EFFECT OF MICROBIAL COMMUNITY ON *SALMONELLA* GROWTH

by

TIFFANY WEN KWAN

(Under the Direction of Margie Lee)

ABSTRACT

Salmonella is a foodborne pathogen that affects humans worldwide. While the virulence factors of this organism have been well-studied, much less is known about its behavior in a mixed community. The intestinal tract is populated with a diverse microbial community, with which *Salmonella* must compete. In order to study how *Salmonella* growth is affected by the presence of naturally-occurring intestinal bacteria, *Salmonella* Typhimurium was cocultured with *Lactobacillus*, *Eubacterium*, and *Bacteroides*, which represent the two largest bacterial groups (Firmicutes and Bacteroidetes) found in the gastrointestinal tract. An anaerobic *in vitro* batch system and complex medium that mimics the chicken cecum were developed for this study. Growth rates were calculated for *Salmonella* and community composition was determined by a combination of traditional culture methods and fluorescence *in situ* hybridization (FISH). A mathematical model was developed in order to further understanding of the effects of metabolism and interaction on microbial growth dynamics.

INDEX WORDS: *Salmonella*, competitive exclusion, intestinal community, microbial competition, mathematical modeling, 16S FISH

EFFECT OF MICROBIAL COMMUNITY ON *SALMONELLA* GROWTH

by

TIFFANY WEN KWAN

B.S., Columbia University, 2009

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2013

EFFECT OF MICROBIAL COMMUNITY ON *SALMONELLA* GROWTH

by

TIFFANY WEN KWAN

Major Professor:	Margie Lee
Committee:	Mark Eiteman
	Caner Kazanci
	John Maurer

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2013

DEDICATION

I would like to dedicate this to my parents, both of whom have made sacrifices so that their children could have access to opportunities they never had.

ACKNOWLEDGEMENTS

This thesis would not be what it is without the help of all those involved.

First, thanks go out to Adriana Pedroso for helping me develop the *ex vivo* chicken cecal medium; Ying Cheng for supplying the reporter strain of *Salmonella*; and Beatriz Kowalski for conducting multiple growth experiments in order to correlate YFP expression to *Salmonella* growth rate.

I would also like to thank the members of my advisory committee—Margie Lee, John Maurer, Mark Eiteman, and Caner Kazanci—for their expertise and helpful guidance and most importantly, their patience. I would especially like to thank my major professor, Margie Lee, for giving me a chance to prove myself and for teaching me to never give up.

Special thanks to Adriana Pedroso and Anne Hurley-Bacon for their endless moral support.

Finally, I must acknowledge Kartik Chandran and Joon Ho Ahn for introducing me to the fascinating world of microbiology.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

LITERATURE REVIEW

The adult human intestine contains an estimated 100 trillion microbial organisms—ten times as many cells as those that make up the human host [1]. In fact, the prokaryotic population found within the gastrointestinal tract is far denser than in any other ecosystem [2]. This microbiome is very important to the host. Some hosts rely on their intestinal microbiome to break down hard-to-digest food [3, 4]. Ruminant diets are comprised of complex plant material that, in addition to host-secreted enzymes, requires the aid of bacteria and fungi in the rumen to be fully broken down [5, 6]. In this manner, the food ingested by the host is made accessible to both itself and the microbiome. Similarly, termites rely on intestinal microbes to aid in digestion of lignocellulose material found in plant matter [7, 8]. In these instances, the host and intestinal microbiota work together for the benefit of both. Not surprisingly, members of the intestinal microbiome are believed to have coevolved with its host [3, 9].

The metabolic networks that exist within the gastrointestinal tract are quite intricate. At such high densities in the gastrointestinal tract, microbes must compete with each other as well as with the host over nutrients and energy sources in order to survive. This fierce competition forces the microorganisms to have unique abilities that allow them to occupy a specific niche within the gastrointestinal tract. Despite this expected diversity, host survival selects for functional redundancy within the microbial community in the intestines to ensure stability and resilience in a dynamic environment [9, 10]. Changes to the intestinal ecosystem, such as dietary

changes, orally administered antibiotics, and intestinal infections, may cause an imbalance in the microbial community and lead to undesirable effects for both the host and its microbiota if metabolic function is disturbed [11-13].

While it is expected that organisms compete with one another for valuable resources, it is less expected for the organisms to cooperate for survival. Indeed there is evidence for metabolic cooperation between individual bacteria occupying the same gastrointestinal tract. Some bacteria can utilize fermentation end products generated by another organism's metabolism. Still others have the capability of breaking down large and complex molecules, making simple substrates available for the rest of the community. Lactate-utilizing *Eubacterium hallii* and *Anaerostipes caccae* were only able to grow on the complex carbohydrate starch when cocultured with *Bifidobacterium adolescentis*, which has the capability to break down the starch and then produce lactate. Another bacterium, *Roseburia* sp. strain A2-183, which does not utilize lactate, grew in the presence of starch when cocultured with *B. adolescentis*. It is believed that the *Roseburia* was able to utilize less complex sugars released by the enzymatic activity of *B. adolescentis* [14].

In a separate study, germ-free mice colonized with *Eubacterium rectale* and/or *Bacteroides thetaiotaomicron*, representatives from the two dominant bacterial phyla found in the large intestines, demonstrated how metabolic pathways of individual bacteria change depending on what is available and who else is present in the community even within a simplified intestinal environment [15]. Whole-genome transcriptional profiling of the bacteria and the mice showed that a complex metabolic interaction existed not only between the two bacterial species but also between bacteria and host. When both bacterial species are present in the gnotobiotic mice, *B. thetaiotaomicron* adapted by up-regulating expression of its glycan-

degrading enzymes and signaling to the host to produce mucosal glycans that only it can utilize. Meanwhile, *E. rectale* responded by decreasing its own production of glycan-degrading enzymes, increasing production of certain amino acid and sugar transporters, and generating butyrate that the host epithelial cells can use.

Despite the high density of bacteria found in the gastrointestinal tract, diversity at the higher order level is low [16]. At the time of a review published by Bäckhed *et al* in 2005, only eight bacterial divisions (or superkingdoms) have been identified in the human intestine, of which five are rare. Two divisions dominate: Cytophaga-Flavobacterium-Bacteroides, which includes the genus *Bacteroides*; and Firmicutes, which includes the genera *Clostridium*, *Eubacterium*, and *Lactobacillus* [3]. The third division, Proteobacteria, is common but not considered to be a dominant group [17, 18]. Other studies have also shown that the large intestine of humans and other mammals are typically dominated by the two bacterial phyla, Firmicutes and Bacteroidetes [18-20]. Similarly, Firmicutes were found to dominate the ileum and cecum of broiler chickens, with Proteobacteria and Bacteroidaceae present as well [21]. Even though bacterial diversity in the gastrointestinal tract is low at the division level, the intestinal microbiota appears to be considerably more diverse at the strain and subspecies level [3, 18].

One division of bacteria, Proteobacteria, is important from a human health standpoint. Although not a dominant group in the gastrointestinal tract, several enteropathogens belong to this group. These include species from the genera *Escherichia*, *Salmonella*, *Shigella*, *Vibrio*, *Campylobacter*, and *Arcobacter*—all of which cause foodborne illness in humans.

Foodborne illness is a problem that affects both developed and developing countries. It is caused by ingestion of infectious or toxic agents through contaminated food or water. The most recent CDC report compiled in 2011 estimates 47.8 million cases of foodborne illness per year in the US alone [22]. Furthermore, the report estimates 128,000 hospitalizations and 3,000 deaths caused by foodborne illness every year in the US [22]. Although a majority of these illnesses (80.3%) have been attributed to unspecified agents (i.e. not one of 31 major foodborne pathogens) [23], bacteria contribute to nearly two-fifths of foodborne illness cases attributed to known pathogens [22]. Nontyphoidal *Salmonella* is the leading bacterial cause of foodborne illness, causing an estimated 1,000,000 cases of illness, 19,000 hospitalizations, and nearly 400 deaths per year in the US alone [22].

Salmonella are rod-shaped, flagellated Gram-negative bacteria capable of causing disease in a number of different hosts. Some species are fully host-adapted, such as *Salmonella* Typhi and *Salmonella* Pullorum, which only colonize the intestinal tracts of humans or chickens, respectively. Other species are highly host-adapted, having a preference for a particular host. Still, some species have wide host specificity, being able to cause illness in a number of different animals.

Salmonella are facultative anaerobes, which means they can grow in the presence and absence of oxygen. This makes them well-adapted to living within the gastrointestinal tract as well as out in the environment, where they wait to colonize their next host. Nearly all *Salmonella* that infect mammals and birds belong to the *Salmonella enterica* species [24]. There are six subspecies of *Salmonella enterica* and over 2500 serovars within those subspecies. Serovars are determined by the carbohydrate structures of lipopolysaccharides and flagella located on the outer surface of the bacterial cell [24].

When *Salmonella* colonizes the intestines of mammals it causes gastroenteritis, which is marked by inflammation of the epithelium and leads to symptoms of vomiting, diarrhea, abdominal pain, and nausea. The severity of infection increases for children and the immunocompromised, where *Salmonella* infection may lead to health complications and sometimes even death. For the most part, however, gastroenteritis is a self-limiting disease and symptoms will disappear in 5-7 days if left untreated [25].

While gastroenteritis is caused by intestinal colonization of *Salmonella*, in the case of typhoid fever the infection becomes systemic in lymph nodes. *Salmonella* species that cause typhoid fever will not elicit an inflammatory response within the intestine, but instead invade M cells [26, 27]. The bacteria then replicate and survive in macrophages and travel from the intestinal lumen to the lymphatics within phagocytic cells. Once in the lymphatics, the bacteria can colonize other sites within the host, such as the liver, spleen, and bone marrow [28]. Symptoms of typhoid fever become apparent in humans 1 to 2 weeks after infection, and include fever, malaise, and abdominal pain with or without headache, muscle pain, nausea, loss of appetite, and constipation [29]. Fever will generally start out mild and worsen with time [29]. Typhoid fever will resolve itself eventually without further complications, but in some cases will cause death. Fecal shedding of the infectious bacteria can continue for months or years even after symptoms are resolved, and a relapse of the disease is possible [29]. *Salmonella enterica* serovars Typhi, Paratyphi, and Sendai cause typhoid fever in humans [25].

For humans in developed countries, *Salmonella* is most commonly contracted through contaminated food. Although a variety of food products can carry *Salmonella*, poultry and poultry products are the major source of foodborne *Salmonella* illnesses. Analysis of data from

around the world between 1988 and 2007 determined that chicken, turkey, eggs and other poultry products accounted for 44% of *Salmonella* infections, while produce attributed 9.6% and beef and dairy products each attributed 7.2% [30].

Poultry meat is contaminated during slaughter and processing, where carcasses come into contact with *Salmonella* found in the fecal matter of infected birds [31]. Mishandling of contaminated eggs may allow small numbers of *Salmonella* to multiply to levels capable of infecting people [32].

Salmonella colonizes the ceca of birds [31, 33]. This is likely due to the relatively slow flow rate in the cecum when compared to the rest of the gastrointestinal tract [31]. Fecal shedding of *Salmonella* can last for weeks after colonization of the intestinal tract, leading to high rates of transmission between birds within a poultry house [31]. *Salmonella* from an infected flock can remain in the poultry house for months after depopulation, as the microorganism is able to survive in the environment until it finds the next susceptible host to infect [34]. Mice in poultry houses may also serve as a reservoir for *Salmonella* and be a source of infection to a healthy flock [35]. In addition to horizontal transmission, some *Salmonella* serovars can infect the ovaries of laying hens, making vertical transmission between hens and eggs an equally important issue [36-39].

There is high variability in *Salmonella* virulence in poultry [33]. *Salmonella*'s ability to infect birds depends on a number of factors, such as age and health of the bird and the *Salmonella* serovar. Susceptibility to *Salmonella* infections also depends on the breed of the bird [33]. *Salmonella enterica* Gallinarum and *Salmonella enterica* Pullorum are avian-specific and cause systemic disease in birds. *S.* Gallinarum causes fowl typhoid and *S.* Pullorum causes pullorum disease [40]. Both serotypes infect poultry lymphatics and cause mortality, with the

former affecting birds of any age and the latter usually causing death in birds no older than 3 weeks of age [41]. Both *S.* Gallinarum and *S*. Pullorum can be transmitted horizontally as well as vertically. Other *Salmonella* serotypes capable of causing death in young chicks are categorized as paratyphoid infections. However, most *Salmonella* serovars important in human health are able to colonize the gastrointestinal tract of chicks without any apparent symptoms of infection. Persistence of *Salmonella* infection and amount of fecal shedding in birds differ among serovars [42].

S. Gallinarum and *S.* Pullorum are highly host-adapted and therefore not a concern in human health. However, *Salmonella* serovars that are associated with human infections are commonly isolated from poultry. *Salmonella enterica* serovars Typhimurium, Enteritidis, and Heidelberg were among the top five serovars most frequently reported in human cases in 2006, as well as the top five serovars isolated from chicken in 2005 [43, 44]. In fact, it is believed that the eradication from poultry of the two avian-specific serotypes, *S.* Gallinarum and *S.* Pullorum, allowed *Salmonella* serovars that are harmful to humans to take their place [45].

Because diseases caused by *S.* Pullorum and *S.* Gallinarum posed a serious economic threat to the poultry industry at the time, a large-scale plan was adopted by the US in 1935 and expanded in 1954 to significantly reduce levels of these *Salmonella* serovars in poultry. Similar control programs were adopted by other countries, and by the mid-1970s both avian-specific serotypes were eliminated from commercial flocks in the US and UK [45]. Since the 1960s, there has been a gradual increase in human infections caused by *Salmonella enterica* Enteritidis present in poultry products [45]. One hypothesis explaining the emergence of *S.* Enteritidis is that the eradication of *S.* Pullorum and *S.* Gallinarum left an ecological niche in poultry for *S.* Enteritidis to fill. Prior to their eradication, *S.* Pullorum and *S.* Gallinarum may have been more

successful than *S.* Enteritidis at colonizing poultry because of the flock immunity generated by the avian-specific serovars. Since all three serovars express the same O9 antigen on their cell surface [45, 46]. It has also been suggested that *S.* Gallinarum and *S.* Pullorum are able to competitively exclude *S.* Enteritidis in the poultry gastrointestinal environment. Before *S.* Enteritidis appeared in poultry flocks, rodents were the only known animal reservoir for *S.* Enteritidis [47].

Interestingly, *Salmonella* Typhimurium, a serotype of major human health concern, is believed to use different colonization strategies in mammals and birds. *S.* Typhimurium requires Type III Secretion System (TTSS) genes to colonize the intestinal tract of calves and cause infection. Mutant strains with transposon insertions in TTSS-1 and TTSS-2 genes were attenuated in the calf model. On the other hand, these same mutant strains had no trouble colonizing 14-day-old chicks [48]. Calves inoculated with wild-type *S.* Typhimurium at 28 days old were susceptible to intestinal colonization followed by epithelial invasion, acute enteritis, and in some cases mortality. In contrast, 3-week-old birds inoculated with wild-type *S.* Typhimurium resulted in heavy cecal colonization and persistent fecal shedding, but no or very little systemic infection [33]. *S.* Typhimurium only caused systemic infection and mortality in young (1 day old) chicks [33]. This suggests that *S.* Typhimurium uses different strategies depending on which host it colonizes.

Young birds are generally more susceptible to *Salmonella* infections [49, 50]. This is believed to be the result of having a simple, non-inhibitory intestinal microbiota as well as having an immature immune system [51]. Chicks of a young age are more susceptible to intestinal colonization by *Salmonella* [33]. Oral inoculation of chicks 24 h after hatching with a

virulent strain of *S.* Typhimurium led to 90% mortality. In contrast, chicks that were challenged 48 h or later after hatching had no mortality [33]. Older birds possess established intestinal microbiota that provides protection against colonization and/or invasion of *Salmonella*. This protection disappears in cases where the microbiota has been disturbed or destroyed, such as during times of stress or after antibiotic treatment [52].

The idea that establishment of an adult intestinal microbiota can provide protection against enteric pathogens is known as the Nurmi principle, or competitive exclusion [52, 53]. This concept has been validated in multiple studies [54-63]. In fact, this protective effect has been widely known and acknowledged for years. Newly-hatched chicks would typically acquire this protective microbiota from their parents through coprophagy. However, chicks are no longer reared with adults in modern mass production practices, delaying the establishment of protective microbiota in the chick gastrointestinal tract [57]. In an effort to promote this type of protection in modern poultry production, competitive exclusion products (e.g. Broilact, Aviguard) consisting of a mixed microbial culture that mimics the intestinal microbiota found in a healthy adult chicken are administered to baby chicks [64, 65].

The mechanisms of competitive exclusion are still unclear. Various mechanisms have been proposed, including metabolic competition, production and secretion of growth-inhibiting compounds, formation of a physical barrier against invasion or competition over attachment sites, and assistance of the host immune system [57]. It is likely that multiple mechanisms are at work when the intestinal microbiota protects the host against enteropathogen colonization.

Competition is fierce in the intestinal environment, making it imperative for invasive species to find a way to compete with both the microbiota and the host over limited nutrients so that it can multiply to high densities and increase its chance for survival and transmission [66]. In

fact, recent studies have suggested that disease caused by *S.* Typhimurium is done so simply to ensure its own survival [67-71]. Type III Secretion Systems (TTSS) help *S.* Typhimurium invade and survive in epithelial cells [72, 73]. These virulence factors trigger intestinal inflammation [74, 75] and causes a respiratory burst that generates tetrathionate, which can be used as a terminal electron acceptor for respiration [69]. Ethanolamine is derived from phospholipids in enterocyte membranes [76], but because it is not easily fermentable it is not a nutrient accessible to most intestinal microorganisms [68]. *S.* Typhimurium can utilize ethanolamine through respiration using tetrathionate as a terminal electron acceptor [68, 69]. This gives *Salmonella* a growth advantage in the intestinal environment. Indeed *S.* Typhimurium was able to outcompete cecal microbiota in the mouse using tetrathionate respiration [68]. Furthermore, diarrhea caused by inflammation removes nutrients and makes the intestinal environment more hostile for *Salmonella*'s competition [73].

Clearly disease caused by *Salmonella* is closely linked to its ability to survive within a hostile and highly competitive environment. While it is important to understand how *Salmonella* causes disease in its host, it is equally as important to understand how *Salmonella* behaves and interacts with resident microbiota from the point of view of survival and replication in the intestines. At the moment, however, research is predominantly focused on *Salmonella* pathogenicity. A PubMed search using the terms "Salmonella pathogenicity" yielded over 6200 results, while a search for "Salmonella competitive exclusion" yielded less than 200 results. *Salmonella* pathogenicity has been comprehensively studied, but much less is known about how *Salmonella* persists in the intestinal environment and its interactions with the intestinal microbiome.

Mathematical modeling is a powerful predictive tool used in a variety of disciplines, from reaction kinetics to economics to population growth. When used in conjunction with scientific research, their predictive capabilities are improved and the models may even provide guidance for future studies. With a robust mathematical model, hypotheses can be tested *in silico* even before running any experiments. Experimental data can be used to confirm and refine the model, which in turn can generate new hypotheses. In this way, *in silico* simulations can be coupled iteratively with experimental work to advance knowledge in complex areas of research such as microbial metabolic networks.

However there are limitations when working with mathematical models. A model that is too simple would not demonstrate anything new, while a model that is too complex would make it difficult to determine which factors are actually affecting the system. It is imperative then to keep the model as simple as possible, only incorporating highly relevant parameters. Another issue with mathematical modeling is lack of data. Many models are designed without a comprehensive dataset. But because it can be quite a useful tool, researchers should not let these challenges deter them from modeling. In 1987 statistician George E. P. Box wrote that "essentially, all models are wrong, but some are useful" [77].

The most basic model for population growth is the exponential curve [78], which has found many uses across disciplines as diverse as biology, physics, finance, and economics. Despite its ubiquity, the exponential growth model is unrealistic because there is no limit to growth. In a natural environment, populations grow at an exponential rate but reach a maximum level due to resource limitation and death. A more realistic growth model is the logistic equation, published by Pierre François Verhulst in 1838 [79]. The logistic growth model mimics the self-

limiting behavior seen in a natural population. The model contains a carrying capacity term, which represents the maximum number a population can reach for a given set of conditions. Another well-known model for microbial growth in an aqueous environment is the Monod equation, which relates the growth rate to the concentration of a limiting substrate [80]. It is commonly used in combination with the exponential growth model to describe microbial growth in a continuous culture. While these growth models are well-studied, they do not address interactions present in a mixed population.

The modeling of growth for a mixed population is made difficult by the fact that when two organisms are competing for the same substrate, complete dominance of the species that is more efficient at obtaining the substrate, and the extinction of the other, will occur [81, 82]. This only results if the competing organisms rely solely on the same substrate. This is rarely the case in the natural environment because organisms are generally able to survive on a variety of nutrients and there are multiple substrates available in the ecosystem. Species of a mixed population can coexist if the degree of overlap in substrate preference is low, allowing the individual species to occupy separate ecological niches. A generalized model of the Lotka-Volterra equations describes very basic interaction between two distinct populations. The model demonstrates a predator-prey, competitive, or mutualistic relationship between the two populations depending on the values of the interaction parameters. Although mathematical models for microbial growth have been used to study wastewater treatment processes, bioreactor systems, pathogen control in food safety, and epidemiology [83-91], there has been very limited use of modeling in the area of microbial metabolic networks.

In an attempt to better understand *Salmonella* in its natural environment and to elucidate how the normal intestinal microbiota competitively exclude enteric pathogens, an *in vitro* system to study *Salmonella* growth and behavior in a mixed community under conditions that mimic the intestinal environment was developed. A mathematical model demonstrating basic interactions between two distinct populations was developed and compared with experimental results. The *in vitro* system can be adapted to study other enteropathogens of interest, and the mathematical model can be modified to study particular microbial interactions within a closed ecosystem.

CHAPTER 2

INTRODUCTION

Although *Salmonella* has been well-studied in the lab and a lot is known about its pathogenicity, much less is known about *Salmonella* growth and behavior in a mixed community. Generally *in vitro* studies of the enteropathogen do not reflect the natural environment in which *Salmonella* is found. *In vivo* models are labor-intensive and expensive. Gnotobiotic, or germ-free, animals are available but it is difficult to guarantee complete sterility and maintain such a status through the entirety of an experiment. Mice, calves, rabbits, and chicks are the more commonly used animal models for biomedical research. One advantage of using mice is knowledge of its genetic background; on the other hand, this is not representative of the natural environment, where genetic variability tends to be high.

Here, a closed anaerobic system was designed to allow for more realistic studies of intestinal bacteria. An *ex vivo* medium containing mucin, a host-produced glycoprotein expected to be a nutritional source for intestinal microbiota, was developed for this purpose. Three intestinal organisms were selected for the coculture study. Two bacterial phyla dominate in the animal intestines: the Cytophaga-Flavobacterium-Bacteroides group and the Firmicutes. *Bacteroides thetaiotaomicron* was chosen to represent the former and *Eubacterium rectale* was chosen to represent the latter. *Lactobacillus gasseri* was chosen because of its prevalence in the small intestines as well as its supposed ability to inhibit enteropathogens and benefit the host. *Bacteroides* and *Eubacterium* are primary degraders capable of breaking apart mucin into small

subunits that can be transported and utilized by the bacteria. On the other hand, *Lactobacillus* and *Salmonella* are secondary degraders, unable to produce the appropriate extracellular enzymes. All four organisms can utilize mucin subcomponents.

The objective of this study was to determine the effect of community composition on *Salmonella* growth in the *in vitro* system designed to mimic conditions found in the chicken cecum. *Salmonella* Typhimurium was cocultured with all possible combinations of *L. gasseri*, *B. thetaiotaomicron*, and/or *E. rectale*. Additionally, *S.* Typhimurium was introduced into "young" (0 h) and "established" (48 h) intestinal communities made up of all three organisms. *Salmonella* growth curves were generated and its growth rates, doubling times, and decay rates were calculated. The composition of mixed communities was determined at mid-exponential, stationary, and decay phases using a combination of fluorescence *in situ* hybridization (16S-FISH) and traditional plate counts. A mathematical model describing growth of two distinct bacterial populations with basic interactions was formulated, and experimental data was compared to model results.

CHAPTER 3

MATERIALS AND METHODS

Ex Vivo Chicken Cecal Medium

An *ex vivo* chicken cecal (EVCC) medium was developed in order to coculture members of the intestinal microbiome including *Salmonella*. The formulation was modified from one used in a published study involving an intestinal culture simulator [92]. Mucin, a glycoprotein secreted by host goblet cells along the intestinal tract, is expected to be a significant nutritional source for intestinal microbes [93, 94]. Phytone peptone, an enzymatic digest of soybean meal, represents the protein component of partially digested chicken feed of which soybean meal is a major constituent [95]. Uric acid was added at a concentration found in cecal contents of White Leghorns [96]. Additionally, 7 amino acids typically supplemented in chicken feed were added at predicted intestinal concentrations (calculated by subtracting amount absorbed by bird from amount fed) [97]. A redox indicator, resazurin, and a reducing agent, cysteine, were also added to enhance anaerobiosis [98].

Stock solutions of resazurin (1000x), hemin (1000x), uric acid (100x, pH 8.8 to 9.0), and amino acid supplement (10x, pH 7) were made. The pH of the basal medium was adjusted to 6.1 ± 0.1 prior to autoclaving. After addition of uric acid and amino acid supplement, the final pH of the EVCC medium was approximately 6.5. For agar plates, 20 g L^{-1} of Bacto agar was added.

TABLE 3.1 *Ex vivo* chicken cecal medium

Compound	Concentration	Manufacturer
Basal medium		
Mucin from porcine stomach (Type III)	2.5 g L^{-1}	Sigma
Phytone peptone	5.0 g L^{-1}	BD
KCl	0.37 g L^{-1}	J.T. Baker
$NaHCO_3$	0.42 g L^{-1}	J.T. Baker
NaCl	1.75 g L^{-1}	J.T. Baker
L-Cysteine HCl H_2O	0.30 g L^{-1}	Fisher
Hemin	10^{-4} g L^{-1}	Frontier Scientific
Resazurin	0.001 g L^{-1}	MP Biomedicals
Uric acid	0.002 g L^{-1}	Sigma
Amino acid supplement		
L-Arginine HCl	0.94 g L^{-1}	Sigma
L-Cysteine HCl H_2O	0.39 g L^{-1}	Fisher
L-Isoleucine	0.60 g L^{-1}	Acros
L-Lysine HCl	0.89 g L^{-1}	Sigma
L-Methionine	0.50 g L^{-1}	Sigma
L-Threonine	0.52 g L^{-1}	Acros

Anaerobic Conditions

All anaerobic work was conducted within a gloveless anaerobe chamber (Coy Lab Products, Grass Lake, MI). The main chamber space was maintained with a gas mixture of 5% H_2 and 95% N_2. Flushing of the handling and glove ports was done using a gas mixture of 10% CO_2 and 90% N_2. A fan box fitted with a desiccant and palladium catalyst (Coy Lab Products), on which O_2 and H_2 molecules react to form H_2O, was used to maintain low oxygen levels in the main chamber. An oxygen and hydrogen analyzer (Coy Lab Products) was used to monitor the concentrations of both gases in the main chamber space.

Equipment and supplies to be used within the anaerobe chamber were placed inside and allowed to equilibrate with the anaerobic environment for several days. This ensured that items and the main chamber were free of oxygen before use. Liquid media was transferred into the

anaerobe chamber immediately after being autoclaved to minimize equilibration time. Media that could not be autoclaved were pre-reduced by bubbling pure nitrogen gas into the liquid for at least 10 min before filter sterilization inside the main chamber.

Organisms were grown anaerobically by inoculating tubes or streaking plates inside the anaerobic chamber and then placing the tubes or plates within an anaerobe canister (Scientific Device Laboratory, Des Plaines, IL) containing a GasPak EZ Anaerobe Sachet (BD, Franklin Lakes, NJ). The canister was removed from the anaerobe chamber and placed within a CO_2-flushed metal paint can before incubation to provide a fail-safe against canister leakage. For broth experiments, 100 ml serum bottles with 13mm rubber stoppers and aluminum crimp caps were used. Introduction of oxygen into these bottles was detected when media containing the redox indicator resazurin became pink in color.

Intestinal Organisms

Three organisms with completed genome sequences were chosen to represent the major groups comprising the microbiome in the gastrointestinal tract: *Lactobacillus gasseri* ATCC 33323, *Eubacterium rectale* ATCC 33656, and *Bacteroides thetaiotaomicron* ATCC 29148. The two bacterial phyla that dominate in the intestines are the Cytophaga-Flavobacterium-Bacteroides (CFB) and the Firmicutes, the latter of which includes the genera *Lactobacillus* and *Eubacterium* (Clostridiales) [3, 18-21]. Furthermore, *B. thetaiotaomicron* and *E. rectale* are considered to be primary degraders of mucin; the genomes of both organisms contain multiple carbohydrate-hydrolases. *L. gasseri* and *Salmonella* Typhimurium, on the other hand, are secondary degraders since neither organism produces extracellular enzymes capable of releasing metabolizable subunits from the complex glycoproteins. *L. gasseri* was maintained on MRS (pH

5.5) agar or broth. *B. thetaiotaomicron* was grown on Bacteroides Bile Esculin agar or Cooked Meat Medium with glucose, hemin, and vitamin K_1 (BBL). *Eubacterium rectale* was maintained on Brain Heart Infusion agar or Cooked Meat Medium with glucose, hemin, and vitamin K_1. All organisms were grown in strict anaerobic conditions, at less than 0 ppm oxygen in an approximate 4% H_2, 96% N_2, and trace CO_2 atmosphere at 40°C.

Salmonella Growth Reporter

The reporter strain *Salmonella* Typhimurium CY1104 was kindly supplied by Dr. Ying Cheng. A fragment comprised of the growth-dependent 16S rRNA promoter found in *S.* Typhimurium SL1344 (*rrnB* P1), open reading frame and ribosomal binding site of yellow fluorescent protein (YFP), the lambda T0 terminator, and the chloramphenicol resistance gene *cat* was constructed and cloned into the Π-dependent low-copy plasmid, pGP704 (Figure 3.1). The resulting plasmid construct, pCY03, was maintained in *E. coli* SM10 λpir. pCY03 was then used as a template for PCR amplification of the fragment containing the YFP transcriptional

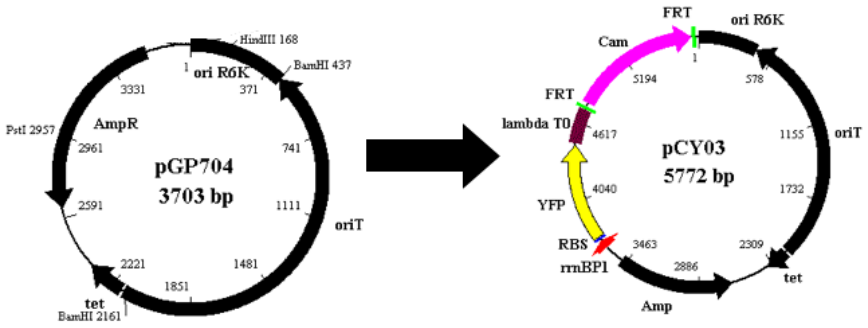

FIGURE 3.1 Insertion of fragment containing growth-dependent YFP gene into low-copy plasmid pGP704

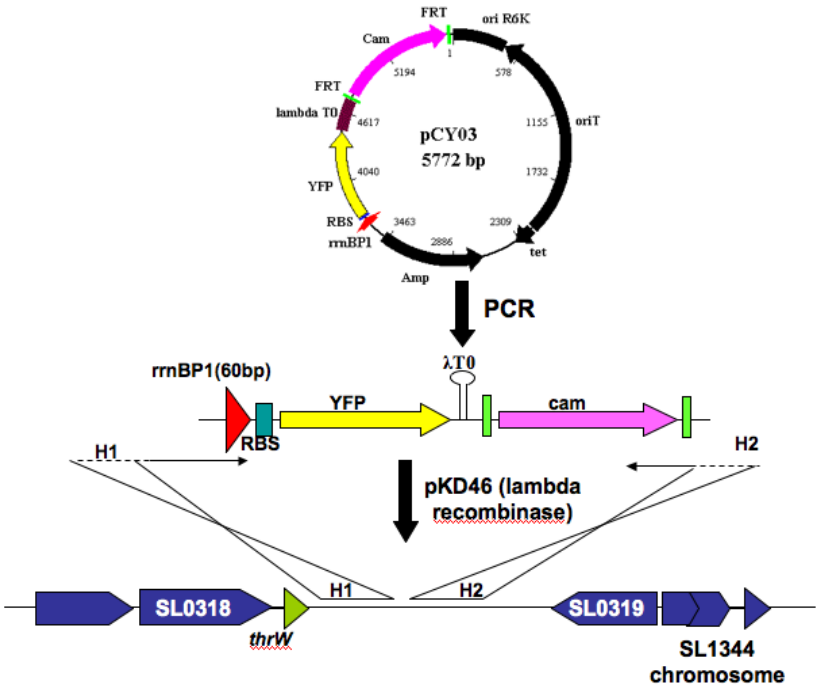

FIGURE 3.2 Insertion of fragment containing growth-dependent YFP gene into *S.* Typhimurium SL1344 genome by homologous recombination

fusion, T0 terminator, and the chloramphenicol resistance cassette by using primers that had 40- to 50-nucleotide tails (H1 and H2 in Figure 3.2) exhibiting perfect homology with the chromosomal site of insertion. The PCR product was inserted into *S.* Typhimurium SL1344 cells by homologous recombination (Figure 3.2) using the λ-Red system [99].

Growth and Preparation of Inocula

All organisms were grown anaerobically and incubated at 40°C on EVCC agar plates (except *L. gasseri*, which was grown on MRS, pH 5.5) 24 - 48 h before the start time of the experiment to reduce lag phase. Colonies were scraped from plates that had no signs of contamination and suspended in 0.9% saline solution to an optical density at 600 nm of 0.30 to 0.45. Suspensions were diluted to desired cell density using 0.9% saline solution and used to

make mixed and pure cultures by adding 1 ml suspension to 99 ml of EVCC broth. Gram stains and KOH tests were conducted on each bacterial suspension to confirm purity.

Salmonella Coculture Study

The purpose of the *Salmonella* coculture study was to see how *Salmonella* growth in EVCC medium was affected by the presence of intestinal organisms. Eight communities comprised of *S.* Typhimurium (S) and all possible combinations of *L. gasseri* (L), *B. thetaiotaomicron* (B), and *E. rectale* (E) at roughly equal initial cell densities were tested. These communities were designated in the following manner, according to their community composition: S, SL, SB, SE, SLB, SLE, SBE, and SLBE. There were three replications for each community in order to quantify biological variation. Each organism was introduced at roughly the same cell density (between 6.70 and 7.51 log cfu ml^{-1}. All communities were set up under strict anaerobic conditions in sealed serum bottles and incubated at 40°C.

Ex Vivo Intestinal Study

An *ex vivo* intestinal study was conducted to see how *Salmonella* growth was affected in a mock intestinal community containing all organisms at initial cell densities mimicking levels found in the small intestines. In 2003 Lu, et al., determined that the ileal (small intestinal) community in broiler birds was dominated by *Lactobacillaceae* (~69%), as opposed to the cecal (large intestinal) community which was dominated by *Clostridiaceae* (~66%) [21]. In addition, to examine how *Salmonella* growth was affected by the age of the mock intestinal community, *S.* Typhimurium CY1104 was introduced into "young" communities (at 0 h) and "established" communities (at 48 h). Two trials were conducted with 3 replications per trial to account for

biological variation. All communities were set up under strict anaerobic conditions in sealed serum bottles and incubated at 40°C.

Sample Collection

Sampling occurred initially every 3 h after the introduction of *S.* Typhimurium CY1104, then every 24 h for 6 days. In order to maintain sterility, the rubber stoppers on serum bottles were wiped with cotton balls soaked in a 10% bleach solution and allowed to dry prior to each sample collection. All samples were collected within the anaerobe chamber and materials were kept in the chamber to reduce introduction of oxygen. Three ml was collected from each bottle using sterile needles and syringes and transferred into sterile Eppendorf tubes containing glycerol (15% final concentration). Samples were mixed well before storing at -80°C.

***Salmonella* Enumeration**

S. Typhimurium CY1104 was enumerated using the spot plate method [100]. Briefly, serial dilutions were made in 1.5-ml Eppendorf tubes with 0.9% NaCl, and 10 µl was spotted onto dry MacConkey agar plates with 3 replications. Plates were incubated overnight at 40°C in ambient atmosphere and *Salmonella* was enumerated the following day.

Growth Rate, Decay Rate, and Doubling Time Calculations

The specific growth rates and decay rates of *S.* Typhimurium CY1104 were calculated using Equation 3.1, where μ, X, and t are the specific growth rate (h^{-1}), cell density (cfu ml^{-1}) and time (h), respectively. Values of ln(X) were plotted against t, and μ was determined by the slope of the best-fit line. Only time points occurring during the bacterial growth phase (i.e. 3, 6, and 9

h) were used to determine the specific growth rate. Time points after 48 h (inclusive) were used to determine the decay rate.

$$\ln(X) = \mu t + C \tag{3.1}$$

$$\mu = \frac{1}{X}\frac{dX}{dt} \tag{3.2}$$

$$\mu \int dt = \int \frac{1}{X} dX \tag{3.3}$$

Equation 3.1 is derived from the definition of specific growth rate (Equation 3.2), which can be rearranged into an indefinite integral (Equation 3.3) and solved to give Equation 3.1. The doubling time (t_D) was calculated from the specific growth rate using Equation 3.4.

$$t_D = \frac{\ln 2}{\mu} \tag{3.4}$$

Microscopic Cell Counts

Total bacterial counts were done at certain time points using an Olympus BH-2 Reflected Light Microscope at 100x (oil) magnification. Samples were washed and resuspended in Phosphate Buffered Saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). One µl of crystal violet was added to every 100 ul of sample and allowed to stand for 5 min. Stained samples were pelleted and resuspended in PBS before being transferred to a Neubauer slide with a counting chamber and coverslip. Photographs of five separate fields were captured with a SPOT RT3 Color camera using SPOT Basic software. Bacterial cells were counted from 25 squares, with no single square exceeding 20 bacteria to avoid over-crowding. Dilutions were made if the cell density was too high to count. Total cell counts were calculated using Equation 3.5, where X is the cell density (cells ml^{-1}), N is the average number of bacterial

cells per square, 1000 mm^3 ml^{-1} is a unit conversion, 1/400 mm^2 is the area of one square, 1/10 mm is the depth of space between slide and coverslip, and D is the dilution factor.

$$X = \frac{N \cdot \left(1000 \frac{mm^3}{ml}\right)}{\left(\frac{1}{400} mm^2\right) \cdot \left(\frac{1}{10} mm\right) \cdot \frac{1}{D}} \quad (3.5)$$

16S Fluorescence *In Situ* Hybridization

The fluorescence *in situ* hybridization (FISH) protocol used in these experiments was modified from that described in Cold Spring Harbor Protocols [101] after determining the proportion of cells that retained probe was highest using the Gram-positive procedure [101]. One ml of bacterial suspension was centrifuged (13,000 x *g* for 5 min) and washed once in 1 ml of PBS (pH 7.4). After discarding the supernatant, bacterial pellets were fixed by first suspending in 500 µl PBS, then adding 500 µl cold ethanol and mixing by inversion. Fixed cells were washed once in PBS and then resuspended in 500 µl of hybridization solution (10% deionized formamide, 0.9 M NaCl, 20 mM Tris-Cl (pH 8.0), 0.01% SDS) to a concentration of approximately 10^6 to 10^7 cfu ml^{-1} and incubated at 37°C for 30 min. Next, 50 µl was subsampled for each hybridization. 5 µl of fluorescently labeled oligonucleotide probe (50 ng μl^{-1}) was added to each 50 µl subsample. Tubes were incubated in the dark at 60°C for 3 h. Following hybridization, the cells were centrifuged (13,000 x *g* for 5 min) and washed twice by resuspending the pellet in 50 µl of warm 0.1X SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) and then incubated at 37°C for 15 min in the dark. For flow cytometry, cells were resuspended in 500 µl of cold PBS (pH 7.4) and kept in the dark on ice. For viewing under the microscope, cells were resuspended in 50 µl PBS and 10 µl was spotted on a slide and allowed to

air dry in the dark. All hybridizations were evaluated with negative controls (un-hybridized samples) and positive controls (pure cultures).

Table 3.3 lists the sequences of 16S oligonucleotide probes used for FISH. Probes were synthesized with fluorescent labels on the 5' end by Integrated DNA Technologies (IDT) or Invitrogen. The Olympus BH-2 microscope is equipped with filters for detection of Cy3 but not Cy5, while the CyAn flow cytometer could only detect Cy5. Therefore Cy3 labeled probes were used to optimize the conditions of hybridization while Cy5 probes were used to quantify the community composition.

TABLE 3.2 Fluorophore excitation and emission wavelengths

Fluorophore	Excitation peak (nm)	Emission peak (nm)
YFP	514	527
Cy3	550	570
Cy5	650	670

TABLE 3.3 16S oligonucleotide probes for FISH

Probe name	Probe sequence	Reference
EUB338	5'-GCTGCCTCCCGTAGGAGT-3'	Amann et al. [102]
Lac722	5'-YCACCGCTACACATGRAGTTCCACT-3'	Sghir et al. [103]
Bac303	5'-CCAATGTGGGGGACCTT-3'	Manz et al. [104]
Erec482	5'-GCTTCTTAGTCARGTACCG-3'	Franks et al. [105]

Fluorescence Microscopy

An Olympus BH-2 Reflected Light Microscope with Fluorescence Attachment was used to visualize fluorescence of YFP and Cy3. The FITC/Cy3 filter set (Chroma 51009) and the Olympus DPlan 100x objective with Type FF immersion oil for fluorescence microscopy (Cargille Laboratories, Cedar Grove, NJ) were used. A SPOT RT3 Color camera and SPOT Basic software were used to digitally capture fluorescent images from the microscope.

Flow Cytometry

Flow cytometry was conducted with a CyAn ADP Analyzer (Beckman Coulter) using the software Summit (ver. 4.3, Beckman Coulter). The 488 nm excitation laser and FL1 (515 to 545 nm) detector were used for YFP. The 633 nm excitation laser and FL8 (655 to 675 nm) detector were used for the Cy5 fluorophore. For each sample, a total of 20,000 events were collected. In some instances, samples were read multiple times using the flow cytometer to look for variation between sample collections. The flow cytometer reported fluorescence as median intensity values of arbitrary units. The cytometer flow rate was adjusted to keep the average event rate between 1000 and 3000 events per second to avoid coincidental detection of bacterial cells. Data was analyzed using FlowJo software (ver. 9.4, TreeStar, Inc.). Particles that did not fall into the expected size and shape of a bacterial cell were not included in the analyses. Positive and negative controls were included with every run. The level of fluorescence in negative controls was used to subtract out background fluorescence from samples of interest.

YFP Expression

For detection of YFP expression, samples at approximate cell densities of 6 log cfu ml^{-1} were pelleted at 13,000 x *g* for 10 min at 4°C and resuspended in 1 ml of 4% (v/v) formalin in PBS (pH 7.4). The samples were incubated in formalin at room temperature for 1 min and then washed twice with PBS. Final suspensions were made in 1 ml cold PBS and kept in the dark on ice until analyzed by flow cytometry. For viewing under the microscope, 10 μl was spotted on a slide and air-dried at room temperature in the dark.

The plasmid containing the YFP transcriptional fusion (pCY03) in *E. coli* SM10 λpir served as a positive control for YFP. Wild-type *S.* Typhimurium SL1344 served as a negative control. Control organisms were grown aerobically and shaken (200 rpm) in LB broth to mid-exponential phase, washed once in PBS (pH 7.4), and stored in freezer stock solution (10 g L^{-1} Bacto peptone (BD), 15% (v/v) glycerol) at -20°C.

Statistical Analysis

Statistical analysis was completed using SAS software. Tukey's test with a significance level (α) of 0.05 was used for pairwise linear contrasts of *Salmonella* growth rates, doubling times, and decay rates across different communities. Linear regression was used to determine the significance of a linear correlation between YFP fluorescence and growth rates of *S.* Typhimurium CY1104.

Mathematical Modeling

Mathematical modeling was performed using Matlab software (ver. 7.10, MathWorks). Three models were developed and their codes are located in the Appendix.

CHAPTER 4

RESULTS

Salmonella Coculture Study with Inoculum at Equal Cell Densities

S. Typhimurium CY1104 was cocultured with different combinations of the three intestinal organisms anaerobically in EVCC medium at initially similar cell densities (Table 4.1). The objective of this study was to determine how the presence of intestinal organisms *L. gasseri, B. thetaiotaomicron*, and/or *E. rectale* might affect the growth of *Salmonella*.

TABLE 4.1 Initial cell densities in *Salmonella* coculture study

Organism	Log cfu ml^{-1}
Salmonella Typhimurium CY1104	5.36 ± 0.05
Lactobacillus gasseri	5.50 ± 0.03
Bacteroides thetaiotaomicron	4.66 ± 0.22
Eubacterium rectale	4.79 ± 0.20

The growth curves of *Salmonella* in each of the communities are given in Figure 4.1. The EVCC medium supports the growth of a pure culture of *S.* Typhimurium CY1104 up to a density of 8 log cfu ml^{-1}. After 6 d of incubation, the final cell density of *Salmonella* ranged from 5.6 to 7.3 log cfu ml^{-1}; the lowest resulting from *Salmonella* cocultured with *B. thetaiotaomicron* and the highest from *Salmonella* cocultured with *L. gasseri* and *B. thetaiotaomicron*. When *Salmonella* was grown alone, the cell density began to drop steadily at 24 h after a relatively

short stationary phase. This trend (short stationary phase, steady drop starting at 24 h) is seen when *Salmonella* is cocultured with only one other intestinal organism. It is important to note, however, that the final cell density of *Salmonella* (at 144 h) is nearly a log lower when cocultured with *B. thetaiotaomicron* than when grown alone or cocultured with *L. gasseri* or *E. rectale*.

On the other hand, *Salmonella* seems to persist better (less sharp drop in death phase and higher final cell densities) when cocultured with two or more organisms. When cocultured with *L. gasseri* and *B. thetaiotaomicron*, *Salmonella* levels remained at about 7.5 log cfu ml^{-1} and never dropped below 7 log cfu ml^{-1}. *Salmonella* cocultured with *L. gasseri* and *E. rectale* was maintained at 7.5 log cfu ml^{-1}, only dropping to 7 log cfu ml^{-1} after 96 h. Cocultured with *B. thetaiotaomicron* and *E. rectale*, *Salmonella* dropped steadily from 8.1 log cfu ml^{-1} to 6.9 log cfu ml^{-1}. In a community with all three intestinal organisms, *Salmonella* persisted at just below 8 log cfu ml^{-1}, and dropping to 6.9 log cfu ml^{-1} after 72 h. Slight oscillations in *Salmonella* were noticeable in a few of the communities, of which the most pronounced occurred when *Salmonella* was cocultured with *L. gasseri*.

The specific growth rates and doubling times of *Salmonella* grown by itself and with different combinations of *L. gasseri*, *B. thetaiotaomicron*, and *E. rectale* at equal starting cell densities are shown in Figure 4.2. The specific growth rate of *Salmonella* ranged from 0.63 to 1.12 h^{-1}. The slowest growth rates of *Salmonella* occurred when grown alone and when cocultured with *L. gasseri*. These slow growth rates were not statistically different from each other, but were statistically different from *Salmonella*'s fastest growth rates, which occurred when *Salmonella* was cocultured with two or more intestinal organisms.

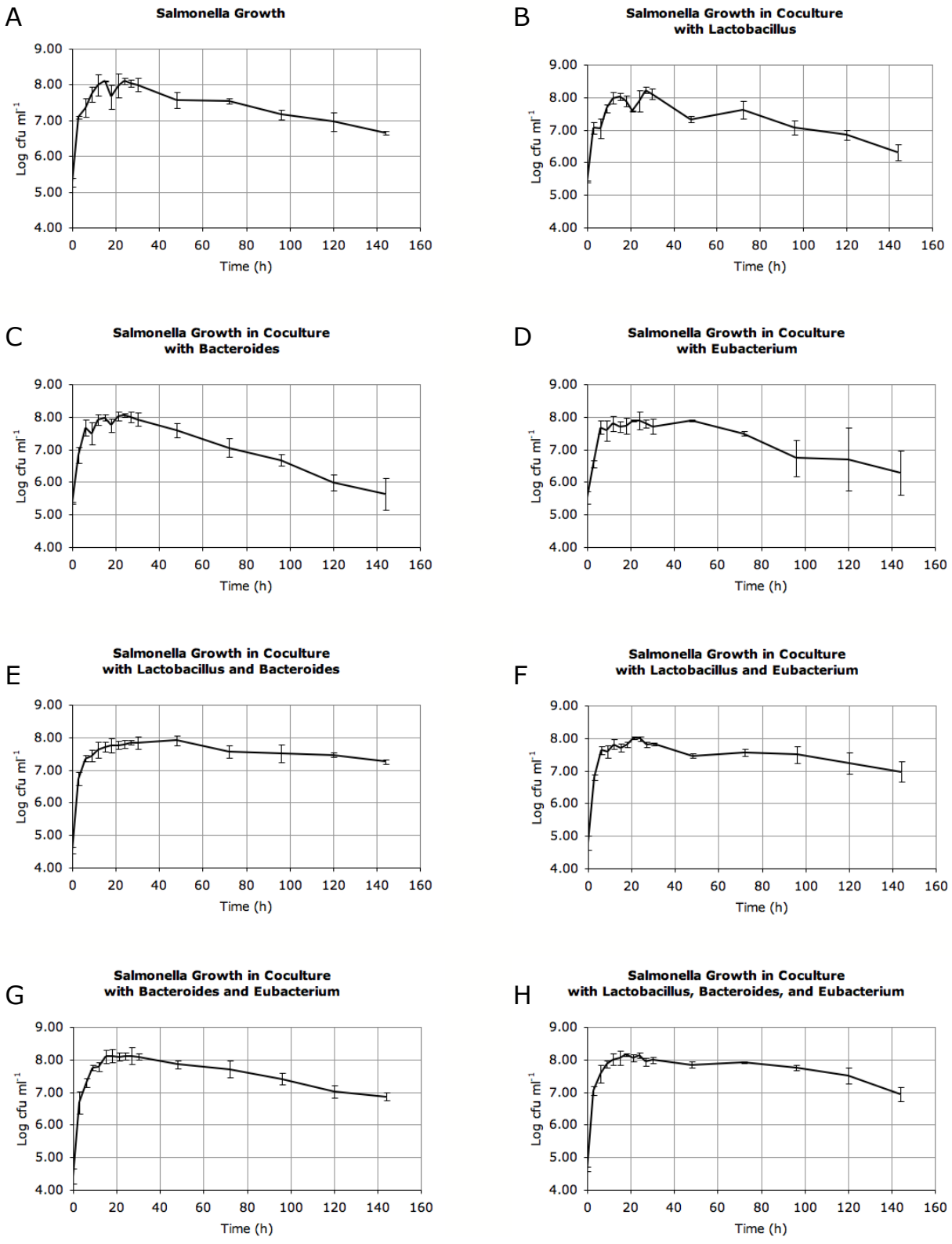

FIGURE 4.1 Growth curves of *Salmonella* when cocultured with equal densities of *Lactobacillus*, *Bacteroides*, and/or *Eubacterium*

The doubling time of *Salmonella* in coculture with different combinations of *L. gasseri*, *B. thetaiotaomicron*, and *E. rectale* at equal starting densities ranged from 37 to 68 min. The quickest doubling times occurred when *Salmonella* was cocultured with at least two intestinal organisms. While the doubling times of *Salmonella* in complex communities (i.e. three or more

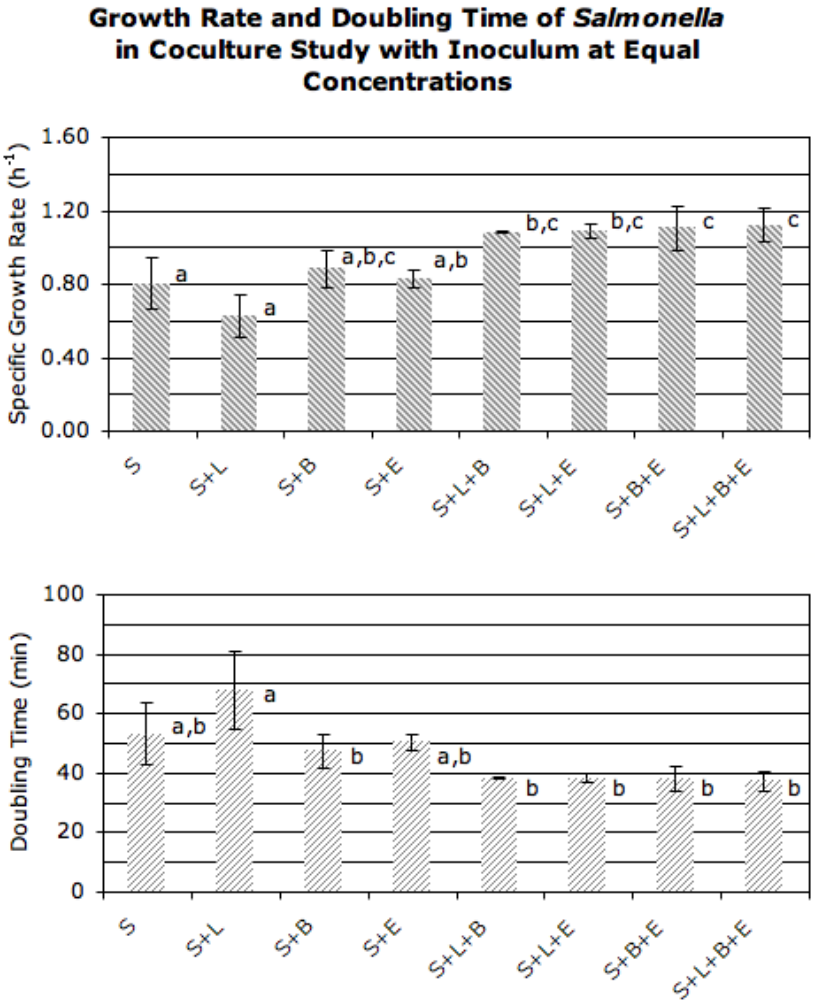

FIGURE 4.2 Growth rates and doubling times of *Salmonella* in coculture study with inoculum at equal cell densities
Communities were comprised of *S.* Typhimurium CY1104 (S), *L. gasseri* (L), *B. thetaiotaomicron* (B), *E. rectale* (E). Bars with the same letter are not significantly different.

total organisms) were not statistically different from one another, they were statistically different from the slowest doubling time, which occurred when *S.* Typhimurium CY1104 was cocultured with *L. gasseri*. *Salmonella* growth rate and doubling time was unchanged when cocultured with *L. gasseri*. On the other hand, *Salmonella* grew faster (i.e. faster growth rate, rapid doubling time) when cocultured with both primary degraders, *B. thetaiotaomicron* and *E. rectale*.

The rate at which *Salmonella* counts declined after 48 h were calculated and results are shown in Figure 4.3. *Salmonella* decay rates ranged from 0.00320 to 0.00076 h^{-1}. The slowest decay rates occurred when *Salmonella* was cocultured with *L. gasseri* and one primary degrader, while *Salmonella* decayed fastest when cocultured with *B. thetaiotaomicron* only.

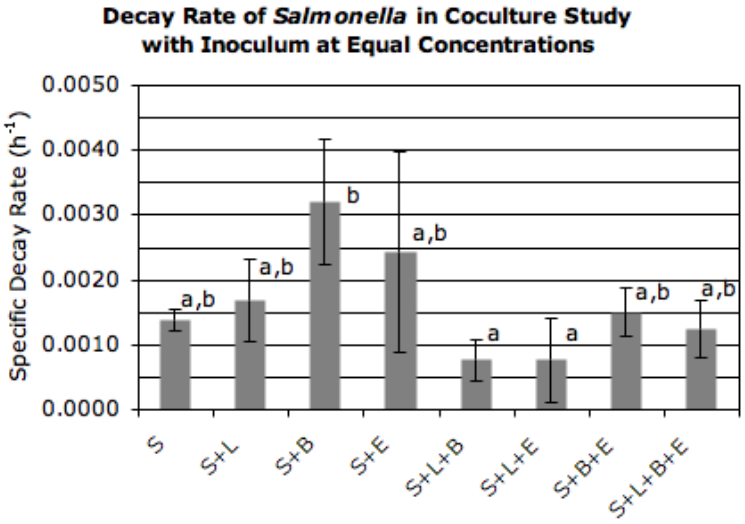

FIGURE 4.3 Decay rates of *Salmonella* in coculture study with inoculum at equal cell densities
Communities were comprised of *S.* Typhimurium CY1104 (S), *L. gasseri* (L), *B. thetaiotaomicron* (B), *E. rectale* (E). Bars with the same letter are not significantly different.

Several observations can be made when looking at this data. First of all, *Salmonella* was able to persist better in the presence of *Lactobacillus* and one or two primary degraders. *Salmonella* final cell densities were highest when cocultured with 2 or more organisms. *Salmonella* growth rates were higher and its decay rates were lower in the more complex communities. Conversely, *Salmonella* decayed quickest and reached its lowest final cell density when cocultured with *Bacteroides* despite having a slightly increased growth rate. This suggests that *Bacteroides* is triggering cell death in *Salmonella*. Furthermore, intense competition was expected between the two secondary degraders in the absence of a primary degrader. Although no inhibition of *Salmonella* was observed when cocultured with *Lactobacillus*, its growth rate and decay rate were slightly affected by the presence of *Lactobacillus*, which suggests possible competition. No inhibition of *Salmonella* suggests that the EVCC medium contains sufficient nutrients that are accessible to *Salmonella*.

Salmonella **in** ***Ex Vivo*** **Intestinal Study**

S. Typhimurium CY1104 was grown in a mixed community comprised of *L. gasseri*, *B. thetaiotaomicron*, and *E. rectale* at initial cell densities that mimic what is found in the small intestines (high *Lactobacillaceae*). Two trials, each with 3 biological replications, were conducted. The initial composition of the communities of both trials is given in Table 4.2. To see if *Salmonella* growth was affected by age of community, *Salmonella* was introduced at two different times, into "young" (at 0 h) and "established" (at 48 h) intestinal communities.

The growth curves of *Salmonella* in *ex vivo* intestinal communities are given in Figure 4.4. When *Salmonella* was inoculated at the same time as the intestinal organisms (young community), there were no apparent differences in *Salmonella* growth between the two trials

TABLE 4.2 Initial cell densities in *ex vivo* intestinal study

Organism	Log cfu ml^{-1}
Trial 1	
S. Typhimurium CY1104	3.45 ± 0.13
L. gasseri	4.15 ± 0.15
B. thetaiotaomicron	4.25 ± 0.13
E. rectale	1.99 ± 0.09
Trial 2	
S. Typhimurium CY1104	3.45 ± 0.13
L. gasseri	3.15 ± 0.15
B. thetaiotaomicron	4.25 ± 0.13
E. rectale	2.99 ± 0.09

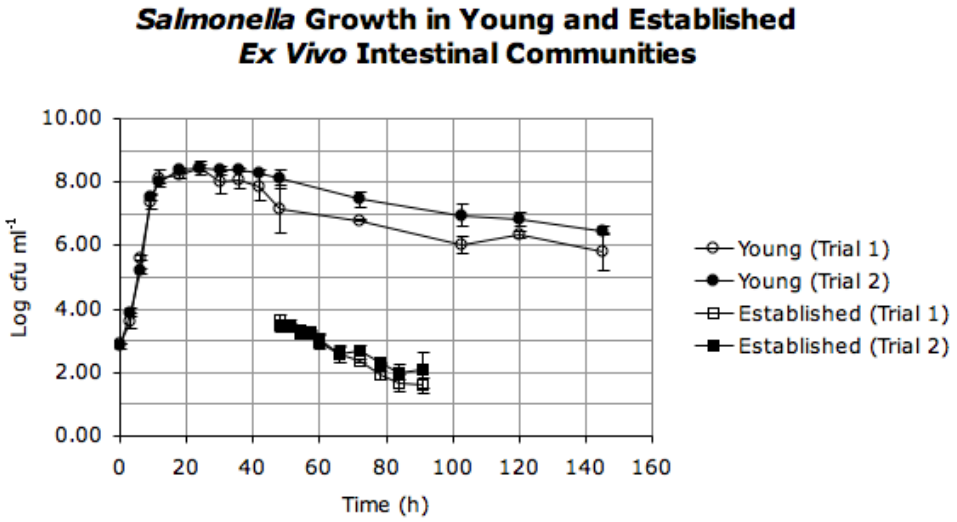

FIGURE 4.4 Growth of *S.* Typhimurium CY1104 in *ex vivo* intestinal communities
S. Typhimurium CY1104 was introduced into young communities at 0 h and established communities at 48 h.

until after 48 h, when *Salmonella* persisted at higher cell densities (0.5 to 1.0 log difference) in the second trial. *Salmonella* numbers did not increase when introduced into established intestinal communities (at 48 h) but immediately started decreasing. Twenty-four hours after introduction

of *Salmonella* into the established communities, there appeared a slight difference of less than 0.5 log cfu ml^{-1} in *Salmonella* levels between the two trials, with *Salmonella* persisting at higher numbers in the second trial.

There was no statistical difference ($p>0.5$) in the specific growth rate or doubling time of *Salmonella* in both trials of the young intestinal communities (Figure 4.5). *Salmonella* doubling

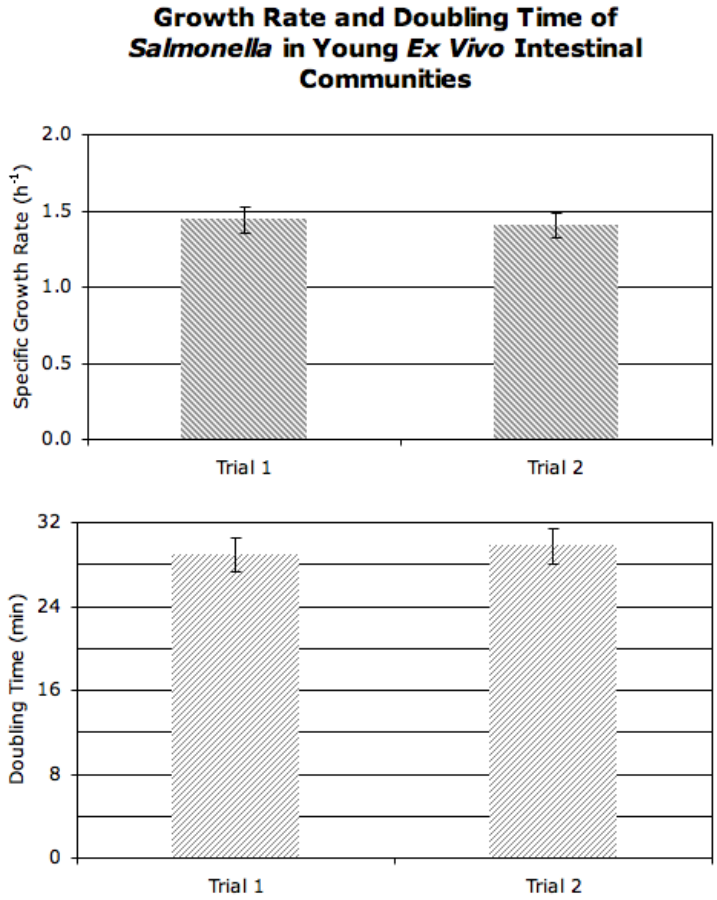

FIGURE 4.5 Growth rate and doubling time of *S.* Typhimurium CY1104 in young *ex vivo* intestinal communities
Communities were comprised of *L. gasseri*, *B. thetaiotaomicron*, and *E. rectale*. *Salmonella* was introduced into the intestinal community at 0 h. The specific growth rates and doubling times did not differ significantly between the two trials ($p>0.5$).

times were 28.9 and 29.8 min for Trial 1 and 2, respectively. The decay rates of *Salmonella* in young *ex vivo* intestinal communities (introduced at 0 h) are shown in Figure 4.6. In young intestinal communities, *Salmonella* numbers declined at a rate of 0.0026 and 0.0025 h^{-1} in Trial 1 and 2, respectively. The specific decay rates were not statistically significant between the two trials ($p>0.5$).

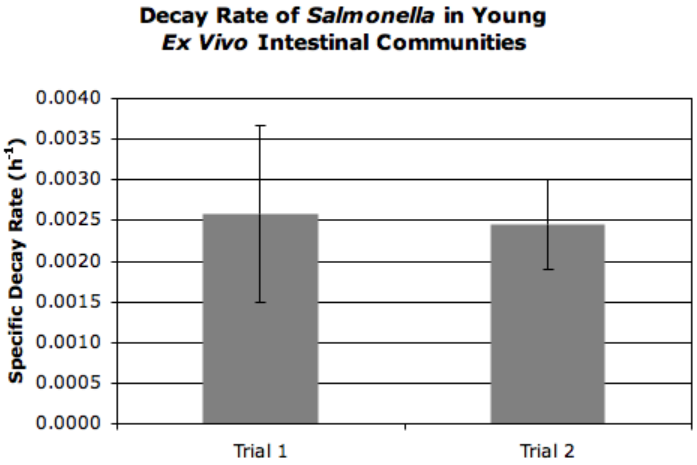

FIGURE 4.6 Decay rate of *S.* Typhimurium CY1104 in young *ex vivo* intestinal communities Communities were comprised of *L. gasseri*, *B. thetaiotaomicron*, and *E. rectale*. *Salmonella* was introduced into the intestinal community at 0 h. Decay rates were not statistically different between the two trials ($p>0.5$).

Salmonella was unable to grow in established intestinal communities (introduced at 48 h) and instead started declining in numbers immediately (Figure 4.4). The specific decay rate of *Salmonella* in established intestinal communities was 0.020 and 0.014 h^{-1} for Trial 1 and 2, respectively (Figure 4.7). There was no statistical difference ($p>0.05$) between the specific decay rates of *Salmonella* in established communities. It should be noted, however, that *Salmonella*

numbers declined at a much higher rate in an established community when compared to growth in a young intestinal community ($p<0.0001$).

The lack of *Salmonella* growth when introduced into established intestinal communities could be due to lack of nutrients, presence of inhibitory compounds, and/or direct microbial interaction or interference. To test the latter two hypotheses, *Salmonella* was grown in EVCC broth mixed with an equal volume of spent EVCC broth (1:1). Spent EVCC broth was taken from Trial 1 samples collected at 48 h, the time at which *Salmonella* was introduced into the *ex vivo* community. The sample was centrifuged at 13,000 *x g* for 5 min, and the supernatant was mixed with fresh EVCC broth. As a control, fresh EVCC broth was mixed with an equal volume

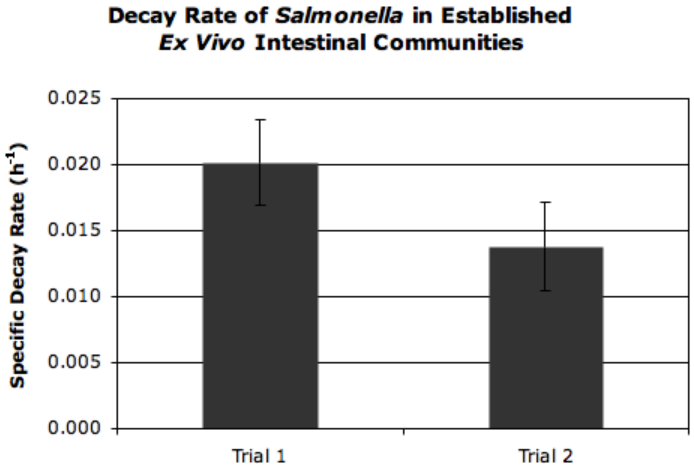

FIGURE 4.7 Decay rate of *S.* Typhimurium CY1104 in established *ex vivo* intestinal communities
S. Typhimurium CY1104 was introduced into established intestinal communities at 48 h. Communities were comprised of *L. gasseri*, *B. thetaiotaomicron*, and *E. rectale*. *Salmonella* was introduced into the intestinal community at 48 h. Decay rates were not statistically different between the two trials ($p>0.05$).

of sterile, deionized, distilled water (1:1). *Salmonella* was enumerated using the spot plate method [100].

As shown in Figure 4.8, *Salmonella* grew in both EVCC broth diluted with water as well as in EVCC broth mixed with spent medium. The lack of growth observed when *Salmonella* was introduced into established *ex vivo* intestinal communities at 48 h might be attributed to the exhaustion of nutrients accessible to *Salmonella*. It should be noted, however, that there is a difference in growth rate between the two media. *Salmonella* grew quicker in EVCC broth diluted with water than in spent EVCC medium. The difference in growth rate might be due to a presence of compounds that are inhibitory to *Salmonella* growth. One possible explanation is a reduction in pH of the spent EVCC medium due to presence of acidic metabolic by-products (i.e. short-chain fatty acids). The pH was not measured or adjusted in this experiment.

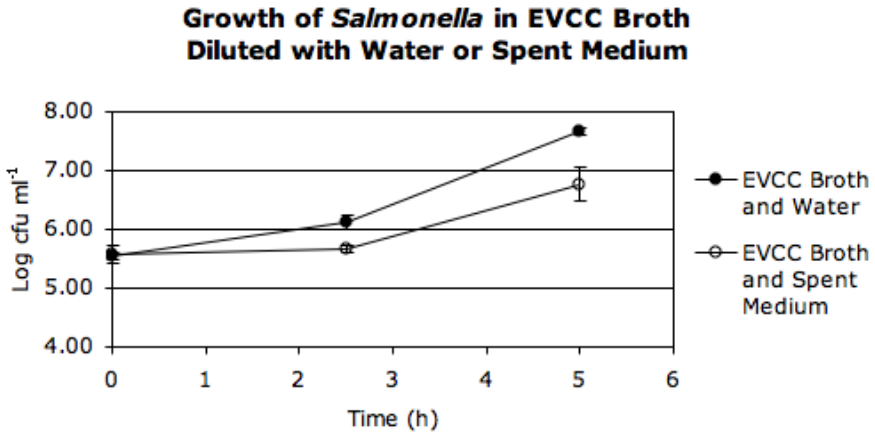

FIGURE 4.8 Growth of *Salmonella* in EVCC broth diluted with water or spent medium

Optimization of 16S FISH to Detect Bacteria in a Mixed Community

In order to determine the abundance of *B. thetaiotaomicron* and *E. rectale*, 16S fluorescence *in situ* hybridization (FISH) was used. Fluorescently labeled oligonucleotide probes targeting the 16S ribosomal RNA of *E. rectale* and *B. thetaiotaomicron* was used to detect the presence of each respective organism in a mixed community. A flow cytometer measured the relative abundance of *E. rectale* and *B. thetaiotaomicron* within each community, and the actual numbers were calculated by multiplying the relative abundances with total microscopic counts.

The protocol for FISH of bacterial cell suspensions described in Cold Spring Harbor Protocols was modified for our use [101]. Percent formamide in the hybridization solution was never changed from 10% since adjusting the hybridization temperature was adequate in controlling stringency. Cold Spring Harbor Protocols recommends two different methods for cell fixation depending on the Gram stain properties of the bacteria. For our purposes, however, we chose to use only the fixation method recommended for Gram-positive bacteria (ethanol fixation) since FISH was applied to mixed cultures containing both Gram-negative and Gram-positive bacteria. Cold Spring Harbor Protocols also states that cells in ethanol fixative can be stored at −20°C for several months. However, we chose to fix bacteria immediately prior to hybridization due to inconsistencies observed from control samples stored in ethanol for various lengths of time.

To verify that probes were getting into individual bacterial cells during protocol optimization, hybridized samples were inspected using fluorescence microscopy before using flow cytometry. At low stringency (45°C hybridization temperature) 2, 4, and 16 h hybridization times were tested on a Gram-negative (*E. coli*) and Gram-positive (*L. gasseri*) species using Cy3-labeled universal bacterial probe EUB338 and the FITC/Cy3 filter set on the fluorescent

microscope. Fluorescent *E. coli* cells were seen in all hybridized samples regardless of hybridization time, suggesting that 2 h was sufficient time for probe hybridization provided there are no issues with permeability. On the other hand, no hybridization was observed for *L. gasseri*. This was attributed to issues of permeability, which have been encountered previously in *Lactobacilli* and other Gram-positive organisms [106-111]. Figure 4.9 shows *E. coli* cells hybridized with Cy3-labeled EUB338 probes.

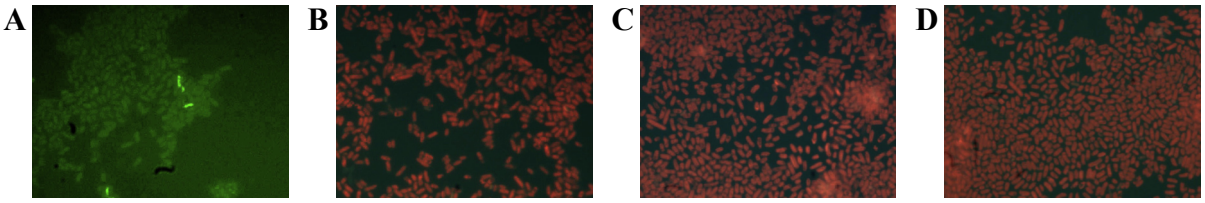

FIGURE 4.9 Cells hybridized with fluorescently-labeled 16S probe
(A) Autofluorescence of *Escherichia coli* (un-hybridized). Fluorescence of *E. coli* hybridized with universal bacterial probe EUB338 after (B) 2 h, (C) 4 h, and (D) 16 h hybridization times.

Enzyme treatment of *L. gasseri* was used to improve permeability of individual cells and promote probe hybridization. Better hybridization was observed under the fluorescence microscope when *L. gasseri* was treated with both proteinase K (100 μg ml^{-1}) and lysozyme (1 mg ml^{-1}) before hybridization with the EUB338 probe, when compared with treatment with only one enzyme or no enzyme treatment at all. Length of enzyme treatment could not be optimized using fluorescence microscopy. Due to the insensitivity of the camera and photobleaching effects, fluorescence could not be reliably quantified through microscopy.

These results were confirmed by flow cytometry using Cy5-labeled EUB338 probes. For *L. gasseri* samples receiving 0, 15, or 30 min of enzyme treatment, the flow cytometer detected

fluorescent cells at 1.42%, 27.5%, or 35.2% of the total population, respectively. However, when *E. rectale* (a Gram positive organism) was treated with enzymes for 0, 30, 60, and 90 min, a noticeable drop in the percentage of fluorescent cells detected by the flow cytometer was observed for treatment times of 30 min and longer (Table 4.3). While enzyme treatment is an effective method to permeabilize bacteria with tough cell walls, caution must be observed when this method is used on mixed cultures because the enzyme treatment may cause other organisms to lose cell wall integrity and rupture.

TABLE 4.3 Effect of length of enzyme treatment on *Eubacterium rectale* hybridization with EUB338 probe

Length of Enzyme Treatment (min)	% Cells Fluorescing (detected by flow cytometry)	
	No Probe	EUB338
0	0.008	72.7
30	0.010	39.6
60	0.003	20.5
90	0.010	25.1

To assess whether a longer hybridization time improved detection of *L. gasseri* by flow cytometry, *L. gasseri* was treated with both enzymes for 15 min and incubated with the universal bacterial probe EUB338 for 3 and 16 h at low stringency (45°C hybridization time). The relative abundance of fluorescent *L. gasseri* detected by the flow cytometer doubled from 36.2% to 74.4% for 3 and 16 h hybridizations, respectively. Interestingly, this only occurred for *L. gasseri* that had been previously fixed and stored in 1:1 ethanol/PBS fixative solution at −20°C for 2.5 months. For *L. gasseri* that had previously fixed and been stored in ethanol/PBS for 1.5 months

or less, the difference in relative abundance as detected by the flow cytometer was not noticeable with longer hybridization times (Table 4.4). *E. rectale* (untreated with enzymes) served as a positive control in these tests. We suspect that the length of storage of bacterial cells in fixative solution may affect cell permeability.

TABLE 4.4 Effect of hybridization time on *Lactobacillus* hybridization with EUB338 probe

Organism (length of storage in fixative solution)	% Cells Fluorescing (detected by flow cytometry)			
	3 h Hybridization		16 h Hybridization	
	No Probe	EUB338	No Probe	EUB338
L. gasseri (2.5 months)	0.073	36.2	0.088	74.4
L. gasseri (1.5 months)	0.043	34.3	0.109	31.0
E. rectale	0.075	85.9	0.048	86.8

Since 16S FISH was to be used on a mixed community, we decided against using this method to enumerate *L. gasseri* given its unique requirements for additional permeabilization steps, since additional measures may have affected other organisms present in the samples and led to inaccurate results. Instead, we chose to optimize hybridization conditions for the Bac303 and Erec482 probes for the enumeration of *B. thetaiotaomicron* and *E. rectale*, respectively.

Sequence similarity between 16S probes and 16S rRNA sequences of each organism (Table 4.5) was calculated using Equation 4.1. To determine the number of mismatches between an oligonucleotide probe and a bacterial small-subunit 16S rRNA sequence, the Basic Local Alignment Search Tool for nucleotides (BLASTN) was used (http://blast.ncbi.nlm.nih.gov/). 16S rRNA sequences were retrieved from The Ribosomal Database Project (RDP) [112].

$$\%similarity = \frac{\#\,matches}{probe\_length} = \frac{probe\_length - \#\,mismatches}{probe\_length} \qquad (4.1)$$

TABLE 4.5 Sequence similarity between 16S rDNA probes and organisms

	EUB338 probe	Entbac probe	Lac722 probe	Bac303 probe	Erec482 probe
S. Typhimurium	100%	100%	48%	59%	37%
L. gasseri	100%	32%	100%	53%	< 28%
B. thetaiotaomicron	100%	28%	48%	100%	< 28%
E. rectale	100%	32%	48%	53%	100%

There were no permeability issues with *E. rectale*. Stringency for probe Erec482 was achieved at a hybridization temperature of 60°C, resulting in high detection of *E. rectale* and relatively low detection of all other organisms by the flow cytometer (Table 4.6). Stringency at a similar level could not be achieved for the probe Bac303. At a hybridization temperature of 65°C for Bac303, the flow cytometer detected both *B. thetaiotaomicron* and *E. rectale* at satisfactory levels, while detection of *L. gasseri* and *Salmonella* were sufficiently low (Table 4.6).

TABLE 4.6 Cross-hybridization levels of Erec482 and Bac303

Organism	% Cells Fluorescing (detected by flow cytometry)			
	No Probe	Erec482	No Probe	Bac303
S. Typhimurium CY1104	0.560	6.38	0.085	20.0
L. gasseri	0.062	6.12	0.187	15.1
B. thetaiotaomicron	0.377	4.20	0.083	86.9
E. rectale	0.043	87.1	0.075	77.5

Experimental samples were hybridized with probes Bac303 and Erec482 at a hybridization temperature of 65°C alongside positive (pure cultures) and negative (un-hybridized) controls with the understanding that hybridization of samples with Bac303 resulted in detection of both *B. thetaiotaomicron* and *E. rectale*.

Determining Community Composition of Mixed Communities

Abundances of *Salmonella*, *L. gasseri*, *B. thetaiotaomicron*, and *E. rectale* were determined using a combination of plate counts, total microscopic counts, and 16S FISH. Data acquired from 16S FISH and flow cytometry was given in percentages of total population. Hence, the percentages had to be multiplied with total cell counts of the corresponding samples in order to determine actual quantities. Abundances of *Salmonella* and *L. gasseri* were determined by plate counts, while abundances of *E. rectale* and *B. thetaiotaomicron* were calculated from 16S FISH results. If present in a sample, *E. rectale* abundance was subtracted from Bac303 data since the probe detected both *B. thetaiotaomicron* and *E. rectale* equally well.

Abundance of *L. gasseri* was originally determined by subtracting *Salmonella*, *B. thetaiotaomicron*, and *E. rectale* numbers from the total cell counts. However, *L. gasseri* numbers would disappear at certain time points using this method. In order to verify these inferences, *L. gasseri* was enumerated using traditional plate counts. MRS agar at an acidic pH of 5.5 was selective against *Salmonella*, and conducting the serial dilutions and plating in the presence of oxygen excluded growth of anaerobic *B. thetaiotaomicron* and *E. rectale*. Viable *L. gasseri* was recovered in samples where the 16S FISH-based method indicated there was no *L. gasseri* present (Figure 4.10). *Lactobacillus* abundance determined using presumptive methods based on 16S FISH resulted in falsely high estimates; in some cases, by 5 log cfu ml^{-1} (data not

shown). As a result of the discrepancies between the two methods, plate counts were considered to be a more reliable method for determining *L. gasseri* abundance.

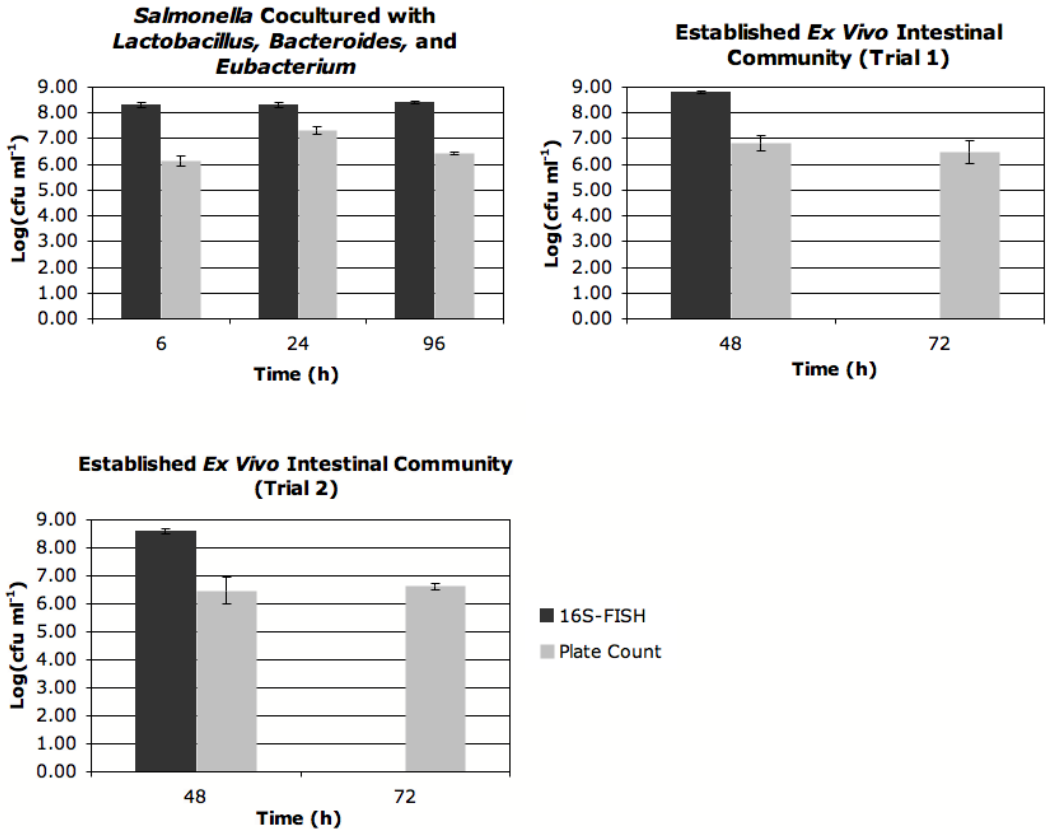

FIGURE 4.10 *Lactobacillus* abundance determined by 16S FISH-based method compared to plate counts

Changes to Community Composition in the Coculture Study

Community composition of the eight communities in the coculture study at three time points (6, 24, and 96 h) representing logarithmic growth, stationary and decay phases was determined using the methods described above. The results are shown in Figure 4.11.

Salmonella was consistently present above 7 log cfu ml^{-1} at 6 h and at about 8 log cfu ml^{-1} at 24 h regardless of community composition. However *Salmonella* numbers at 96 h varied, ranging from 6.7 to 7.8 log cfu ml^{-1}, depending on which organisms were present. When grown alone or with *Lactobacillus*, *Salmonella* was at approximately 7 log cfu ml^{-1} at 96 h. In the presence of one primary degrader (*Bacteroides* or *Eubacterium*), *Salmonella* was below 7 log cfu ml^{-1} at 96 h. Interestingly, *Salmonella* seemed to persist better when cocultured with two or more organisms, as it was present at greater than 7 log cfu ml^{-1} at 96 h.

L. gasseri did not reach high cell densities when grown alone with *Salmonella*, barely surpassing 6 log cfu ml^{-1} at 24 h. When grown in coculture with *Salmonella* and *Eubacterium*, *Lactobacillus* reached 6.1 logs at 6 h and remained at those numbers at 24 and 96 h. However in the presence of *Bacteroides, Lactobacillus* was able to reach higher numbers: 6.8 logs (SLB community) and 7.3 logs (SLBE community) at 24 h. *Lactobacillus* grew to higher levels in the presence of one or both primary degraders, suggesting its reliance on these organisms for nutrients.

B. thetaiotaomicron was present at the highest levels, consistently surpassing 8 log cfu ml^{-1} regardless of community. *Bacteroides* was present at levels above 8 log cfu ml^{-1} at 24 and 96 h when cocultured with *Salmonella* only as well as with *Salmonella* and *Eubacterium*. When cocultured with *Salmonella* and *Lactobacillus*, *Bacteroides* barely reached 8 log cfu ml^{-1}. *Bacteroides* growth was most affected by the presence of all three organisms, dropping below 7 log cfu ml^{-1} at 24 h and appearing at about 8 log cfu ml^{-1} at 96 h. We cannot rule out that this dipping behavior seen of *Bacteroides* in the SLBE community is not an artifact of the methods used to determine abundance. Nevertheless, the data does suggest that *Bacteroides* has no problem competing with *Salmonella*, even in the presence of *Lactobacillus* or *Eubacterium*.

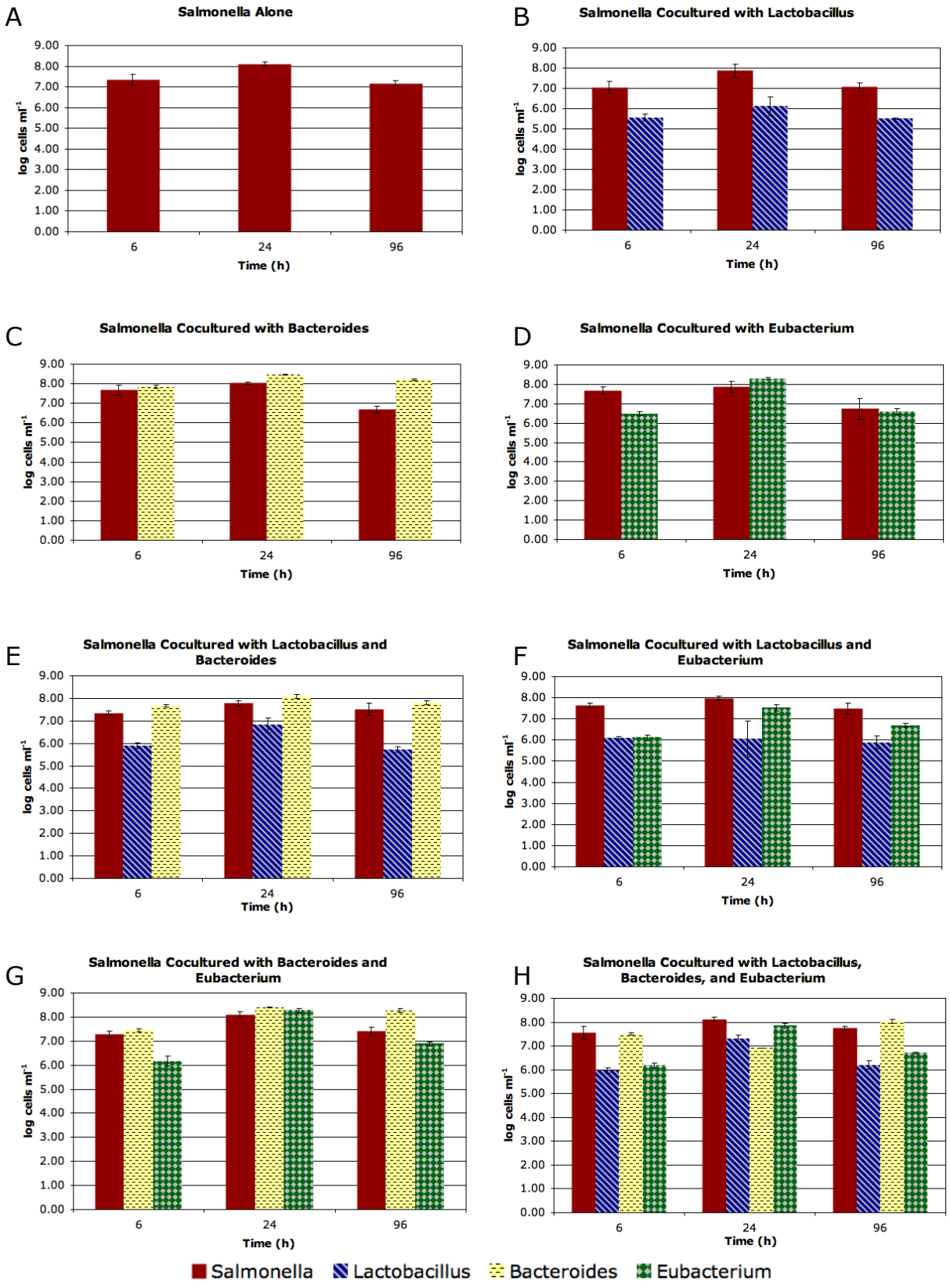

FIGURE 4.11 Composition of communities in *Salmonella* coculture study

E. rectale reached its highest cell densities when cocultured with *Salmonella* only and with *Salmonella* and *Bacteroides*, reaching 8.3 log cfu ml^{-1} at 24 h. On the other hand, in the presence of *Lactobacillus*, *Eubacterium* never reached 8 log cfu ml^{-1}. This suggests that *Eubacterium* faced more resource competition in the presence of *Lactobacillus*. Compared to the other organisms at 96 h, *Eubacterium* was present at the lowest levels (i.e. between 6.6 and 6.9 logs).

Changes to Community Composition in the *Ex Vivo* Intestinal Study

Community composition of the *ex vivo* intestinal study at three time points representing logarithmic growth, stationary and decay phases was determined using the methods described above. For samples in which a "young" community was challenged with *Salmonella* (at 0 h), samples at 6, 24, and 72 h were analyzed. For samples in which an "established" community was challenged with *Salmonella* (at 48 h), samples at 6, 48, and 72 h were analyzed. The results are shown in Figure 4.12.

Salmonella increased 3 log cfu ml^{-1} between 6 and 24 h to a cell density of 8.4 log cfu ml^{-1} in both trials involving "young" intestinal communities. When compared with the results of the 4-member community in the coculture study, *Salmonella* was able to reach the same maximum cell density despite a lower starting inoculum in the *ex vivo* studies (about 2 log difference). *Salmonella* was unable to grow, however, in the "established" intestinal communities.

Lactobacillus was unable to reach cell densities in the *ex vivo* intestinal communities similar to what resulted in the 4-member community in the coculture study. In fact, the maximum level of *Lactobacillus* seems to depend on the size of the inoculum. In Trial 1 of the

"young" intestinal community, *Lactobacillus* was introduced at 4.2 log cfu ml^{-1} and reached 6.1 log cfu ml^{-1} at 24 h. In Trial 2 of the "young" intestinal community, *Lactobacillus* was introduced at 3.2 log cfu ml^{-1} and reached 5.1 log cfu ml^{-1} at 24 h. In the 4-member coculture study, *Lactobacillus* was introduced at 5.5 log cfu ml^{-1} and reached 7.3 log cfu ml^{-1} at 24 h. *Lactobacillus* growth was affected by whether *Salmonella* was present or not. *Lactobacillus* reached higher cell densities at 72 h in the "established" intestinal communities where *Salmonella* was not present for the first 48 h. This suggests that *Lactobacillus* is indeed competing with *Salmonella*.

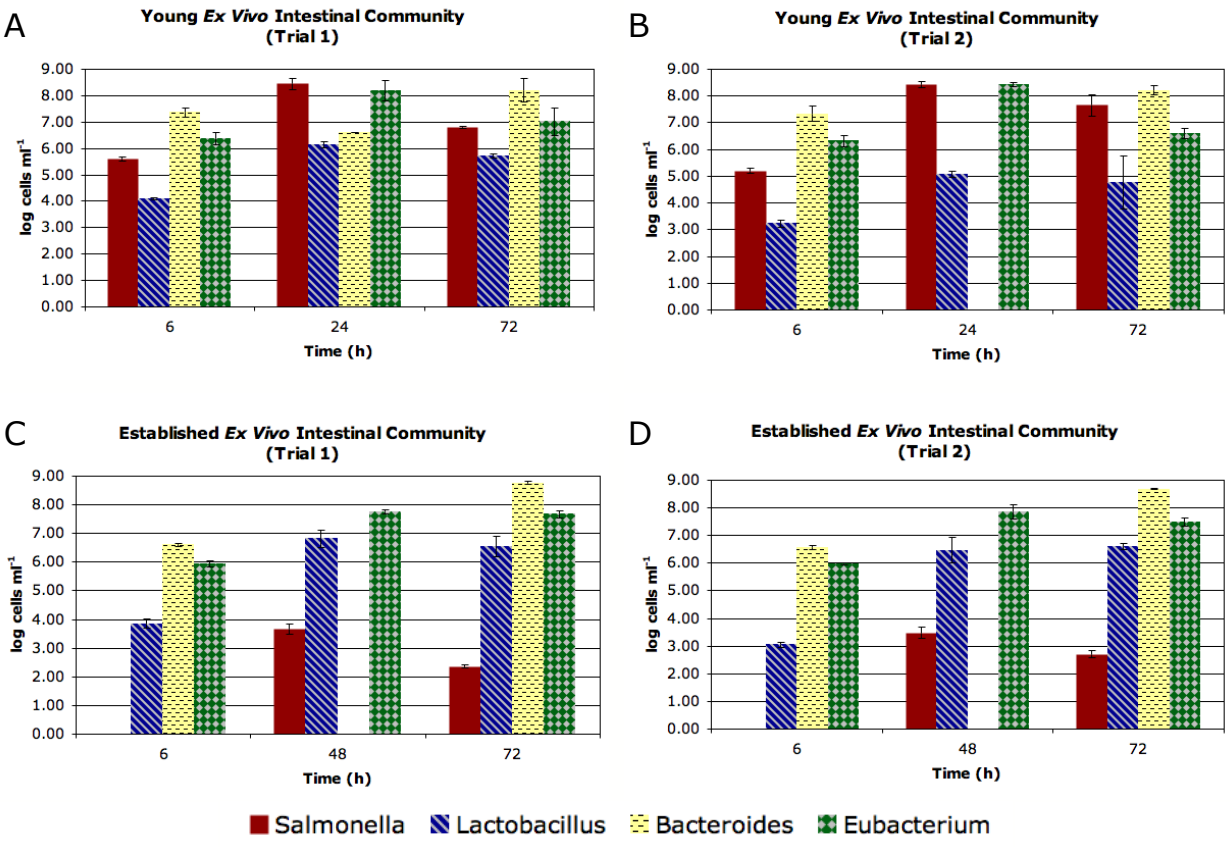

FIGURE 4.12 Composition of communities in *ex vivo* intestinal study

Bacteroides growth also appeared to depend strongly on the presence of *Salmonella*. When *Bacteroides* had to compete with *Salmonella* in the "young" intestinal communities, *Bacteroides* was present at 7.3 log cfu ml^{-1} at 6 h and 8.2 log cfu ml^{-1} at 72 h. In the "established" communities, where *Salmonella* was absent during the first 48 h, *Bacteroides* was present at 6.6 log cfu ml^{-1} at 6 h and 8.7 log cfu ml^{-1} at 72 h. *Bacteroides* was present at higher numbers at 72 h in the "established" intestinal communities, suggesting that *Bacteroides* is better able to persist in the absence of growing *Salmonella*. However, *Bacteroides* seemed to grow more quickly if *Salmonella* was present early on, reaching a higher cell density at 6 h in the presence of *Salmonella*. *Bacteroides* was introduced into both young and established communities at 4.3 log cfu ml^{-1}. It appears as though presence of *Salmonella* may have a positive effect on *Bacteroides* early on (perhaps competition forces *Bacteroides* to become more efficient), but may have detrimental effects later on. Both organisms have a negative effect on the final cell density of one another when they are cocultured together.

Consistent with behavior observed in the 4-member coculture study, *Bacteroides* numbers dropped at the time point representing stationary phase, in some cases disappearing completely. This strange behavior is believed to be an artifact of the methods used to determine abundance.

Unlike *Lactobacillus*, the initial cell densities of *Eubacterium* did not seem to affect its growth potential. Despite the 1 log difference in inoculum between the two trials, at 6 h *Eubacterium* was present at 6.3 log cfu ml^{-1} in the "young" intestinal communities, while in the "established" communities *Eubacterium* was present at a slightly lower 5.9 log cfu ml^{-1}. Like *Bacteroides*, *Eubacterium* appeared to persist better in the absence of growing *Salmonella*. In the

"established" intestinal communities, *Eubacterium* was present at or above 7.5 log cfu ml^{-1} while it was present at or below 7.0 log cfu ml^{-1} in the "young" intestinal communities.

In summary, *B. thetaiotaomicron* and *E. rectale* both reached higher cell densities early on (at 6 h) in the presence of *Salmonella*. Furthermore, *Bacteroides* and *Eubacterium* persisted at higher cell densities at 72 h when actively growing *Salmonella* was not present. This behavior suggest that the presence of *Salmonella* exerts a competitive force on the two primary degraders of mucin, driving them to increase their nutrient uptake rate and hence growth rate. Since these studies were done in a closed, batch system, once nutrients ran out cell death set in. In the presence of *Salmonella*, intense competition led to faster depletion of nutrients and presumably bacteria reached the decay phase quicker as a result. Both *Bacteroides* and *Eubacterium* were present at 72 h in the "young" intestinal communities (i.e. in the presence of growing *Salmonella*) at lower numbers than in the corresponding "established" intestinal communities.

Correlating YFP Expression to *Salmonella* Growth Rate

A growth-dependent yellow fluorescent protein (YFP) gene (linked to *Salmonella* 16S rRNA promoter) was inserted into the *S.* Typhimurium SL1344 genome. This growth reporter strain was developed for the purpose of rapidly determining the growth rate by measuring YFP expression and comparing to a reference curve. Several growth experiments were conducted with the *Salmonella* reporter strain to provide for a range of growth rates. *S.* Typhimurium CY1104 was grown in pure culture in shaken flasks (aerobically) in complex medium (LB broth) at 30°C and 37°C as well as in minimal medium with glucose or glycerol as the main carbohydrate source. The growth rates of the *Salmonella* reporter strain were calculated from the logarithmic growth phase of a growth curve, and YFP fluorescence was measured by flow cytometry. Using

linear regression, YFP fluorescence was correlated with growth rates and a reference curve was generated (Figure 4.13). The reference curve demonstrated good fit to the reference data with an $R^2 > 0.94$.

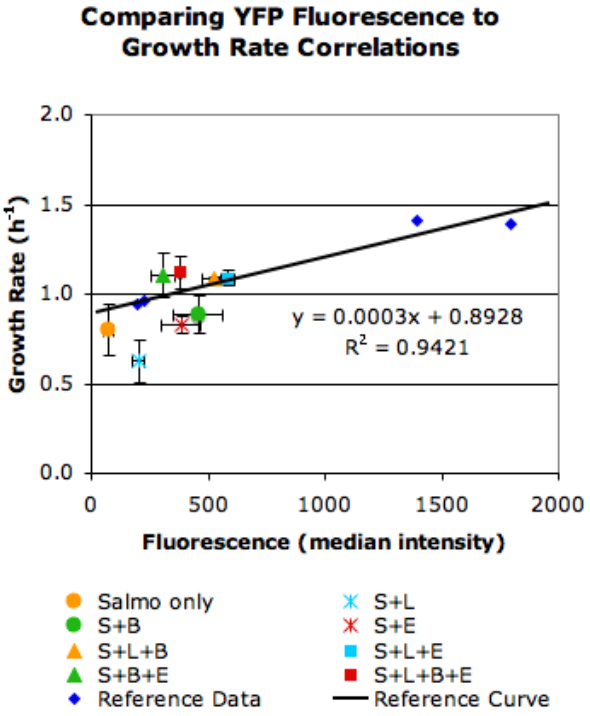

FIGURE 4.13 Correlation of YFP fluorescence to growth rate in the *Salmonella* reporter strain
Reference curve was generated from reference data, which were collected in monoculture growth experiments under aerobic conditions. Experimental data refers to mid-log samples from the coculture experiment. Communities were comprised of *S.* Typhimurium CY1104 (S), *L. gasseri* (L), *B. thetaiotaomicron* (B), *E. rectale* (E).

To test how robust this reference curve is, YFP expression of *Salmonella* at mid-exponential phase from the coculture experiment was measured by flow cytometry. YFP expression of *Salmonella* from each community and its corresponding growth rate were plotted alongside the reference curve in Figure 4.13 for comparison.

Correlation of YFP fluorescence and growth rate of the *Salmonella* reporter strain in two communities (when cocultured with *L. gasseri* and *B. thetaiotaomicron*, and with *L. gasseri* and *E. rectale*) appeared to be in excellent agreement with the reference curve. Other points did not fall far from the reference curve. One correlation in particular, *Salmonella* cocultured with *L. gasseri*, appeared well below the reference curve. This suggests that presence of *L. gasseri* may falsely raise the fluorescence signal. It is important to note that growth rates were calculated as an average, taking into account the entire logarithmic growth phase, while YFP expression gives an instantaneous snapshot of ribosomal gene expression.

Mathematical Modeling

A simple growth model, hereafter referred to as the Basic Model, was developed to determine which factors affect growth dynamics the most. In the Basic Model, two distinct populations, X_1 and X_2, grow on the same substrate S. The cell densities of X_1 and X_2 are defined by Equations 4.2 and 4.3, respectively, each of which contain growth and decay terms. The growth term is based on the Monod equation, which is frequently used to model organism growth in an aqueous environment [80]. Substrate concentration is defined by Equation 4.4 and its rate of depletion is dictated by organism growth. Parameter definitions are given in Table 4.7. The Basic Model demonstrates that growth dynamics rely solely on nutrient availability and initial cell densities.

$$\frac{dX_1}{dt} = \left(r_{1,\max} \frac{S}{K_1 + S} \right) X_1 - d_1 X_1 \tag{4.2}$$

$$\frac{dX_2}{dt} = \left(r_{2,\max} \frac{S}{K_2 + S} \right) X_2 - d_2 X_2 \tag{4.3}$$

$$\frac{dS}{dt} = -\frac{1}{Y_1} \left(r_{1,\max} \frac{S}{K_1 + S} \right) X_1 - \frac{1}{Y_2} \left(r_{2,\max} \frac{S}{K_2 + S} \right) X_2 \tag{4.4}$$

$$\frac{dS_C}{dt} = -S_C \left(r_S \frac{X_2}{K_S + X_2} \right) \tag{4.5}$$

$$\frac{dS}{dt} = nS_C \left(r_S \frac{X_2}{K_S + X_2} \right) - \frac{1}{Y_1} \left(r_{1,\max} \frac{S}{K_1 + S} \right) X_1 - \frac{1}{Y_2} \left(r_{2,\max} \frac{S}{K_2 + S} \right) X_2 \tag{4.6}$$

$$\frac{dX_1}{dt} = \left(r_{1,\max} \frac{S}{K_1 + S} \right) X_1 - d_1 X_1 - a_1 X_1 X_2 \tag{4.7}$$

$$\frac{dX_2}{dt} = \left(r_{2,\max} \frac{S}{K_2 + S} \right) X_2 - d_2 X_2 - a_2 X_1 X_2 \tag{4.8}$$

TABLE 4.7 Definition of model parameters

Parameter	Definition
X_1	Cell density of organism 1 (secondary degrader)
X_2	Cell density of organism 2 (primary degrader)
$r_{i,max}$	Maximum growth rate of X_i
d_i	Decay rate of X_i
K_i	Monod coefficient of X_i
S	Simple substrate concentration
S_C	Complex substrate concentration
Y_i	Conversion efficiency of X_i (substrate to cells)
r_s	Maximum rate of substrate conversion (S_C to S)
K_s	Substrate conversion coefficient
n	# Simple substrate molecules per molecule of complex substrate
a_1	Competition coefficient (antagonism against X_1)
a_2	Competition coefficient (antagonism against X_2)

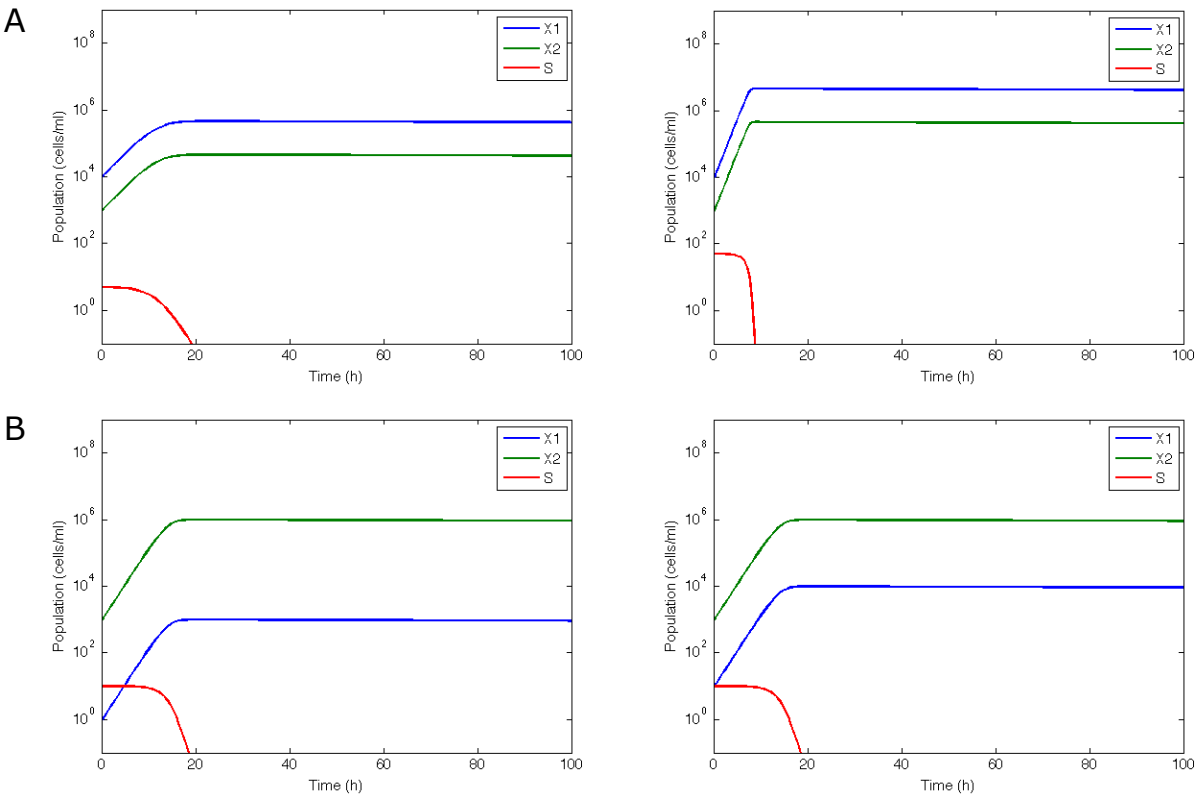

FIGURE 4.14 Effect of initial substrate concentration and initial cell density on maximum cell density in the Basic Model
The maximum cell density is dependent on (a) initial substrate concentration and (b) initial cell density. From left to right in row (a) S_0 = 5, 50. From left to right in row (b) $X_{1,0}$ = 1, 10. Graphs were generated from the Basic Model with the following parameter values: $X_{1,0}$ = 1e4, $X_{2,0}$ = 1e3, S_0 = 10, $r_{1,max} = r_{2,max}$ = 1, $K_1 = K_2$ = 10, $d_1 = d_2$ = .001, $Y_1 = Y_2$ = 1e5

The maximum level to which either population reached depends on how much substrate is available initially in the closed system. Both populations grow to higher cell densities when initial substrate concentration S_0 is increased 10-fold (Figure 4.14a). The maximum cell density of an organism is also affected by its initial cell density. The growth curve of organism X_1 is shifted one log when its initial cell density $X_{1,0}$ is increased 10-fold (Figure 4.14b). A high decay

rate also had an effect on the maximum cell density, but at more realistic values the maximum cell density remained dependent on substrate availability and initial cell density.

This model was expanded to include two types of substrate and a primary degrader. Complex substrate S_C cannot be utilized by either organism, but can be converted into simple substrate S that both populations can use. In this model, hereafter referred to as the Primary Degrader Model, one of the organisms (X_2) is a primary degrader and its presence is necessary to facilitate the conversion of complex substrate into simple substrate. In the Primary Degrader Model, population growth is defined by Equations 4.2 and 4.3, while complex and simple substrate concentrations are defined by Equations 4.5 and 4.6. A schematic of the metabolic relationship in the Primary Degrader Model is given in Figure 4.15.

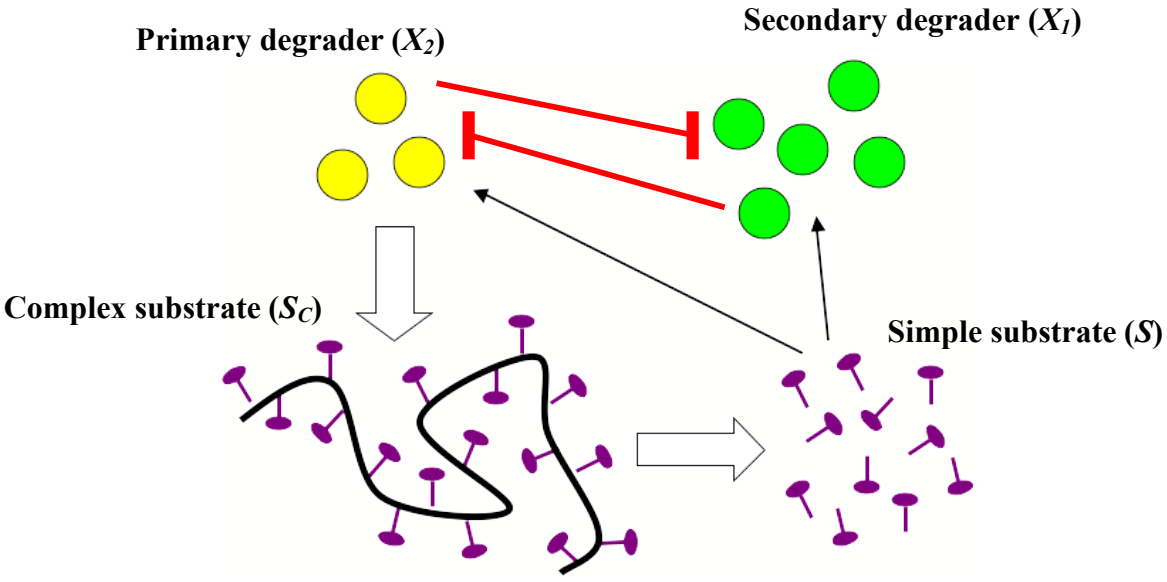

FIGURE 4.15 A schematic of the Primary Degrader and Inhibition Models
Two organisms are grown on the same simple substrate, which is released from the complex substrate by the primary degrader. This is described by the Primary Degrader Model, which does not include any interaction terms between the organisms. The Inhibition Model incorporates antagonism between the two organisms (shown in red).

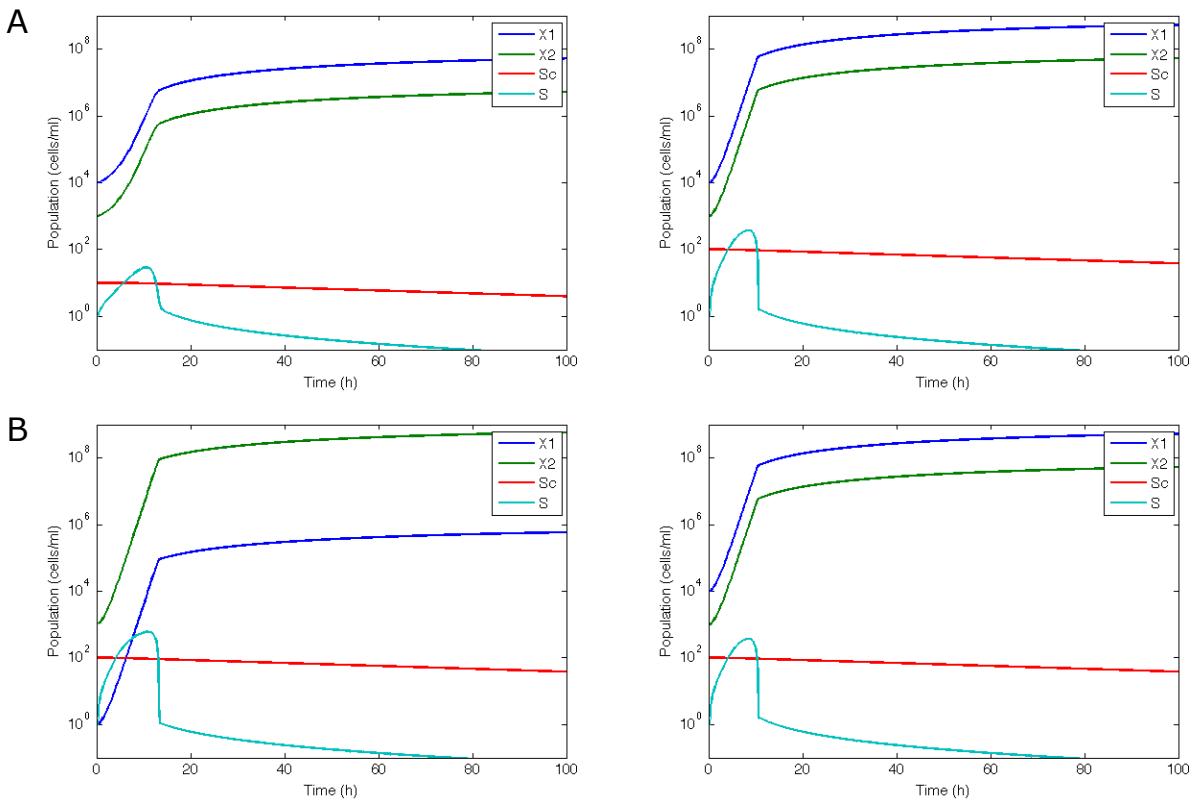

FIGURE 4.16 Effect of initial substrate concentration and initial cell density on maximum cell density in the Primary Degrader Model
The maximum cell density is dependent on (a) initial complex substrate concentration and (b) initial cell density. From left to right in row (a) $S_{C,0}$ = 10, 100. From left to right in row (b) $X_{1,0}$ = 1, 100. Graphs were generated from the Primary Degrader Model with the following parameter values: $X_{1,0}$ = 1e4, $X_{2,0}$ = 1e3, $S_{C,0}$ = 100, S_0 = 1, $r_{1,max} = r_{2,max}$ = 1, $K_1 = K_2$ = 10, $d_1 = d_2$ = .001, r_S = .01, K_s = 1e4, $Y_1 = Y_2$ = 1e5, n = 100

In the Primary Degrader Model, the maximum cell density is still determined by substrate availability and initial cell densities, just as in the Basic Model (Figure 4.16). A 10-fold increase in the initial complex substrate concentration $S_{C,0}$ increases the maximum levels of both organisms. A higher initial cell density of organism X_1 raises its maximum cell density. No inhibition was observed in this model against either organism. While competition over substrate is apparent, growth is only prevented in extreme cases where growth rate is unrealistically low or

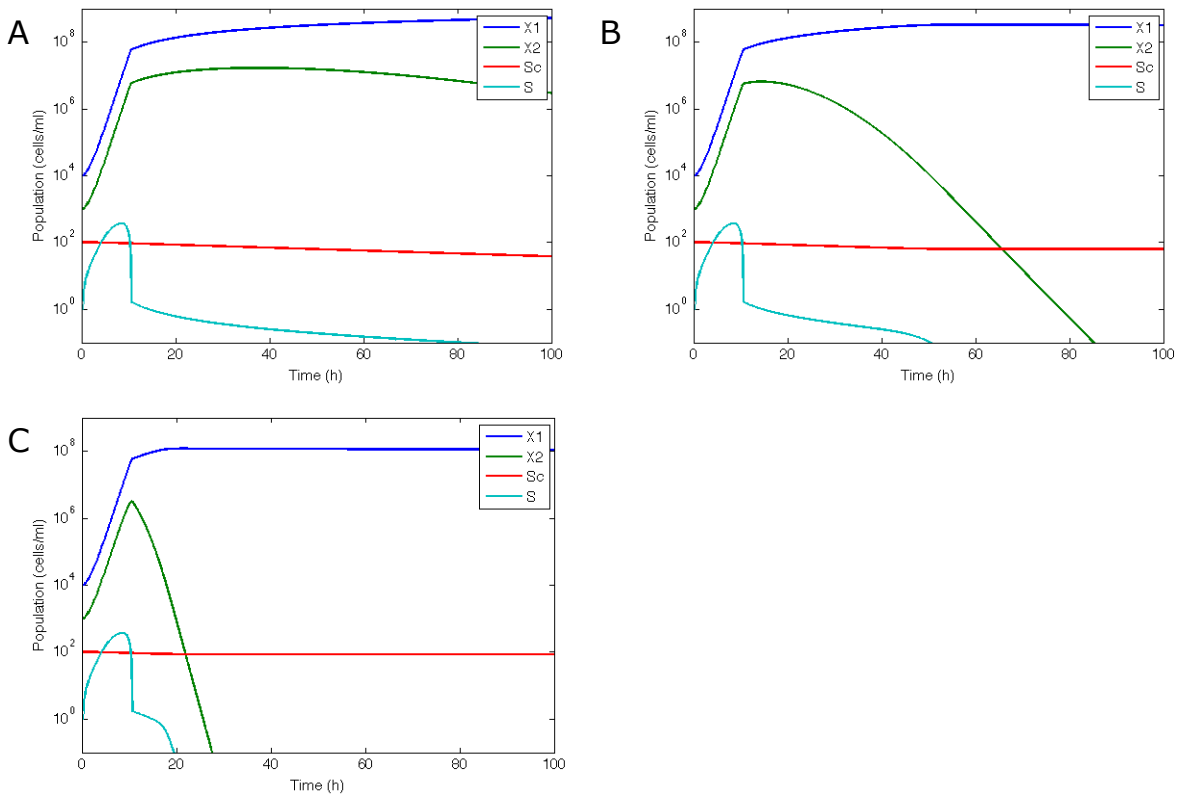

FIGURE 4.17 Growth dynamics of a two-member population in the Inhibition Model The ability of an organism to grow and reach stationary phase depends on the strength of antagonism between the two organisms and its initial cell density. Competition coefficients a_1 and a_2 were equal, and their values were (a) 1e-10, (b) 1e-9, (c) 1e-8. Graphs were generated from the Inhibition Model with the following parameter values: $X_{1,0}$ = 1e4, $X_{2,0}$ = 1e3, $S_{C,0}$ = 100, S_0 = 1, $r_{1,max} = r_{2,max}$ = 1, $K_1 = K_2$ = 10, $d_1 = d_2$ = .001, r_S = .01, K_s = 1e4, $Y_1 = Y_2$ = 1e5, n = 100

decay rate is much greater than the growth rate. At high initial cell density of one or both organism(s), substrate concentration may be too low to support growth of either organism. This behavior is expected in a closed, batch system.

Finally, interaction terms were introduced into the growth equations of the Primary Degrader Model. Only negative values for the interaction coefficients a_1 and a_2 were tested in

this model, hereafter referred to as the Inhibition Model, to see if inhibition was possible. The Inhibition Model is defined by Equations 4.5 through 4.8 and described by Figure 4.15.

When the competition coefficients a_1 and a_2 are equal for both organisms, secondary degrader X_1 has no trouble replicating and reaching stationary phase (Figure 4.17). Interestingly, however, growth inhibition of primary degrader X_2 can be achieved at sufficiently high values of a_1 and a_2. This is suspected to a result of the difference in initial cell densities (i.e. $X_{1,0} > X_{2,0}$) since all other parameters, including growth rate, efficiency of obtaining substrate, and decay rate, were the same for the two organisms.

Since substrate availability depends on presence of the primary degrader, inhibition of the secondary degrader X_1 is expected to occur when primary degrader X_2 does not replicate or is present at low numbers. This hypothesis was tested by decreasing the growth rate and initial cell

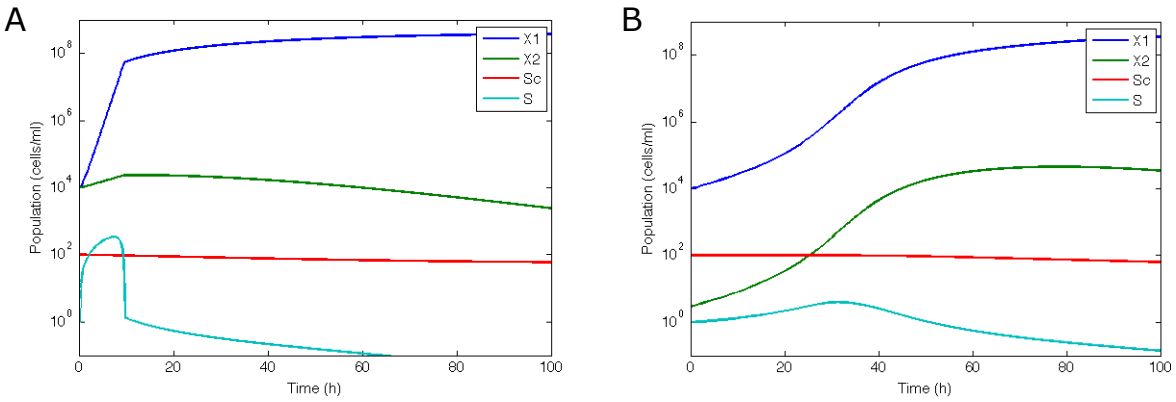

FIGURE 4.18 Growth of secondary degrader in spite of slow-growing primary degrader in the Inhibition Model
Secondary degrader X_1 is able to grow and reach stationary phase even when primary degrader X_2 (a) is not growing or (b) is present at low numbers. (a) $r_{2,max} = 0.1$, (b) $X_{2,0} = 3$. Graphs were generated from the Inhibition Model with the following parameter values: $X_{1,0} = X_{2,0} = 1e4$, $S_{C,0} = 100$, $S_0 = 1$, $a_1 = a_2 = 1e\text{-}10$, $r_{1,max} = r_{2,max} = 1$, $K_1 = K_2 = 10$, $d_1 = d_2 = .001$, $r_S = .01$, $K_s = 1e4$, $Y_1 = Y_2 = 1e5$, $n = 100$

density of X_2 independently at a competition coefficient value that does not inhibit growth of either organism. Figure 4.18 demonstrates that this was not the case, however. When primary degrader X_2 does not replicate due to a low growth rate, or is present at low numbers due to a lower initial cell density, secondary degrader X_1 has no difficulty growing. In all cases, simple substrate was present initially at low levels (S_0 = 1), but apparently this was enough to allow the secondary degrader X_1 to replicate and reach stationary phase.

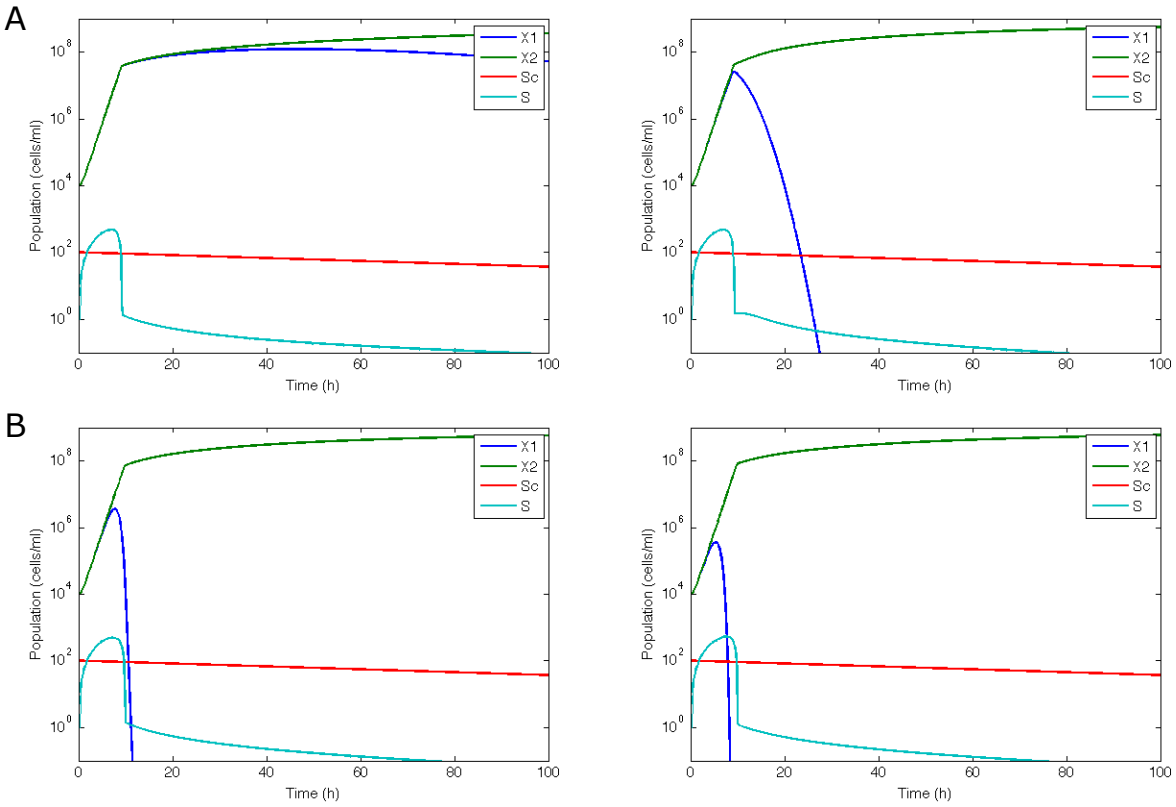

FIGURE 4.19 Inhibition of secondary degrader in the Inhibition Model
Secondary degrader X_1 is inhibited when antagonism against it is sufficiently high. From left to right in row (a) a_1 = 1e-10, 1e-8 and a_2 = 0. From left to right in row (b) a_1 = 1e-7, 1e-6 and a_2 = 1e-9, 1e-8. Graphs were generated from the Inhibition Model with the following parameter values: $X_{1,0} = X_{2,0}$ = 1e4, $S_{C,0}$ = 100, S_0 = 1, $r_{1,max} = r_{2,max}$ = 1, $K_1 = K_2$ = 10, $d_1 = d_2$ = .001, r_S = .01, K_s = 1e4, $Y_1 = Y_2$ = 1e5, n = 100

To see if inhibition of the secondary degrader X_1 was possible at all, several other scenarios were tested using the Inhibition Model. Inhibition of X_1 is observable when antagonism is unidirectional ($a_2 = 0$) and the intensity of antagonism was sufficiently high (Figure 4.19a). Inhibition against X_1 is also possible in bidirectional antagonism, provided the degree of antagonism against X_1 is sufficiently greater than the antagonism against X_2 (i.e $a_1 > a_2$) (Figure 4.19b).

Both the Basic Model and the Primary Degrader Model demonstrate that the maximum cell density a population can reach in a closed batch system is dictated by the amount of substrate that is available and the initial cell density. This behavior was seen most notably when *L. gasseri* was cocultured with all three organisms at different initial cell densities (3.2, 4.2, 5.5 log cfu ml^{-1}) in Trials 1 and 2 of the *ex vivo* study and the coculture study, and the cell density at 24 h was approximately 2 logs higher than its starting concentration.

The Primary Degrader Model and the Inhibition Model further demonstrate that inhibition is difficult to achieve in a two-member mathematical model with a simple metabolic relationship and even with antagonistic interactions. Growth inhibition of the secondary degrader was achieved when antagonism was sufficiently high and directed against the organism. These observations may explain why no inhibition of *Salmonella* was seen in our experimental system.

CHAPTER 5

DISCUSSION

Not much is known about *Salmonella* growth and behavior in a mixed culture that is representative of the intestinal microbiome. Being pathogen of human concern, *Salmonella* has been the subject of many studies. Its pathogenic mechanisms (colonization, adhesion, and invasion) are well-characterized. However, most studies are performed in a laboratory setting using growth conditions and media that do not accurately reflect what *Salmonella* encounters in its natural environment.

The *Salmonella* genome contains transcriptional regulators that allow the organism to respond quickly and appropriately to a wide range of environments. *Salmonella* is typically found in the animal intestinal tract, but can survive in the environment until it finds its next host to colonize. *Salmonella* is equipped with sensor systems that detect changes to temperature, osmolarity, and pH. In the intestinal tract among a diverse bacterial community, *Salmonella* encounters additional challenges like antimicrobial peptides, oxidative stress, and nutrient starvation [113]. Clearly *Salmonella* behavior is dictated by its environment, making it important to study the organism under realistic conditions and as a member of a mixed community.

Studies of *Salmonella* growth in a mixed culture are not very common. There are multiple studies on the ability of a characterized or uncharacterized mixed culture to inhibit *Salmonella* [114-116]. Other studies focus on enhancing enrichment and isolation of *Salmonella* [117]. No studies on exactly how a mixed community affects *Salmonella* growth can be found.

It is not uncommon for growth and expression experiments to be conducted using the same medium used to maintain the organism. Maintenance media is usually nutrient-rich and not recommended for physiological studies since the purpose of maintenance media is to keep the organism alive. However, microorganisms can adapt relatively quickly, and extensively passaged organisms may have altered genomes [118]. This is the case for laboratory strain *S.* Typhimurium LT2, which is avirulent due to an rpoS mutation [119, 120]. Nevertheless, these laboratory strains are typically used in physiological studies of the organism.

Furthermore, *Salmonella* is a facultative aerobe, capable of growing both in the presence and absence of oxygen. For ease, most studies are conducted at the bench in the presence of oxygen. Within the animal intestinal tract, however, oxygen levels are low if not absent. Most importantly, in order to fully understand the behavior of *Salmonella* in its natural environment, one must consider the presence of a complex microbiome in order to account for microbial and metabolic interactions.

In this study, the organisms and growth environment were selected to more accurately represent the conditions of the chicken cecum because *Salmonella* is known for its ability to maintain a stable population in this diverse bacterial community. *Salmonella* growth rate (doubling time) and decay rate were affected by the community composition. When grown alone under these *ex vivo* conditions, *Salmonella*'s doubling time was three times that of its fastest doubling time (17 min) when grown in LB medium in the presence of oxygen [121]. Despite these differences, *Salmonella* reached a maximum cell density of about 8 log cfu ml^{-1} regardless of community composition. This is most likely due to the carrying capacity of the closed system (100 ml of EVCC broth) and is limited by the organism's growth rate under such conditions.

As expected, presence of a primary degrader (*Bacteroides* or *Eubacterium*) seemed to free more accessible nutrients for the secondary degraders (*Salmonella* and *Lactobacillus*). Direct competition between *Salmonella* and *Lactobacillus*, *Bacteroides*, and *Eubacterium* was observed. The ability of the primary degraders, *Bacteroides* and *Eubacterium*, to access nutrients and compete with the secondary degraders differed. The cell densities of *Eubacterium* were less affected than those of *Bacteroides* by the presence of *Salmonella* or *Salmonella* and *Lactobacillus*. *E. rectale* has been shown to decrease production of glycan-degrading enzymes and increase production of amino acid and sugar transporters in coculture [15].

In future studies, it will be important to focus on more realistic conditions when determining *Salmonella* behavior in its natural environment. The EVCC medium can be modified to contain less peptone in order to reveal *Salmonella* growth dynamic and behavior in a nutrient-limited environment. Expanding the timescale by converting the batch culture system to a continuous culture will allow for more realistic studies of *Salmonella* persistence and steady state growth dynamics in a mixed community. In order to obtain a more complete picture of microbial interactions and metabolic networks, data such as growth and decay rates as well as transcriptional profiles can be collected from other members of the mixed community.

CHAPTER 6

CONCLUSION

The objective of this study was to determine the effect of community composition on *Salmonella* growth in the *ex vivo* system developed to mimic conditions found in the chicken cecum. *Salmonella* Typhimurium was cocultured with three other organisms representative of major bacterial groups found in the animal intestine: *Lactobacillus gasseri*, *Bacteroides thetaiotaomicron*, and *Eubacterium rectale*. *Salmonella* growth rates (doubling times) and decay rates were calculated, and community composition was determined using a combination of methods. A mathematical model describing the growth of two distinct populations with basic interaction was formulated and compared with the experimental data.

The coculture study demonstrated that *Salmonella* growth and decay rates, but not its maximum cell density, was affected by which organisms were present in the community. *Salmonella* doubling time was slower when grown under these *ex vivo* conditions than under ideal lab conditions. Direct competition between *Salmonella* and all other organisms was detected as expected. Furthermore, presence of primary degraders of mucin, *Bacteroides* and/or *Eubacterium*, freed more nutrients for the rest of the community. From community composition data, *Bacteroides* and *Eubacterium* appeared to differ in ability to compete with *Salmonella* and *Lactobacillus*. In fact, *Eubacterium* has been shown to increase production of amino acid and sugar transporters while decreasing production of glycan-degrading enzymes in coculture [15].

When *Salmonella* was introduced into an "established" community (at 48 h), it was unable to grow, suggesting that other organisms may have depleted all accessible nutrients by that time.

It is my hope that future studies to advance understanding of *Salmonella* growth dynamics and behavior in its natural environment will be conducted under more realistic conditions. The EVCC medium and anaerobic *in vitro* system developed for this study can be modified and used in future studies of other enteropathogens. Furthermore, mathematical modeling can be a useful tool to predict behavior and explain complex interactions. The models developed here can be expanded and modified to analyze metabolic and microbial interactions.

LITERATURE CITED

1. Savage, D.C., *Microbial ecology of the gastrointestinal tract.* Annual Reviews in Microbiology, 1977. **31**(1): p. 107-133.

2. Whitman, W.B., D.C. Coleman, and W.J. Wiebe, *Prokaryotes: the unseen majority.* Proceedings of the National Academy of Sciences, 1998. **95**(12): p. 6578-6583.

3. Bäckhed, F., et al., *Host-bacterial mutualism in the human intestine.* Science, 2005. **307**(5717): p. 1915-1920.

4. Björnhag, G., *Adaptations in the large intestine allowing small animals to eat fibrous foods*, in *The Digestive System in Mammals*, D.J. Chivers and P. Langer, Editors. 1994, Cambridge University Press. p. 287-309.

5. Akin, D.E. and R. Benner, *Degradation of polysaccharides and lignin by ruminal bacteria and fungi.* Applied and Environmental Microbiology, 1988. **54**(5): p. 1117-1125.

6. Forsberg, C.W., K. Cheng, and B.A. White, *Polysaccharide degradation in the rumen and large intestine*, in *Gastrointestinal Microbiology*, R.I. Mackie and B.A. White, Editors. 1997, Springer US. p. 319-379.

7. Breznak, J.A. and A. Brune, *Role of microorganisms in the digestion of lignocellulose by termites.* Annual Review of Entomology, 1994. **39**(1): p. 453-487.

8. Brune, A. and M. Ohkuma, *Role of the termite gut microbiota in symbiotic digestion*, in *Biology of Termites: A Modern Synthesis*, D.E. Bignell, Y. Roisin, and N. Lo, Editors. 2011, Springer. p. 439-475.

9. Ley, R.E., D.A. Peterson, and J.I. Gordon, *Ecological and evolutionary forces shaping microbial diversity in the human intestine.* Cell, 2006. **124**(4): p. 837-848.

10. Lozupone, C.A., et al., *The convergence of carbohydrate active gene repertoires in human gut microbes.* Proceedings of the National Academy of Sciences, 2008. **105**(39): p. 15076-15081.

11. Edlund, C. and C.E. Nord, *Effect on the human normal microflora of oral antibiotics for treatment of urinary tract infections.* Journal of Antimicrobial Chemotherapy, 2000. **46**(suppl 1): p. 41-48.

12. Waaij, V., *The ecology of the human intestine and its consequences for overgrowth by pathogens such as Clostridium difficile.* Annual Reviews in Microbiology, 1989. **43**(1): p. 69-87.

13. Guarner, F. and J.R. Malagelada, *Gut flora in health and disease.* The Lancet, 2003. **361**(9356): p. 512-519.

14. Belenguer, A., et al., *Two routes of metabolic cross-feeding between Bifidobacterium adolescentis and butyrate-producing anaerobes from the human gut.* Applied and Environmental Microbiology, 2006. **72**(5): p. 3593-3599.

15. Mahowald, M.A., et al., *Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla.* Proceedings of the National Academy of Sciences, 2009. **106**(14): p. 5859-5864.

16. Hugenholtz, P., B.M. Goebel, and N.R. Pace, *Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity.* Journal of Bacteriology, 1998. **180**(18): p. 4765-4774.

17. Seksik, P., et al., *Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon.* Gut, 2003. **52**(2): p. 237-242.

18. Eckburg, P.B., et al., *Diversity of the human intestinal microbial flora.* Science, 2005. **308**(5728): p. 1635-1638.

19. Turnbaugh, P.J., et al., *A core gut microbiome in obese and lean twins.* Nature, 2008. **457**(7228): p. 480-484.

20. Ley, R.E., et al., *Evolution of mammals and their gut microbes.* Science, 2008. **320**(5883): p. 1647-1651.

21. Lu, J., et al., *Diversity and succession of the intestinal bacterial community of the maturing broiler chicken.* Applied and Environmental Microbiology, 2003. **69**(11): p. 6816-6824.

22. Scallan, E., et al., *Foodborne illness acquired in the United States—major pathogens.* Emerging Infectious Diseases, 2011. **17**(1): p. 7-15.

23. Scallan, E., *Foodborne illness acquired in the United States—unspecified agents.* Emerging Infectious Diseases, 2011. **17**(1).

24. Fierer, J. and D.G. Guiney, *Diverse virulence traits underlying different clinical outcomes of Salmonella infection.* Journal of Clinical Investigation, 2001. **107**(7): p. 6.

25. Coburn, B., G.A. Grassl, and B.B. Finlay, *Salmonella, the host and disease: a brief review.* Immunology and Cell Biology, 2006. **85**(2): p. 112-118.

26. Merrell, D.S. and S. Falkow, *Frontal and stealth attack strategies in microbial pathogenesis.* Nature, 2004. **430**(6996): p. 250-256.

27. Weinstein, D.L., et al., *Differential early interactions between Salmonella enterica serovar Typhi and two other pathogenic Salmonella serovars with intestinal epithelial cells.* Infection and Immunity, 1998. **66**(5): p. 2310-2318.

28. Bhan, M.K., R. Bahl, and S. Bhatnagar, *Typhoid and paratyphoid fever.* The Lancet, 2005. **366**(9487): p. 749-762.

29. Parry, C.M., et al., *Typhoid fever.* New England Journal of Medicine, 2002. **347**(22): p. 1770-1782.

30. Greig, J. and A. Ravel, *Analysis of foodborne outbreak data reported internationally for source attribution.* International Journal of Food Microbiology, 2009. **130**(2): p. 77-87.

31. Barrow, P., J.M. Simpson, and M.A. Lovell, *Intestinal colonisation in the chicken by food-poisoning Salmonella serotypes; microbial characteristics associated with faecal excretion.* Avian Pathology, 1988. **17**(3): p. 571-588.

32. Humphrey, T., *Growth of salmonellas in intact shell eggs: influence of storage temperatures.* Veterinary Record, 1990. **126**(12): p. 292.

33. Barrow, P., et al., *Observations on the pathogenesis of experimental Salmonella typhimurium infection in chickens.* Research in Veterinary Science, 1987. **42**(2): p. 194.

34. Cooper, G.L., et al., *Invasiveness and persistence of Salmonella enteritidis, Salmonella typhimurium, and a genetically defined S. enteritidis aroA strain in young chickens.* Infection and Immunity, 1994. **62**(11): p. 4739-4746.

35. Henzler, D. and H. Opitz, *The role of mice in the epizootiology of Salmonella enteritidis infection on chicken layer farms.* Avian Diseases, 1992. **36**(3): p. 625-631.

36. Gast, R.K., J. Guard-Bouldin, and P.S. Holt, *Colonization of reproductive organs and internal contamination of eggs after experimental infection of laying hens with Salmonella Heidelberg and Salmonella Enteritidis.* Avian Diseases, 2004. **48**(4): p. 863-869.

37. Gast, R.K., et al., *Colonization of specific regions of the reproductive tract and deposition at different locations inside eggs laid by hens infected with Salmonella Enteritidis or Salmonella Heidelberg.* Avian Diseases, 2007. **51**(1): p. 40-44.

38. O'Brien, J., *Salmonella enteritidis infection in broiler chickens.* The Veterinary Record, 1988. **122**(9): p. 214.

39. Snoeyenbos, G., C. Smyser, and H. Van Roekel, *Salmonella infections of the ovary and peritoneum of chickens.* Avian Diseases, 1969. **13**(3): p. 668-670.

40. Shivaprasad, H., *Fowl typhoid and pullorum disease.* Revue scientifique et technique (International Office of Epizootics), 2000. **19**(2): p. 405.

41. *The Poultry Site*. Quick Disease Guide 2000-2012 [cited 2012 December 9]; Available from: http://www.thepoultrysite.com/diseaseinfo.

42. Smith, H.W. and J. Tucker, *The virulence of Salmonella strains for chickens: their excretion by infected chickens.* Journal of Hygiene, 1980. **84**(03): p. 479-488.

43. Foley, S., A. Lynne, and R. Nayak, *Salmonella challenges: prevalence in swine and poultry and potential pathogenicity of such isolates.* Journal of Animal Science, 2008. **86**(14 suppl): p. E149-E162.

44. CDC, *Salmonella surveillance: annual summary, 2006*, 2008, US Department of Health and Human Services, CDC: Atlanta, GA.

45. Bäumler, A.J., B.M. Hargis, and R.M. Tsolis, *Tracing the origins of Salmonella outbreaks.* Science, 2000. **287**(5450): p. 50-52.

46. Foley, S.L., et al., *Population dynamics of Salmonella enterica serotypes in commercial egg and poultry production.* Applied and Environmental Microbiology, 2011. **77**(13): p. 4273-4279.

47. Edwards, P. and D. Bruner, *The occurrence and distribution of Salmonella types in the United States.* The Journal of Infectious Diseases, 1943. **72**(1): p. 58-67.

48. Morgan, E., et al., *Identification of host-specific colonization factors of Salmonella enterica serovar Typhimurium.* Molecular Microbiology, 2004. **54**(4): p. 994-1010.

49. Bailey, J.S., *Integrated colonization control of Salmonella in poultry.* Poultry Science, 1988. **67**(6): p. 928-932.

50. Lahellec, C. and P. Colin, *Relationship between serotypes of Salmonellae from hatcheries and rearing farms and those from processed poultry carcases.* British Poultry Science, 1985. **26**(2): p. 179-186.

51. Barrow, P., M. Jones, and N. Thomson, *Salmonella*, in *Pathogenesis of Bacterial Infections in Animals*, C. Gyles, et al., Editors. 2010, Wiley-Blackwell: Ames, Iowa.

52. Nurmi, E., L. Nuotio, and C. Schneitz, *The competitive exclusion concept: development and future.* International Journal of Food Microbiology, 1992. **15**(3-4): p. 237-40.

53. Nurmi, E. and M. Rantala, *New aspects of Salmonella infection in broiler production.* Nature, 1973. **241**(5386): p. 210-211.

54. Impey, C. and G. Mead, *Fate of salmonellas in the alimentary tract of chicks pre-treated with a mature caecal microflora to increase colonization resistance.* Journal of Applied Microbiology, 1989. **66**(6): p. 469-475.

55. Pivnick, H., *The Nurmi concept and its role in the control of salmonellae in poultry.* Developments in Food Microbiology, 1982. **1**: p. 41-70.

56. Rantala, M. and E. Nurmi, *Prevention of the growth of Salmonella infantis in chicks by the flora of the alimentary tract of chickens.* British Poultry Science, 1973. **14**(6): p. 627-630.

57. Schneitz, C., et al., *Competitive exclusion in the young bird: challenge models, administration and reciprocal protection.* International Journal of Food Microbiology, 1992. **15**(3-4): p. 241-4.

58. Cameron, D. and J. Carter, *Evaluation of the efficacy of Broilact® in preventing infection of broiler chicks with Salmonella Enteritidis PT4.* International Journal of Food Microbiology, 1992. **15**(3): p. 319-326.

59. Salvat, G., et al., *Use of a competitive exclusion product (Broilact®) to prevent Salmonella colonization of newly hatched chicks.* International Journal of Food Microbiology, 1992. **15**(3): p. 307-311.

60. Cameron, D., J. Carter, and P. Mansell. *Evaluation of Aviguard against a Salmonella enteritidis infective model in broiler chickens*. in *Western Poultry Disease Conference*. 1996.

61. Cameron, D., et al. *Floor-pen efficacy study with Aviguard against Salmonella typhimurium DT104 colonization in turkeys*. in *Proc. International Symposium on Salmonella and Salmonellosis*. 1997. Ploufragan.

62. Nakamura, A., et al., *Evaluation of Aviguard, a commercial competitive exclusion product for efficacy and after-effect on the antibody response of chicks to Salmonella.* Poultry Science, 2002. **81**(11): p. 1653-1660.

63. Impey, C., G. Mead, and S. George, *Evaluation of treatment with defined and undefined mixtures of gut microorganisms for preventing Salmonella colonization in chicks and turkey poults.* Food Microbiology, 1984. **1**(2): p. 143-147.

64. Hirn, J., et al., *Long-term experience with competitive exclusion and salmonellas in Finland.* International Journal of Food Microbiology, 1992. **15**(3): p. 281-285.

65. Wierup, M., H. Wahlström, and B. Engström, *Experience of a 10-year use of competitive exclusion treatment as part of the Salmonella control programme in Sweden.* International Journal of Food Microbiology, 1992. **15**(3): p. 287-291.

66. Lawley, T.D., et al., *Host transmission of Salmonella enterica serovar Typhimurium is controlled by virulence factors and indigenous intestinal microbiota.* Infection and Immunity, 2008. **76**(1): p. 403-416.

67. Sekirov, I., et al., *Salmonella SPI-1-mediated neutrophil recruitment during enteric colitis is associated with reduction and alteration in intestinal microbiota.* Gut Microbes, 2010. **1**(1): p. 30.

68. Thiennimitr, P., et al., *Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota.* Proceedings of the National Academy of Sciences, 2011. **108**(42): p. 17480-17485.

69. Winter, S.E., et al., *Gut inflammation provides a respiratory electron acceptor for Salmonella.* Nature, 2010. **467**(7314): p. 426-429.

70. Stecher, B., et al., *Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota.* PLoS Biology, 2007. **5**(10): p. e244.

71. Barman, M., et al., *Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract.* Infection and Immunity, 2008. **76**(3): p. 907-915.

72. Coburn, B., et al., *Salmonella enterica serovar Typhimurium pathogenicity island 2 is necessary for complete virulence in a mouse model of infectious enterocolitis.* Infection and Immunity, 2005. **73**(6): p. 3219-3227.

73. Santos, R.L., et al., *Life in the inflamed intestine, Salmonella style.* Trends in Microbiology, 2009. **17**(11): p. 498-506.

74. Tsolis, R.M., et al., *Contribution of Salmonella typhimurium virulence factors to diarrheal disease in calves.* Infection and Immunity, 1999. **67**(9): p. 4879-4885.

75. Zhang, S., et al., *The Salmonella enterica serotype typhimurium effector proteins SipA, SopA, SopB, SopD, and SopE2 act in concert to induce diarrhea in calves.* Infection and Immunity, 2002. **70**(7): p. 3843-3855.

76. Koichi, K., F. Michiya, and N. Makoto, *Lipid components of two different regions of an intestinal epithelial cell membrane of mouse.* Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism, 1974. **369**(2): p. 222-233.

77. Box, G.E. and N.R. Draper, *Empirical model-building and response surfaces.* 1987: John Wiley & Sons.

78. Malthus, T.R., *An essay on the principle of population as it affects the future improvement of society, with remarks on the speculations of Mr. Godwin, M. Condorcet, and other writers.* St. Paul's Church-Yard, London, 1798.

79. Verhulst, P., *Notice sur la loi que la popolation poursuit dans son accroissement.* Correspondance mathématique et physique, 1838. **10**: p. 113-121.

80. Monod, J., *The growth of bacterial cultures.* Annual Reviews in Microbiology, 1949. **3**(1): p. 371-394.

81. Gauss, G.F., *The Struggle for Existence.* 1934, Baltimore: Williams and Wilkins.

82. Yoon, H., G. Klinzing, and H. Blanch, *Competition for mixed substrates by microbial populations.* Biotechnology and Bioengineering, 1977. **19**(8): p. 1193-1210.

83. Jones, J., et al., *Mathematical modelling of the growth, survival and death of Yersinia enterocolitica.* International Journal of Food Microbiology, 1994. **23**(3): p. 433-447.

84. McClure, P., et al., *Modelling the growth, survival and death of microorganisms in foods: the UK food micromodel approach.* International Journal of Food Microbiology, 1994. **23**(3): p. 265-275.

85. Gombert, A.K. and J. Nielsen, *Mathematical modelling of metabolism.* Current Opinion in Biotechnology, 2000. **11**(2): p. 180-186.

86. Bishop, S. and M. Stear, *Modeling of host genetics and resistance to infectious diseases: understanding and controlling nematode infections.* Veterinary Parasitology, 2003. **115**(2): p. 147-166.

87. Boudrant, J., et al., *Mathematical modelling of cell suspension in high cell density conditions: Application to L-lactic acid fermentation using Lactobacillus casei in membrane bioreactor.* Process Biochemistry, 2005. **40**(5): p. 1641-1647.

88. Schulze-Horsel, J., et al., *Infection dynamics and virus-induced apoptosis in cell culture-based influenza vaccine production—flow cytometry and mathematical modeling.* Vaccine, 2009. **27**(20): p. 2712-2722.

89. Kim, S., H. Park, and K. Chandran, *Propensity of activated sludge to amplify or attenuate tetracycline resistance genes and tetracycline resistant bacteria: A mathematical modeling approach.* Chemosphere, 2010. **78**(9): p. 1071-1077.

90. Schmidt, J.K., et al., *A novel concept combining experimental and mathematical analysis for the identification of unknown interspecies effects in a mixed culture.* Biotechnology and bioengineering, 2011. **108**(8): p. 1900-1911.

91. Ni, B.-J., et al., *Evaluating four mathematical models for nitrous oxide production by autotrophic ammonia-oxidizing bacteria.* Biotechnology and Bioengineering, 2012. **110**(1): p. 153-163.

92. Ruiz-Perez, F., et al., *Use of a continuous-flow anaerobic culture to characterize enteric virulence gene expression.* Infection and Immunity, 2004. **72**(7): p. 3793-3802.

93. Gérard-Champod, M., et al., *Development and validation of a continuous in vitro system reproducing some biotic and abiotic factors of the veal calf intestine.* Applied and Environmental Microbiology, 2010. **76**(16): p. 5592-600.

94. Roberton, A.M. and R.A. Stanley, *In vitro utilization of mucin by Bacteroides fragilis.* Applied and Environmental Microbiology, 1982. **43**(2): p. 325-30.

95. National Research Council, S.o.P.N., *Nutrient requirements of poultry*1994: National Academies Press.

96. Beck, J. and T. Chang, *Measurement of uric acid levels in chicken cecal contents.* Poultry Science, 1980. **59**(6): p. 1193-1196.

97. Babinszky, L., et al., *Determination of amino acid digestibility with different methods in birds.* Slovak Journal of Animal Science, 2006. **39**(1-2): p. 74-78.

98. Ljungdahl, L. and J. Wiegel, *Working with anaerobic bacteria*, in *Manual of Industrial Microbiology and Biotechnology*, A. Demain and N. Solomon, Editors. 1986, American Society for Microbiology: Washington, DC. p. 84-96.

99. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* Proceedings of the National Academy of Sciences, 2000. **97**(12): p. 6640-6645.

100. Miles, A. and S. Misra, *The estimation of the bactericidal power of blood.* Journal of Hygiene, 1938. **38**(6): p. 732-749.

101. Parsley, L.C., M.M. Newman, and M.R. Liles, *Fluorescence in situ hybridization of bacterial cell suspensions.* Cold Spring Harbor Protocols, 2010. **2010**(9): p. pdb.prot5493.

102. Amann, R., et al., *Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations.* Applied and Environmental Microbiology, 1990. **56**(6): p. 1919-1925.

103. Sghir, A., D. Antonopoulos, and R. Mackie, *Design and evaluation of a Lactobacillus group-specific ribosomal RNA-targeted hybridization probe and its application to the study of intestinal microecology in pigs.* Systematic and Applied Microbiology, 1998. **21**(2): p. 291-296.

104. Manz, W., et al., *Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment.* Microbiology, 1996. **142**(5): p. 1097-1106.

105. Franks, A.H., et al., *Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes.* Applied and Environmental Microbiology, 1998. **64**(9): p. 3336-45.

106. Quevedo, B., et al., *Phylogenetic group- and species-specific oligonucleotide probes for single-cell detection of lactic acid bacteria in oral biofilms.* BMC Microbiology, 2011. **11**(1): p. 14.

107. Meier, H., et al., *Specific oligonucleotide probes for in situ detection of a major group of Gram-positive bacteria with low DNA G+C content.* Systematic and Applied Microbiology, 1999. **22**(2): p. 186-196.

108. Macnaughton, S., A. O'Donnell, and T. Embley, *Permeabilization of mycolic-acid-containing actinomycetes for in situ hybridization with fluorescently labelled oligonucleotide probes.* Microbiology, 1994. **140**(10): p. 2859-2865.

109. Kenzaka, T., et al., *rRNA-targeted fluorescent in situ hybridization analysis of bacterial community structure in river water.* Microbiology, 1998. **144**(8): p. 2085-2093.

110. Harmsen, H.J.M., et al., *A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent in situ hybridization.* Microbial Ecology in Health and Disease, 1999. **11**(1): p. 3-12.

111. Beimfohr, C., et al., *In situ identification of Lactococci, Enterococci and Streptococci.* Systematic and Applied Microbiology, 1993. **16**(3): p. 450-456.

112. Cole, J., et al., *The Ribosomal Database Project: improved alignments and new tools for rRNA analysis.* Nucleic Acids Research, 2009. **37**(Suppl 1): p. D141-D145.

113. Shen, S. and F.C. Fang, *Integrated stress responses in Salmonella.* International Journal of Food Microbiology, 2012. **152**(3): p. 75-81.

114. Corrier, D., et al., *Control of Salmonella typhimurium colonization in broiler chicks with a continuous-flow characterized mixed culture of cecal bacteria.* Poultry Science, 1995. **74**(6): p. 916-924.

115. van der Wielen, P.W., et al., *Competitive exclusion of Salmonella enterica serovar Enteritidis by Lactobacillus crispatus and Clostridium lactatifermentans in a sequencing fed-batch culture.* Applied and Environmental Microbiology, 2002. **68**(2): p. 555-559.

116. Lloyd, A., R. Cumming, and R. Kent, *Prevention of Salmonella typhimurium infection in poultry by pretreatment of chickens and poults with intestinal extracts.* Australian Veterinary Journal, 2008. **53**(2): p. 82-87.

117. Rhodes, P., L. Quesnel, and P. Collard, *Growth kinetics of mixed culture in Salmonella enrichment media.* Journal of Applied Microbiology, 1985. **59**(3): p. 231-237.

118. Cha, T.-a., et al., *Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains.* Journal of Virology, 1996. **70**(1): p. 78-83.

119. Swords, W.E., B.M. Cannon, and W.H. Benjamin Jr, *Avirulence of LT2 strains of Salmonella typhimurium results from a defective rpoS gene.* Infection and Immunity, 1997. **65**(6): p. 2451-2453.

120. Wilmes-Riesenberg, M.R., J.W. Foster, and R. Curtiss, *An altered rpoS allele contributes to the avirulence of Salmonella typhimurium LT2.* Infection and Immunity, 1997. **65**(1): p. 203-210.

121. Aldea, M., et al., *Surface density of major outer membrane proteins in Salmonella typhimurium in different growth conditions.* Journal of General Microbiology, 1980. **120**(2): p. 355-367.

APPENDIX A

BASIC MODEL (MATLAB CODE)

```
% This is the Basic Model
% 2-member population (X1, X2)
% Grown on substrate (S)
% No interaction
% Batch (closed) system.


function model00

time = 100;

% Initial conditions
X10 = 1e5;  % X1: organism 1 (cells/ml)
X20 = 1e3;  % X2: organism 2 (cells/ml)
S0 = 10;    % S: simple substrate (g/L)

% ODE
Q_init = [ X10 X20 S0];
time = [ 0 time ];
[T,Q] = ode45(@ode_model, time, Q_init);

% Plotting the data
semilogy(T,Q,'linewidth',2);
legend('X1','X2','S');
xlabel('Time (h)');
ylabel('Population (cells/ml)');

end


% ========== Differential Equation Model ==============

function dQ = ode_model(T,Q) % set of differential equations

X1 = Q(1);
X2 = Q(2);
S = Q(3);
```

```
% Define parameters
r1max = 1;  % rmax: max growth rate (1/h)
r2max = 1;
K1 = 10;    % K: Monod coefficient (g/L)
K2 = 10;
D1 = .001;  % D: death rate (1/h)
D2 = .001;
Y1 = 1e5;   % Y: conversion efficiency (cells/(g/L))
Y2 = 1e5;

% Differential equations
dX1 = (r1max*S/(K1+S))*X1 - D1*X1;
dX2 = (r2max*S/(K2+S))*X2 - D2*X2;
dS = -((r1max*S/(K1+S))*X1/Y1) - ((r2max*S/(K2+S))*X2/Y2);

% Passing derivatives to ODE solver
dQ = [ dX1; dX2; dS ];

end
```

APPENDIX B

PRIMARY DEGRADER MODEL (MATLAB CODE)

```
% This is the Primary Degrader Model
% 2-member population (X1, X2), X2 is a primary degrader
% Complex substrate (Sc) is converted into simple substrate (S) by X2
% Organisms can only grow on simple substrate S
% No interaction
% Batch (closed) system.


function model02

time = 100;

% Initial conditions
X10 = 1e4;  % X1: organism 1 (cells/ml)
X20 = 1e3;  % X2: organism 2 (cells/ml)
Sc0 = 100;  % Sc: complex substrate (g/L)
S0 = 1;     % S: simple substrate (g/L)

% ODE
time = [ 0 time ];
Q_init = [ X10 X20 Sc0 S0];
[T,Q] = ode45(@ode_model, time, Q_init);

% Plotting the data
semilogy(T,Q,'linewidth',2);
legend('X1','X2','Sc','S');
xlabel('Time (h)');
ylabel('Population (cells/ml)');

end


% =========== Differential Equation Model ==============

function dQ = ode_model(T,Q) % set of differential equations

X1 = Q(1);
X2 = Q(2);
Sc = Q(3);
S = Q(4);
```

```
% Define parameters

r1max = 1;  % rmax: max growth rate (1/h)
r2max = .001;
K1 = 10;    % K: Monod coefficient (g/L)
K2 = 10;
D1 = .001;  % D: death rate (1/h)
D2 = .001;
rS = .01;   % rS: max conversion rate of substrate (1/h)
Ks = 1e4;   % Ks: substrate conversion coefficient (cells/ml)
Y1 = 1e5;   % Y: conversion efficiency (cells/(g/L))
Y2 = 1e5;
n = 100;    % n: # S per Sc

% Differential equations
dX1 = (r1max*S/(K1+S))*X1 - D1*X1;
dX2 = (r2max*S/(K2+S))*X2 - D2*X2;
dSc = -Sc*(rS*(X2/(Ks+X2)));
dS= n*Sc*(rS*(X2/(Ks+X2)))-((r1max*S/(K1+S))*X1/Y1)-((r2max*S/(K2+S))*X2/Y2);

% Passing derivatives to ODE solver
dQ = [ dX1; dX2; dSc; dS ];

end
```

APPENDIX C

INHIBITION MODEL (MATLAB CODE)

```
% This is the Primary Degrader Model
% 2-member population (X1, X2), X2 is a primary degrader
% Complex substrate (Sc) is converted into simple substrate (S) by X2
% Organisms can only grow on simple substrate S
% Competitive interaction (antagonism)
% Batch (closed) system.


function model04

time = 100;

% Initial conditions
X10 = 1e4;  % X1: organism 1 (cells/ml)
X20 = 1e4;  % X2: organism 2 (cells/ml)
Sc0 = 100;  % Sc: complex substrate (g/L)
S0 = 1;     % S: simple substrate (g/L)

% ODE
time = [ 0 time ];
Q_init = [ X10 X20 Sc0 S0];
[T,Q] = ode45(@ode_model, time, Q_init);

% Plotting the data
semilogy(T,Q,'linewidth',2);
legend('X1','X2','Sc','S');
xlabel('Time (h)');
ylabel('Population (cells/ml)');

end


% ========== Differential Equation Model ==============

function dQ = ode_model(T,Q) % set of differential equations

X1 = Q(1);
X2 = Q(2);
Sc = Q(3);
S = Q(4);
```

```
% Define parameters
a1 = 1e-6;  % a: antagonistic coefficient
a2 = 1e-8;
r1max = 1;  % rmax: max growth rate (1/h)
r2max = 1;
K1 = 10;    % K: Monod coefficient (g/L)
K2 = 10;
D1 = .001;  % D: death rate (1/h)
D2 = .001;

rS = .01;   % rS: max conversion rate of substrate (1/h)
Ks = 1e4;   % Ks: substrate conversion coefficient (cells/ml)
Y1 = 1e5;   % Y: conversion efficiency (cells/(g/L))
Y2 = 1e5;
n = 100;    % n: # S per Sc

% Differential equations
dX1 = (r1max*S/(K1+S))*X1 - D1*X1 - a1*X1*X2;
dX2 = (r2max*S/(K2+S))*X2 - D2*X2 - a2*X1*X2;
dSc = -Sc*(rS*(X2/(Ks+X2)));
dS= n*Sc*(rS*(X2/(Ks+X2)))-((r1max*S/(K1+S))*X1/Y1)-((r2max*S/(K2+S))*X2/Y2);

% Passing derivatives to ODE solver
dQ = [ dX1; dX2; dSc; dS ];

end
```