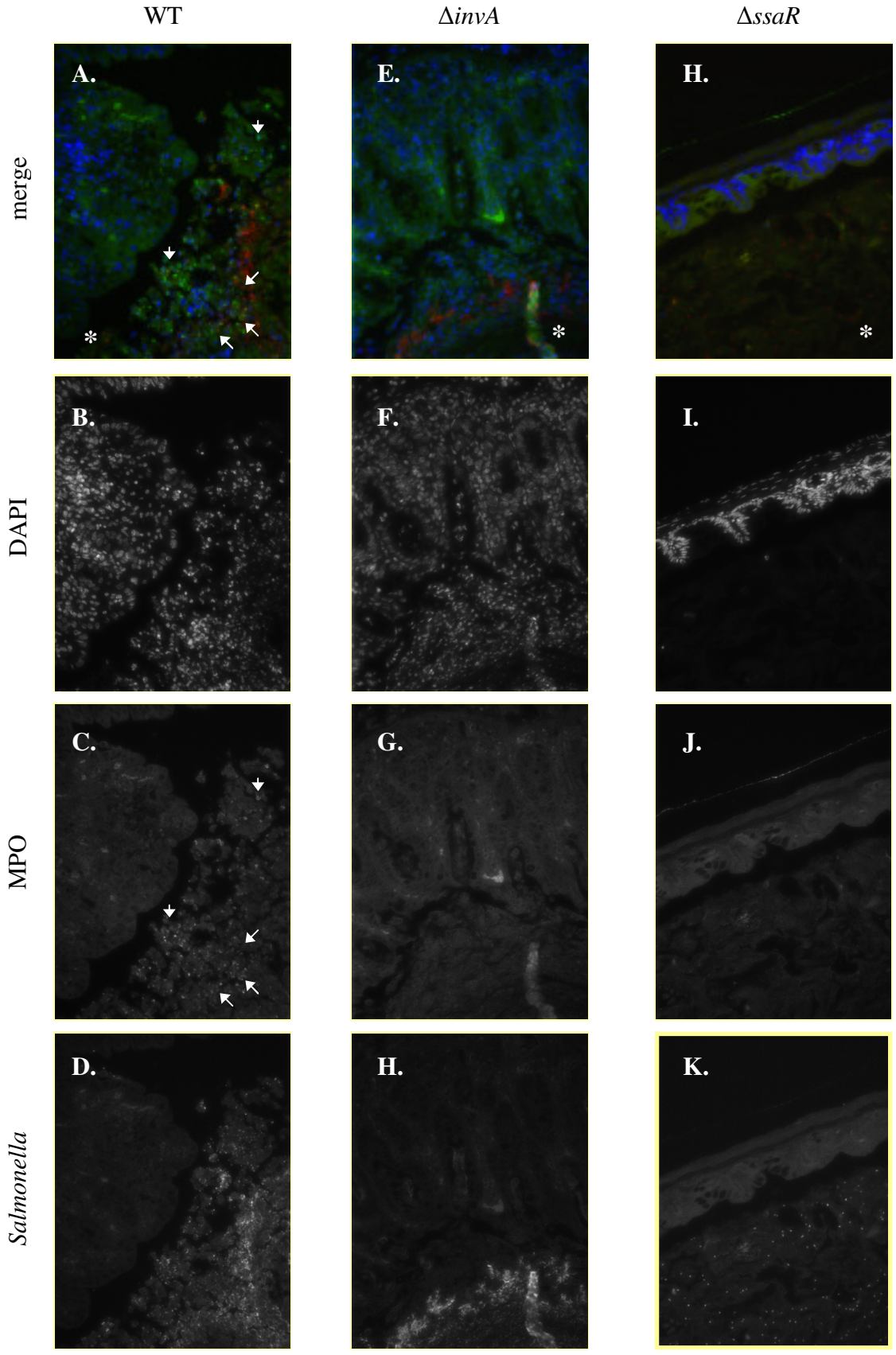
Day 3



Day 4



Day 5



Day 6

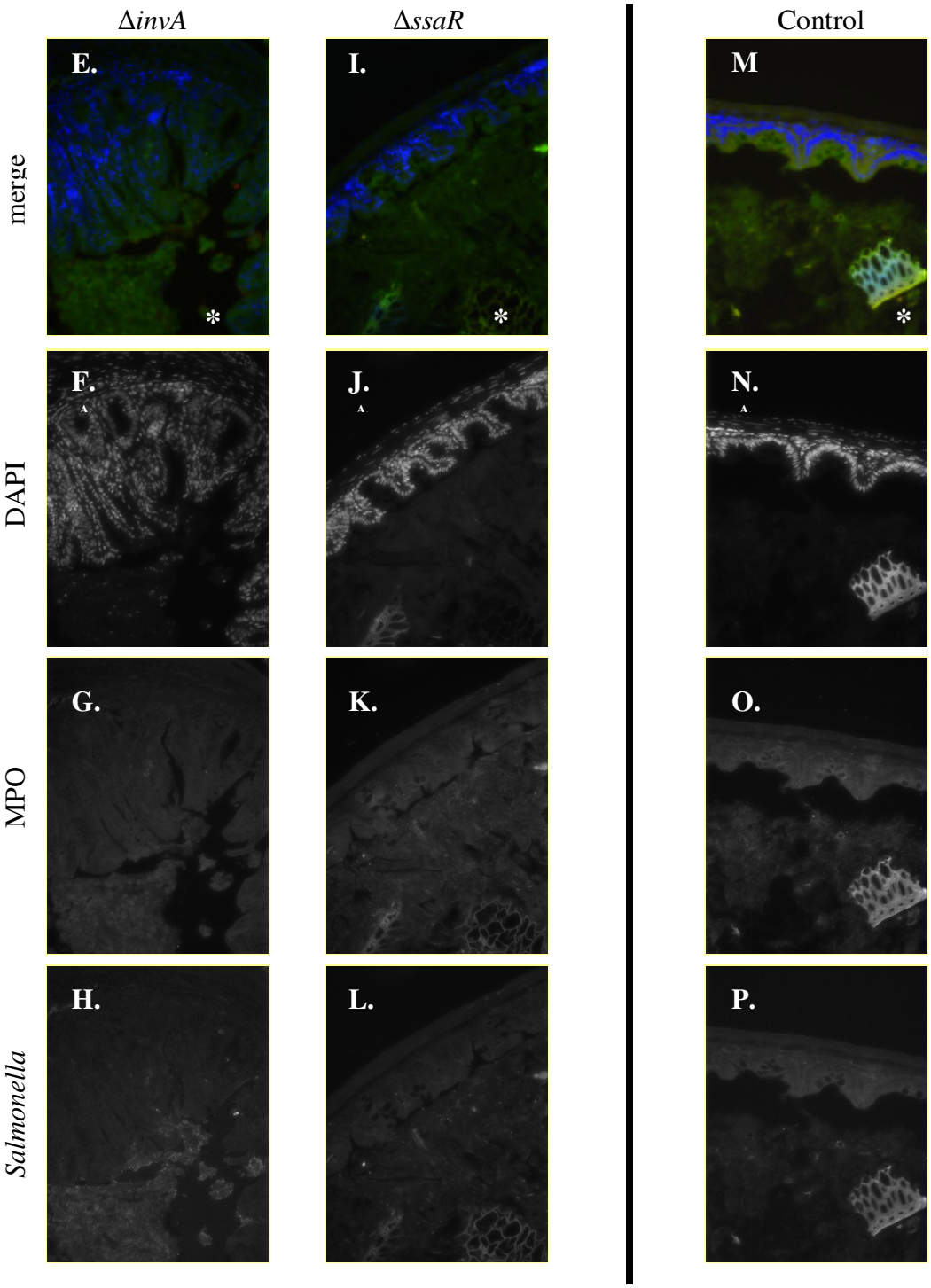

Δ*invA*
Δ*ssaR*
Control
merge
DAPI
MPO
Salmonella
E.
I.
M
F.
J.
N.
G.
K.
O.
H.
L.
P.

Figure 4.6 Murine infection with WT, but not Δ*invA* and Δ*ssaR S*. Typhimurium results in cecal infiltration of neutrophils

Mice were treated with 450 mg/L streptomycin in drinking water for 2 days. Following antibiotic withdrawal, mice were infected with $2.7X10^8$ cfu of specified *S*. Typhimurium strain. 2 to 8 ice per group were used. Formalin-fixed, paraffin embedded cecum sections were stained for nuclear DNA (with DAPI [4',6'-diamidino-2-phenylindole]; blue in merge), neutrophil myeloperoxidase (green in merge), and *Salmonella* LPS (red in merge). Ceca of the mice infected with WT *S*. Typhimurium exhibit positive staining for neutrophils starting at day 3 post infection and until the end of the infection time course (granular staining surrounding nuclei in the lumen; A-D in day 3, 4, and 5 panels). Ceca of the mice infected with each of the mutant strains did not stain positive for neutrophils for the whole duration of the infection time course (E-H and I-L in day 1, 3, 4, 5 and 6 panels). All of the infected tissues stained positive for *Salmonella* throughout the infection time-course. Asterisks indicate lumen in merge images; arrows indicate positive staining of myeloperoxidase granules. Images are pseudocolour and are shown at 200X magnification.

persisting until day 5, at which point all WT-infected mice were sacrificed (Figure 4.6, Days 1-5, A-D). The presence of neutrophils correlated with the increase in pathological indices and microbiota perturbations observed during WT *S*. Typhimurium infection. In contrast, no positive neutrophil staining was observed in the lumina of the mice infected with either Δ*invA* or Δ*ssaR S*. Typhimurium strains for the duration of the infection (Figure 4.6, Days 1-6, E-H, I-L).

4.4 Discussion

As the role of the resident intestinal microbiota in the progression of enteric infections gains increasing attention, convenient models for dissecting the particulars of the host-pathogen-microbiota interactions are needed. We present here a low dose streptomycin pre-treatment model of murine salmonellosis that retains unaltered numbers of the host microbiota while supporting the establishment of WT *S*. Typhimurium-induced intestinal inflammation (Sekirov et al., 2008). Using this model we investigated the contribution of *Salmonella* pathogenicity islands 1 and 2 to induction of murine typhlitis and microbiota perturbations over 6 days of infection. While previously described murine model of intestinal salmonellosis (Barthel et al., 2003) provided a great tool for the in vivo assessment of the contribution of *S*. Typhimurium virulence factors and host defenses to the progression of the infection, the massive dose of

streptomycin used to pre-treat the mice in this model greatly decreased the numbers of the resident microbiota, thus precluding a fair assessment of its role in the infection progression. The use of the model system presented here will allow a better assessment of the role of the microbiota in the infection progression, as well as of the impact of *S*. Typhimurium on the microbiota.

Upon assessment of *S*. Typhimurium colonization dynamics we noted that all three strains colonized the murine ceca equally successfully as early as day 1 p.i. (Figure 4.1). Colonization by the WT and Δ*invA* strains remained unchanged over the time course of infection, while Δ*ssaR* strain showed a significant decline in numbers at the later time points. These colonization dynamics are similar to the situation in the high streptomycin dose pre-treatment model (Coburn et al., 2005; Coombes et al., 2005a).

Upon assessment of the intestinal pathology induced in our model, some differences were noted compared to the previously described high dose streptomycin pre-treatment model. Although previous in vivo work indicated that early conspicuous murine typhlitis (at day 2 post infection) can be induced by SPI-1 alone (Coburn et al., 2005; Coombes et al., 2005a; Hapfelmeier et al., 2004), we now show that in the presence of adequate numbers of the intestinal microbiota SPI-1 is insufficient to induce significant inflammatory changes in the murine cecum (Figure 4.2). The presence of SPI-2 alone was also shown to be insufficient to induce intestinal inflammation early in infection, similar to the situation in high dose streptomycin pre-treatment model, with only the WT strain causing significant typhlitis at day 3 p.i. In the previously used model, WT *S*. Typhimurium-induced typhlitis reached its maximum intensity by 48 hrs p.i., persisting at the same level until day 5 p.i. (Coburn et al., 2005; Coombes et al., 2005a). In contrast to that, we have found that in the presence of high numbers of host

microbiota, inflammation took a longer time to develop, reaching significantly high intensity at day 3 p.i., and continuing to mount until 4 days p.i., at which point it reached a plateau.

When the relative contribution of SPI1 and SPI2 to the intestinal inflammation was examined at the later time points, it was noted that while infection with Δ*ssaR S.* Typhimurium failed to induce notable typhlitis for the duration of the infection, infection with ΔinvA strain resulted in significant inflammatory changes at days 5 and 6 p.i. (Figure 4.2). A similar contribution of SPI2 to later stages of the intestinal inflammation was also noted in the high dose streptomycin pretreatment model in some studies (Coburn et al., 2005; Coombes et al., 2005a), while others have demonstrated that both SPI1 and SPI2 alone are able to induce significant typhlitis at the later time points of infection (Hapfelmeier et al., 2005). This apparent disparity could be explained by the use of different SPI2 mutant strains in the aforementioned studies; the mutant strain used in our study was in the same apparatus component as that used by Coburn and colleagues and Coombes and colleagues. The use of high doses of other antibiotics to reduce the intestinal microbiota of mice has rendered them equally susceptible to *S.* Typhimurium-induced intestinal inflammation as the use of streptomycin, with SPI1 mutant strain inducing intestinal pathology at later stages of infection (Woo et al., 2008).

In conclusion, in this murine model of intestinal salmonellosis, *S.* Typhimurium colonization dynamics mimic those previously described in the high dose streptomycin pre-treatment model, while there are some unique features of intestinal inflammation. SPI1 was shown to be insufficient for the induction of significant intestinal inflammation throughout the time-course of infection, while SPI2 provoked significant typhlitis starting at day 5 p.i. The presence of both SPI1 and SPI2 was necessary to produce typhlitis of maximum intensity. The observed disparities in the presence and intensity of typhlitis between this model and the high

dose streptomycin pre-treatment model are likely attributable to the presence of high numbers of indigenous microbiota in our model system, highlighting its contribution to the progression of the infection.

The majority of the studies in the high dose streptomycin pre-treatment mouse model focused on the role of either the bacterial or host factors in the progression of the murine intestinal salmonellosis and did not examine the effect of the infection on the microbiota. However, previously published results on the effect of enteric infections on the microbiota have indicated that infection-associated inflammation acts to extensively modify and reduce indigenous microbes (Lupp et al., 2007; Stecher et al., 2007). We sought to evaluate the effect of infection with the three *S.* Typhimurium strains on the total numbers and composition of the murine intestinal microbiota. Interestingly, we found that while inflammatory changes associated with WT infection induced extensive perturbations to the murine microbiota (as previously described in both the low dose (Sekirov et al., 2008) and the high dose streptomycin pre-treatment models (Stecher et al., 2007)), inflammation induced during infection with Δ*invA* strain failed to affect the host microbiota in the same way (Figures 4.3, 4.4, 4.5). Although Stecher and colleagues have previously indicated that they did not see microbiota alterations during infection with a *S.* Typhimurium strain deficient in SPI1 (Stecher et al., 2007), they also reported that in their studies the SPI1 mutant strain did not cause any inflammatory changes in the murine intestine, and consequently no perturbations in the microbiota were expected. Another study in a different mouse strain with no antibiotic pre-treatment indicated that no inflammation was associated with a 3 day long infection with either a SPI1 or a SPI2 *S.* Typhimurium mutant (although mutants in different virulence genes were used), while there occurred partial changes to the microbiota following infection with a SPI2 mutant (Barman et al.,

2008). The reported partial changes were in the proportion of some of the microbiota species and no alterations to the total numbers of the microbiota were observed, while infection with a WT *S*. Typhimurium strain caused a 95% decrease in microbiota at day 7 p.i.

Our finding that infection-induced intestinal inflammation is not always associated with pronounced microbiota perturbations prompted two possible explanations. Either the extent of the inflammation induced by the Δ*invA* strain was not as great as that induced by the WT *S*. Typhimurium, and consequently failed to perturb the microbiota, or there were qualitative differences between the two host inflammatory responses. Although the degree of inflammation induced by Δ*invA* was less than that induced by WT at each day of infection, typhlitis induced by Δ*invA* at day 5 p.i. was not significantly different than WT-induced typhlitis at day 3 p.i. And yet there were no reduction in microbiota numbers, no increase in the proportion of γ-Proteobacteria and no change in microbiota appearance at day 5 p.i. with Δ*invA* strain. Accordingly, we conclude that it was more likely that the differences in the type of inflammatory response induced by the two *S*. Typhimurium strains are responsible for the differences in the effect on microbiota.

S. Typhimurium infection is known to induce a strong inflammatory cellular response (Tam et al., 2008), while the presence of neutrophils, macrophages and dendritic cells occurs in the inflamed murine intestinal tract (Bishop et al., 2008; Coburn et al., 2005; Valdez et al., 2008). Neutrophils have been observed in high numbers in the intestinal tissues of human patients and have been shown to dominate the stool leukocyte population (Tukel et al., 2006). Coburn and colleagues have shown that neutrophils are differentially recruited by the WT, Δ*invA* and Δ*ssaR S*. Typhimurium strains into the ceca of high dose streptomycin pre-treated mice. This prompted us to hypothesize that variations in neutrophil recruitment by the different *S*.

Typhimurium strains might also occur in this model system, potentially accounting for the observed differences in the effects on the intestinal microbiota. Indeed, when neutrophil infiltration into the lumina of the diseased ceca was evaluated, it was observed that while WT infection resulted in a massive neutrophil influx starting at day 3 p.i. and persisting until the end of the infection time-course, no luminal neutrophils were present in the mice infected with the Δ*invA*, or the Δ*ssaR* strains (Figure 4.6).

Neutrophils induce a pronounced oxidative stress that is very damaging to many bacterial species. The presence of reactive oxygen species is particularly detrimental to strictly anaerobic bacteria. Consequently *S.* Typhimurium-induced neutrophil influx could potentially account for the preferential elimination of the anaerobic bacteria from the CFB phylum observed during WT *S.* Typhimurium infection (Sekirov et al., 2008; Stecher et al., 2007). Failure of the Δ*invA* mutant to induce neutrophil influx into the lumina of the infected mice could explain the absence of severe perturbations to the intestinal microbiota despite the presence of significant inflammation.

Presented here is a murine model of enteric salmonellosis that provides a tool for elucidating the details of the host-pathogen-microbiota interactions during *S.* Typhimurium infection. This model has increased our understanding of the relative roles of SPI1 and SPI2 in the generation of intestinal inflammation and points to their contributions to the profound perturbations in the host intestinal microbiota observed during *S.* Typhimurium infection. Our results indicate that while inflammation induced during infection with WT *S.* Typhimurium produces severe disturbances in the host intestinal microbiota, inflammatory changes associated with a Δ*invA* infection fail to generate the same effect. WT *S.* Typhimurium-induced neutrophil infiltration was implicated as one of the potential mediators of microbiota perturbations.

Since this research was initiated, a different murine model of intestinal salmonellosis

(in a FvB mouse strain) not requiring any antibiotic pre-treatment was presented (Barman et al., 2008), which also provides an opportunity for studying all three components involved in enteric infection. We believe, however, that although in this model system pre-treatment with a low dose of an antibiotic is required, it is still of value due to the fact that it is in a C57BL/6 mouse strain, which has been sequenced and consequently offers more opportunities for the study of host component through generation of complete and conditional knockout strains.

CHAPTER 5:
DISCUSSION

The principal theme of this research was to start elucidating the role of the microbiota in the progression of the murine intestinal *S.* Typhimurium infection and to begin uncovering the mechanisms behind the impact that *S.* Typhimurium exerts upon the intestinal microbiota.

When this research was initiated there were very few published culture-independent studies on the effects of antibiotics on the intestinal microbiota. Even fewer examined the effects of microbiota perturbations on host susceptibility to infection. In the first two parts of this research we demonstrate that different antibiotics have distinct, dose-dependent effects on the intestinal microbiota. These effects can at times traverse beyond the expected perturbations in the intestinal environment and into systemic sites. Antibiotic-induced disturbances in microbiota composition predispose the host to increased severity of infectious colitis, as observed in the case of *S.* Typhimurium infection. These findings underscore the importance of a healthy and balanced intestinal microbiota in host response to infection.

The third part of this research begins to uncover the particulars of the pathogen and host factors responsible for the perturbations in the microbiota observed during infection with enteric pathogens. We establish that in the presence of high numbers of host intestinal microbiota a mutant in *Salmonella* pathogenicity island 2 (SPI2) is unable to cause significant typhlitis throughout the time-course of the infection, while a SPI1 mutant promotes significant late typhlitis. The presence of both SPI1 and SPI2 is shown to be necessary to induce inflammatory changes of maximum severity. Upon examination of the effect of infection with the three *S.* Typhimurium strains on the microbiota, it is shown that not all cases of intestinal inflammation are associated with extensive perturbations to the intestinal microbiota, as inflammation induced by infection with a SPI1 mutant fails to trigger the same microbiota perturbations as those observed during WT infection. Neutrophils, which are differentially recruited by the WT and

SPI1 mutant strains, are implicated as one potential agent detrimentally affecting the host microbiota.

A clear understanding of the interactions occurring in the host-pathogen-microbiota axis is necessary for the advancement of our theoretical knowledge of microbial pathogenesis and its practical application to the design of prophylactics and therapeutics. Research presented herein adds to the current pool of knowledge on the progression of the enteric salmonellosis and opens up new avenues for future explorations.

5.1 Intestinal inflammation and microbiota

Salmonella-induced murine colitis and associated microbiota perturbations were discussed in chapters 3 and 4 of this thesis. The association of intestinal inflammation and microbiota perturbations has been described in a number of disease states (Lupp et al., 2007; Sartor, 2008; Stecher et al., 2007; Whary et al., 2006) and so the cause and effect aspects of this relationship warrant further discussion. As microbiota changes associated with acute *Salmonella* colitis are the focus of this thesis, the discussion will concentrate on the involvement of the innate immune system in inflammation and microbiota perturbations.

5.1.1 Mechanisms of intestinal inflammation

Intestinal inflammation, which is the centerpiece of IBD and infectious enterocolitis, can develop through a number of pathways, all leading to the occurrence of characteristic mucosal injury. Immune cell-mediated inflammatory changes have long been recognized and appreciated, and in recent years the contribution of the intestinal epithelium itself to the initiation of the inflammatory response has been described.

Immune cell-mediated inflammation

There are a number of inflammatory cells residing in the intestinal mucosa. Macrophages, DCs and neutrophils play a central role in modulation of colitogenic host response in reaction to an infection or other noxious stimulus.

DCs are a very heterogeneous cell population that resides in a number of intestinal lymphoid tissues, and in the lamina propria of the intestinal tract, where they send out projections into the intestinal lumen. They play a central role in the induction of oral tolerance

through their interactions with regulatory and effector T-cells. Intestinal DCs normally contribute to the prevention of excessive immune response to microbiota- and food-derived antigens. However, a dysregulation of intestinal DCs function was implicated as a factor in IBD pathogensis. Although details of the exact mechanisms underlying their role in this process are still largely elusive, abnormal cytokine production and T-cell priming are contributing factors (Niess, 2008).

Intestinal macrophages are phenotypically different from their systemic counterparts and are located in the lamina propria of the intestine, just below the epithelium. The main difference between systemic and intestinal macrophages lies in the hyporesponsiveness of the latter to normal inflammatory stimuli, which allows them to control the resident bacterial population without the coincidental induction of a damaging inflammatory response. However, in an injured epithelium intestinal macrophages show increased respiratory burst and production of pro-inflammatory cytokines leading to tissue damage and recruitment of additional inflammatory cells (Platt and Mowat, 2008).

Neutrophils are active participants in the pathogenesis of many diseases where inflammation is the key feature, including infectious enterocolitis. They infiltrate the mucosa and lumina of the diseased tissues in response to a stimulus from the assaulted intestinal epithelium, such as IL8 and pathogen-elicited epithelial chemoattractant (PEEC), or hepoxilin A_3 (Mrsny et al., 2004). N-formylated peptides released by bacteria are also potent neutrophil chemoattractants. Once recruited, neutrophils employ several strategies to combat the intruding microorganisms, such as phagocytosis, induction of oxidative stress, and the release of inflammatory mediators and anti-microbial substances. The passage of massive numbers of neutrophils through the epithelial tight junctions was implicated in the pathogenesis of epithelial erosion. Conversely,

disruption of tight junctions contributes to the translocation of bacterial products and further stimulation of neutrophil recruitment (Reaves et al., 2005).

Epithelium-mediated inflammation

An active role for the intestinal epithelium in the generation of an inflammatory response was proposed after TLR expression was found along the length of the intestinal tract. While in a normal state TLRs remain relatively unresponsive to the myriad of bacteria overlying the mucosa, in a state of infection, injury or another assault they initiate a cascade of events that contribute to the induction of inflammatory host response. TLR4 (agonist is LPS), TLR2 (agonist is peptidoglycan), TLR5 (agonist is flagellin) and TLR9 (agonist is unmethylated DNA) are mainly implicated in the induction of inflammation by activation of pro-inflammatory cytokine secretion, which promotes recruitment of inflammatory cells, as well as increasing apoptosis and impairing repair processes in the epithelium (Gribar et al., 2008; Reaves et al., 2005).

5.1.2 Inflammation-mediated assault on the intestinal microbiota

A role for neutrophils

Significant microbiota depletion during inflammatory response has been shown to occur during *Citrobacter rodentium* and *S.* Typhimurium infections, and intestinal inflammation elicited by both these pathogens is characterized by neutrophil infiltration (Khan et al., 2006; Tukel et al., 2006). Neutrophil infiltration into the colonic mucosa is also the hallmark of IBD (Naito et al., 2007) and is a prominent feature of dextran sodium sulfate (DSS) – induced colitis used to model IBD in laboratory animals (Stevceva et al., 2001). A decrease in the microbial population was observed both in IBD patients (Sartor, 2008) and in murine DSS-induced colitis

(Lupp et al., 2007). Oxidative stress induced by neutrophils would be deleterious to most microorganisms, especially the obligate anaerobes that represent the majority of the intestinal microbiota population; consequently a role for neutrophils in inflammation-associated microbiota depletion was hypothesized.

No neutrophil influx was expected during infection with the SPI2 mutant in our model system, as high numbers of the indigenous microbiota (albeit of modified composition) protect the mice from instigation of significant colitis in response to *S.* Typhimurium infection. The apparent absence of luminal neutrophils during infection with the SPI1 mutant, which did produce intestinal inflammation, was more conspicuous. However, the absence of SipA, a SPI1 effector that has been shown to induce neutrophil influx across the intestinal epithelium (Lee et al., 2000) and is not secreted by the Δ*invA S*. Typhimurium strain, could account for this observation. Interestingly, invasion of the intestinal epithelium was not required for transepithelial signaling to neutrophils to occur in *Salmonella* infection of epithelial cell monolayers (McCormick et al., 1995), and so neutrophil recruitment during *S*. Typhimurium infection might be induced prior to initiation of extensive interactions with the intestinal epithelium. Consequently a model emerges in which *S*. Typhimurium provokes recruitment of neutrophils that induce oxidative stress in the infected lumen, inadvertently killing a large population of indigenous anaerobic bacteria. *S*. Typhimurium, however, is better equipped to survive this attack and invade the intestinal epithelium following the reduction in colonization resistance from the intestinal microbiota (Figure 5.1). In this manner, neutrophil recruitment appears to be directly beneficial to *S.* Typhimurium, illuminating additional facets of the question "who drives the neutrophil influx during enteric salmonellosis?" (Tukel et al., 2006).

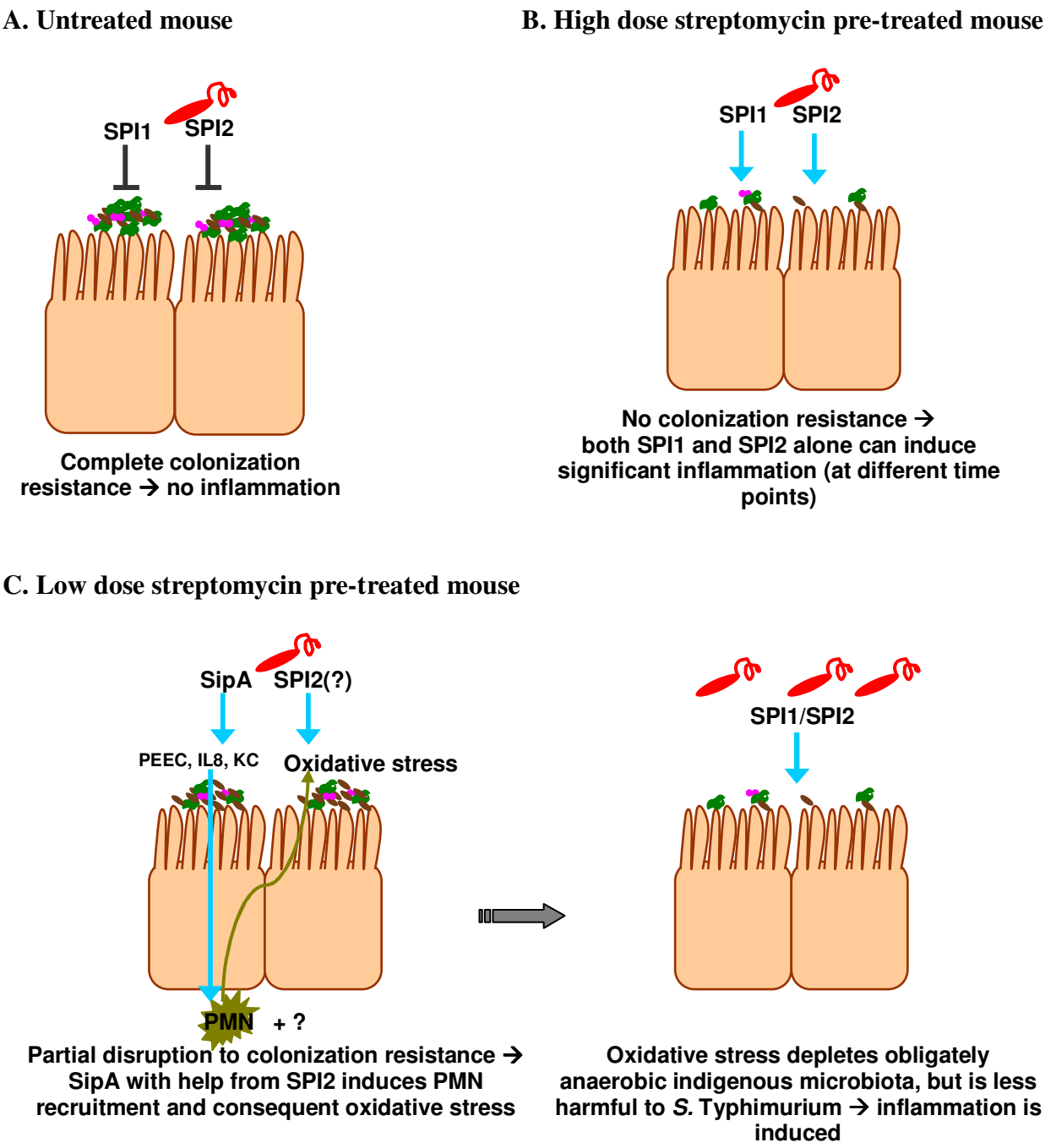


Figure 5.1 Proposed models for host microbiota-*S.* Typhimurium interactions during murine infection
A. During infection of an untreated mouse, *S.* Typhimurium encounters complete colonization resistance which prevents it from successfully employing either SPI1 or SPI2 to induce colitis. B. During infection of a mouse pre-treated with a high dose of streptomycin, which obliterates over 90% of the microbiota, *S.* Typhimurium encounters no colonization resistance and can successfully induce colitis by either SPI1 or SPI2 during different time points of the infection progression. C. During infection of a mouse pre-treated with a low dose of streptomycin, with a dense microbial community of a modified composition, *S.* Typhimurium experiences a partial colonization resistance, which can then be successfully overcome through neutrophil recruitment and possibly other factors. Both SPI1 and SPI2 are needed to induce significant colitis of maximum severity.

The question remains, however, as to why a SPI2 mutant that has a functional SPI1 T3SS and is able to secrete SipA induced neither the neutrophil recruitment, inflammation or microbiota depletion early in infection. This observation demonstrates that some features of SPI2-associated virulence contribute to SipA-induced neutrophil recruitment in a densely colonized intestinal tract. Additionally, although neutrophils appear to play a role in microbiota depletion associated with *S*. Typhimurium infection, they are likely not the sole agent driving this effect. Additional aspects of *S*. Typhimurium-induced host response must, therefore, be involved in microbiota perturbations during infection. And while SPI1 T3SS might be the principal instigator of microbiota perturbations, having both SPI1- and SPI2- associated virulence strategies must be necessary for the production of a successful disturbance. The fact that SPI2 is expressed while *S*. Typhimurium is still found in the intestinal lumen (Brown et al., 2005) contributes to this hypothesis. The final model stemming from these observations is SPI1-induced neutrophil recruitment, with the help of yet unknown contributing factors from SPI2, playing an important part in the elimination of the highly oxygen-sensitive indigenous microbiota, while the more oxygen-resistant *S*. Typhimurium remains relatively unharmed and is then able to proceed with its virulence strategy. In the absence of SPI2, SPI1 alone is unable to promote the host response required for the ultimate depletion of the intestinal microbiota, cannot proceed with its virulence strategy and ultimately its clearance from the infected host begins. In the absence of SPI1, SPI2 can still persist in the infected intestine in high numbers and initiate inflammatory changes in the intestine later on in infection through different mechanisms. The induced inflammatory response, however, does not result in the depletion of the indigenous intestinal microbiota. In the presence of both SPI1 and SPI2 (i.e. the WT strain), both

inflammatory pathways converge to promote the host microbiota depletion and the intestinal inflammation of maximal severity.

A role for the intestinal epithelium

It has been shown that the two T3SS of *S.* Typhimurium promote distinct components of the intestinal inflammatory changes: SPI1-associated pathology was characterized by diffuse cecal inflammation that proceeded independently of MyD88 signaling, while SPI2 induced focal mucosal inflammation that required MyD88 (Hapfelmeier et al., 2005). These findings were observed in the high dose streptomycin pre-treatment model, in which a SPI2 *S.* Typhimurium mutant is able to induce significant typhlitis early in infection. In our low dose streptomycin pre-treatment model a SPI2 mutant was unable to induce significant intestinal inflammation at any of the assessed time points, while a SPI1 mutant induced significant, albeit less severe than WT, typhlitis late in infection. The inability of a SPI1 mutant to produce typhlitis of the same magnitude as that seen in WT infection underscores the need for all signaling pathways involved in response to WT *S.* Typhimurium to converge to elicit all of the aspects of the host inflammatory response. The fact that only infection with the WT strain elicited profound microbiota alterations implicates signaling pathways activated by the SPI1 virulence factors as the principal driving force behind the microbiota depletion observed during WT infection. It follows then that MyD88-independent inflammation, associated with the SPI1 T3SS, could contribute to microbiota disturbance associated with *S.* Typhimurium infection. Manipulation of NFκB signaling pathways by SPI1 effectors delivered to the cytosol of the intestinal epithelial cells could result in the induction of inflammatory changes detrimental to the intestinal microbiota. Alternatively, epithelial TLRs and NLRs whose function is not dependent on the

MyD88 adaptor protein (such as TLR4, which can activate downstream pathways through TIRAP and TRAM adaptor proteins (Horng et al., 2001; Yamamoto et al., 2003) could be involved in the induction of the detrimental inflammatory response. SPI1-mediated disruption of epithelial tight junctions (Boyle et al., 2006) could also contribute to microbial translocation and promote further neutrophil recruitment.

5.2 Microbiota contributions to host metabolism

It is now well accepted that the intestinal microbiota provide a significant contribution to host metabolism (Martin et al., 2007; Nicholson et al., 2005). The input of the microbial residents to certain metabolic pathways can even surpass that of the host (Gill et al., 2006), which is not surprising considering that the microbiota greatly outnumber the host cells in the gastrointestinal tract. Different microbiota components have different metabolic abilities, with a consequent impact on the pool of micrometabolites found in the intestinal tract. Antibiotic-induced microbiota perturbations demonstrated in Chapter 2 of this thesis were shown to promote host susceptibility to an invading pathogen (Chapter 3). One of the possible mechanisms for the induction of this increased susceptibility is a change in host metabolism associated with microbiota perturbations (Figure 5.2).

5.2.1 Antibiotics and nutrient absorption

The use of antibiotics is rampant in our society while the full complexity of their effects upon the host is not clearly understood. Yet these effects clearly extend beyond the intended clinical consequences. It has been shown that vancomycin treatment (albeit at doses considerably higher than the ones used clinically or in this study), exerts a profound impact on

host metabolism (Yap et al., 2008), highlighting its interrelationship with the gut microbiota. For instance, fecal excretion of various dietary compounds normally metabolized by the gut microbial constituents was noted. Thus antibiotic treatment resulted in a reduction of nutrient bioavailability to the host. This could impact on the homeostasis of colonic epithelium, as well as that of remote organ systems. Microbiota perturbations associated with the use of other antibiotics are likely to present with similar consequences. These antibiotic-induced disturbances in host metabolism are bound to influence the overall host well-being and might impact on the general resistance or susceptibility to invading pathogens, involving both the local and the systemic aspects of the disease.

Gut microbiota have been implicated in the absorption of cholesterol (Gustafsson et al., 1975), which is the major component of lipid rafts (Simons and Ikonen, 1997). It has been shown that dietary lipids contribute to raft composition and stability (Puskas and Kitajka, 2006), and consequently microbiota might also be involved in the process through its role in lipid absorption. Along with many other functions, lipid rafts have long been implicated as portals for intracellular pathogen entry into host cells (Rosenberger et al., 2000), while *Salmonella*, *Shigella* and enteropathogenic *E. coli* (EPEC) have been shown to require cholesterol for successful T3SS effector translocation (Hayward et al., 2005). Antibiotic-induced alterations in microbiota composition might impact on cholesterol metabolism, as well as the composition and stability of lipid rafts, thus modifying host susceptibility to infection with pathogens whose entry/virulence strategy is reliant on cholesterol.

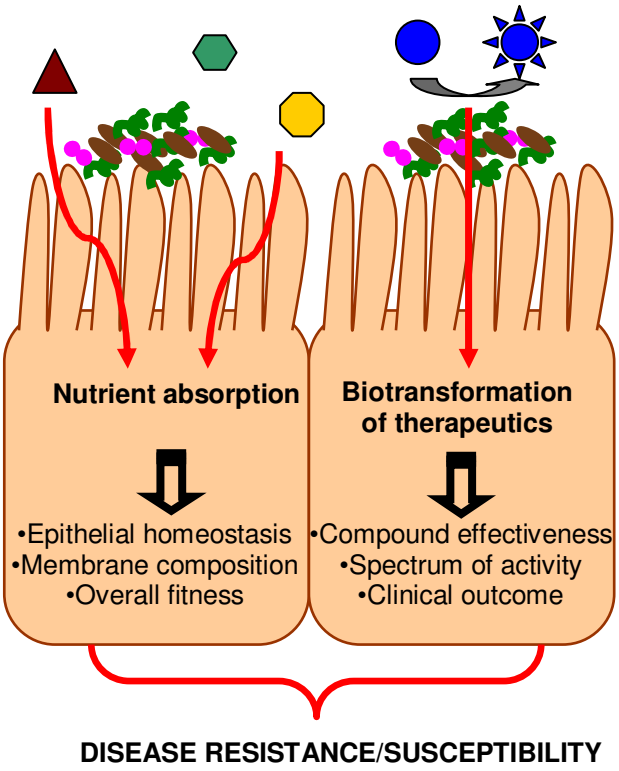


Figure 5.2 Microbiota contributions to host metabolism and their effects on disease susceptibility
Microbial constituents are very important for host nutrient absorption, affecting caloric bioavailability and homeostasis at the intestinal mucosa. In addition, members of the microbial consortium metabolize incoming xenobiotics, including therapeutics, affecting their effectiveness and spectrum of activity. The combined outcome of these functions is liable to affect host susceptibility to disease, as well as response to treatment.

5.3 A synthesized view of host-microbiota-pathogen tripartite relationship

Gastrointestinal infection is an intricate process of multifaceted interactions between three main components – the host, the pathogen and the host's intestinal bacterial community. These interactions are ongoing and continuously evolving throughout the infection process, either leading to the resolution of infection, a chronic pathogen colonization of the infected individual, or perpetuating a vicious cycle culminating in the demise of the host (Figure 5.3). Resolution of details contributing to this byzantine web of interfaces is still in its infancy, but its thorough understanding is required to provide us with proper tools for control of gastrointestinal infections.

A number of factors from each of the three participants contribute to the eventual outcome of the gastrointestinal infection process. The host's overall fitness, genetic make-up and

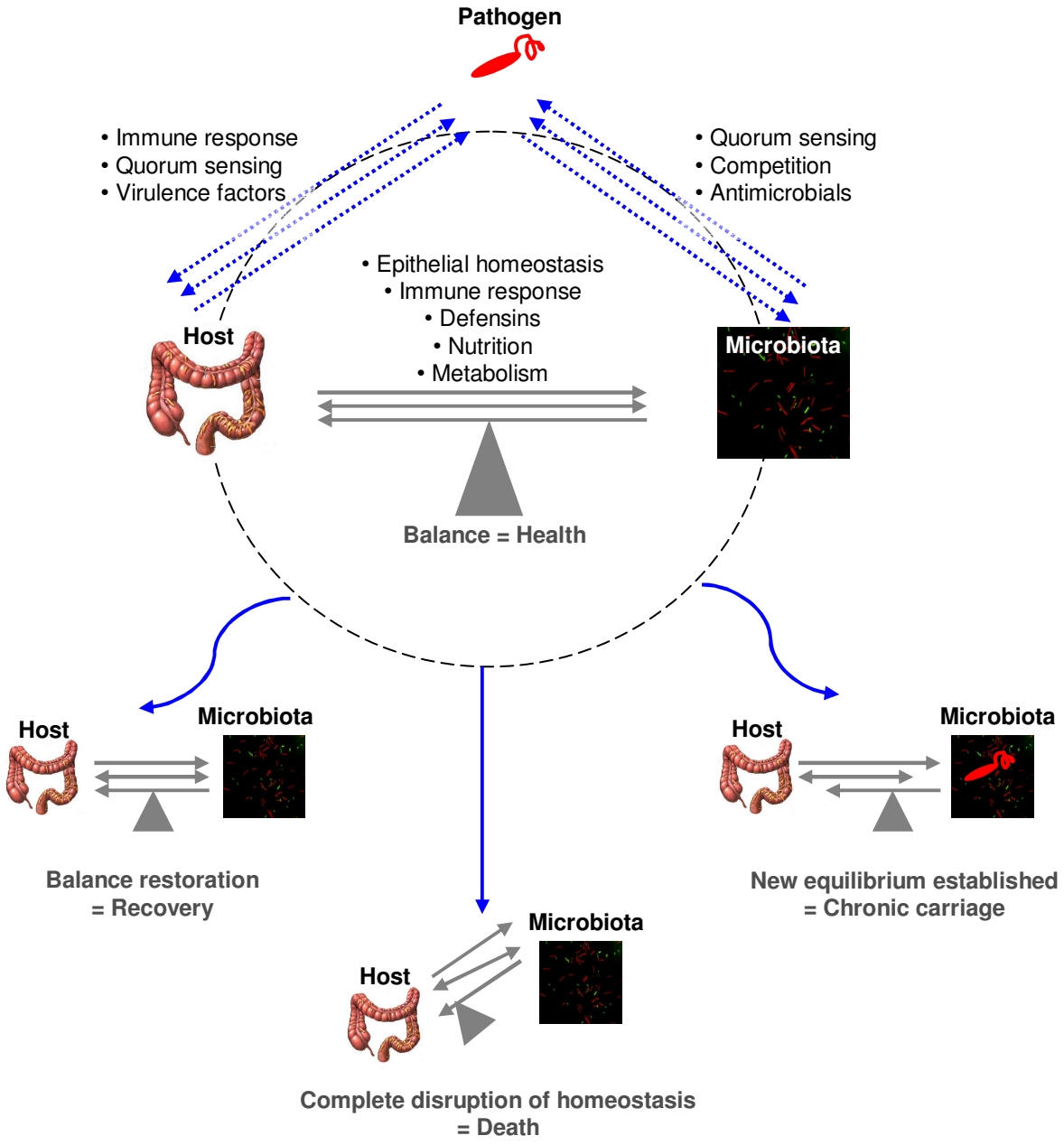


Figure 5.3 Host-microbiota-pathogen interactions and outcomes
A healthy host exists in a state of balance with his intestinal microbiota, wherein the microbiota contribute to normal host immunity, homeostasis at the intestinal mucosa and metabolism, and the host provides it with a convenient habitat, while keeping the microbial numbers in check. An invading pathogen interacts with both the host and the microbiota, enacting a disturbance in the host-microbiota balance through its virulence strategy. If both the host and the indigenous microbiota are resilient, the balance is restored with a consequent recovery at the intestinal mucosa. If the host-microbiota balance is disturbed too severely leading to the complete disruption of intestinal homeostasis, death of the host will likely follow. If the pathogen carves out a niche for itself within the indigenous microbial community, a new equilibrium between the host and the microbiota will eventually establish, resulting in chronic carriage of the pathogen.

the choice of therapy to treat the infection will contribute to the generation of the immune response. The individual variations in microbiota composition and stability, and any pre-existing perturbations to it will contribute to colonization resistance, resilience of the intestinal mucosa, and will also affect the generation of the immune response. The virulence factors carried by the pathogen will determine its interactions with the host, the microbiota, and its ability to promote a detrimental to microbiota host response. The initial stability of the intestinal microbiota population, as well as its ability to recover to steady state following an insult, are underlying the progression of many of the interactions happening between the players of this "ménage à trois", positioning it at the corner stone of the host's health. In view of this, future research in this area will greatly contribute to our knowledge of enteropathogenesis.

5.4 Future Directions:

Research presented herein underscores the importance of a balanced intestinal microbial community in the progression of intestinal salmonellosis and by analogy in infectious enterocolitis in general. The mounting interest in this area of research has developed only recently and many possibilities for exploration are available.

1. The effects of the most commonly used therapeutics on the intestinal microbiota

These studies demonstrate the importance of assessing the effect of antimicrobial agents on the indigenous host microbiota; however the effects of only three example antibiotics are evaluated. Future studies should focus on assessing the effects of additional commonly used antimicrobials, including antifungal agents and potentially antivirals, as both fungi and viruses

have been shown to contribute to the make-up of the intestinal microbial community. The effort, however, should not be limited to only antimicrobials, as additional commonly used medicines, including herbal remedies, are likely to interfere with the intestinal microbial community, either through direct interactions or through modulation of host immune status, hormonal and nutritional balance, etc. The advance of sequencing technologies and development and improvement of additional culture-independent techniques, such as quantitative PCR, RFLP and microarrays, would be able to facilitate a detailed evaluation of the effects of many agents upon the intestinal microbial community.

Clear understanding of the interactions of the commonly used therapeutic agents with the host microbiota will help to ameliorate drug regiments by addressing any potential post-therapy imbalances in the microbial community through the use of pre-, pro-, and syn-biotics. Maintenance of a balanced microbial community has the potential to avert complications associated with many therapies, antimicrobial and otherwise, such as secondary infections, overgrowth of opportunistic members of the microbiota, etc.

2. Host factors involved in the infection-associated microbiota perturbations, during intestinal salmonellosis and other infectious colitis; the effects of *S*. Typhimurium on the intestinal microbiota in chronic enteric salmonellosis

In recent years the contribution of the indigenous microbial members to the tripartite interaction of the host, the microbiota and the invading pathogen has received more and more attention and was attributed an increasingly greater importance. It is imperative to conduct detailed studies on the interactions of the commonly encountered pathogens with the microbiota in order to elucidate the mechanisms behind these interactions and how they translate into the

detrimental effects on the host. Detailed information on the interactions between the host microbiota and an invading pathogen would also be indispensable for accurately devising mathematical models describing them (Kim et al., 2007).

Since the proposed low dose antibiotic model of intestinal salmonellosis allows one to focus on the interactions between the invading *S.* Typhimurium and the intestinal microbial community, it provides an opportunity to answer many questions regarding the intestinal phase of the *S.* Typhimurium-induced disease. Extending the low dose model into the natural resistance-associated macrophage protein 1 (Nramp1) positive mice (which are able to limit systemic *S.* Typhimurium infection and are a model of chronic salmonellosis), would allow evaluation of the interactions between *S.* Typhimurium and the microbiota at later time points of infection, and how these later interactions contribute to the progression of the intestinal inflammation. Studies involving additional mutant *S.* Typhimurium strains would allow us to elucidate in more detail the contribution of different *S.* Typhimurium virulence factors to the establishment and maintenance of host inflammatory response.

It would also be possible to evaluate the contribution of various host factors to the progression of the intestinal salmonellosis through the use of genetically modified mouse strains, especially conditional intestinal knock-outs – the low dose antibiotic model would allow to study the contribution of the host factors in the presence of a dense microbial community, a setting which is more akin to the human intestinal salmonellosis than the high dose antibiotic model.

3. The role of different microbiota constituents in quorum-sensing interactions with *S.* Typhimurium

Revealing the identity of the microbiota constituents involved in quorum sensing interactions with *S.* Typhimurium (and with other pathogenic organisms) will improve our knowledge of such infection processes as colonization and persistence.

Infections of mice pre-treated with various antibiotics with specific spectra of activity with WT *S.* Typhimurium and *S.* Typhimurium deficient in quorum sensing would allow assessment of which bacterial species are important in the establishment of *S.* Typhimurium colonization. Extending these studies into Nramp1 positive mice will evaluate the contribution of different microbiota constituents to the establishment of persistent *S.* Typhimurium intestinal infection.

Knowing which microbial members interact with *S.* Typhimurium during infection can lead to the design of new pre-, pro-, and syn-biotics. Signaling pathways modified through quorum sensing mechanisms will likely be involved in pathogenesis and might provide targets for the design of antimicrobial therapies.

4. Metagenomic assessment of microbiota perturbations during *S.* Typhimurium infection and other infectious colitis

Due to time constraints imposed on these studies it was only possible to assess the broad changes in microbiota populations using culture-independent techniques. However, the many new advances in sequencing technologies would permit a more thorough and detailed assessment of *S.* Typhimurium-associated microbiota changes, as well as changes occurring during infection with other enteric pathogens. Techniques such as RFLP (restriction fragment length

polymorphisms) and DDGE (denaturing detergent gradient gel electrophoresis) would allow convenient comparisons of changes in overall microbial communities pre- and post-infection. DNA arrays, as well as carefully designed probes and primers for FISH and quantitative PCR would provide an opportunity to characterize variations in specific groups of bacteria at different phylogenetic levels. Detection of changes in all the members of the microbial community, as well as alterations in microbial gene expression would be made possible with the use of sequencing technologies. Innovations in both the culture-independent techniques used to assess the complex microbial communities, as well as in mathematical models and softwares available for analysis of metagenomic data (Frank and Pace, 2008; Kurokawa et al., 2007), provide an opportunity to ask and answer unique and fascinating questions regarding the role of microbiota in enteric infections.

CHAPTER 6:
MATERIALS AND METHODS

6.1 Bacterial strains used in this study

Salmonella Typhimurium SL1344 (WT) (Hoiseth and Stocker, 1981), *invA* mutant (invA::kan SB103, defective in a structural gene of the SPI-1 type 3 apparatus; 'Δ*invA*') (Galan and Curtiss, 1991) and *ssaR* mutant (ΔssaR, in frame deletion of a structural gene of the SPI-2 type 3 apparatus; 'Δ*ssaR*') (Brumell et al., 2001) were grown overnight shaking (200rpm) in Luria-Bertani broth (LB) with 100ug/ml streptomycin overnight (ON).

6.2 *In vivo* experiments

6.2.1 Mice

Inbred C57Bl6 female mice (Jackson Laboratory, Bar Harbor, Maine, USA) were housed in the animal facility at the University of British Columbia in direct accordance with guidelines drafted by the University of British Columbia's Animal Care Committee and the Canadian Council on the Use of Laboratory Animals and infected at 4.5-5.5 weeks of age. Mice were fed a standard sterile chow diet (Laboratory Rodent Diet 5001, Purina Mills, St. Louis, Missouri) ad libitum throughout the experiments.

6.2.2 Mouse infections

Mice were treated with streptomycin (Sigma) at 150, 300 and 450 mg/L and vancomycin (Sigma) at 50 and 100 mg/L for 2 days in drinking water. As mice drink, on average, 3 ml of liquid per day (Bing & Mendel, 1931), the average consumed dose of antibiotics was calculated (Table 2.1). Control mice were given sterilized not acidified drinking water without antibiotics. After 2 days the antibiotics were withdrawn and mice were infected with the appropriate strain of *S*. Typhimurium at $2.7x10^8$ cfu/mouse by oral gavage. Uninfected control mice were given 100µl

of sterile LB broth. At an indicated time point post-infection the mice were euthanized by CO_2 asphyxiation and tissues were harvested aseptically for further evaluation.

6.2.3 Tissue collection and culturable bacteria enumeration

Ceca, colons and spleens were collected in 1 ml of sterile PBS on ice and homogenized with a MixerMill 301 (Retsch, Newtown, PA, USA). Serial dilutions of the homogenates were plated on LB or Xylose Lysine Deoxycholate (XLD) (Oxoid) agar plates containing 100 µg/ml streptomycin to enumerate *S.* Typhimurium colonization. Serial dilutions were plated on LB, McConkey (Oxoid), Kanamycin Esculin Azide (EMD chemicals) and ROGOSA (Oxoid) agar plates to enumerate colonization by culturable aerobes, *Enterobacteriaceae*, *Enterococci*/D-*Streptococci,* and *Lactobacilli* respectively. All plates, except ROGOSA, were incubated aerobically at 37^{o}C ON; ROGOSA plates were incubated in anaerobic chambers with GasPack Plus anaerobic system envelopes (BD) for 2 days.

6.3 Histology

6.3.1 Histopathology

Cecal tips were fixed in 10% neutral buffered formalin overnight and then placed into 75% ethanol. Fixed tissues were embedded in paraffin and cut into 5 µm sections by Wax-it Histology Services (Vancouver, BC, Canada). Tissues were stained with hematoxylin and eosin (H&E) using standard techniques by Wax-it Histology Services and UBC Histology Laboratory. Pathological scores were assigned as previously described (Coburn et al., 2005). Briefly, scores were assigned to indicators of pathology in the lumen (presence of necrotic epithelial cells and inflammatory infiltrate), surface epithelium (presence of regenerative changes, desquamation,

ulceration, neutrophils), mucosa (presence and abundance of crypt abcesses, presence of mucinous plugs and granulation tissue) and submucosa (presence and extent of mononuclear and neutrophil infiltration, edema).

Pathology images were taken using Zeiss Axioskop 2 microscope.

6.3.2 Immunofluorescence

Cecal tips were fixed in 10% neutral buffered formalin overnight and then placed into 75% ethanol. Fixed tissues were embedded in paraffin and cut into 5 µm sections by Wax-it Histology Services (Vancouver, BC, Canada).

Tissue sections were deparaffinized in xylene and rehydrated by successive incubations in 100% ethanol, 95% ethanol, 70% ethanol and water. Following rehydration tissue sections were subjected to antigen retrieval by steaming in citrate buffer for 30 min. Subsequently the tissues were blocked with 2% normal goat serum (NGS) in TTPBS-BSA (PBS containing 0.2% Triton X-100, 0.05% Tween 20 and 1% BSA) for 1 hr at room temperature. Primary antibodies (see Table 6.1) were diluted in TTPBS-BSA and incubated overnight at 4^{o}C. Tissue sections were washed with TTPBS and then incubated for 1 hr at room temperature with secondary antibodies (see Table 6.1) conjugated to Alexa 568 and 488. The sections were washed with TTPBS and then mounted in ProlongGold containing DAPI (Invitrogen).

Sections were viewed with an Olympus 1X81 microscope.

Table 6.1 Antibodies used in these studies

Antibody (anti-)	**Species**	**Dilution**	**Source**
Myeloperoxidase (MPO)	rabbit	1:200	Thermo Scientific
Salmonella LPS	mouse	1:1000	BioDesign, Saco, ME
Rabbit AlexaFluor® 488	goat	1:500	Molecular Probes
Mouse AlexaFluor® 568	goat	1:500	Molecular Probes

6.4 ELISAs

Cecum homogenates were centrifuged for 10 min at 13000X*g* twice, supernatants were collected and stored at -80˚C. The levels of TNF-α, MCP-1, IL-6 (BD Biosciences) and KC (R&D Systems), were determined by ELISAs according to manufacturer's instructions. Cytokine levels were normalized to total protein in the samples, as determined by Bradford assay.

6.5 Culture-independent microbiota characterization

6.5.1 Sample collection

A 1:10 dilution of each organ homogenate was stored in 3.7% formalin at 4 °C until use.

6.5.2 SYBR green DNA staining

Between 2 to 40 ul of samples (depending on the total numbers of bacteria in a "pilot" mouse for each group, attempting to achieve 50-200 bacteria/field of view with 40 ul of sample being the maximal usable volume) were diluted in 1 ml PBS and filtered onto Anodisc 25 filters (Whatman International Ltd) with a pore size of 0.2 uM and 2.5 cm diameter. After complete drying, each sample was stained with 0.25 uL SYBR green (Invitrogen) in 100 uL PBS for 15 minutes in the dark. Filters were dried and mounted on glass slides with 30uL antifade solution (50% PBS, 50% glycerol, 0.1% p-phenylenediamine) and viewed with an Olympus 1X81 microscope. Three fields were randomly chosen, the number of cells counted and averaged.

6.5.3 Fluorescent *in situ* hybridizations (FISH)

Between 5 to 100 uL of the samples (volume determined from SYBR results as volume yielding 100-200 bacteria/field of view with 100 ul of sample being the limit of detection) were diluted in 1 mL PBS and filtered onto a polycarbonate membrane filter (Nucleopore Track-Etch Membrane, Whatman International Ltd). The samples were dehydrated on the filter by soaking for 3 minutes each in 50%, 80% and 100% EtOH. After air-drying, the filter was submersed in 100 uL hybridization solution (0.9 M NaCL, 0.1 M TRIS pH 7.2, 30% Formamide, 0.1% SDS) containing 250 ng each of the general EUB338 probe (5' GCT GCC TCC CGT AGG AGT 3') (Amann et al., 1990) fluorescently labelled with Texas Red, and 250ng of either CFB286 probe (5' TCC TCT CAG AAC CCC TAC 3') (Weller et al., 2000) or GAM42a probe (5' GCC TTC CCA CAT CGT TT 3') (Manz et al., 1992) labelled with fluorescein. The hybridization was carried out overnight at 37°C in the dark. After incubation the filters were washed for 15 minutes at 37°C on a shaker first in 15 mL hybridization solution (0.9 M NaCL, 0.1 M TRIS pH 7.2, 30 % Formamide, 0.1 % SDS) and then in 15 mL buffer (0.9 M NaCL, 0.1 M TRIS pH 7.2). After complete drying, the filters were mounted on microscope slides with 10 uL antifade solution (50 % glycerol, 50 % PBS, 0.1 % p-phenylenediamine) and viewed and counted as described above. The percent composition of CFB and γ-Proteobacteria phyla was determined by dividing the numbers obtained for these phyla (CFB286 and GAM42a probes respectively) over the numbers obtained for all Eubacteria (EUB338 probe).

6.5.4 Microbiota appearance evaluation

Cecal tips were fixed in 3% PFA for 3 hrs at RT and then washed three times for 10 min with PBS. Fixed tissues were frozen and sectioned into 5 μm sections by Wax-It Histology Services (Vancouver, British Columbia).

Cryo-sections were permeabilized with 0.1% Triton-PBS for 5 min and then blocked and stained with α- *Salmonella* LPS and DAPI as described in section 6.3.2. Microbiota in tissue lumina were imaged with Olympus 1X81 microscope.

6.6 Statistical analysis

One-way ANOVA with Bonferroni post test or Kruskal-Wallis with Dunn's post test was performed using a 95% confidence interval. Non-parametric t-tests are two-tailed Mann-Whitney tests using a 95% confidence interval. All analyses were performed using GraphPad Prism version 4.0. Differences were considered to be significant with $p<0.05$ or smaller.

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Appendix 1 – List of Publications

Primary research

Sekirov I, Tam NM, Jogova M, Robertson ML, Li Y, Lupp C, Finlay BB. **Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection**. Infect Immun. 2008 Oct;76(10):4726-36.

Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB. **Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of *Enterobacteriaceae*.** Cell Host Microbe. 2007 Aug 16;2(2):119-29.

Asper DJ, Sekirov I, Finlay BB, Rogan D, Potter AA. **Cross reactivity of enterohemorrhagic Escherichia coli O157:H7-specific sera with non-O157 serotypes.** Vaccine. 2007 Nov 28;25(49):8262-9

Arikawa E, Cheung C, Sekirov I, Battell ML, Yuen VG, McNeill JH. **Effects of endothelin receptor blockade on hypervasoreactivity in streptozotocin-diabetic rats: vessel-specific involvement of thromboxane A2.** Can J Physiol Pharmacol. 2006 Aug-Sep;84(8-9):823-33.

Gruenheid S, Sekirov I, Thomas NA, Deng W, O'Donnell P, Goode D, Li Y, Frey EA, Brown NF, Metalnikov P, Pawson T, Ashman K, Finlay BB. **Identification and characterization of NleA, a non-LEE-encoded type III translocated virulence factor of enterohaemorrhagic *Escherichia coli* O157:H7.** Mol Microbiol. 2004 Mar;51(5):1233-49.

Galipeau D, Arikawa E, Sekirov I, McNeill JH. **Chronic thromboxane synthase inhibition prevents fructose-induced hypertension.** Hypertension. 2001 Oct;38(4):872-6.

Reviews

Coburn B*, Sekirov I*, Finlay BB. **Type III secretion systems and disease.** Clin Microbiol Rev. 2007 Oct;20(4):535-49.

Sekirov I, Finlay BB. **Human and microbe: united we stand.** Nat Med. 2006 Jul;12(7):736-7.

* authors contributed equally to this work

Appendix 2 – Animal Ethical Approvals

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A05-1082
Investigator or Course Director: Brett B. Finlay
Department: Michael Smith Laboratories

Animals:

Mice nramp -/- 40
Mice SHIP -/- 43
Mice C3H/He 114
Mice 129/Sv 40
Mice Transgenic mice - various 147
Mice C57/BL6 or BALB/c 80
Mice BALB/c 160
Mice C57/BL6 408
Mice CD1 286

Start Date: October 1, 2005 **Approval Date: August 25, 2008**

Funding Sources:

Funding Agency: Genome British Columbia
Funding Title: The Pathogenomics of Innate Immunity (PI2)

Funding Agency: Genome British Columbia
Funding Title: The Pathogenomics of Innate Immunity (PI2)

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Novel therapeutics that boost innate immunity to treat infectious disease

Funding Agency: The Foundation for the National Institutes of Health
Funding Title: Novel therapeutics that boost innate immunity to treat infectious diseases

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Novel therapeutics that boost innate immunity to treat infectious disease

Funding Agency: The Foundation for the National Institutes of Health

Funding Title: Novel therapeutics that boost innate immunity to treat infectious diseases

Funding Agency: The Foundation for the National Institutes of Health
Funding Title: Novel therapeutics that boost innate immunity to treat infectious diseases

Unfunded title: N/A

The animal care committee has examined and approved the use of animals for the above experimental project

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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