A MOUSE MODEL FOR STUDYING GASTROINTESTINAL MICROBIAL ECOLOGY

By

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ABSTRACT

The mammalian gastrointestinal (GI) tract harbors a complex microbial ecosystem with hundreds of bacterial species at total levels as high as 10^{10-12} cells/g tissue. These bacteria play an important role in the health of the host as well as in causing disease, but studies on the roles of the GI microflora are hindered by the lack of a suitable model system. This thesis characterizes and uses a defined flora mouse model to study interactions between exogenous bacteria and the GI microflora.

The first part of the thesis characterizes a defined flora mouse model consisting of immunodeficient C.B-17 SCID mice colonized with the eight bacterial strains of the altered Schaedler flora (ASF). Specific and sensitive molecular methods were developed to accurately estimate the levels of different ASF strains. Distribution of specific ASF strains along the GI tract was dependent on the oxygen tolerance of the strains. Fecal levels of different strains, which are typically used as a model for colonic bacteria, were different from colonic levels, although all colonic strains were present in feces. ASF strains were persistent in the mouse GI tract even in the face of long-term exposure to other bacteria.

The second part of the thesis evaluates the presence of different ASF strains in inbred and outbred mouse models from different vendors to establish the potential for widespread use of these strains as representatives of the mouse GI microflora. Seven of the eight strains were persistent in both inbred and outbred mice from all vendors studied. This indicates the potential for the ASF strains to be used as a model system even in mouse models that do not harbor a defined flora.

The third part of the thesis explores ecological interactions following infection by an intestinal pathogen, *Citrobacter rodentium*, in defined flora C.B-17 SCID mice. Bulk levels of the pathogen and the ASF strains in different regions of the large intestine and their spatial localization across the luminal-mucosal cross-section were assessed. *C. rodentium* exhibited accelerated dynamics, and infection was followed by increases in levels of specific ASF strains and in total bacterial levels in infected mice as compared to control mice. Different ASF strains were observed to have specific spatial niches that did not vary between infected and control mice. These results suggest that the role of the microflora in preventing colonization of pathogens is a complex process that potentially involves more than one bacterial species.

Thesis Supervisor: Martin F. Polz
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Chapter 1

Introduction

INTRODUCTION

The discovery that the human gastrointestinal (GI) tract harbors a large number of microorganisms came over a century ago and led to two schools of thought. Pasteur, Metchnikoff and others postulated a positive role and viewed the bacteria favorably, while Nenchi and others believed that the bacteria were a source of constant irritation and ideally, humans should be devoid of any bacteria (Schaedler, 1983). Our growing understanding of the microflora and its interactions with the mammalian host over the last century demonstrates that both these points of view have validity. Research over the last 50 years has shown that the GI tract microbial ecosystem is highly diverse and potentially plays a key role in host's nutrition, intestinal development, prevention of disease, as well as a detrimental role in causing disease, through a variety of mechanisms, many of which we are only beginning to understand.

The GI tract ecosystem is diverse in terms of physiochemical properties and potential substrates for microbial metabolism. There is a gradient in oxygen levels in the GI tract, from a high level of oxygen in the proximal colon to anaerobic conditions in the colon (He, 1999). There is also variation in motility, water content and levels of epithelial shedding. Additionally, there is a distinction in conditions between the mucosal layer and the lumen of the GI tract. The complex mammalian diet ensures a high level of substrate diversity that could be exploited by the microflora (Savage, 1977).

All these factors make the GI tract a site for potentially high microbial diversity and numbers. This is indeed seen to be true, with the total levels of bacteria in the GI tract as high as 10^{10-12}/g tissue in the colon. The bacterial load in the GI tract is thought to exceed the total eukaryotic cell number in the human body by an order of magnitude (Savage, 1977)! There is

no accurate estimate of the diversity of bacterial species in the GI tract yet. Studies of the mammalian gut microflora using both culture-based and molecular biological methods have estimated the diversity to be in the range of several hundred species, most of which are obligate anaerobes (Moore, 1974, Savage, 1977, Foster, 1984, Suau, 1999). Commonly isolated species include lactobacilli, clostridia, bacteroides, staphylococci, bifidobacteria and various coliforms (Dubos, 1965, Savage, 1977, Suau, 1999). Suau *et al.* also observed that 60-80% of fecal bacteria seen under a microscope cannot be cultivated. This stresses the need for the development of culture-independent methods and model systems with limited diversity for studying the gastrointestinal microflora.

The role of complex microflora in the nutrition and metabolism of the host is one of the important effects of the microflora. The diet of mammals consists of a mixture of simple and complex carbohydrates, whereas mammalian capabilities of absorption are limited to simple monosaccharides like glucose and galactose. Microbes digest the dietary polysaccharides, which would otherwise be excreted unused, into monosaccharides and ultimately into utilizable short-chain fatty acids such as acetate, propionate and butyrate. Thus, the microbiota in the GI tract play a significant role in assisting mammals in their caloric intake (Falk, 1998, Savage, 1986, Hooper, 2002).

A role in the development of the host has also been proposed for the GI microflora. The intestinal morphology of germfree animals, with very regular ileal villi that have reduced crypt depth, is distinct from that of animals with a normal microflora (Gordon, 1971). Colonization by a single commensal organism, *Bacteroides thetaiotaomicron*, in germfree mice leads to a wide array of transcriptional changes in the mouse small intestinal cells including different levels of expression of the genes for several important intestinal functions

such as nutrient absorption, xenobiotic metabolism and postnatal intestinal maturation (Hooper, 2001).

The GI microflora is believed to have a significant role in preventing disease through a phenomenon termed colonization resistance. Several studies have observed that a reduction in the complexity of the microflora leads to a higher susceptibility of the host to infections and a lower infectious dose (Wells, 1982, Kennedy, 1985, Vollaard, 1994, Hudault, 2001, Waidmann, 2003). A hypothesis put forward to explain this phenomenon is that exogenous pathogens have to compete with the intestinal microflora for attachment to the gastrointestinal mucosa (Kennedy, 1987, Vollaard, 1994). However, the complexity of the microflora makes detailed ecological studies of such interactions between the GI microflora and pathogens difficult. Due to this limitation, such competition has only been shown to occur in cell culture models where colonization of human epithelial cell surfaces with lactobacilli or bifidobacteria prevents pathogens from attaching (Bernet, 1993, Resta-Lenert, 2003). A study to assess colonization resistance to *E. coli* in germfree mice monoassociated with *Bacteroides vulgatus* observed that although *B. vulgatus* reduced the severity of disease, no competition for attachment was observed (Waidmann, 2003).

While several of the effects of the microflora are beneficial to the host, GI bacteria are also believed to be involved in causing disease in susceptible hosts through autoimmune responses and opportunistic pathogenesis. A proposed harmful effect of the intestinal microflora is in the pathogenesis of inflammatory bowel diseases (IBD). Studies have indicated that IBD could be caused by an abnormal immune response to the intestinal microbiota (Sellon, 1998, Madden, 2002, Bouma, 2003). This reasoning is reinforced by the fact that germfree mice do not suffer from IBD, while colonization of germfree mice by a

complex microbiota leads to IBD in susceptible models (Sartor, 1997). Intestinal bacteria have also been implicated in opportunistic pathogenesis that occurs when the intestinal ecosystem is disturbed by factors such as antibiotic therapy or immunosuppressant use, leading to the overgrowth of organisms such as *Clostridium difficile* that cause colitis (Falk, 1998).

Studies on the GI microflora and its interaction with pathogens and the host are hindered by the complexity of the microflora. A simple model system that is representative of the species diversity of the complex flora and is functional would be of immense use in studying the ecology of microbial interactions in the GI tract, and the effects of these interactions. One such model system is the set of eight bacterial species called the altered Schaedler flora (ASF) (Orcutt, 1987, Dewhirst, 1999). The eight bacterial strains include lactobacilli (ASF360, ASF361), clostridia and related species (ASF356, ASF500, ASF502), bacteroides (ASF519), eubacteria (ASF492) and a spirillum (ASF457). These bacterial strains have been used to colonize germfree rodents and protect them from opportunistic infections for over two decades. Since their phylogeny has been characterized based on 16S rRNA gene sequence, this provides the opportunity to design sensitive and specific molecular biological assays to identify and quantify the different ASF species.

The overall goal of this thesis was to put forth defined flora mice colonized with the altered Schaedler flora as a model system for studying aspects of the microbial ecology of the mammalian GI tract, and gain insight into pathogen-GI microflora interactions through experiments in the model. The first part of the thesis (**Chapter 2**) details the characterization of the defined flora C.B-17 SCID mouse model. Specific quantitative PCR (QPCR) assays capable of estimating as few as 10 cells were developed for each ASF bacterial strain and the

distribution of each strain along the length of the GI tract, from the esophagus to distal colon, was explored. Since oxygen levels in the GI tract decrease from the stomach to the colon (He, 1999), one of the objectives was to understand whether ASF strain distribution and abundance along the GI tract correlates with oxygen tolerance of specific ASF strains. A second objective was to compare fecal levels of the different ASF strains with levels in the colon. Fecal levels of bacteria are typically used as a model for colonic levels due to the difficulty of sampling the colon (Rumney and Rowland, 1992). However, the similarity of the levels of the microbiota in the colon and feces is not well understood. Another aspect that was examined was the persistence of the ASF species in mice that were originally defined flora, but have been maintained under less stringent conditions for several years. The detection of ASF species in such mice would indicate the ability of the strains to stably colonize the GI tract even in the face of competition from other bacteria flora.

The second part of the thesis (**Chapter 3**) assesses the distribution of ASF strains in inbred and outbred mouse models from several commonly used vendors. The utility of ASF strains as a representative model of the mouse GI tract flora would be greatly enhanced if they were commonly found in animal models in widespread use. Indeed, if they were universally present, they could be used as indicator organisms to compare the microflora in different groups of animals to ensure consistency between experiments. ASF strains were used by vendors to colonize germfree breeding stocks of animal models, but the production stocks of the animals are not maintained under strict gnotobiotic conditions and are subject to competition from other organisms present in food, water and the environment. The presence and abundance of different ASF strains in C.B-17 SCID and Swiss Webster mice from three large vendors was evaluated.

The final part of the thesis (**Chapter 4**) involves a detailed analysis of the ecological interactions between an exogenous pathogen, *Citrobacter rodentium*, and the ASF strains in C.B-17 SCID mice over a period of 21 days following infection with *C. rodentium.* Both bulk scale effects, in terms of overall levels /g tissue, and spatially resolved effects, in terms of the localization of the different ASF strains in the luminal-mucosal cross-section, are assessed through the use of QPCR and fluorescence *in situ* hybridization respectively. The motivation behind this experiment was to understand if overall levels and spatial niches of the normal microbiota potentially change in response to an enteric infection with a microbial pathogen. Results from this study could shed light on the potential role of competition for adhesion to the mucosal wall as a mechanism responsible for the colonization properties of the GI microbiota.

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Chapter 2

Spatial distribution and stability of the eight microbial species of the Altered Schaedler Flora in the gastrointestinal tract of mice

Spatial distribution and stability of the eight microbial species of the Altered Schaedler Flora in the gastrointestinal tract of mice

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ABSTRACT

The overall complexity of the microbial community in the gastrointestinal (GI) tract of mammals has hindered observations of dynamics and interactions of individual bacterial populations. Yet such information is crucial for understanding the diverse disease-causing and protective roles gut microbiota play for their hosts. Here, we determine the spatial distribution, inter-animal variation and persistence of bacteria in the most complex defined flora (gnotobiotic) model system to date, *viz.* mice colonized with the eight strains of the Altered Schaedler flora (ASF). Quantitative PCR protocols based on the 16S rRNA sequence of each ASF strain were developed and optimized to specifically detect as few as 10 copies of each target. Total numbers of the ASF strains were determined in the different regions of the GI tract of three C.B-17 SCID mice. Individual strain abundance was dependent on oxygen-sensitivity, with aerotolerant *Lactobacillus murinus* ASF361 present at 10^5-10^7 cells/g tissue in the upper GI tract and obligate anaerobic ASF strains being predominant in the cecal and colonic flora at 10^8-10^{10} cells/g tissue. The variation between the three mice was small for most ASF strains, except for *Clostridium* sp. ASF502 and *Bacteroides* sp. ASF519 in the cecum. A comparison of the relative distribution of the ASF strains in feces and the colon indicated large differences suggesting that fecal bacterial levels may provide a poor approximation of colonic levels. All the ASF strains were detected by PCR in the feces of C57BL/6 Restricted Flora mice, which had been maintained in an isolator without sterile food, water, or bedding for several generations, providing evidence for the stability of these strains in the face of potential competition by bacteria introduced into the gut.

INTRODUCTION

The mammalian body harbors large microbial populations on the skin, in the oral cavity and in the genital and gastrointestinal (GI) tracts. The number of bacteria is thought to exceed the number of mammalian cells by as much as a factor of 10 and a majority of bacteria are present in the GI tract (26). Culture-dependent studies of the bacterial populations in the gut of mice have indicated about 10^9 bacteria/g tissue in the stomach and a gradient of bacterial density along the intestine, with the numbers changing from 10^7 bacteria/g tissue in the small intestine to 10^{11} -10^{12} bacteria/g tissue in the cecum and colon (5, 6, 26). No culture-independent estimates of bacterial density are available for the GI tract; however, comparison of direct counts and culturing indicates that between 40-80% of anaerobic bacteria in fecal matter have not been cultured (18). Although the culturability of bacteria in the GI tract exceeds that of many other environments studied (2), the lack of culture-independent cell counts indicates that the gut remains a poorly characterized ecosystem.

The nature of the GI tract changes along its length, with reduced flow rates and oxygen levels as ingested matter travels from the stomach to the large intestine. These factors, combined with the abundance of substrates, make the gut an ecosystem that could support a complex bacterial community with high spatial heterogeneity (5, 9, 26). Indeed, in culture-based studies, the bacterial populations mirror this gradient along the length of the gut. Aerotolerant bacteria such as enterococci and lactobacilli are predominant in the stomach and small intestine and obligate anaerobic species such as clostridia and bacteroides

are predominant in the large intestine (6). Overall, anaerobes are thought to exceed facultative aerobes by a ratio of 1000:1 (5).

Both culture-based and molecular ecological studies have indicated that the gut microflora are very diverse. Studies of the human fecal flora using culture-based methods have suggested that over 400 species could be present (20). While culture-based assessment of species diversity can be misleading due to subjectivity in defining species, recent molecular studies have indicated a similarly high level of diversity. Analysis of a 16S rRNA clone library constructed from human fecal DNA found 82 phylotypes, where phylotypes were defined as sequences with greater than 98% similarity (32). A similar study of the pig GI tract used amplification products from pooled ileal, cecal and colonic DNA from a pig to generate a 16S rRNA clone library. 375 phylotypes were detected when phylotypes were defined as sequences with greater than 97% similarity (19).

The GI microflora are thought to have a major effect on the host but the extent and mechanisms of influence of the GI microbiota on the host are only beginning to be understood (11). Several studies have indicated a role for the gastrointestinal microflora in providing essential nutrients such as Vitamin K (27), differentiation of the host intestine (11), immune development of the host (7) and protection of the host from pathogenic infections (10, 15, 30, 33). Comparison of gnotobiotic mice colonized with a single bacterial species with germfree mice have shown a marked effect on gene expression in the tissues of the small intestine (12) and a significant increase in the survival rate following infection with a pathogen (13). On the other hand, the microflora are also postulated to play a role in the progression of diseases. An example of the potentially harmful nature of the microbiota are their role in the development of inflammatory bowel disease (IBD) and cancer (3, 25). While

the role of *Helicobacter hepaticus* in causing persistent hepatitis and liver cancer in mice has been known since the early 1990s, recent studies in immune dysregulated (C.B-17 SCID) gnotobiotic mice have shown that infection with *Helicobacter hepaticus* may result in IBD (3).

Major limiting factors in studying interactions of the GI microbiota with the host are both the complexity and potential variation of bacterial populations. Thus, defined flora (gnotobiotic) animal models have become useful tools for the study of the effects of the microbiota on the host (7, 8) and germfree mice inoculated with a single bacterial species have been used as the simplest gnotobiotic model system for the study of bacterial effects. Another simplifying approach involves the use of fecal microflora as a surrogate for the study of the colonic microflora (23, 26). The representative nature of the fecal flora has not been established (23) and a recent study comparing the qualitative molecular fingerprints of the colonic mucosal flora with fecal flora found significant differences (35). Thus, the development of a widely available defined-flora mouse model that is more complex, and thus more realistic, than monoassociated models would assist in the study of the consortium effects of the gut microflora.

Here, we introduce a novel application of mice colonized with the eight-member altered Schaedler flora as a model system for quantitatively studying the microecology of the murine gut. The original Schaedler flora, developed in 1965, was used to colonize germfree mice and consisted of eight aerobic and aerotolerant species isolated from mice. These flora were selected on the basis of their persistence from generation to generation in germfree mice and their ability to restore cecal morphology, similar to that of normal mice, in ex-germfree animals (29). It was later modified to include a spiral bacterium as well as more extremely

oxygen sensitive (EOS) strains to make it representative of the nature of the gastrointestinal flora (21). The defined flora substantially reduced the cecal size of germfree mice indicating their functional similarity to the complex microbiota of normal mice. This defined flora, known as the altered Schaedler flora (ASF), has become the standard flora to colonize germfree rodents worldwide (21). Several members of this flora were also detected in a recent study that assessed the diversity of the mouse GI microbiota using clone libraries (24). Because gnotobiotic immunodeficient (SCID) mice colonized with the ASF strains have recently been used as a model system to study development of IBD (3), a characterization of the ASF strains in this mouse model would greatly enhance its utility. The bacteria in the ASF have been phylogenetically characterized based on their 16S rRNA gene sequence (4). The aerotolerant bacterial species, ASF360 and ASF361, are lactobacilli. ASF360 clusters with *L. acidophilus* while the 16S gene sequence of ASF361 is identical to *L. murinus* and *L. animalis*. ASF519 is related to *Bacteroides distasonis* while ASF356, ASF502 and ASF492 fall in the *Clostridium* cluster XIV, the last being identical to *Eubacterium plexicaudatum*. ASF457 is a spiral-shaped bacterium that clusters with the *Flexistipes* species while ASF500 is not closely related to any of the sequences in the database and clusters with the low G+C gram positive bacteria (4).

The goals of this study were to (1) develop protocols for the quantification of the ASF strains based on their 16S rRNA genes, (2) assess the variation of the ASF strains along the length of the gastrointestinal tract, (3) evaluate the differences between mice and (4) compare fecal levels to colonic levels.

MATERIALS AND METHODS

Strain culture. Eight bacterial strains comprising the ASF were obtained from Taconic (Germantown, NY). Strains ASF360 and ASF361 were grown on MRS agar (Difco Labs, MI) under microaerobic conditions generated using the Campypak system (Remel, KS). The rest of the ASF strains were cultured as recommended (22) on Schaedler agar supplemented with 5% defibrinated sheep's blood (Remel, KS) in an anaerobic glove box (Coy Laboratories, MI) containing a 10% CO_2-10% H_2–80% N_2 atmosphere at 34-35°C. Schaedler agar plates were pre-reduced for 2 days prior to inoculation of the Extremely Oxygen Sensitive (EOS) strains, which require an anaerobic glove box for successful culture. However, even under these conditions, EOS strains ASF500 and ASF502 did not grow well. Whole cell lysates of these two strains (kind gift of Dr. Bruce Paster, Forsyth Institute, Boston, MA) were used to obtain genomic DNA for testing the specificity of the primers and for generation of QPCR standards (see below). Liquid cultures were grown at 37°C in MRS broth (ASF360 and ASF361) or in Schaedler broth supplemented with 5% heat inactivated fetal calf serum (ASF356 and ASF519).

Mice. Six-week old female C.B-17 SCID mice inoculated with the ASF strains were obtained from Taconic (Germantown, NY). This age was chosen based on the fact that the GI microflora reach their climactic level after four weeks (5, 6). The mice were transferred into an autoclaved SCID cage upon receipt and fed autoclaved food and water. In order to avoid contamination of the mice with additional species of bacteria, the mice were euthanised after 1 day of arrival at the animal facility, as opposed to the standard 72 h period for recovery from transportation stress. The stability of the ASF strains in a more complex biota was also assessed using fecal pellets obtained from 9-month old female C57BL/6 restricted flora (RF)

mice. The original founders of the RF breeding colony were defined flora before being bred and maintained in an isolator without sterile food, water, or bedding. Hence, they may have acquired a more complex flora than the original defined flora. There is evidence for a more complex microbiota in these mice from terminal Restriction Fragment Length Polymorphism (t-RFLP) analysis of their fecal bacterial DNA (unpublished results). The RF mice used in this experiment were maintained in a cube isolator (CRL, MA) with HEPA filtered air and were fed non-sterile feed and water. All mice were maintained and handled as per IACUC guidelines.

Sample collection. Three C.B-17 SCID mice were euthanized by CO_2 asphyxiation and their entire gastrointestinal tract was collected aseptically. The GI tract was divided into five anatomically defined segments- esophagus, stomach, small intestine, cecum and colon. The stomach was incised, rinsed free of ingesta, separated into the glandular and non-glandular portions and 0.5-cm samples were taken from each portion. The cecum was divided into the basal (including the ileocecal valve) and the apical halves, with the loss of some of the cecal ingesta. 0.5cm samples were then collected from the ileocecal valve and the apical cecum. The small intestine was sub-divided into six equal-length segments. Due to approximation errors and the accuracy of the measurement method, the last segment was consistently longer than the others. The colon was sub-divided into 3 equal segments. Full thickness samples, which include the tissue as well as luminal contents, consisting of the first 0.5 cm of each intestinal segment were collected. Prior to sectioning of the colon, formed fecal pellets were expelled from the colon and collected for analysis from each mouse. All the samples were weighed immediately and stored at –80°C prior to DNA extraction.

DNA extraction. (i) Tissue samples. DNA was extracted from tissue samples with the DNEasy Tissue kit, using a modification of the DNEasy tissue extraction protocol (Qiagen, CA) to ensure maximal lysis of both Gram-positive and Gram-negative bacteria. Following overnight digestion of tissue samples with proteinase K (20µl, 12mAU) and buffer ATL (Qiagen, CA), the sample was centrifuged and DNA was isolated from the supernatant. Briefly, the supernatant was treated with 400µg RNase A for 2 min at room temperature, incubated with a guanidium isothiocyanate-based buffer AL for 10 min at 70°C and applied to a spin column. Following washes with buffer AW1 and buffer AW2, the DNA was eluted in elution buffer AE. The pellet from the centrifugation of the lysed tissue was subjected to an extraction using the DNEasy protocol for Gram-positive bacteria, to ensure recovery of any possibly recalcitrant Gram-positive bacteria. Briefly, the pellet was resuspended in a lysozyme buffer (20mM Tris.Cl, pH 8.0, 2mM EDTA, 1.2% Triton X-100), incubated at 85°C for 10 min to inactivate the proteinase K and 3.6 mg of lysoyme (Sigma, MO) was added to the resuspended pellet. Following a 45-min incubation at 37°C, 400 µg of RNase A was added to the lysate and incubated at room temperature for 2 min. Proteinase K (25 µl, 15 mAU) and buffer AL were added to the lysate and incubated at 70°C for 30 min. Following addition of 200µl of ethanol, the lysate was applied to a spin column, washed with buffers AW1 and AW2 and eluted in elution buffer AE as above. DNA from both the extractions was pooled and stored at -80°C prior to use for quantitative PCR.

(ii) Fecal pellet samples Since bead-beating protocols have been shown to be more effective in extracting bacterial DNA from fecal samples (34), a bead-beating protocol adapted from Stahl *et al.* (31) was used for the fecal pellet samples. Briefly, the fecal pellet samples were resuspended in Phosphate Buffer Saline (pH 7.4). Tris-saturated phenol (pH

7.4) (500µl), 0.1mm Zirconium beads (0.5g) and 20% Sodium Dodecyl Sulfate (50µl) were added to the tube and shaken at 5000 rpm for 2 min on a Bead beater (Biospec, OK). This step was repeated after a 10 min incubation at 60°C, following which the supernatant was transferred to a new tube and an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added. The tube was mixed by inversion and centrifuged for 5 min at 12000×g. The supernatant was transferred to a new tube and the same procedure was repeated twice. Following this, an equal volume of chloroform (Sigma, MO) was added to the aqueous phase, the tube was mixed by inversion and centrifuged at 12000×g for 5 min. DNA from the supernatant was then precipitated using 0.1 volume of 3M Sodium acetate and a volume of isopropanol equal to that of the aqueous phase. The DNA was pelleted by centrifugation at 15000×g for 10 min, washed with 70% ethanol, dried under a vacuum using a SpeedVac (Thermo Corporation, MA) and resuspended in Tris-chloride buffer. Losses in extraction of different samples were kept constant by using constant volumes at all the steps.

The modified Qiagen Tissue protocol was compared with the bead beating protocol by extracting DNA from identical mixtures of *Lactobacillus murinus* ASF361 (Gram positive) and *Citrobacter rodentium* (Gram negative) using both protocols and quantifying the number of bacteria in the extracted DNA. The two protocols yielded comparable results for both Gram positive and Gram negative bacteria (data not shown).

Primers. (i) Primer design. A secondary structure based alignment of the 16S rRNA gene sequences of the ASF strains (4) was made against the *E. coli* 16S rRNA gene using Seqlab (Genetics Computing Group, WI). Candidate primers were designed based on sequence stretches in the alignment unique for each ASF strain. These candidate primers were checked against Genbank using the BLAST algorithm (1) and hits that were perfect

matches only for the ASF strain in question or other identical 16S sequences in the Genbank database were selected. If non-ASF sequences had perfect matches for the primers, forward and reverse primers that did not match the same non-ASF sequence were preferentially chosen. The other parameters considered in designing the primers, besides specificity, were primer length (preferably 18-22 bases), dissociation temperature (>58°C) and amplicon size (optimally <200bp). Primer dissociation temperatures and amplicon sizes in this range are recommended to enhance QPCR performance. The melting temperatures of the selected sequences were determined using the PrimerExpress software (Applied Biosystems, CA). Primers were synthesized and PAGE-purified by IDT DNA Technologies (Coralville, IA).

(ii) Specificity testing. The specificity of the primers was tested by amplifying DNA from the target organism in a background of fecal pellet DNA from restricted flora mice and primers that only yielded a band of the expected size were selected for QPCR. Amplifications were performed using the AmpliTaq kit (Applied Biosystems, CA) on a Robocycler (Stratagene, CA) with the following conditions: 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C. After PCR, the amplification products were run on a 2% agarose gel (Invitrogen, CA) in Tris Borate EDTA buffer and visualized on the EagleEye Gel documentation system (Stratagene, CA).

QPCR standards. Linearized plasmids containing the 16S rRNA gene of each ASF strain were used as standards. The 16S rRNA gene from each of the ASF strains was amplified using bacteria-specific 16S primers 27F and 1492R (17) and cloned into a pCR2.1 plasmid vector using the Topo-TA cloning kit (Invitrogen, CA). The plasmids were sequenced using the BigDye terminator kit (Applied Biosystems, CA) to ensure that the primer regions were not altered due to *Taq* polymerase error. Prior to use for generating

standard curves, the plasmids were linearized with the restriction enzyme *Bam*HI (NEB, MA) that cuts the vector exactly once and does not have a recognition site in the 16S rRNA genes of the ASF strains. The concentrations of the linearized plasmid were determined on a Synergy HT microplate fluorescence reader (Bio-Tek, VT) using the PicoGreen kit (Molecular Probes, OR) and dilutions of known concentrations of λ DNA as standards.

QPCR optimization. (i) $MgCl_2$ optimization. Increasing the concentration of $MgCl_2$ in the QPCR reaction can make the reaction more efficient, but can also lead to non-specific amplification. To optimize the $MgCl_2$ concentration, primers were tested in a range of $MgCl_2$ concentrations (2-5 mM in increments of 0.5 mM) against all the ASF strains in a background of fecal pellet DNA from restricted flora mice to ensure an absence of non-specific amplification. PCR conditions were the same as those used for testing specificity. Amplification products were analyzed on a 2% agarose gel (Invitrogen, CA) and visualized using the EagleEye gel documentation system (Stratagene, CA). The highest $MgCl_2$ concentration that did not yield non-specific amplification products (4 mM) for any of the ASF strains was used as the optimal concentration for all reactions.

(ii) Primer concentration. Higher primer concentrations can increase the efficiency of the QPCR reaction, but can also lead to primer dimer formation. All combinations of 100 nM, 200 nM and 300 nM forward and reverse primer concentrations were tested with approximately 10^6 copies of plasmid template. QPCR reactions were performed in duplicate for each of the nine possible primer concentration pairs, along with corresponding no-template controls, using the SYBR Green core reagents kit (Applied Biosystems, CA) and the QPCR conditions specified below. The primer concentrations yielding the lowest threshold cycle value for the plasmid standard and highest threshold cycle value for the no-

template control were chosen as the optimal concentrations for QPCR. Where no significant change between the different primer concentrations was seen, i.e. intra-assay variation was similar to inter-assay variation, the lowest concentrations (100 nM, 100 nM) were considered optimal. None of the primers formed primer dimers at the optimal concentrations.

QPCR reaction conditions. All reactions were performed in triplicate on the DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research, MA) in a 25μl reaction volume using the SYBR Green core reagents kit (Applied Biosystems, CA) and the following reaction conditions: 95°C 10 min, 40 cycles of: 95°C 30 sec, 60°C 45 sec, 72°C 75 sec. The $MgCl_2$ concentration, optimized as specified above, was kept constant at 4.0 mM for all the reactions. Melting curves were generated post-QPCR to test for purity by identification of the characteristic melting peak. This was done by measuring the fluorescence of the amplification product every 0.1°C while increasing the temperature from 50°C to 95°C. Standard curves for individual ASF strains were generated using a ten-fold dilution series of the linearized plasmid standard ranging from 1-10^8 copies. The fluorescence level for determining the threshold cycle (C_T) was set at 0.01 fluorescence units in all reactions. It was observed that the small intestinal samples and some of the ileocecal samples had a very high background of eukaryotic DNA, which increased the fluorescence background in the QPCR reactions. These samples were diluted ten-fold for QPCR.

Assessment of persistence of ASF strains in RF mice. To determine whether the ASF strains persisted in RF mice, DNA extracted from fecal pellets was amplified with the strain-specific primers using the conditions specified in Table 1 and the following amplification conditions: 3 min at 94°C, 35 cycles of 1 min at 94°C, 45 sec at 60°C, 75 sec at 72°C. After PCR, the amplification products were run on a 2% agarose gel (Invitrogen, CA)

in 0.5 x Tris Borate-EDTA electrophoresis buffer and visualized on the EagleEye Gel documentation system (Stratagene, CA).

rRNA operon number determination. The rRNA operon number in the different ASF strains was determined to enable correlation of copies of the gene to number of bacteria. Where the ASF strain could be grown in liquid culture and high-quality genomic DNA extracted (ASF356, ASF360, ASF361 and ASF519), a Southern hybridization-based protocol for the estimation of operon number was used. 1-2 μg of genomic DNA extracted from the individual ASF strains was digested overnight with different restriction enzymes (six-cutters [*Bam*HI, *Bgl*II, *Eco*RI *and Hin*dIII] and four-cutters [*Hha*I *and Nci*I]) that did not have a recognition site in the probe region. The digested genomic fragments were run for 4-5 h on a 0.7% agarose gel at 5-6 V/cm and then electrophoretically transferred onto a Zeta-probe GT membrane (BioRad, CA) using the Trans-Blot SD semi-dry transfer cell (BioRad, CA). The DNA was crosslinked to the membrane in a UV crosslinker (Stratagene, CA). The probe consisted of a 500-bp fragment of the 16S rRNA from each ASF strain amplified using the 16S rRNA primers 27F and 519R (17) and gel-purified. The probe was labeled by random priming (Amersham, CA) using [^{32}P]dATP (Perkin Elmer Life Sciences, MA). Unincorporated [^{32}P]dATP was removed by purification with MicroBio-Spin 30 columns (BioRad, CA). The membrane was hybridized with the probe for 14 h in a hybridization buffer containing 50% formamide (Amersham, CA), 6X SSC, 5X Denhardt's solution and sheared salmon sperm DNA (100μg/ml) (Brinkmann, NY). The membrane was then subjected to two low-stringency washes for 30 min (in 2X SSC, 0.1%SDS at 37°C and 1.5X SSC, 0.1%SDS at 50°C respectively) and one high-stringency wash for 1 h at 65°C (1X SSC, 0.1%SDS). A phosphor-storage plate was exposed to the membrane for 5-30 min and

scanned on a Cyclone Phosphor Imager (Perkin Elmer Life Sciences, MA). The amount of radioactive label in the bands was quantified using Optiquant software (Perkin Elmer Life Sciences, MA) and a comparison of the number and intensity of the bands for the different restriction enzymes was used to determine a consensus copy number.

Two of the strains (ASF457 and ASF492) could not be grown in sufficient quantities for Southern hybridizations. Thus, rRNA operon number was estimated by correlation of microscopic cell counts with target copy number estimated by QPCR. Cells were collected from either from a liquid culture or a pure culture on Schaedler agar plates. Cells collected from plates were resuspended in Schaedler broth. Following vortexing to disrupt any cell clumps, two replicates of 100 µl were spun down at 8000xg for 10 min and DNA was extracted from the resultant cell pellet using the DNeasy tissue kit (Qiagen, CA). Two replicates of 1/100 and 1/50 dilutions of the cell suspension were made in 0.2-µm-filter sterilized PBS (pH 7.4), fixed with 100 µl formaldehyde, stained with 4´-6-Diamidine-2´-phenylindole-dihydrochloride (DAPI) (Sigma, MO) and collected on a Nucleopore membrane (Millipore, MA). Cell numbers were estimated using an Axioskop epifluorescence microscope (Carl Zeiss, NY). QPCR was performed on 5 replicates from each of the two cell pellet samples, using the conditions described above, and the number of copies in the template were estimated using the average C_T numbers. The number of copies from QPCR was divided by the number of cell-equivalents used as template, as calculated using the cell counts, to yield an estimate of the operon copy number. The average of the values obtained for the two replicates is reported as the estimated copy number. The method was tested using *Lactobacillus murinus* ASF361, for which the copy number had been estimated using the Southern hybridization protocol to be 6 copies. The QPCR-based protocol yielded an

estimate of 5.79 copies, indicating its validity. While the protocol yields an estimate of the copy number, some error may be present due to the variability in bacterial counts and losses due to DNA extraction. ASF500 and ASF502 (both most closely related to *Clostridium* spp.) did not grow sufficiently on plates to allow the use of even the alternate method. Hence, the average rRNA operon number of the *Clostridium* group (11.2) reported on the rRNA operon number database (16) was used in all calculations of cell numbers.

RESULTS

Assay development. All primers were sensitive and specific for the strain they were designed to target. Comparison against Genbank using BLAST (1) produced hits only against the ASF strains for four of the primer combinations, while the remaining four also hit some closely-related sequences (≥ 99% or more sequence similarity) from intestinal clone libraries (Table 1). Amplification of each ASF strain in a background of fecal pellet DNA resulted in all cases in only one band of the same size as the strain-specific PCR product . Further, melting curves obtained for QPCR products amplified from DNA of pure strains, fecal pellet DNA from RF mice and GI samples were identically shaped for each primer pair. The products were also sequenced to confirm their identity. All these results indicate the specificity of the primers for the strain they amplify.

The sensitivity of the primers was determined by QPCR of a dilution series over eight orders of magnitude of the plasmid standards for each strain. The slope of the standard curve, which is an indicator of amplification efficiency (a slope of –3.3 = 100% efficiency), was in the range of –3.3 to –3.77 for most of the ASF strains, except ASF502 which had a slope of –4.65 (an efficiency of about 60%) (Table 1). The intercept, which is an estimate of the threshold cycle for the detection of 1 copy of the target gene, was less than 40 cycles for all the strains except ASF457 (40.96) and ASF502 (48.25). These considerations give confidence in the high sensitivity of the assay.

The rRNA operon number of the ASF strains was estimated to enable the correlation of QPCR results to cell numbers (Table 1). While the operon numbers for most of the ASF strains were estimated by either Southern hybridization or a QPCR-based method, two of the ASF strains (ASF500 and ASF502) that were closely related to the *Clostridium* group did not

grow to adequate levels for the use of either method. The average operon number of the *Clostridium* group from the rRNA operon number database (16) was used for all the calculations for these strains.

Spatial distribution of ASF flora. The total numbers (mean ± standard deviation) of the ASF strains varied over several orders of magnitude over the length of the GI tract as determined by averaging the sum of all ASF strains for the three mice (Fig. 1A). The esophagus harbored about 3.76×10^6 ($\pm 2.54 \times 10^6$) cells/g while the number fell to about 1.59×10^6 ($\pm 1.04 \times 10^6$) cells/g in the glandular stomach. Surprisingly, no bacteria were detected in the non-glandular stomach. The number of bacteria increased in the small intestine to about 1.26×10^7 ($\pm 2.34 \times 10^7$) cells/g. A sharp increase in numbers was evident at the ileocecal valve, where total levels climbed to 8.30×10^9 ($\pm 5.47 \times 10^9$) cells/g in the cecum and remained at about 5.78×10^8 ($\pm 4.76 \times 10^8$) cells/g and 1.13×10^9 ($\pm 3.09 \times 10^8$) cells/g in the colon and feces respectively. Overall, the diversity of strains and relative distribution changed substantially over the GI tract. The aerotolerant anaerobe, *Lactobacillus murinus* ASF361 comprised close to 100% of the total cells in the esophagus, about 50% in the stomach and small intestine and declined to about 0.01% in the cecum and colon. However, Mouse #3 showed a deviation from the other two mice, with ASF361 accounting for about 0.2% of the total bacteria in the cecum and colon. The anaerobes were generally absent in the esophagus and some were present at low numbers in the stomach. They, however, increased sharply over the length of the intestine, from about 50% in the small intestine to about 99.99% in the cecum and colon.

Four of the ASF strains, *Clostridium* sp. ASF356 (Fig. 1B), *Flexistipes* sp. ASF457 (Fig. 1C), *Eubacterium plexicaudatum* ASF492 (Fig. 1D) and low-G+C-content gram

positive sp. ASF500 (Fig 1E), showed a similar distribution over the length of the gastrointestinal tract in all three mice. These four strains were present at low levels (10^4- 10^6 cells/g) in the small intestine and increased sharply at the ileocecal valve (10^8- 10^9 cells/g), sustaining this high level in the colon. Surprisingly, the extremely oxygen sensitive (EOS) strain ASF500 was also seen at low levels (10^2- 10^3 cells/g) in the esophagus of all three mice. Aerotolerant *Lactobacillus murinus* ASF361 (Fig. 1F) maintained populations of 10^5 - 10^7 cells/g over the length of the GI tract of all three mice, with about 10^6 cells/g in the esophagus, 10^4 - 10^6 cells/g in the stomach, 10^5 - 10^7 cells/g in the small intestine and cecum and falling to lower levels in the colon. The apical cecum (C2) of Mouse #3 had higher levels of *Lactobacillus murinus* ASF361 along with lower levels of the other strains when compared to Mouse #1 and Mouse #2. The other lactobacillus, ASF360, was absent in most regions of the gut and had very low levels of 10^2 cells/g when present.

ASF502 (Fig.1G) and ASF519 (Fig. 1H) showed greater variation between the three mice. The distribution of *Bacteroides* sp. ASF519 was similar for all three mice in the stomach (10^4-10^5 cells/g), small intestine (10^5-10^6 cells/g) and colon (10^7-10^9 cells/g). In the cecum, however, Mouse #2 had much higher numbers as compared to Mouse #1 and Mouse #3 (10^8 v/s 10^4-10^6 cells/g). The distribution of ASF502 varied between the three mice in the small intestine. Mouse #3 had higher numbers of ASF502 in the stomach (10^6 cells/g v/s 10^4 cells/g) and small intestine (10^7 cells/g v/s 10^6 cells/g) as compared to Mouse #1 and Mouse #2. However, the numbers were comparable for all three mice in the cecum (10^9 - 10^{10} cells/g) and colon (10^7-10^8 cells/g), with Mouse #1 having lower levels (10^6 cells/g) at the ileocecal junction.

Comparison of fecal and intestinal bacterial distribution. The levels of individual ASF strains in fecal matter varied over 3 orders of magnitude between different ASF species, but were similar for all three mice. The non-parametric Kruskal-Wallis test indicated no significant differences between the fecal distributions for the three mice (P=0.75, KW statistic = 0.564) and hence the ASF strains had the same population distribution in the feces of all three mice. ASF502 and ASF519 were present at about 10^9 cells/g feces while ASF361 and ASF457 were present at about 10^8 cells/g feces. ASF492 and ASF500 were present at about 10^7 cells/g and ASF356 was present at about 10^6-10^7 cells/g feces (Fig. 2).

Since fecal bacteria are often used as a surrogate for studying the colonic microbiota, we compared the levels of the bacteria in feces to levels in the colon. This indicated a considerable difference in the distribution of the ASF species in the colon and feces. The data were normalized to make the samples more comparable since per gram values for tissue are not comparable to feces. The numbers of the different ASF strains in each section of the colon and in feces was divided by the numbers of *Lactobacillus murinus* ASF361 in the same section. The ratios in the different sections were then compared for similarity. The fecal numbers of the ASF strains were within an order of magnitude of ASF361 numbers. However, the colonic ratios varied greatly, with *Flextipes* sp. ASF457, *Clostridium* sp. ASF502 and *Bacteroides* sp. ASF519 present at 2-3 orders of magnitude higher than ASF361. The other strains were 1-2 orders of magnitude higher in abundance (Table 2). Thus, there was only poor correspondence between the fecal and colonic levels of the ASF strains.

Stability of ASF flora in restricted flora mice. PCR with ASF primers of fecal pellet DNA from C57BL/6 RF mice yielded the correct-sized bands for all the ASF strains

(Fig. 3). The PCR products were sequenced and found to be identical to the ASF strain sequences. This demonstrated the persistence of the ASF strains in the GI tract of RF mice, which have been bred and maintained in an isolator without sterile food, water, or bedding for 3 years.

DISCUSSION

Here, we present the ASF defined flora mouse as a model system for the study ofhost-bacterial and inter-species interactions in the gut. Defined flora models are invaluable in studying the effects of the microflora on the mammalian host in a manner not limited by the complexity and mouse-to-mouse variability that the full microbial complement entails (8). Recent studies have generally utilized the simplest defined flora models, colonized with a single bacterial species such as *Bacteroides thetaiotaomicron* (12) and *E. coli* (13), to assess the role of the microflora in host development and colonization resistance to *Salmonella* infections respectively. However, a more complex but easily quantifiable model system would be invaluable for many applications. The ASF model may be such a system for several reasons. First, mice colonized with the ASF strains have normal GI physiology, as shown by the similarity of their cecal size to normal mice and their resistance to opportunistic pathogens. Second, the ASF flora is stable. Even when the defined flora mice were maintained in clean conditions but without sterile food, water, or bedding over several years, allowing other bacteria to colonize the gut, we detected the ASF strains in the feces of the mice by PCR methods. Further, a recent study assessing the diversity of the mouse GI microbiota using clone libraries found several of the ASF strains to be present, indicating that they are stable members of the complex mouse microbiota (24). Third, ASF mice are widely available and with the increased routine use of QPCR, the model system is easily accessible. Since the constituents of the flora were chosen for both historical and functional reasons, they might not be ideal for all studies. In particular, several of the metabolic groups of intestinal bacteria are not represented in this model. However, additional bacterial strains can be added to this model to address various questions of interactions. Further, observations by

studies using this model can serve as the basis for developing hypotheses to test in mice with a complex microflora. The choice of mouse strain in our study was influenced by its availability and prior use to test the pathogenesis of *Helicobacter hepaticus* in the development of IBD, in a defined non-pathogenic microbial background (3). Since the mice lack both T and B cells, they afford some ability to separate the effects of bacterial interactions from specific immune responses of the host. Thus, they represent a powerful model for studying the role of the microecology of the gut in the pathogenesis of diseases such as IBD.

QPCR has proven a robust and sensitive method for the quantification of bacterial species in complex environmental communities. QPCR assays developed for the quantification of the different ASF strains are specific for the strains, as shown both by a search against Genbank using BLAST and by amplifying the strain of interest in a complex background. The assays performed well in all regions of the GI tract, but the small intestine samples exhibited a high background. This was likely due to the higher number of mammalian cells, and hence mammalian DNA, which intercalated the fluorescent dye used to detect and quantify PCR amplification products. Dilution of these samples prior to the quantification reduced the problem but led to a higher detection limit (~10^{4-5} copies v/s 10^{3-4} copies in other regions) and higher intra-assay variation (0.08-1.67 CV). Some of the cecal samples also had to be treated similarly. In contrast, the values obtained in the colon showed low intra-assay variation (0.04-0.74 CV).

Culture-based methods have previously been used to study the distribution of bacteria in the different parts of the GI tract. A comparison of total bacterial counts in feces with culture results indicated that 20-40% of fecal bacteria were culturable by the methods used (18).

Thus, the culturability of intestinal bacteria is better than other environmental systems, where the value is typically around 0.01% (2, 14). Culture-based studies in mice (28) and humans (5) have indicated that the esophagus has about 10^7 cells/g, the stomach about 10^3-10^9 cells/g, the small intestine about 10^5-10^8 cells/g (predominantly lactobacilli) and the large intestine about 10^{10} -10^{12} cells/g (predominantly anaerobes). These numbers compare favorably with our results of a total of 10^6-10^7 cells/g in the esophagus and stomach, although we did not detect any bacteria in the non-glandular stomach where the expected number and type of bacteria would be similar to the esophagus (28). Since the non-glandular stomach lacks a mucus layer and the bacteria are loosely attached, one of the possible factors leading to this result could be the loss of bacteria due to the wash that we subjected the stomach to in order to remove the ingesta. Given that the sensitivity of our assays was of the order of 10 cell/reaction, our lower limit of detection would be about 10^3 cells/g in the non-glandular stomach. In the small and large intestine, we observed about 10^6-10^8 cells/g and 10^8-10^{10} cells/g respectively. Like the culture-based studies, we also observed a sharp thousand-fold increase in bacterial numbers across the ileocecal valve. Thus, the QPCR-based data corresponds well with culture-based studies and the ASF strains reach near natural levels in the defined flora model.

Oxygen sensitivity of the individual ASF strains appears to be a predominant factor in their distribution along the GI tract and a good proxy for overall community patterns. The increasingly anaerobic nature of the GI tract from the stomach to the large intestine has recently been shown in live mice using non-invasive electron paramagnetic resonance (EPR) methods (9). The esophagus and stomach are aerobic, with a decreasing gradient of oxygen along the small intestine and the cecum and colon are anaerobic. Based on the results of

previous culture-based studies (28), the aerotolerant *Lactobacillus* sp. ASF360 and ASF361 would be expected to have high numbers in the esophagus, stomach and small intestine. *Lactobacillus murinus* ASF361 does follow this trend, with high numbers in these regions (10^6-10^8 cells/g wet weight, 100% of esophageal bacteria, about 50% of small intestine bacteria). These high levels are maintained in the cecum, with a decrease over the length of the colon (10^4-10^7 cells/g wet weight). The anaerobic ASF strains would be expected to have high numbers in the cecum and colon (>10^9 cells/g) and low numbers (<10^5 cells/g) elsewhere, as seen by culture-based studies (6). Indeed, the distribution of *Clostridium* sp. ASF356, *Flexistipes* sp. ASF457, *Eubacterium* sp. ASF492, low G+C strain ASF500, *Clostridium* sp. ASF502 and *Bacteroides* sp. ASF 519 follows this trend. There was a dramatic increase in the populations of the anaerobic ASF strains at the ileocecal valve and the populations were maintained at high levels of 10^8-10^{10} cells/g (wet weight) in the cecum and colon. Thus, the patterns seen by QPCR correspond well with culture-based studies but afford higher throughput and accuracy between samples. Furthermore, oxygen sensitivity of the strains appears to explain the overall distribution of the ASF strains at the current spatial resolution of the assay.

Factors other than oxygen-sensitivity may also play a role in regulation of population abundance. Mice are coprophagic and ingestion of fecal matter may cause detection of transient populations especially in the upper GI tract. This, for example, may explain the low levels of anaerobic populations (10^5-10^7 cells/ g) in the stomach and small intestine of all mice. However, existence of anaerobic microniches cannot be discounted at this point and may be a more likely explanation for the presence of ASF500 in the esophagus. This strain is extremely oxygen sensitive and was the only anaerobe to be present in the esophagus of all

three mice. Competition due to high niche overlap may explain why one of the lactobacilli was only present at trace amounts in the gut of all three mice. *Lactobacillus* sp. ASF360 was detected only in very low numbers ($<10^3$ cells/g wet weight) in a few regions of the GI tract. However, we were able to detect ASF360 by PCR in fecal pellet samples from C57BL/6 RF mice (Fig. 3), indicating that it was a stable colonizer of ex-gnotobiotic strains of mice. To determine this discrepancy and to further differentiate the distribution of the ASF strains, more detailed studies with finer resolution will need to be performed.

Inter-mouse variation of the total bacterial numbers/g was relatively moderate. Higher levels of bacteria were seen in the stomach and small intestine of Mouse #3 while the stomach of Mouse #1 has relatively fewer anaerobic ASF strain than the other two mice. Fecal matter consumed prior to the dissection is a possible cause for this increase in Mouse #3, since the levels of bacteria were also high in the stomach. Individual strains showed low inter-mouse variability over the length of the GI tract, with the exception of *Clostridium* sp. ASF502 and *Bacteroides* sp. ASF519. ASF502 exhibited high inter-mouse variation in the small intestine, with higher numbers in the small intestine of Mouse #3 as compared to Mouse #1 and Mouse #2. This mirrored the total bacterial distribution discussed above and coprophagy is also a likely cause for this difference. ASF519 showed high inter-mouse variation in the cecum. Mouse #2 had 2-3 orders of magnitude higher levels of ASF519 as compared to Mouse #1 and Mouse #3. This was confirmed by repeating the quantification. However, the numbers of ASF519 become comparable for all three mice in the colon. Overall, the inter-mouse variation is within an order of magnitude and the few differences that were observed between the three mice could be due to differences in feeding or

coprophagy. Moreover, the colon, which is often the region of interest in studying the microecology of the gut, showed little inter-mouse variation for the different ASF strains.

The lack of accessibility of the gut for easy sampling has resulted in the frequent use of fecal matter as a surrogate for the colonic flora. However, the utility of this approximation may not be suitable for all purposes, since the qualitative and quantitative correspondence of the fecal bacterial distribution to the colonic biota has not been studied in detail (23). Zoetendal et al. (35) provided evidence that the human colonic mucosal flora and fecal flora are significantly different based on molecular community fingerprints obtained by Denaturant Gradient Gel Electrophoresis (DGGE). Our study is, to the best of our knowledge, the first to use quantitative molecular methods to compare the quantitative distribution of bacteria in fecal matter and the colon. Our results show that even in the limited diversity of the ASF model, the microflora of the colon is only poorly reflected in fecal matter. The distribution of ASF strains in feces was similar for all three mice and all the ASF strains (except ASF360) were present in feces varying within an order of magnitude. The colonic distribution, however, varied over three orders of magnitude, with anaerobes exceeding the aerotolerant ASF361 by a thousand-fold. In contrast to this, the levels of ASF361 in feces were comparable to the levels of the anaerobes. Thus, our results, in conjunction with the study of Zoetendal et al. (35), suggest that the fecal microflora differs quantitatively from the colonic microflora and that the fecal flora may be an inappropriate surrogate for some questions of GI ecology.

The role of the microbiota in the host remains difficult to assess largely due to the complexity of the gut ecosystem. Thus, the characterization of defined flora mice colonized with the ASF flora, along with the highly specific and sensitive assays developed for the

accurate quantification of the ASF, should provide a useful tool for the study of a wide range of questions in gastrointestinal microecology. Examples of questions that may be addressed with this model system include population dynamics that drive colonization resistance to pathogens, understanding the role of the normal microbiota in the prognosis of diseases such as IBD and the spread of antibiotic resistance.

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gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl. Environ. Microbiol. **68**:3401-7.

FIGURE LEGENDS

FIG. 1. The distribution in different sections of the GI tract of (A) total bacterial cells/g, (B) ASF356, (C) ASF457, (D) ASF492, (E) ASF500, (F) ASF361, (G) ASF502 and (H) ASF519, is shown here for the three defined flora C.B-17 mice. Mouse#1 is represented by solid circles, Mouse#2 by open squares and Mouse#3 by an open diamond. Section E1: esophagus, S1-2: stomach, I1-6: small intestine, C1-2: ileocecal junction and distal cecum and L1-3: colon.

FIG. 2. Fecal levels of the ASF strains in three defined flora C.B-17 mice were determined by QPCR. Mouse#1: white column, Mouse#2: grey column with shading and Mouse#3: black column.

FIG. 3. Specific PCR products obtained by amplification of fecal DNA extracted from C57BL/6 restricted flora mice using the ASF-specific primers. These mice, which originally harbored the defined flora, have been bred and maintained under non-sterile conditions for 3 years. Lanes 1, 10: 100bp ladder, lanes 2-9: RF fecal pellet DNA amplified with ASF356 (417bp), ASF360 (131bp), ASF361 (182bp), ASF457 (95bp), ASF492 (167bp), ASF500 (285bp), ASF502 (427bp), ASF519 (429bp) specific primers (expected size), respectively.

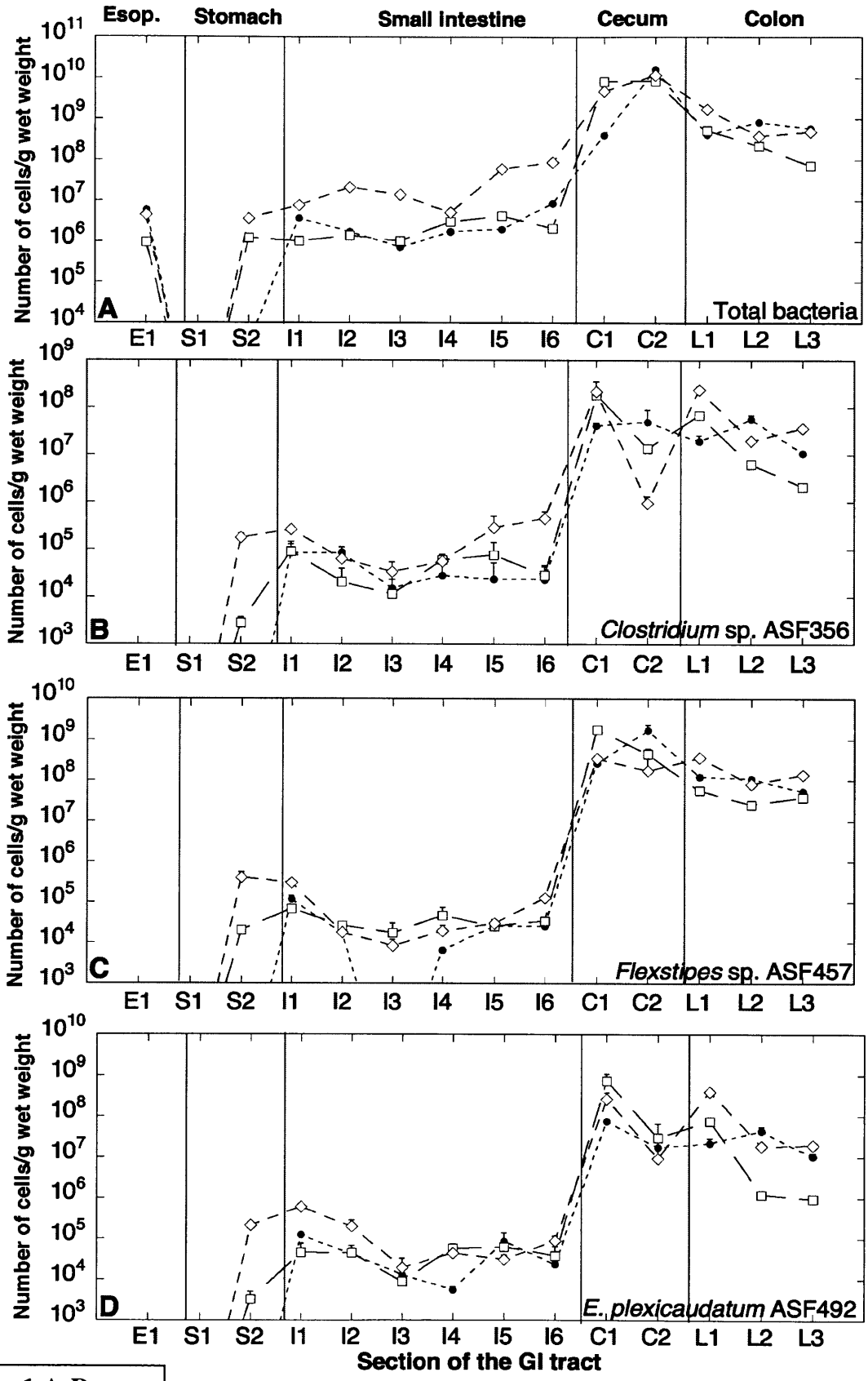
Esop.
Stomach
Small intestine
Cecum
Colon
Number of cells/g wet weight
A
Total bacteria
B
Clostridium sp. ASF356
C
Flexstipes sp. ASF457
D
E. plexicaudatum ASF492
E1 S1 S2 I1 I2 I3 I4 I5 I6 C1 C2 L1 L2 L3
Section of the GI tract

Figure 1 A-D

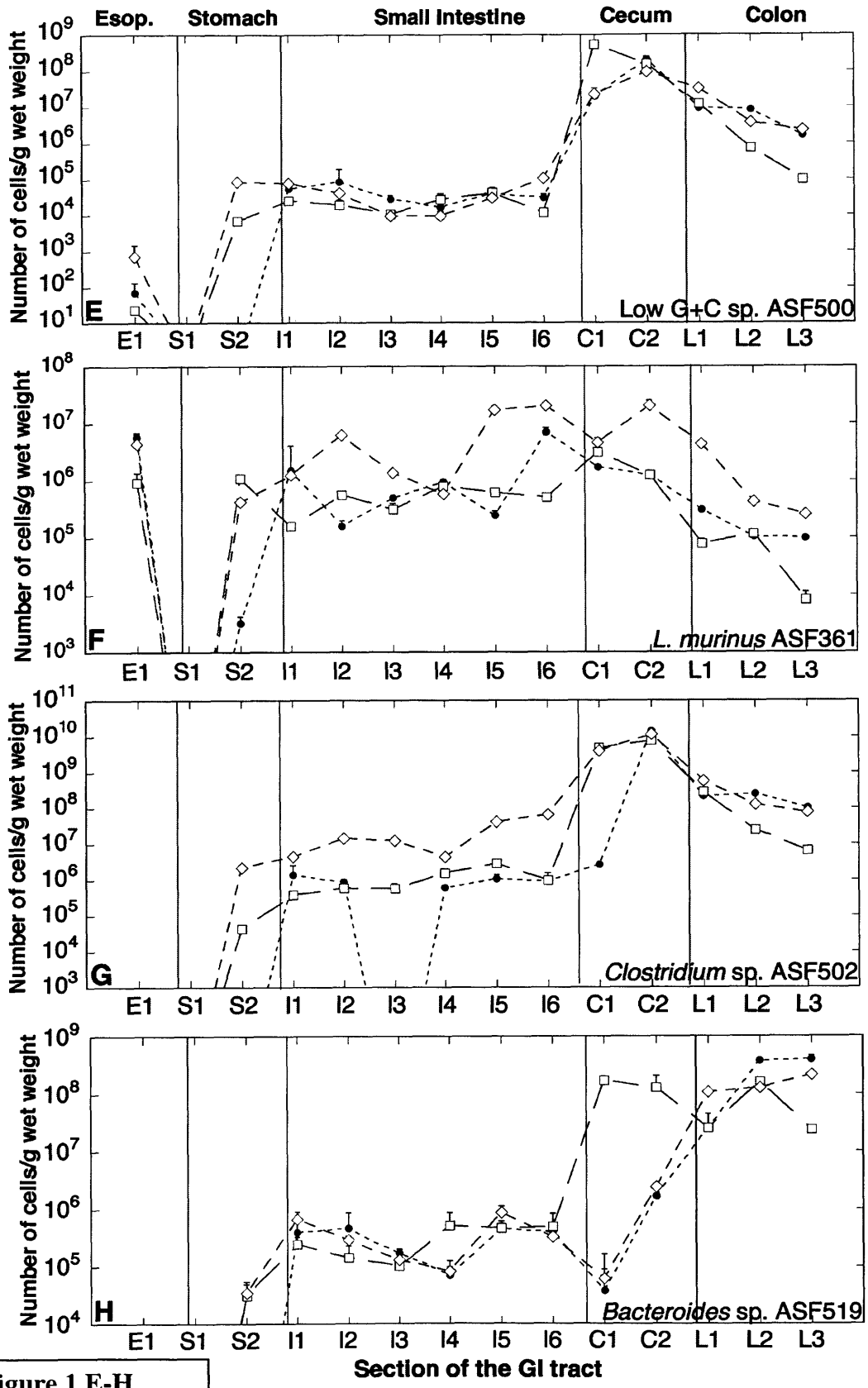
Esop.
Stomach
Small Intestine
Cecum
Colon
Number of cells/g wet weight
E
Low G+C sp. ASF500
F
L. murinus ASF361
G
Clostridium sp. ASF502
H
Bacteroides sp. ASF519
E1 S1 S2 I1 I2 I3 I4 I5 I6 C1 C2 L1 L2 L3
Section of the GI tract

Figure 1 E-H

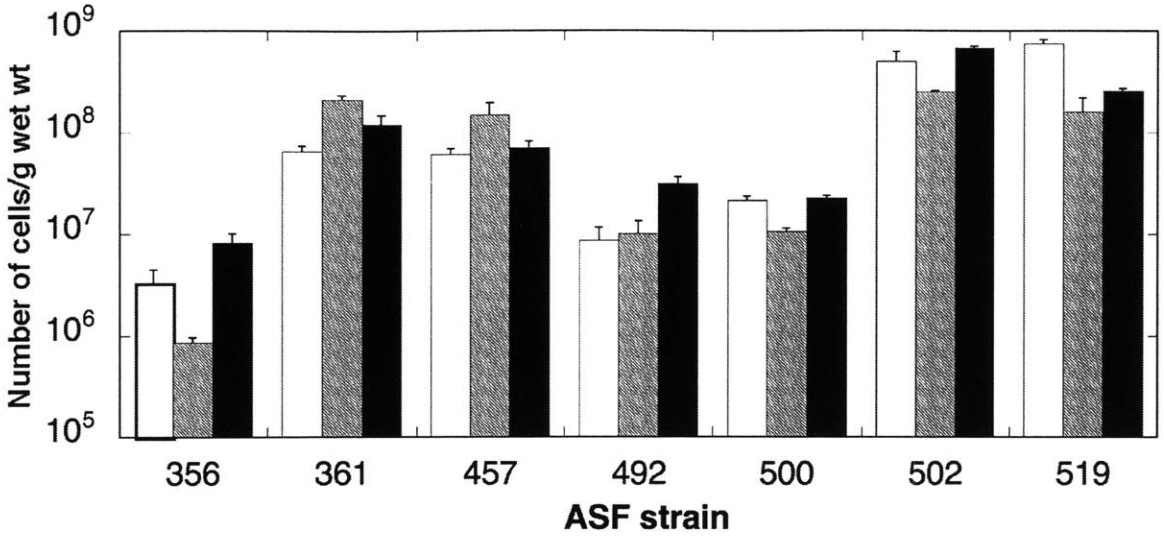
Number of cells/g wet wt
10^9
10^8
10^7
10^6
10^5
356
361
457
492
500
502
519
ASF strain

Figure 2

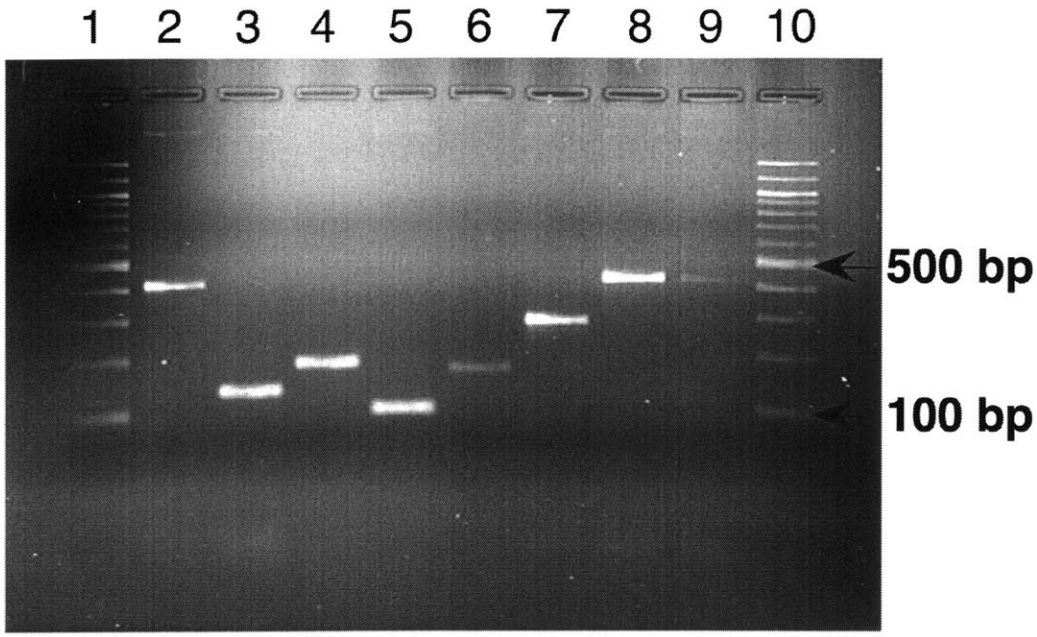

Figure 3

TABLE 1. Primer sequences, specificity, rRNA copy number and QPCR parameters[a] for the ASF strains

Strain name	Species/group	Primer[b]	Slope of standard curve	Intercept of standard curve	Specificity[c]	Operon number
ASF356	*Clostridium* sp.	356-144F CGGTGACTAATACCGCATACGG (100)	-3.50	35.83	NH[d]	5[e]
		356-538R CCTTGCCGCCTACTCTCCC (100)				
ASF360	*Lactobacillus* sp.	360-81F CTTCGGTGATGACGCTGG (300)	-3.49	37.26	Both primers hit *L. intestinalis* and 99-100% identical clones	4[e]
		360-189R GCAATAGCCATGCAGCTATTGTTG (200)				
ASF361	*Lactobacillus murinus*	361-278F GCAATGATGCGTAGCCGAAC (200)	-3.30	32.97	Both primers hit *L. animalis* and 99-100% identical clones	6[e]
		361-435R GCACTTTCTTCTCTAACAACAGGG (300)				
ASF457	*Flexistipes* gp.	457-130F CCGAAAGGTGAGCTAATGCCGG (100)	-3.77	40.96	NH	14[f]
		457-219R GGGACGCGAGTCCATCTTTC (100)				
ASF492	*Eubacterium plexicaudatum*	492-57F CTGCGGAATTCCTTCGGGG (100)	-3.72	38.15	NH	4[f]
		492-204R CCCATACCACCGGAGTTTTC (100)				
ASF500	Low-G+C-content gram positive gp.	500-183F GTCGCATGGCACTGGACATC (200)	-3.45	34.52	Reverse primer hits environmental clones and human colonic clones	11.2[g]
		500-445R CCTCAGGTACCGTCACTTGCTTC (200)				
ASF502	Clostridium sp.	502-195F CGGTACCGCATGGTACAGAGG (200)	-4.65	48.25	NH	11.2[g]
		502-600R CAATGCAATTCCGGGGTTGG (300)				
ASF519	*Bacteroides* sp.	519-834F CACAGTAAGCGGCACAGCG (200)	-3.78	38.57	Forward primer hits uncultured pig GI and environmental clones, reverse primer hits identical mouse clone	5[e]
		519-1243R CCGCTCACACGGTAGCTG (200)				

[a] $MgCl_2$ concentration used in the QPCR mixtures was 4.0 mM .

[b] The primer designation (F, forward; R, reverse), sequence, and nanomolar concentration of the primer used in QPCR (in parentheses) are shown.

[c] Specificity determined by BLAST on 1st July, 2003.

[d] NH = No other hits.

[e] Estimated using Southern blotting

[f] Estimated using QPCR , see Materials and Methods.

[g] Average for *Clostridium* spp. from rRNA Operon Copy Number Database (16).

TABLE 2. Comparison of the ratios[a] of the ASF strains in different section of the colon and in fecal matter.

	L1[b]			L2			L3			F1		
Strain	M#1	M#2	M#3	M#1	M#2	M#3	M#1	M#2	M#3	M#1	M#2	M#3
356	62.4	875.7	54.1	547.6	53.9	46.5	110.6	253.7	142.4	0.1	0.0	0.1
361	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
457	392.5	738.3	84.9	1060.7	213.9	190.7	554.4	4499.1	524.1	0.9	0.7	0.6
492	69.9	966.4	89.9	424.0	10.3	42.5	104.8	113.1	75.5	0.1	0.0	0.3
500	30.1	154.2	7.4	81.0	6.1	8.7	16.7	11.8	8.9	0.3	0.1	0.2
502	675.2	3632.5	130.5	2388.8	209.1	289.1	1022.8	787.6	298.2	7.7	1.2	5.7
519	82.1	324.2	24.6	3597.2	1357.4	296.2	4027.7	2847.9	826.6	11.4	0.8	2.1

[a] Ratios were obtained by dividing the numbers of the strains in each section by the numbers of ASF361 in the same section

[b] L1-3: proximal to distal colon, F1: fecal sample, M#1-3: Mouse1-3

Chapter 3

Distribution and abundance of the altered Schaedler flora in outbred and inbred mouse models

Distribution and abundance of the altered Schaedler flora in outbred and inbred mouse models

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ABSTRACT

The mammalian gastrointestinal tract harbors a complex microflora that is present at levels as high as 10^{10-12} cells/g tissue and has hundreds of species of bacteria. The gut microflora has an important role in the health and development of the host, as well as in causing disease. Detailed analyses of the role of the microflora in its postulated functions are hindered by the lack of a representative model system that is also functional. The eight bacterial strains of the altered Schaedler flora could be one such model system. The validity and utility of the ASF models system would be further enhanced if the ASF strains were stable in the mouse GI tract in the face of competition due to other microflora found in different mouse models from commonly used commerical vendors. This study assessed the prevalence and abundance of ASF strains in the feces of inbred C.B-17 SCID and outbred Swiss Webster mice from Taconic, Charles River Laboratories and Harlan. Other than *Eubacterium plexicaudatum* ASF492, which is only found in Taconic defined flora C.B-17 SCID mice, all the other ASF strains were detected in most of the animals assessed. Specific patterns were seen in the quantitative distribution of *Lactobacillus* sp. strain ASF360 and *Mucispirillum schaedleri* ASF457 in terms of their levels in C.B-17 SCID (higher ASF457, lower ASF360) and Swiss Webster (lower ASF457, higher ASF360) mice. The total levels of the ASF strains in the feces of mice with a flora of unknown complexity were as high as 20% of the total levels in defined flora C.B-17 SCID mice from Taconic. These results indicate that the ASF strains are detectable in both inbred and outbred mouse models from several vendors and could potentially be utilized as a universal model system for assessing the microbial ecology of mice from different vendors.

BACKGROUND

The gastrointestinal tract of mammals harbors a complex microbial ecosystem with possibly hundreds of species of bacteria (Moore, 1974, Savage, 1977, Xu, 2003). These bacteria are present at total levels as high as 10^{10-12} cells/gram tissue in the colon and are thought to outnumber mammalian cells in the human body by an order of magnitude (Savage, 1977). The microflora are known to playing an important role in the health of the host by interacting with the host both locally and systemically. The GI tract microflora are involved with the development of the host immune system and in aiding host nutrition (Falk, 1998, Hooper, 2002). There is also evidence that the complex microbiota acts as an ecological barrier in preventing pathogens from establishing themselves in the GI tract and causing disease (Kennedy, 1987, Vollaard, 1994). On the other hand, the GI tract microflora can also be associated with causing disease in the host, either through opportunistic pathogenesis (Falk, 1998), or by being one of the factors triggering an uncontrolled inflammatory response (Bouma, 2003). Due to these multiple positive and negative effects, different aspects of the GI microflora interaction with the mammalian host have been the focus of study for several decades.

There are several obstacles to studying the role of the GI tract microflora in mammalian health and development. Over 99% of the bacterial species in the GI tract are obligate anaerobes that are difficult to culture (Savage, 1977, Berg, 1996). Further, the high level of diversity renders the study of the microflora by traditional culture-based, as well as molecular biological methods difficult. In addition, non-destructive sampling of the intestinal tract is cumbersome. This has necessitated the use of several different model systems to study the role of the intestinal microflora in the host, as well as the changes in bacterial

populations in the GI tract in response to different factors (Rumney, 1992). Both *in vitro* models, where the intestinal bacteria interact in a laboratory setup, and *in vivo* models involving animal species have been used to study the role of the intestinal microflora. Some of the most commonly used *in vitro* model systems include continuous flow reactors incubated with either specific members of the intestinal flora or the entire fecal flora (Rumney, 1992). Such models have been used to study the ecology and metabolism of the colonic microflora (Macfarlane, 1998). Chemostats have yielded interesting insights into the reduction in population levels of specific bacterial strains in the presence of other intestinal bacterial strains (Freter, 1983). However, the GI tract is spatially diverse with structured microenvironments whereas chemostats are homogeneous environments. Further, although bacterial interactions are important, it is in the context of the host that the effect of these interactions often needs to be assessed. This has led to the use of *in vivo* models in order to dissect the host effects of the GI microflora. These models typically involve small animals colonized with known bacterial components or with the entire complex microbiota. The most common species of animals used are mice (Rumney, 1992), rats (Rath, 2002), ferrets (Marini, 2004) and pigs (Leser, 2002), although other models such as zebrafish are increasingly being used (Rawls, 2004).

Mouse models have been especially useful in helping to understand the role of the intestinal microflora. One factor driving this use is the availability of genetically engineered mouse models used to study the immune system. These models can help elucidate the interactions between the microflora and the host immune system. Another important advantage of small animal models is the ability to derive and maintain them germfree, as germfree animals can be colonized with specific bacterial strains to study a variety of roles of

the intestinal bacteria (Gordon, 1971, Falk, 1998). Studies have shown that germfree mice associated with a single commensal bacterial species have a markedly different transcriptional profile in the intestine (Hooper, 2001) and are less susceptible to infection by pathogens (Hudault, 2001) when compared to germfree mice.

While germfree mice inoculated with a single bacterial strain provide information on host effects, the gut ecosystem in reality consists of several interacting microbes. Thus, a more complex functional flora would complement information derived from mono-associated mouse models and provide a deeper understanding of ecological effects observed in animals maintained in a barrier or conventional state. One such model system is the Altered Schaedler Flora (ASF) that consists of eight bacterial strains that are representative of the composition of the complex microflora (Dewhirst, 1999). The original Schaedler flora was selected in 1965 from among several autochthonous isolates of bacteria from the GI tracts of mice, with the purpose of colonizing germfree mice to protect them from opportunistic infections. This set of organisms, however, consisted primarily of aerobic and aerotolerant bacterial species. The composition of the strains was later modified in order to make them more representative of the predominantly anaerobic nature of the GI tract microflora (Orcutt, 1987). The eight strains of the ASF are *Lactobacillus murinus* strain ASF361, *Lactobacillus* sp. strain ASF360, *Clostridium* sp. strain ASF356, *Clostridium* sp. strain ASF500, *Clostridium* sp. strain ASF502, *Eubacterium plexicaudatum* strain ASF492, *Bacteroides* sp. strain ASF519 and *Mucispirillum schaedleri* strain ASF457 (Dewhirst, 1999, Robertson, *in press*).

Detailed bacterial population analyses of the ASF strains are especially tractable due to the development of culture-independent molecular methods that sensitively and

specifically quantify each ASF strain (Sarma-Rupavtarm, Chapter 2). If these strains were prevalent at high levels in laboratory mouse models, the ASF strains could serve as a universal model flora in determining changes to the GI tract ecosystem following different insults. Although major vendors used these strains to colonize their breeding stocks of mice (Orcutt, 1987), the mice are often not maintained under strict barrier conditions and the stability of the ASF strains in the face of a challenge by other enteric organisms is not well characterized. Clone library studies assessing the diversity of the mouse GI microflora have observed the presence of individual ASF strains (Salzman, 2001, Schuppler, 2004). However, no study has systematically assessed the prevalence of the ASF strains in the GI tracts of mice from different vendors.

The overall goal of this study was to evaluate the prevalence of the ASF strains in the fecal flora of both inbred immunodeficient C.B-17 SCID and outbred Swiss Webster mice from three different vendors. The specific aims of the study are to 1) assess which of the ASF strains were present in the GI tracts of mice from the vendors, and 2) enumerate the levels of individual ASF strains to compare their abundance.

MATERIALS AND METHODS

Mice. 4 to 6-week old female C.B-17 SCID (inbred) (n=2) and Swiss Webster (outbred) (n=2) mice were ordered from Taconic (Germantown, NY), Charles River Laboratories (Wilmington, MA) and Harlan (Indianapolis, IN). The C.B-17 SCID mice from Taconic had a defined flora while all other mice harbored a flora of unknown complexity. The mice were euthanatised the same day they arrived at the facility. All mice were maintained and handled as per MIT IACUC guidelines.

Samples. Mice were euthanatized by CO_2 asphyxiation and their entire gastrointestinal tract was collected aseptically. One formed fecal pellet was collected from the distal colon. The cecum was divided into the apical region and the ileocecal valve and 0.5 cm sections were collected from the two regions. Also, 0.5 cm sections were collected from the proximal colon and the distal colon. All samples were weighed and stored at –80°C. Fecal pellets were used for DNA extraction while the other samples were stored for future use.

Fecal pellet DNA extraction. A modified bead-beating protocol as described previously is used (Sarma-Rupavtarm, Chapter 2). Briefly, the fecal pellet was resuspended by vortexing in Phosphate Buffer Saline (pH 7.4). Tris-saturated phenol (pH 7.4) (500µl), 0.1mm Zirconium beads (0.5g) and 20% Sodium Dodecyl Sulfate (50µl) were added to the tube and shaken at 5000 rpm for 2 min on a Bead beater (Biospec, OK). After a 10 min incubation at 60°C, the bead-beating step was repeated. The supernatant was then transferred to a new tube and an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added. The tube was mixed by inversion and centrifuged for 5 min at 12000×g. The supernatant was transferred to a new tube and the same procedure was repeated twice.

Following this, an equal volume of chloroform (Sigma, MO) was added to the aqueous phase, the tube was mixed by inversion and centrifuged at 12000×g for 5 min. DNA from the supernatant was then precipitated using 0.1 volume of 3M Sodium acetate and a volume of isopropanol equal to that of the aqueous phase. The DNA was pelleted by centrifugation at 15000×g for 10 min, washed with 70% ethanol, dried under a vacuum using a SpeedVac (Thermo Corporation, MA) and resuspended in Tris-chloride buffer. Losses in extraction of different samples were kept constant by using constant volumes at all the steps.

QPCR. Primers specific for each ASF strain and reaction conditions for QPCR have been described previously (TABLE 1). All reactions were performed in triplicate on the DNA Engine Opticon Continuous Fluorescence Detection System (Bio-Rad Labs, MA) in a 25 μl reaction volume. The $MgCl_2$ concentration was kept constant at 4 mM in all reactions, as previously optimized (Sarma-Rupavtarm, Chapter 2). The reaction conditions were as follows: 95°C 10 min, 40 cycles of: 95°C 30 sec, 60°C 45 sec, 72°C 75 sec. Melting curves were generated post-QPCR to test for purity by identification of the characteristic melting peak. This was done by measuring the fluorescence of the amplification product every 0.1°C while increasing the temperature from 50°C to 95°C. Standard curves for individual ASF strains were generated using a ten-fold dilution series of the linearized plasmid standard ranging from 1-10^8 copies. The fluorescence level for determining the threshold cycle (C_T) was set at 0.01 fluorescence units in all reactions. Fecal pellet DNA was diluted 1 in 5 prior to use in QPCR to prevent the effects of any inhibitory substances in the extracted DNA.

RESULTS

Prevalence of ASF strains in fecal matter of mice from different vendors. Most of the ASF strains were detected in both C.B-17 SCID and Swiss mice from Taconic, Harlan and Charles River Laboratories (CRL) (TABLE 2). The most noticeable difference was in the distribution of *Eubacterium plexicaudatum* strain ASF492, which was detectable only in Taconic C.B-17 SCID mice. On the other hand, *Lactobacillus* sp. strain ASF360 was detectable in all the mice assessed except for Taconic C.B-17 SCID mice. *Bacteroides* sp. strain ASF519 was present in all the mice except Harlan Swiss mice. *Clostridium* sp. strain ASF356, *Lactobacillus murinus* strain ASF361, *Mucispirillum schaedleri* strain ASF 457, *Clostridium* sp. strain ASF500 and *Clostridium* sp. strain ASF502 were present in both mouse backgrounds from all three vendors.

Total levels of ASF strains in mice from different vendors. Cumulative levels per gram feces wet weight of the ASF strains varied over an order of magnitude between the different strains of mice. Levels of the ASF strains were highest in Taconic C.B-17 SCID mice, which are maintained under strict gnotobiotic conditions by the vendor, at 6.6×10^{10} cells/g wet weight feces. Since the eight ASF strains are the only bacteria present in the GI tract of the Taconic SCID mice, the total bacteria/g feces in these mice could be thought of as the maximum population level that the ASF strains can reach in the feces of mice. When the total levels/g feces wet weight of ASF strains were expressed as a percentage of the levels in Taconic SCID mice, the values ranged from a low of 8.16% in Taconic Swiss Webster mice to a high of 20.57% for CRL Swiss Webster mice (Figure 1). The total levels of the ASF strains in CRL and Harlan Swiss Webster mice, when expressed as a percentage of Taconic SCID levels, were seen to be higher than the levels in CRL and Harlan SCID levels. A major

contributor to these differences is the fact that *Lactobacillus* sp. strain ASF360 is present at levels 3-4 orders of magnitude higher in Swiss Webster mice than in SCID mice.

Quantitative distribution of ASF strains in C.B-17 SCID v/s Swiss Webster Mice.

There was a high level of variation in the numbers of the specific ASF strains in the feces of mice when compared across different backgrounds and vendors (Figure 2, A-C). *Lactobacillus* sp. strain ASF360 was seen to be present at levels 2-3 orders of magnitude higher in Swiss mice than in SCID mice from all vendors, while *Mucispirillum schaedleri* strain ASF457 was consistently present at levels 1-2 orders of magnitude higher in SCID mice as compared to Swiss mice from all vendors. In contrast to this, no specific patterns were seen in the distribution of *Clostridium* sp. strain ASF356, *Clostridium* sp. strain ASF500, *Clostridium* sp. strain ASF502, *Lactobacillus murinus* strain ASF361 and *Bacteroides* sp. strain ASF519 between SCID and Swiss mice from the same vendor. *Lactobacillus murinus* strain ASF361 and *Bacteroides* sp. strain ASF519 were present at high levels ($> 10^8$ cells/g feces wet weight) irrespective of vendor or background.

DISCUSSION

We present an assessment of the stability of the eight bacterial strains of the altered Schaedler flora in the mouse gastrointestinal tract flora of both inbred C.B-17 SCID mice and outbred Swiss Webster mice from three different vendors, *viz.* Taconic, Harlan and Charles River Laboratories (CRL). The altered Schaedler flora consists of bacterial strains that were isolated from the GI tracts of laboratory mice and are believed to be autochthonous members of the mouse GI flora (Orcutt, 1987, Dewhirst, 1999, Macpherson, 2004). Several vendors used the ASF strains to colonize their barrier-maintained breeding stocks, but the production stocks are not maintained under barrier conditions, leaving them susceptible to acquiring other bacterial strains from food, water, animal care workers and the environment. Of the three vendors used in this study, Taconic maintains its production stock C.B-17 SCID mice under strict barrier conditions and hence, the mice are known to be defined-flora, harboring only the ASF strains. The Swiss Webster mice from Taconic are not maintained in isolators and expected to have other non-pathogenic bacteria. Charles River Laboratories does not maintain its production stock of C.B-17 SCID mice in isolators and only checks them for pathogens. The same is true for the Swiss Webster mice from CRL. Harlan does not specify the conditions under which it maintains its production stocks of mice.

Most of the ASF strains were detected in both C.B-17 SCID and Swiss Webster mice from all three vendors. The strains present in all mice tested are *Clostridium* sp. strain ASF356, *Clostridium* sp. strains ASF500 and ASF502, *Lactobacillus murinus* strain ASF361 and *Mucispirillum schaedleri* strain ASF457. *Bacteroides* sp. strain ASF519 was present in all mice tested except for Swiss Webster mice from Harlan. These findings are consistent

with a recent clone library assessment of the microflora of FvB and C57/B6 mice that found ASF360, ASF361, ASF502 and ASF519 among the clones screened (Salzman, 2002).

Some of the ASF strains showed interesting patterns in their distribution in different mouse models. *Eubacterium plexicaudatum* strain ASF492 was found only in defined-flora SCID mice from Taconic and was absent in the other mice tested. This could indicate that ASF492 is not truly autochthonous and is easily displaced when challenged by other incoming bacterial species. On the other hand, *Lactobacillus* sp. strain ASF360 was present in all mice tested except the defined-flora C.B-17 SCID mice from Taconic. This finding is surprising, as it would be expected that a bacterial strain that is unable to colonize in a simple defined-flora background would be easily out-competed in the presence of other bacterial species. It is unlikely that the immunodeficiency of C.B-17 SCID mice is responsible for the absence of this strain, as the C.B-17 SCID mice from Harlan and CRL harbor ASF360, albeit at relatively low levels (Figure 1 B, C). One possible explanation for this observation is that ASF360 is inhibited in the presence of some component of the ASF strains and the introduction of additional species of bacteria suppresses this competitor, allowing for the persistent colonization of ASF360. Since ASF360 and ASF492 are not both detectable in the same mouse sample, the possibility exists that ASF360 is suppressed by ASF492 in defined flora mice. Further experiments that assess the possibility of competition between these two strains in germfree mice could provide more information on this possible explanation. Another possibility is that multiple strains of lactobacilli have the same 16S rRNA sequence that is targeted by the primers specific for ASF360, and colonization capabilities differ between these strains. This hypothesis can be tested by isolating the lactobacilli from the

different mice on selective media and analyzing the biochemical and genetic differences between the isolates that have the 16S rRNA sequence as ASF360.

The total levels of ASF strains in the different non-defined-flora mouse backgrounds from the different vendors were less than the total levels in defined-flora C.B-17 SCID mice from Taconic. Since the defined-flora SCID mice harbored only the eight ASF strains, it would be expected that the total levels of bacteria in the feces of these mice would be similar to the maximum possible bacterial levels in feces. Based on this assumption, the levels of total ASF strains in the non-defined-flora mice were calculated as a percentage of the defined-flora levels, and indicated that the ASF strains represented between 6.35-20.57% of the approximate total bacterial levels. This indicates that the ASF strains represent a sizeable portion of the total fecal bacteria, despite competition from other strains acquired. Typically, bacterial strains acquired from the environment are aerobic and would not be expected to constitute a significant proportion of the GI tract microflora.

The quantitative distribution of the different ASF strains in the mice tested varied widely depending on the strain, background and vendor, but was similar in the two mice of the same genetic background from the same vendor. Consistent differences in ASF strain abundance depending on the genetic background of the mice were observed for *Lactobacillus* sp. strain ASF360 and *Mucispirillum schaedleri* ASF457. ASF360 was present at 3-4 orders of magnitude higher levels in outbred Swiss Webster mice than in C.B-17 SCID mice from both CRL and Harlan mice. ASF457, on the other hand, was present at 1-2 orders of magnitude higher levels in C.B-17 SCID mice than in Swiss Webster mice. It has been seen that ASF457 is present at high levels in the mucus layer of C.B-17 SCID mice (Sarma-Rupavtarm, Chapter 4) and possible explanations for the lower levels include a functional

immune system in Swiss Webster mice and the potential presence of competing species that colonize the mucus layer and suppress the levels of ASF457. The levels of the other ASF strains were similar within an order of magnitude between the different mice tested.

Given the representative nature of the composition of the ASF strains vis-à-vis the complex microflora, the detection of seven of the eight ASF strains in the GI tract of h inbred and out-bred mice from several vendors further validates the use of the ASF strains as a model system. Further, these strains are often present at high levels and can represent a significant portion of the total bacteria in feces. Also, studying the changes in ASF strain levels can provide insight into the microecology of the GI tract in diseases. We believe that the ASF strains can be used as an indicator flora to study changes in the overall GI tract microbiota even in mice that do not harbor a defined-flora. Another potential significance of our results is in the use of the ASF strains to standardize the background microflora between different groups of mice of the same genetic background for use in infection studies, as well as between different stocks of mice used in replicate experiments. While other approaches such as a comprehensive 16S rRNA-based phylochip to assess the diversity of the mouse GI tract microflora are being developed (Wilson, 2002), the accessibility and cost-effectiveness of PCR and, increasingly, QPCR makes the monitoring of the ASF strains as an indicator flora very attractive and approachable.

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FIGURE LEGENDS

Figure 1. The relative proportion of the total fecal levels of the ASF strains in different mouse models are expressed as a percentage of the total fecal levels of the ASF strains in defined flora C.B-17 SCID mice.

Figure 2. Fecal levels of the different ASF strains in C.B-17 SCID and Swiss Webster mice from a)Taconic, b) Harlan and c) Charles River Laboratories. 356: *Clostridium* sp. ASF356, 361: *Lactobacillus murinus* ASF361, 457: *Mucispirillum schaedleri* ASF457, 492: *Eubacterium plexicaudatum* ASF492, 500: *Clostridium* sp. ASF500, 502: *Clostridium* sp. ASF502 and 519: *Bacteroides* sp. ASF519.

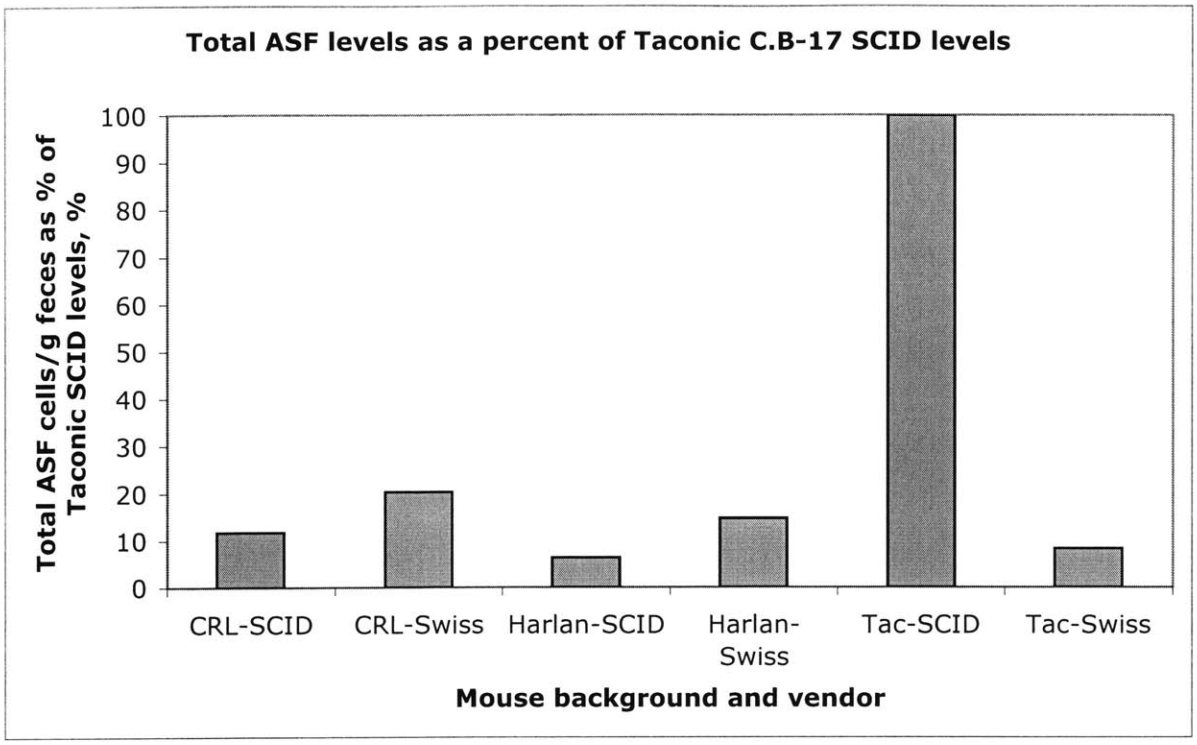
Total ASF levels as a percent of Taconic C.B-17 SCID levels
Total ASF cells/g feces as % of Taconic SCID levels, %
100
90
80
70
60
50
40
30
20
10
0
CRL-SCID
CRL-Swiss
Harlan-SCID
Harlan-Swiss
Tac-SCID
Tac-Swiss
Mouse background and vendor

Figure 1

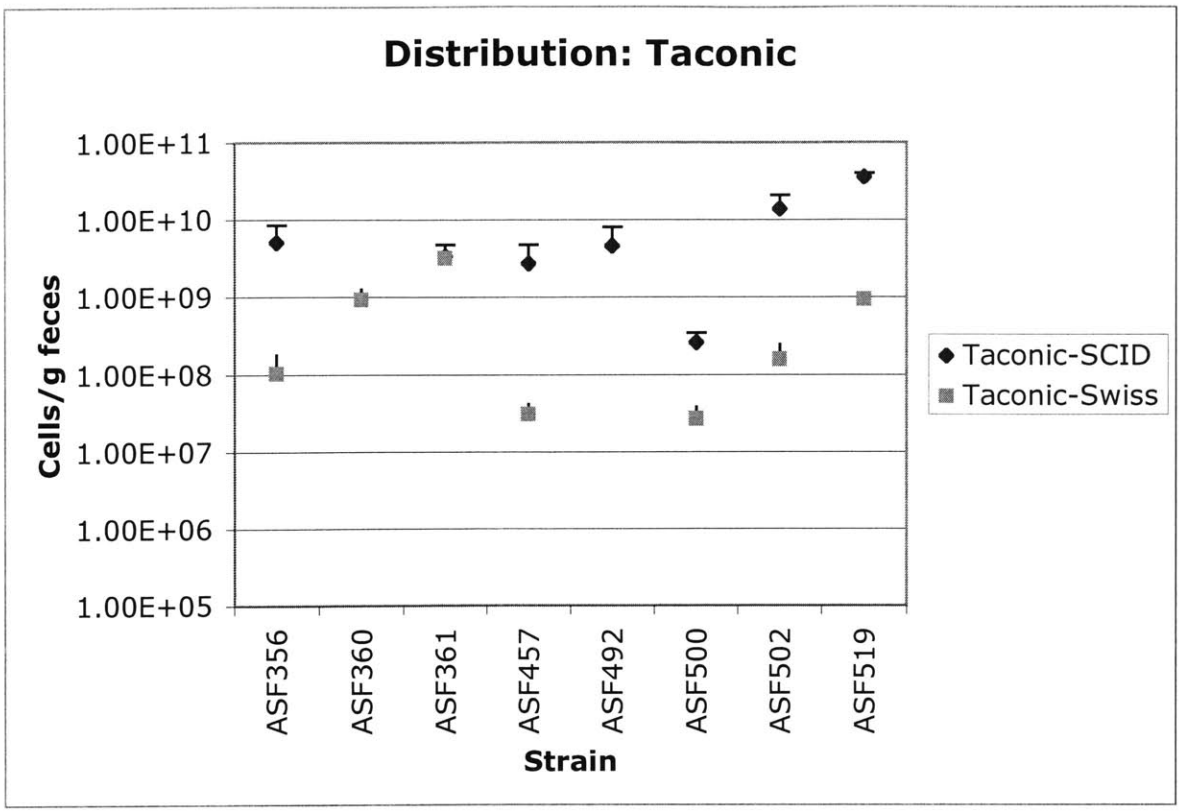
Distribution: Taconic
1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
Cells/g feces
ASF356
ASF360
ASF361
ASF457
ASF492
ASF500
ASF502
ASF519
Strain
Taconic-SCID
Taconic-Swiss

Figure 2(A)

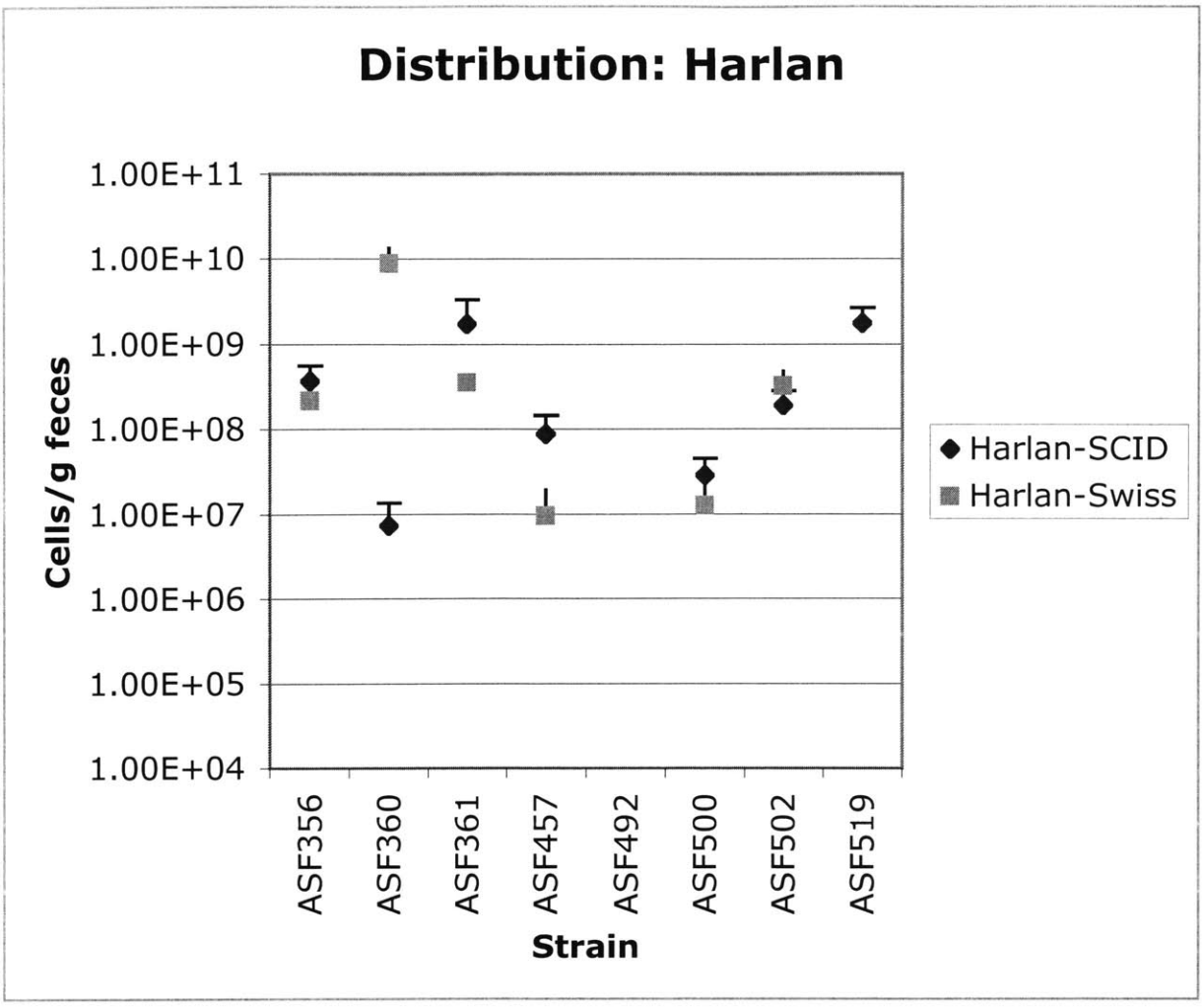
Distribution: Harlan
1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
Cells/g feces
ASF356
ASF360
ASF361
ASF457
ASF492
ASF500
ASF502
ASF519
Strain
Harlan-SCID
Harlan-Swiss

Figure 2(B)

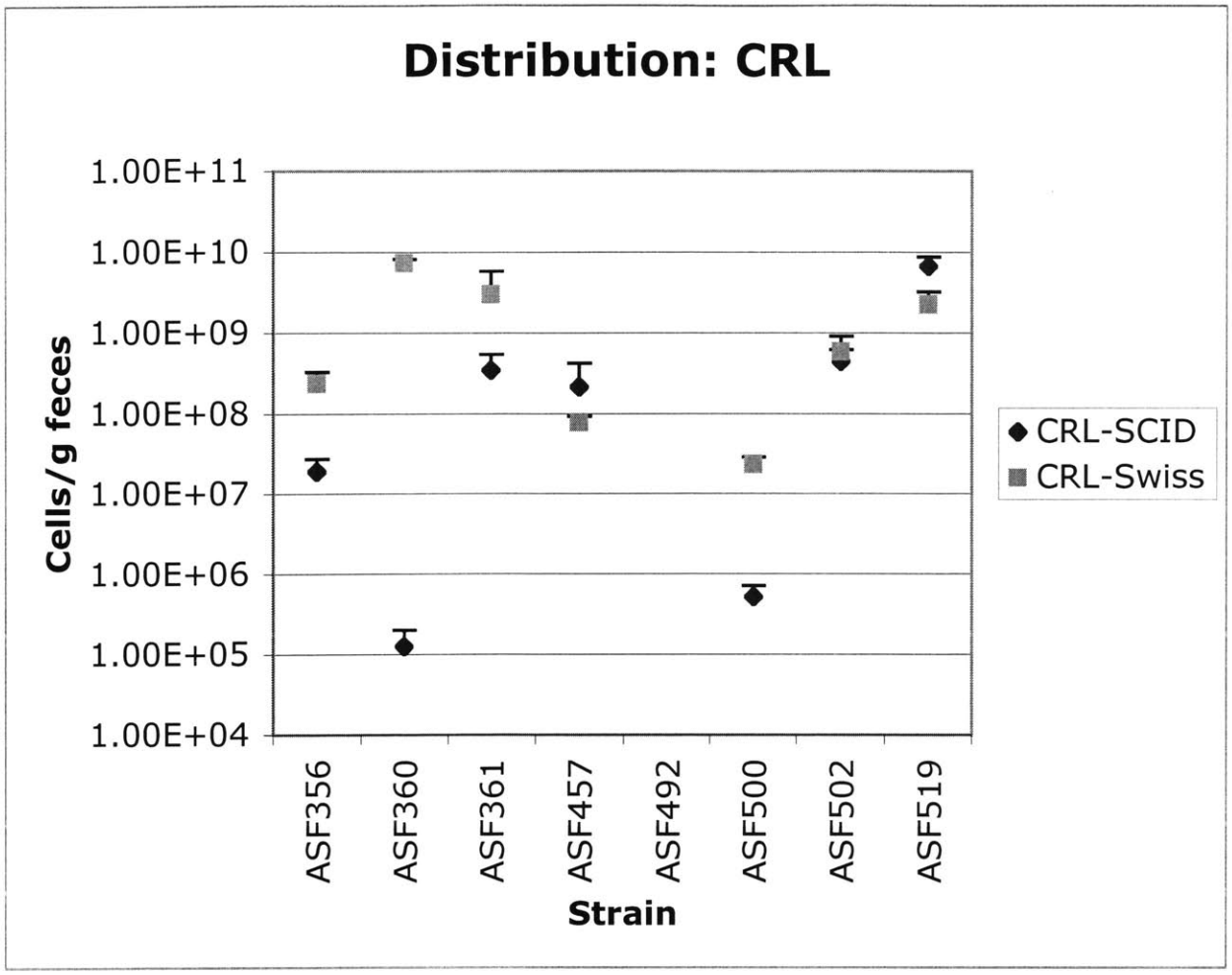
Distribution: CRL
1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
Cells/g feces
ASF356
ASF360
ASF361
ASF457
ASF492
ASF500
ASF502
ASF519
Strain
CRL-SCID
CRL-Swiss

Figure 2(C)

TABLE 1. Primer sequences and QPCR parameters[a] used in the study.

Strain name Species/group	Forward and reverse primers (concentration used in QPCR reaction, nM)
Clostridium sp. ASF356	356-144F CGGTGACTAATACCGCATACGG (100)
	356-538R CCTTGCCGCCTACTCTCCC (100)
Lactobacillus sp. ASF360	360-81F CTTCGGTGATGACGCTGG (300)
	360-189R GCAATAGCCATGCAGCTATTGTTG (200)
Lactobacillus murinus ASF361	361-278F GCAATGATGCGTAGCCGAAC (200)
	361-435R GCACTTTCTTCTCTAACAACAGGG (300)
Flexistipes gp. ASF457	457-130F CCGAAAGGTGAGCTAATGCCGG (100)
	457-219R GGGACGCGAGTCCATCTTTC (100)
Eubacterium plexicaudatum ASF492	492-57F CTGCGGAATTCCTTCGGGG (100)
	492-204R CCCATACCACCGGAGTTTTC (100)
Low-G+C-content gram positive gp. ASF500	500-183F GTCGCATGGCACTGGACATC (200)
	500-445R CCTCAGGTACCGTCACTTGCTTC (200)
Clostridium sp. ASF502	502-195F CGGTACCGCATGGTACAGAGG (200)
	502-600R CAATGCAATTCCGGGGTTGG (300)
Bacteroides sp. ASF519	519-834F CACAGTAAGCGGCACAGCG (200)
	519-1243R CCGCTCACACGGTAGCTG (200)

[a] $MgCl_2$ concentration was 4 mM in all reactions, as optimized in Sarma-Rupavtarm, Chapter 2

.

TABLE 2. Prevalence of ASF strains in mice from different vendors[c]

Species	Charles River Labs		Harlan Sprague Dawley		Taconic	
	SCID[a]	SW[b]	SCID	SW	SCID	SW
Clostridium sp. strain ASF356	+	+	+	+	+	+
Lactobacillus sp. strain ASF360	+	+	+	+	-	+
Lactobacillus murinus strain ASF361	+	+	+	+	+	+
Mucispirillum schaedleri strain ASF457	+	+	+	+	+	+
Eubacterium plexicaudatum strain ASF492	-	-	-	-	+	-
Clostridium sp. strain ASF500	+	+	+	+	+	+
Clostridium sp. strain ASF500	+	+	+	+	+	+
Bacteroides sp. strain ASF519	+	+	+	-	+	+

[a] C.B-17 SCID mice (inbred)
[b] Swiss Webster mice (outbred)
[c] as assessed by QPCR, detection limit ~5000 cells/g feces

Chapter 4

Bulk and microscale effects of infection by an intestinal pathogen on a defined microbiota in the GI tract of mice

Bulk and microscale effects of infection by an intestinal pathogen on a defined microbiota in the GI tract of mice

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ABSTRACT

The mammalian gastrointestinal microbiota is a complex ecosystem, with effects in the well-being of the host, as well as in causing disease. The role of the microbiota in the development and nutrition of the host is increasingly being characterized. An important function of the GI microbiota is in its ability to prevent infections by limiting the ability of exogenous pathogens to colonize and cause disease through a phenomenon termed colonization resistance. While in-depth study of microbiota-pathogen interactions such as colonization resistance has been difficult due to the complexity of the microbiota, the recent characterization of a defined flora model system involving C.B-17 SCID mice colonized with the eight bacterial strains of the altered Schaedler flora provides a useful tool for in-depth ecological studies in the GI tract. This study aims to explore the ecology of infection of an intestinal pathogen, *Citrobacter rodentium* in the defined flora C.B-17 SCID mouse model, with the goal of studying overall population levels, as well as the microscale spatial localization of the ASF strains and the pathogen in the large intestine using quantitative PCR and fluorescence *in situ* hybridization respectively. The population dynamics of *C. rodentium* infection were accelerated as compared to the results of prior studies in literature. Infection by *C. rodentium* caused changes in the levels of specific ASF strains at early time points post-infection. Surprisingly, levels of the individual ASF strains and total bacterial levels were higher in infected mice than control mice. The ASF strains assessed by FISH primarily localized either in mucus (*Mucispirillum schaedleri* ASF457), at the lumen-mucus interface (clostridial strains ASF356 and ASF500) or in the lumen (*Bacteroides* sp. ASF519). The localization of the ASF strains was similar in both infected and control mice, with the two

most abundant bacterial strains, ASF457 and ASF519, being present in dominant in different spatial niches.

BACKGROUND

The mammalian gastrointestinal (GI) tract is a complex ecosystem with considerable variation in environmental conditions. There are marked differences in the physiochemical characteristics of the GI tract both longitudinally and across. Longitudinally, there is variation in the oxygen tension, which decreases from the ileum to the colon (He, 1999). The flow rate of the digesta and the water content are also higher in the small intestine than in the colon. In addition, the rate of epithelial cell shedding is ten times higher in the small intestine than in the large intestine (Xu, 2003). Across the cross-section of the intestine, the main difference in conditions is between the lumen and the mucosal layer. In the lumen, bacterial cells are susceptible to being removed from the system along with the digestive bolus as it moves through the GI tract due to peristalsis. The bacterial cells in the mucosal layer, being either embedded in the layer or attached to it, are relatively protected from removal by peristalsis but are exposed to the mucosal immune system, as well as removal by epithelial shedding (Savage, 1977).

The number of bacterial species that could be supported by such a complex ecosystem is vast. Indeed, there is no accurate estimate of the number of species of bacteria in the mammalian GI tract, with values in literature ranging from 400 to 1000 (Moore, 1974, Gordon, 2003). Further, total bacterial numbers are as high as $10^{10\text{-}11}$ cells/g colonic tissue, with the number of bacterial cells in mammals thought to exceed the number of mammalian cells by an order of magnitude (Savage, 1977). The bacteria in the GI tract are important to the health and development of the host. The bacterial role in aiding mammalian nutrition (Savage, 1986) and the development of the immune system (Hooper, 2001) is increasingly the focus of attention.

An important potential role of the bacteria in the GI tract is the ecological barrier they represent to the establishment of pathogens, both opportunistic and constitutive. The GI tract is second only to the respiratory tract in terms of the surface area exposed to external influences (Bengmark, 1998), with exogenous organisms constantly entering the GI tract via food and water. In the absence of factors preventing their persistent colonization, exogenous bacteria would be expected to establish themselves in the ecologically diverse GI tract. However, despite the constant influx of exogenous organisms, the microflora of adult humans has been seen to be stable over a period of as long as 6 months in the absence of major dietary shifts (Zoetendal, 1998, Zoetendal, 2003).

The intact intestinal microbiota, in combination with host factors such as GI motility and mucosal immunity, seemingly forms a barrier that limits exogenous pathogens and endogenous opportunistic pathogens such as *Clostridium difficile* that overgrow and cause disease when the equilibrium of the flora is disrupted. This concept has been termed "colonization resistance" and the role of the microbiota in effecting the resistance is not well characterized. Differences in complexity of the microbiota influence the susceptibility of the host to infections and the extent of disease caused. Following infection by intestinal pathogens, germfree mice suffered higher mortality rates and had lower LD_{50} values than mono-associated or defined flora mice (Huduault, 2001, Wells, 1982). Infection by *Candida albicans* in antibiotic-treated hamsters, presumably bearing a simpler microflora, predisposed the hamsters to overgrowth and systemic dissemination of the pathogen (Kennedy, 1986). Antibiotic-treated mice also showed higher mortality than conventional mice when infected with intestinal pathogens (Hentges, 1986). One recent study examined the role of colonization by *Bacteroides vulgatus* in germfree mice in protecting the mice from colitis

caused by *E. coli* mpk (Waidmann, 2003). While *B. vulgatus* prevented colitis, it was seen that *E. coli* numbers and spatial localization *in situ* were not affected due to the presence of *B. vulgatus*. These studies indicate a role for the complex microflora in colonization resistance and ameliorating the severity of disease.

Studies on mechanisms behind colonization resistance are hindered by the complexity of the microflora, which makes it difficult to observe and interpret any interactions between microbes by both culture based and molecular biological methods. Over 60% of the hundreds of species of bacteria in the GI tract are not culturable by current methods used and greater than 99.99% of the GI tract bacterial cells are obligate anaerobes (Suau, 1999, Berg, 1996). Complex model systems, such as antibiotic treated mammals, have been used in the past for gastrointestinal microbial studies, providing valuable insight into the role of the resident flora in preventing infection (Hentges, 1986, Kennedy, 1986), but exhaustive studies of interactions are not possible due to the complexity. Hence, detailed ecological studies on the interaction between a pathogen and the intestinal flora have been limited to simple models systems such as mono-associated mice (Waidmann, 2003) or cell culture studies wherein colonization of a Caco2 cell monolayer surface by commensal strains is seen to exclude pathogens (Bernet, 1993, Resta-Lenert, 2003). However, such models have the disadvantage of being functionally different from the complex GI tract microbiota. A simple defined flora model system that is functional and replicates the composition of the GI tract flora would ideally complement studies in mono-associated and complex systems. One such functional model system is the group of eight bacteria called the altered Schaedler flora, whose members were isolated from mice and are thought to be autochthonous species (Orcutt, 1987, Sarma-Rupavtarm, Chapter 2 and 3). The flora, as characterized on the basis of the 16S

rRNA gene sequence, consists of clostridia (strains ASF 356, ASF 500 and ASF502), lactobacilli (strains ASF360 and ASF361), *Eubacterium plexicaudatum* strain ASF492, *Mucispirillum schaedleri* strain ASF457 (previous thought to be a *Flexistipes* sp., FOX paper) and *Bacteroides* sp. strain ASF519 (Dewhirst, 1999). Molecular methods for the sensitive and specific quantitation of the ASF strains have been developed and the distribution of the bacteria along the GI tract has been characterized (Sarma-Rupavtarm, Chapter 2). The phylogenetic characterization of the strains enables the development of fluorescence *in situ* hybridization methods to assess the microscale spatial localization of the bacterial community across the cross-section of the GI tract.

The overall objective of this study is to explore whether infection by a colonic pathogen leads to spatial interactions and competition for space between the pathogen and the host microflora, potentially resulting in changes in the bulk population levels and/or differences in localization across the luminal-mucosal interface. Since the adult GI tract ecosystem is a mostly stable community varying mainly with diet and age, competition for adhesion sites with a successful invading species would be expected to displace the resident bacteria from their spatial niche, leading to higher potential for washout and hence, lower population sizes post-infection. Competition for adherence sites has been proposed as a hypothesis for the mechanism behind colonization resistance (Kennedy, 1987, Vollaard, 1994, Savage, 1978, Hopkins, 2003). The goal of this study is to shed light on potential mechanisms by which the intestinal microbiota cause colonization resistance. In the study, immunodeficient defined flora C.B-17 SCID mice harboring the altered Schaedler flora were infected with the mouse pathogen, *C. rodentium*. Since the mice lack both B and T cells, their use in the study provides some ability to separate the effects of pathogen-host microflora

interactions from specific immune responses of the host. Further, the C.B-17 SCID mouse model colonized with ASF strains has been previously characterized and validated as a close approximation of mice harboring a complex flora (Sarma-Rupavtarm, Chapter 2). The mouse pathogen *Citrobacter rodentium* is used in the study since it causes mild pathology and has a short time course of infection. *C. rodentium* causes disease by attachment and effacement of epithelial cells, similar to human enteropathogenic *E. coli* infections, (Nataro, 1998). This mechanism, as opposed to the toxinogenic infections caused by pathogens such as *Clostridium difficile*, provides an opportunity to evaluate any possible pathogen-intestinal flora competition for adherence sites.

The population levels and spatial distribution of the different ASF strains and *C. rodentium* were studied at different time points post-infection. The chief goals of this study are to 1) assess the variation in the bulk numbers/g of tissue of *C. rodentium* and the ASF strains in different regions of the large intestine over several time points in infected and control mice, and 2) assess the microscale spatial localization of the bacterial community across the GI tract cross section in infected and control mice.

METHODS

Bacterial strains: *Citrobacter rodentium* DBS120 *kan*^{r} was grown overnight in LB broth supplemented with kanamycin. Bacterial concentration was determined by measuring absorbance at 600 nm. Cells were pelleted by centrifugation and resuspended in autoclaved water to a concentration of 10^9 cells/ ml for inoculation in mice.

Mice: Six-week old female C.B-17 SCID mice harboring a defined flora were ordered from Taconic (Germantown, NY). Isolator cubes (CRL, MA) were cold-sterilized with Clidox and the mice were housed in them for 72 hours prior to the experiment in order to acclimatize them to the surroundings and remove the effect of transportation stress on the experiment. Air entering the isolator cubes was filtered through HEPA filters. Autoclaved food and water were supplied to the mice during the course of the experiment and handling of the mice was performed under a sterile laminar flow hood. Instruments were autoclaved prior to use used in the experiment. Surfaces were decontaminated with Quatricide during animal handling.

Mice were divided into the experimental group (n= 20) and the control group (n =12). Isolators housing the experimental group to be infected with *C. rodentium* were located in a quarantine facility while those housing the control group were housed in the cleanest facility. This was done to prevent any potential contamination of mouse stocks with *C. rodentium* in the clean facility. In order to minimize the chances of contamination during cage changing, mice were house at a maximum of 3 per cage (for the experimental groups to be euthanized on Days 3, 6 and 14 PI) and mice to be euthanized on Day 21 PI were housed singly. Cage changes were kept to a minimum. All husbandry and experimentation was performed as per protocols approved by the MIT Institutional Animal Care and Use Committee.

Oral infection of mice: Mice in the experimental group were intragastrically inoculated with 100 µl of autoclaved water containing 10^8 cells of *C. rodentium* DBS 120 suspension while mice in the control group received 100 µl of autoclaved water. Inoculum dosage was confirmed by plating dilutions on LB-*kan* plates.

Sample collection: Mice from the control and infected group were euthanized at the specified time points by CO_2 asphyxiation. The GI tract from the ileum to the rectum was excised and samples were collected from the ileocecal valve, proximal colon and distal colon. Each sample consisted of 1 cm of tissue as measured along the longitudinal axis, of which 0.5 cm was collected for DNA extraction and the remaining 0.5cm was embedded in disposable plastic cassettes with OCT (Tissue-Tek) for hybridization studies using fluorescent probes. The sections in OCT were flash-frozen in liquid nitrogen immediately after collection. Care was taken to exclude any formed fecal matter from the tissue samples. Formed fecal pellets were collected from the colon of the mice for DNA extraction. Instruments were heat-sterilized prior to use for each sample and control mice were dissected prior to the infected group to prevent any contamination. Tissue and fecal samples were weighed and stored at –80°C prior to use for DNA extraction.

DNA extraction: (i) Tissue samples. DNA was extracted from tissue samples with the DNEasy Tissue kit, using a modification of the DNEasy tissue extraction protocol (Qiagen, CA) to ensure maximal lysis of both Gram-positive and Gram-negative bacteria, as previous detailed (Sarma-Rupavtarm, Chapter 2). Briefly, following overnight digestion of tissue samples with proteinase K (20µl, 12mAU) and buffer ATL (Qiagen, CA), the sample was centrifuged and separated into supernatant and pellet. The supernatant was RNase

treated and DNA was extracted using the Qiagen DNEasy protocol for tissues. The pellet from the centrifugation of the lysed tissue was subjected to an extraction using the DNEasy protocol for Gram-positive bacteria, to ensure recovery of any possibly recalcitrant Gram-positive bacteria. DNA from both the extractions was pooled and stored at -80°C prior to use for quantitative PCR.

(ii) Fecal pellet samples DNA was extracted from fecal pellets as previously detailed (Sarma-Rupavtarm, Chapter 2). Briefly, the fecal pellet samples were resuspended in Phosphate Buffer Saline (pH 7.4). Tris-saturated phenol (pH 7.4) (500µl), 0.1mm Zirconium beads (0.5g) and 20% Sodium Dodecyl Sulfate (50µl) were added to the tube and shaken at 5000 rpm for 2 min on a Bead beater (Biospec, OK). After a 10 min incubation at 60°C, the bead-beating step was repeated following which the supernatant was transferred to a new tube and an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added. The tube was mixed by inversion and centrifuged for 5 min at 12000×g. The supernatant was transferred to a new tube and the same procedure was repeated twice. Following this, an equal volume of chloroform (Sigma, MO) was added to the aqueous phase, the tube was mixed by inversion and centrifuged at 12000×g for 5 min. DNA from the supernatant was then precipitated using 0.1 volume of 3M Sodium acetate and a volume of isopropanol equal to that of the aqueous phase. The DNA was pelleted by centrifugation at 15000×g for 10 min, washed with 70% ethanol, dried under a vacuum using a SpeedVac (Thermo Corporation, MA) and resuspended in Tris-chloride buffer. Losses in extraction of different samples were kept constant by using constant volumes at all the steps.

Primer design and specificity testing. Primers sequences specific for the ASF strains were obtained from a previous study (Sarma-Rupavtarm, Chapter 2). Primers for the

quantitative PCR (QPCR) of *Citrobacter rodentium espB* gene were modified from published oligonucleotide sequences targeting the *Citrobacter rodentium espB* gene (McKeel, 2002) in order to increase their dissociation temperature to a level optimal for QPCR (> 58°C), as evaluated by using the PrimerExpress software (Applied Biosystems, CA). The specificity of the primers was tested *in silico* by checking against Genbank by using the BLAST algorithm and experimentally by amplifying DNA from *C. rodentium* in a background of fecal pellet DNA from defined flora mice. The primers were also tested against *E. coli* and yielded no amplicon. Primers espB-F and espB-R were synthesized and PAGE-purified by IDT DNA Technologies (Coralville, IA). Amplifications were performed using the Platinum PCR kit (Invitrogen, CA) on a Robocycler (Stratagene, CA) with the following conditions: 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C. After PCR, the amplification products were run on a 2% agarose gel (Invitrogen, CA) in Tris Borate EDTA buffer and visualized on the EagleEye Gel documentation system (Stratagene, CA). Primers used in this study are detailed in Table 1.

QPCR assay development Linearized plasmids containing the 16S rRNA gene of each ASF strain were generated as specified in a previous study (Sarma-Rupavtarm, Chapter 2). For *C. rodentium*, the *espB* gene was amplified and cloned into a pCR2.1 plasmid vector using the Topo-TA cloning kit (Invitrogen, CA). The plasmid was sequenced using the BigDye terminator kit (Applied Biosystems, CA) to ensure that the primer regions were not altered due to *Taq* polymerase error. Prior to use for generating standard curves, the plasmid was linearized with the restriction enzyme *Bam*HI (NEB, MA) that cuts the vector exactly once outside the cloned *espB* gene. The concentrations of the linearized plasmid were determined on a Synergy HT microplate fluorescence reader (Bio-Tek, VT) using the

PicoGreen kit (Molecular Probes, OR) and dilutions of known concentrations of λ DNA as standards.

QPCR optimization. (i) $MgCl_2$ optimization. $MgCl_2$ concentrations as previously optimized were used for QPCR of the ASF strains (Sarma-Rupavtarm, Chapter 2). *C. rodentium* levels were quantified using Platinum® SYBR® Green qPCR SuperMix-UDG kit which contains a working concentration of 3 mM $MgCl_2$. Hence, *C. rodentium* specific primers were tested at this $MgCl_2$ concentration in a background of DNA extracted from fecal pellets from restricted flora mice, which harbor the ASF strains and a more complex unknown flora, to ensure the absence of non-specific amplification. PCR conditions were the same as those used for testing specificity. Amplification products were analyzed on a 2% agarose gel (Invitrogen, CA) and visualized using the EagleEye gel documentation system (Stratagene, CA).

(ii) Primer concentration. Primer concentrations, while increasing the efficiency of QPCR reactions, can lead to primer dimer formation. Optimal primer concentrations for QPCR of the ASF strains were obtained from Sarma-Rupavtarm (Chapter 2). For *C. rodentium* QPCR, all combinations of 100 nM, 200 nM and 300 nM forward and reverse primer concentrations were tested with approximately 10^6 copies of plasmid template bearing the *espB* gene. QPCR reactions were performed in duplicate for each of the nine possible primer concentration pairs, along with corresponding no-template controls, using the Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen, CA) and the QPCR conditions specified below. The primer concentrations yielding the lowest threshold cycle value for the plasmid standard and highest threshold cycle value for the no-template control

(300nM each of forward and reverse primer) were chosen as the optimal concentrations for QPCR. None of the primers formed primer dimers at the optimal concentrations.

QPCR reaction conditions. All QPCR reactions were performed in triplicate on the DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research, MA). Levels of ASF strains were quantified in a 25µl reaction volume using the SYBR Green core reagents kit (Applied Biosystems, CA) and the following reaction conditions: 95°C 10 min, 40 cycles of: 95°C 30 sec, 60°C 45 sec, 72°C 75 sec. The $MgCl_2$ concentration was kept constant at 4.0 mM for all the reactions. QPCR reactions for *C. rodentium* were also performed in triplicate on the Opticon System using the Invitrogen Platinum QPCR Supermix (Invitrogen, CA), with a reaction volume of 25µl and the following reaction conditions: 95°C 2 min, 40 cycles of: 95°C 30 sec, 60°C 45 sec, 72°C 75 sec. Melting curves were generated post-QPCR to test for purity by identification of the characteristic melting peak. This was done by measuring the fluorescence of the amplification product every 0.1°C while increasing the temperature from 50°C to 95°C. Standard curves for individual ASF strains and *C. rodentium* were generated using a ten-fold dilution series of the linearized plasmid standard ranging from $1\text{-}10^8$ copies. The fluorescence level for determining the threshold cycle (C_T) was set at 0.01 fluorescence units in all reactions. Some of the tissue DNA samples had a very high background of eukaryotic DNA, which increased the fluorescence background in the QPCR reactions. These samples were diluted ten-fold prior to assay by QPCR.

Fluorescence *in situ* hybridization. i) Probes. Oligonucleotide probe sequences for *in situ* hybridization of the ASF strains and *C. rodentium* were either designed in this study or obtained from literature (Table 2). Probes for *Mucispirillum schaedleri* ASF457 and

Clostridium sp. strain ASF356 were designed by identifying sequences unique to each strain in a 16S rRNA secondary structure-based alignment of all eight ASF strains. Candidate probes were selected based on published optimality criteria (Hugenholtz, 2001). Selected candidate probe sequences were assessed for specificity by comparison against Genbank using the BLAST algorithm and results indicating a perfect match only for the target organism were chosen as FISH probes. Probes were ordered from ThermoElectron Corporation GMBH (Bremen, Germany) and diluted in autoclaved milliQ water to a concentration of 0.5 μg/μl. The universal eubacterial probe was labeled with Marina blue, probes for the different ASF strains were labeled with Cy3 and the enterobacteriaceae probe for *C. rodentium* was labeled with Oregon Green. The probes were used at 50 ng/μl working concentration in the hybridization buffer.

ii) Sample preparation. Tissue samples embedded in OCT (Tissue-Tek) were cut on a cryostat in 5μm thick sections onto Superfrost Plus Gold slides (Erie Scientific, NY). Samples and slides were kept at –20°C or lower throughout the sectioning process, following which they were stored at –80°C prior to hybridization.

iii) *In situ* hybridization. Whole cell hybridization protocols for fecal matter and freshwater samples were modified for tissue (Barc, 2004, Hugenholtz, 2001). Briefly, cryosectioned colonic samples were fixed for 3 h in 4% paraformaldehyde solution (Ph 7.2) prepared in PBS (Hugenholtz, 2001). Following fixation, the slides were washed for 3 min in PBS (pH 7.2) and 5 min in TE-HIS buffer (100mM Tris-Cl, 50mM EDTA). The sections were treated for 10 min at room temperature in a 1mg/ml lysozyme (EM Sciences, NJ) solution in TE-HIS buffer. Following this, the samples were dehydrated for 3 min each in 50%, 80% and 95% ethanol and air-dried. Hybriwell hybridization chambers (Molecular

Probes/Invitrogen, CA) were affixed onto the samples to facilitate hybridization without buffer evaporation. Hybridization buffer containing 102 mM NaCl, 20 mM Tris-HCl, 0.01% SDS and 30% formamide used for all hybridizations, since this buffer composition has been seen to work well when hybridizing with several probes with different dissociation temperatures on a single section (Hugenholtz, 2001). For optimization of hybridization conditions, two different sets of conditions (35°C hybridization/ 37°C wash (Barc, 2004) and 46°C hybridization/48°C wash (Hugenholtz, 2001)) were assessed on an artificial mixture of *Lactobacillus murinus* ASF361 (Gram positive) and *Citrobacter rodentium* (Gram negative). The two strains were grown overnight in MRS broth (Difco, MI) and LB broth (Difco, MI) respectively, and harvested by centrifugation at 8000xg for 5 min. The cell pellets were resuspended in sterile water, pooled and spotted onto SuperFrost Plus slides (Erie Scientific, NY). The cells were hybridized with the universal EUB338, lactobacilli-specific LAB158 and enterobacteriaceae-specific ENTER1532 probes using the above protocol for 2 h. The 35°C hybridization was washed for 15 min at 37°C with Wash Buffer I containing 65 mM NaCl, 5 mM Tris-HCl, 5 mM EDTA and 0.01% SDS (Barc, 2004), while the 46°C hybridization was washed for 15 min at 48°C in Wash Buffer II containing 102 mM NaCl, 20 mM Tris-HCl and 0.01% SDS (Hugenholtz, 2001). Following washing, the samples were rinsed in ice cold water and air-dried. The samples were cover-slipped after the addition of Vectashield Hardset medium with DAPI (Vector Labs, CA). The higher hybridization/wash temperature conditions yielded a strong specific signal while nonspecific hybridization was seen with the lower temperature set. Thus, all hybridizations were performed for 2 h at 46°C, followed by a 15 min wash in Buffer II.

iv) Immunofluorescence for *C. rodentium*. Briefly, tissue sections were subjected to heat-induced epitope retrieval in sodium citrate buffer at pH 6.0, followed by incubation with the primary antibody at 1:1500 dilution for 1 hour. Sections were subsequently washed with PBS and incubated with goat anti-rabbit secondary antibody conjugated to Alexa Fluor 488 dye (Molecular probes) at 1:100 dilution for 30 minutes. DAPI was used as a counter stain.

v) Fluorescence microscopy and image analysis. Hybridized samples were viewed on a Zeiss Axioskop epifluorescence microscope (Carl Zeiss, NY) with filters with the following excitation emission characteristics: 360 nm/460 nm (DAPI, Marina Blue), 470 nm/535 nm (Oregon Green) and 546 nm/580 nm (Cy3). Images were captured with a Nikon D70 digital camera (Nikon, NY) in 8-bit TIFF format. Image analysis was performed using Photoshop 7.0 (Adobe, CA). All images are shown in false color here.

Statistical analysis. Cell numbers from infected and control groups were analyzed using Microsoft Excel for statistically significant differences using Analysis of Variance (ANOVA). Statistical significance was set at $P < 0.05$.

RESULTS

***C. rodentium* infection in Defined Flora C.B-17 SCID mice.** *Citrobacter rodentium* levels assessed by QPCR over the course of the study in three regions of the large intestine, *viz.* ileocecal valve, proximal colon and distal colon, showed differences depending on the day of sampling and the region sampled. *C. rodentium* reached levels of greater than 10^5 cells/g tissue by the first post-infection (PI) sampling time point at Day 3 in all three regions, but only reached a peak level of 10^{7-8} cells/g tissue in all three regions of the large intestine (**Figure 1A, B & C**). *C. rodentium* was present at a level of over 10^7 cells/g tissue by Day 3 PI in the ileocecal valve and stayed at this level over the course of the infection study (**Figure 1A**). In the proximal colon, *C. rodentium* levels increased by an order of magnitude over the course of the infection, from about 10^6 cells/g tissue at Day 3 to about 10^7 cells/g by Day 6 and were stable at this level (**Figure 1B**). Average *C. rodentium* levels were at 10^6 to 10^7 cells/g tissue in the distal colon over the course of the infection. However, while the numbers did not vary much between different infected mice at Day 3 and Day 6 PI, there was over 2 orders of magnitude difference (10^6 to 10^8) between different mice at both Day 14 and Day 21 (**Figure 1C**). Surprisingly, the number of cells of the pathogen per gram of tissue were highest upstream of the distal colon in the ileocecal valve and proximal colon throughout the course of the infection, although the distal colon has been seen to be the site of pathology due to *C. rodentium*-induced colitis.

Despite the substantial levels of *C. rodentium,* infection in the C.B-17 SCID mice led only to low levels of disease in the mice, as observed on Day 14 PI. Examination of hematoxylin and eosin stained colonic tissue showed a low level of colonic hyperplasia in the infected mice as compared to the control mice (**Figure 2**). The formed fecal pellets in the

mice euthanized on Day 14 PI were soft, but the mice showed no obvious behavioral signs of disease.

Bulk population effects of *C. rodentium* infection on altered Schaedler flora. Bulk levels of all the ASF strains were enumerated in the ileocecal valve, proximal colon and distal colon for all four temporal data points. Infection by *C. rodentium* was associated with subtle changes in the bulk population levels of specific ASF strains that varied both over the time course of the infection and in the different regions of the GI tract assayed. Surprisingly, all changes involved an increase in the level of the ASF strain in infected mice as compared to control mice. Total bacterial levels were higher in infected mice than in control mice in all three colonic regions at Day 3 PI, and in the proximal and distal colon on Day 6 PI. Levels of total bacteria were similar in both groups after Day 6 PI.

The most significant differences in abundance were seen in the ileocecal valve at Day 3 PI. Levels of *Clostridium* sp. strains ASF356 (10^{9-10} cells/g vs. 10^{8} cells/g) and ASF500 (10^{8} cells/g vs. 10^{7} cells/g), *Lactobacillus murinus* ASF361 (10^{7} cells/g vs. 10^{6} cells/g), *Mucispirillum schaedleri* ASF457 (10^{10} cells/g vs. 10^{9} cells/g) and *Bacteroides* sp. strain ASF519 (10^{10} cells/g vs. 10^{9} cells/g) were all seen to be significantly higher ($P<0.05$) in infected mice than in control mice (**Figure 3**). No significant differences were seen in the ileocecal valve at Day 3 PI for *Eubacterium plexicaudatum* ASF492 and *Clostridium* sp. strain ASF502. *Lactobacillus* sp. strain ASF360 was not detected in any of the mice, consistent with previous observations (Sarma-Rupavtarm, Chapter 2).

There were no significant differences in bacterial levels at Day 6, Day 14 and Day 21 PI (**Supplementary figures, S1-4**) with the exception of low levels of *Eubacterium plexicaudatum* ASF492 and *Clostridium* sp. strain ASF500 in control mice on Day 6 PI,

which were probably abnormal and due to animal to animal variation. No contaminants were detected in feces from the mice by culture tests. However, the numbers were at previously observed levels in the control mice on day 14 PI and beyond. Other than these changes, no significant differences were observed between infected and control mice for any of the ASF strains, and the levels of the ASF strains in the two groups were statistically indistinguishable in the regions studied on the different sampling time points.

Microscale effects on altered Schaedler flora. Infection with *C. rodentium* did not cause any changes in the localization of the ASF strains that could be observed by comparing the infected and control mice. The intestinal cross-section could be divided into three regions where bacteria appeared to be localized, in the lumen, at the lumen-mucus interface or inside the mucus. Different ASF strains showed distinct spatial localization across the mucosal-luminal cross-section that was consistent over time, and between the infected and control groups. Interestingly, bacterial colonization was patchy and some regions of the mucus or the lumen appeared not to be colonized by any bacteria.

Specific ASF strains were located consistently in one or more of the three regions. However, no differences were seen between infected and control mouse sections in the location of the ASF strains assayed for by FISH. The two most abundant ASF strains appear to have different spatial niches. *Mucispirillum schaedleri* ASF457, whose bulk levels as assessed by QPCR assays were an order of magnitude higher than most of the other ASF strains in different sections of the large intestine, was the only ASF strain localized within the mucus layer (**Figure 4A&B**). *M. schaedleri* ASF457 was also the only bacterium identified close to the base of the crypts, embedded deeply in the mucus layer. The other abundant ASF strain, *Bacteroides* sp. strain ASF519, was primarily localized in the lumen, unattached to the

mucus layer (**Figure 4C&D**). Other ASF strains assayed for were also present at specific locations across the mucosal-luminal continuum. *Clostridium* sp. strain ASF356 was seen in the lumen or attached to the mucus or, but never in the mucus layer (**Figure 4E**). *Clostridium* sp. strain ASF500 was present attached to or near the mucus layer (**Figure 4F**).

Surprisingly, *C. rodentium* was not conclusively detected in sections from infected mice either by FISH with the *Enterobacteriaceae* probe or immunofluorescence with anti-*Citrobacter rodentium* antibodies. Bulk *C. rodentium* levels as measured by QPCR assays would indicate greater than 10 cells per field even in the ileocecal valve sections where *C. rodentium* makes up less than 0.1% of the total population.

DISCUSSION

Studies on the interaction of the GI microbiota with exogenous pathogens have primarily focused on the disease-ameliorating role of the bacteria. There is an abundance of evidence in literature suggesting a role for the GI microbiota in reducing the severity of disease caused and increasing the needed infectious dose of the pathogen (Wells, 1982, Kennedy, 1986, Hudault, 2001, Hopkins, 2003, Vollaard, 1994). The mechanisms behind the role of the microbiota are not well understood and one proposed hypothesis invokes competition for adherence sites between the exogenous pathogen and the intestinal microbiota (Kennedy, 1987). However, studying the microecology of infection in depth in an ecosystem of hundreds of species of bacteria is an intractable problem. Experimental setups to study GI microbiota-pathogen interactions have involved either simplification of the microbiota by antibiotic treatment or simple defined flora models such as mono-associated germfree mice. While both approaches provide insight into the role of the complex flora as a whole or of two-species interactions, the former cannot resolve fine-scale interactions whereas the latter are difficult to generalize, as one species is not representative of the diversity of the complex flora. To complement these two approaches, our study used gnotobiotic mice with a more complex defined flora that is representative of the abundant intestinal bacterial species. The mouse pathogen, *Citrobacter rodentium*, was chosen since it causes a mild self-limiting disease and prior studies have indicated that the infection reaches a peak by day 14 post-infection. A short time line of infection is critical to defined flora experiments in order to minimize the potential for contamination from cage changes and other husbandry requirements. The aim of this study was to examine the ecology of infection by a pathogen in the GI tract in a defined flora mouse model which, while sacrificing some

level of complexity, allows for analysis of both bulk population dynamics as well as microscale spatial effects of infection.

The dynamics of *C. rodentium* infection in the C.B-17 defined flora model were different from previously observed results in other mouse models. Surprisingly, the levels of *C. rodentium* in defined flora mice in this study reached a high level of 10^{6-7}cells/g tissue by Day 3 PI and increased only by an order of magnitude by Day 6 PI. The variation over the course of the experiment was within one order of magnitude in the ileocecal valve and proximal colon, while there was higher variability in the distal colon on the later sampling dates. In addition, *C. rodentium* levels were consistently observed to be higher upstream of the distal colon, which is seen to be the site of pathology. While no studies have assessed the colonization dynamics of *C. rodentium* in defined flora immunodeficient mice, studies conducted in immunodeficient RAG k/o mice harboring a flora of unknown complexity have indicated an increase in *C. rodentium* levels from about 10^3 CFU/colon (colon weight is approximately 0.1 grams) at Day 4 PI to about 10^8 CFU/colon (Vallance, 2002). In studies of immunocompetent mice harboring a flora of unknown complexity, *C. rodentium* levels increased from less than 10^4 CFU/colon at Day 4 PI to about 10^8 CFU/colon at Day 6 PI (Vallance, 2003). Thus, *C. rodentium* reaches a high level of colonization in defined flora immunodeficient mice at an earlier time point post infection than immunodeficient or immunocompetent mice with a flora of unknown complexity.

One possible explanation for the observed dynamics in our experiment is the difference in complexity between the intestinal flora in mice in previous studies and the defined flora mice used in our study. The vendor used in our study, Taconic, is the only one that maintains production stock immunodeficient SCID mice in strict defined flora conditions (Sarma-

Rupavtarm, Chapter 3). It is highly likely that the mice in other studies, from vendors other than Taconic, harbored a more complex flora than the eight ASF strains. In particular, facultatively anaerobic organisms such as *E. coli* are known to colonize the GI tract of animals, although only as a small proportion of the colonic flora. While the altered Schaedler flora is representative of the obligate anaerobic and aerotolerant components of the complex microbiota, it does not include a facultatively anaerobic strain. From a competition standpoint, it is possible that the facultatively anaerobic *C. rodentium* faces the biggest challenge from a facultative member of the complex microbiota. Potentially, resistance to colonization of a pathogen mediated by the intestinal flora could be effected by competition, possibly for a spatial niche, between the pathogen and components of the microbiota physiologically most similar to the pathogen. Colonizing defined flora mice with *E. coli* prior to *Citrobacter rodentium* infection would provide more information on the validity of this hypothesis. Alternately, the defined flora mice could be infected with an anaerobic pathogen and assessed for competition between the pathogen and the obligate anaerobes among the ASF strains.

Another interesting observation from our results is that the ileocecal valve is a primary site of colonization of *C. rodentium*, with high levels of the pathogen being seen by Day 3 PI. This corroborates the results of a recent study in specific pathogen free C57/BL6 mice, where the infection dynamics were studied with a genetically engineered *C. rodentium* strain that expressed a bioluminescent pathway (Wiles, 2004). Since the distal colon is a site where pathology due to *C. rodentium* infection is commonly seen (Luperchio, 2001), it is interesting to observe that *C. rodentium* levels are consistently higher in the ileocecal valve and

proximal colon, potentially indicating that tissue/organ specificity of *C. rodentium* is not necessarily correlated with pathogenesis.

The effect of *C. rodentium* infection on bulk levels of ASF strains is nuanced and unexpected. Our study indicates that infection by *C. rodentium* leads to statistically significant increases ($P< 0.05$) in the levels of specific ASF strains in infected mice as compared to control mice at Day 3 PI. Further, the total number of bacteria/g of tissue was higher in infected mice than in control mice at Day 3 PI. The contribution of *C. rodentium* levels to this increase was minimal, with the pathogen representing less than a few percent of the total cells. A statistically significant difference in levels was also seen in feces from infected and control mice at Day 3 PI (data not shown). This dynamic changed over the course of the infection, with the total numbers of cells/g tissue similar after Day 6 PI in both infected and control mice.

A potential reason for the increase in the overall bacterial numbers could be the presence of additional nutrients from an increased sloughing of epithelial cells in response to infection. The GI tract ecosystem has been called a precarious alliance between the host immune system and the intestinal microbiota, wherein a tolerance is developed for the intestinal bacteria (McCracken, 2002). In this context, the increase in resident bacterial populations may be one of the factors that could potentially cause a breakdown in the equilibrium between the host and the microflora in susceptible individuals. This may have implications in diseases of immune system dysregulation, such as inflammatory bowel disease, where, for reasons not understood, the body mounts an uncontrolled inflammatory response to the intestinal microbiota antigens.

The microscale spatial architecture data from FISH indicates that the ASF bacteria have specific niches in both infected and control mice. While this could indicate that infection with *C. rodentium* does not detectably alter the spatial architecture of the ASF strains across the GI cross-section, it is difficult to make this assertion, given the lack of strong results correlating the spatial colocalization of *C. rodentium* with ASF strains. A prior study that examined the microscale interactions between an *E. coli* strain (exogenous) and *B. vulgatus* (monoassociated with mice) observed that the exogenous strains did not overlap with or displace the resident bacterial species (Waidmann, 2003).

While immunofluorescence data indicated a few stained cells at the lumen-mucus interface, the high background and low number of cells that stained made definitive identification of the cells as *C. rodentium* difficult. Similar results were observed with FISH, where a signal was visible in a few sections, but in a high background, precluding definitive identification. The immunofluorescence results could potentially be due to the usage of frozen sections, on which the protocol has not been optimized. In future experiments, *C. rodentium* cells will be immobilized in OCT, flash frozen and sectioned similar to tissue, and the immunofluorescence protocol will be optimized on these sections prior to use on sectioned tissue. The FISH results for *C. rodentium* could potentially be due to the relative short length of the probe, which was 15 nucleotides long., which could also potentially explain the high background seen. The probe will be lengthened prior to use in future experiments in order to increase specificity.

The spatial localization of the ASF strains indicated that strains were likely to be present at one or more of three regions: the lumen, the lumen-mucus interface or inside the mucus layer. Two of the most abundant ASF strains as measured by QPCR, *Bacteroides* sp. strain

ASF519 and *Mucispirillum schaedleri* ASF457, appear to have different niches wherein *M. schaedlerii* ASF457 is primarily located in or at the mucus layer and *Bacteroides* sp. strain ASF519 is primarily free-living in the lumen.

The role of the microbiota in colonization resistance is hard to assess due to the complexity of the bacterial species. This study indicates that infection may initially be associated with an increase in total bacterial numbers, and the relative stability of the microbiota in response to infection, combined with the accelerated dynamics of infection, in the ASF mouse model suggests that colonization resistance effects may potentially be dependent on bacteria similar in physiology to the pathogen. Studies with anaerobic pathogens in the ASF model will provide more insight into the potential relationship physiological similarities and colonization resistance. Further, the understanding derived on the spatial distribution of some of the ASF strains will help in the development of testable microecological hypotheses on the role of mucosal vs. luminal bacteria. Thus, future experiments in the defined flora mouse models on pathogen-microflora-host interactions hold the potential to yield insight into the role of our microbial cohabitants in preventing disease.

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FIGURE LEGENDS

Figure 1. The levels of *C. rodentium* (cells/g tissue) over the four sampling days post-infection as measured by QPCR in a) ileocecal valve, b) proximal colon and c) distal colon, are shown here. The three different infected mice from which the samples were collected after euthanizing them are represented by a filled circle (●), filled square (■) and filled diamond (◆).

Figure 2. Defined flora C.B-17 SCID mice infected with *C. rodentium* exhibit a low level of hyperplasia, as seen in hematoxylin & eosin-stained colonic sections from mice euthanized on Day 14 post-infection. The two leftmost panels are from control mice, while the middle and rightmost panels are from infected mice.

Figure 3. The levels of *C. rodentium* and the seven ASF strains (cells/g tissue) measured in the ileocecal valve of infected and control mice by QPCR on Day 3 PI are shown. C. rod: *C. rodentium*, 356: *Clostridium* sp. ASF356, 361: *Lactobacillus murinus* ASF361, 457: *Mucispirillum schaedleri* ASF457, 492: *Eubacterium plexicaudatum* ASF492, 500: *Clostridium* sp. ASF500, 502: *Clostridium* sp. ASF502 and 519: *Bacteroides* sp. ASF519. Infected mice are represented by a filled circle (●), filled square (■) and filled diamond (◆) while control mice are represented by a light-colored upward triangle (▲) and downward triangle (▼).

Figure 4. Representative images from intestinal sections hybridized with labeled probes specific for a) & b) *Mucispirillum schaedleri* ASF457 (40X and 100X magnification), c) & d) *Bacteroides* sp. strain ASF519 (40X and 100X magnification), e) *Clostridium* sp. strain ASF356 (100X magnification) and f) *Clostridium* sp. strain ASF500 (100X magnification) are shown here. *M. schaedleri* ASF457 is seen localized in the mucus layer deeply embedded

in the crypt at 100X magnification. All images are in false color, with blue representing EUB338 and DAPI fluorescence, and red representing an ASF strain-specific probe fluorescence.

Supplemental figure 1. The levels of *C. rodentium* and the seven ASF strains (cells/g tissue) measured by QPCR in a) proximal colon and b) distal colon of infected and control mice on Day 3 PI are shown. C. rod: *C. rodentium*, 356: *Clostridium* sp. ASF356, 361: *Lactobacillus murinus* ASF361, 457: *Mucispirillum schaedleri* ASF457, 492: *Eubacterium plexicaudatum* ASF492, 500: *Clostridium* sp. ASF500, 502: *Clostridium* sp. ASF502 and 519: *Bacteroides* sp. ASF519. Infected mice are represented by a filled circle (●), filled square (■) and filled diamond (◆) while control mice are represented by a light-colored upward triangle (▲) and downward triangle (▼).

Supplemental figures 2-4 The levels of *C. rodentium* and the seven ASF strains (cells/g tissue) measured by QPCR at Day 6 PI **(Figure S2)**, Day 14 PI **(Figure S3)** and Day 21 PI **(Figure S4)** in a) proximal colon and b) distal colon, and c) ileocecal valve of infected and control mice are shown. C. rod: *C. rodentium*, 356: *Clostridium* sp. ASF356, 361: *Lactobacillus murinus* ASF361, 457: *Mucispirillum schaedleri* ASF457, 492: *Eubacterium plexicaudatum* ASF492, 500: *Clostridium* sp. ASF500, 502: *Clostridium* sp. ASF502 and 519: *Bacteroides* sp. ASF519. Infected mice are represented by a filled circle (●), filled square (■) and filled diamond (◆) while control mice are represented by a light-colored upward triangle (▲) and downward triangle (▼).

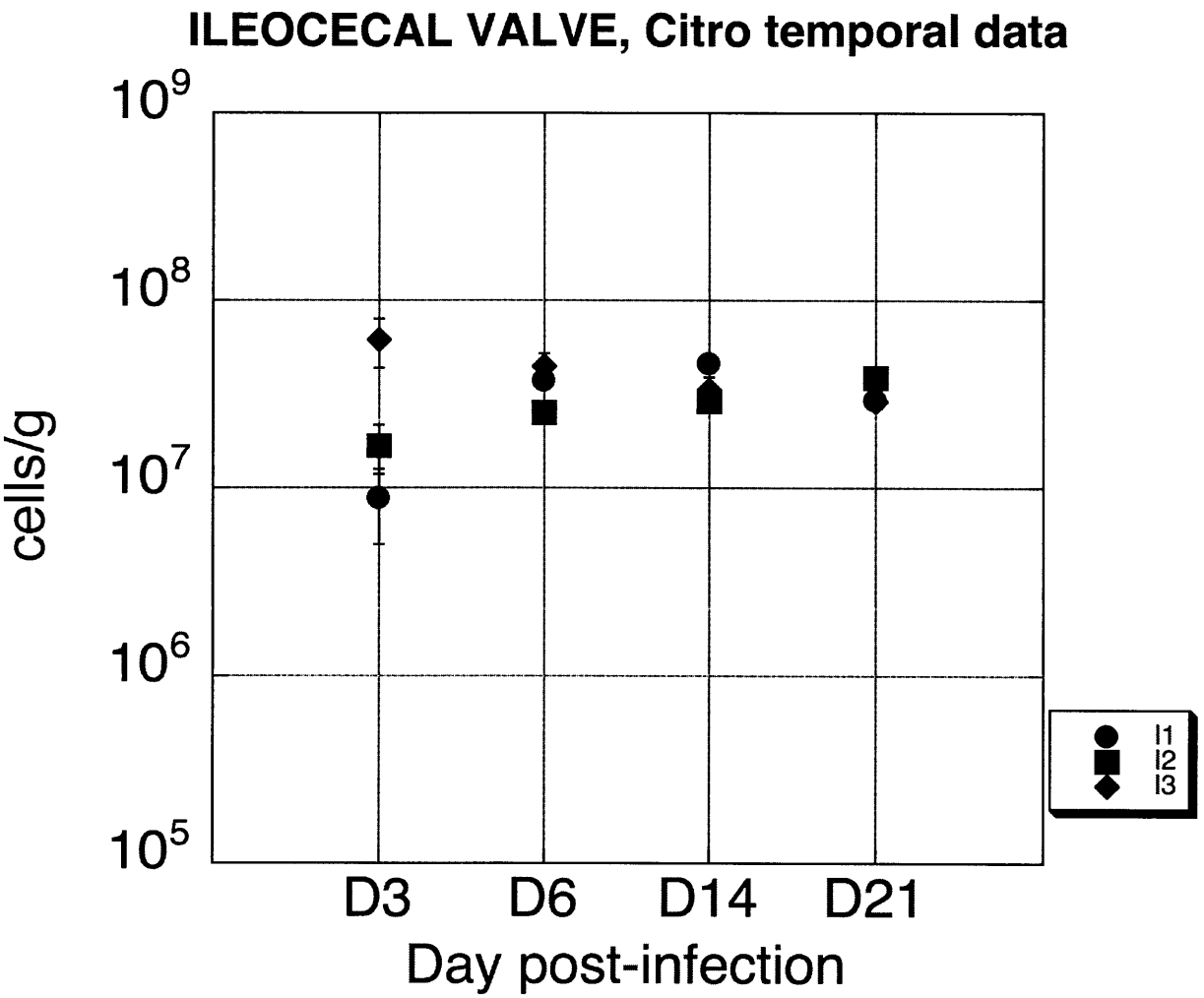
ILEOCECAL VALVE, Citro temporal data
10^9
10^8
10^7
10^6
10^5
cells/g
D3
D6
D14
D21
Day post-infection
I1
I2
I3

Figure 1(A)

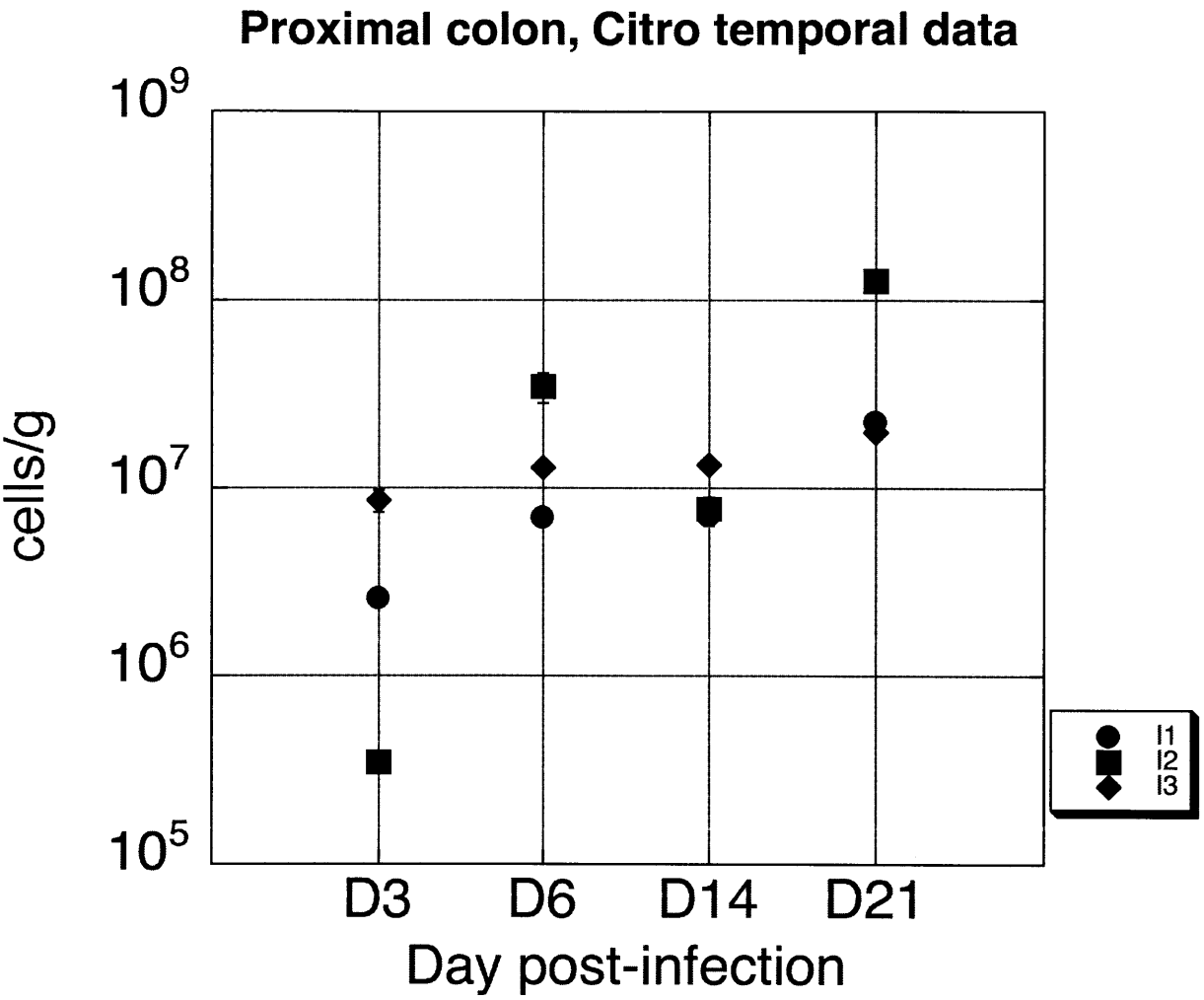
Proximal colon, Citro temporal data
10^9
10^8
10^7
10^6
10^5
cells/g
D3
D6
D14
D21
Day post-infection
I1
I2
I3

Figure 1(B)

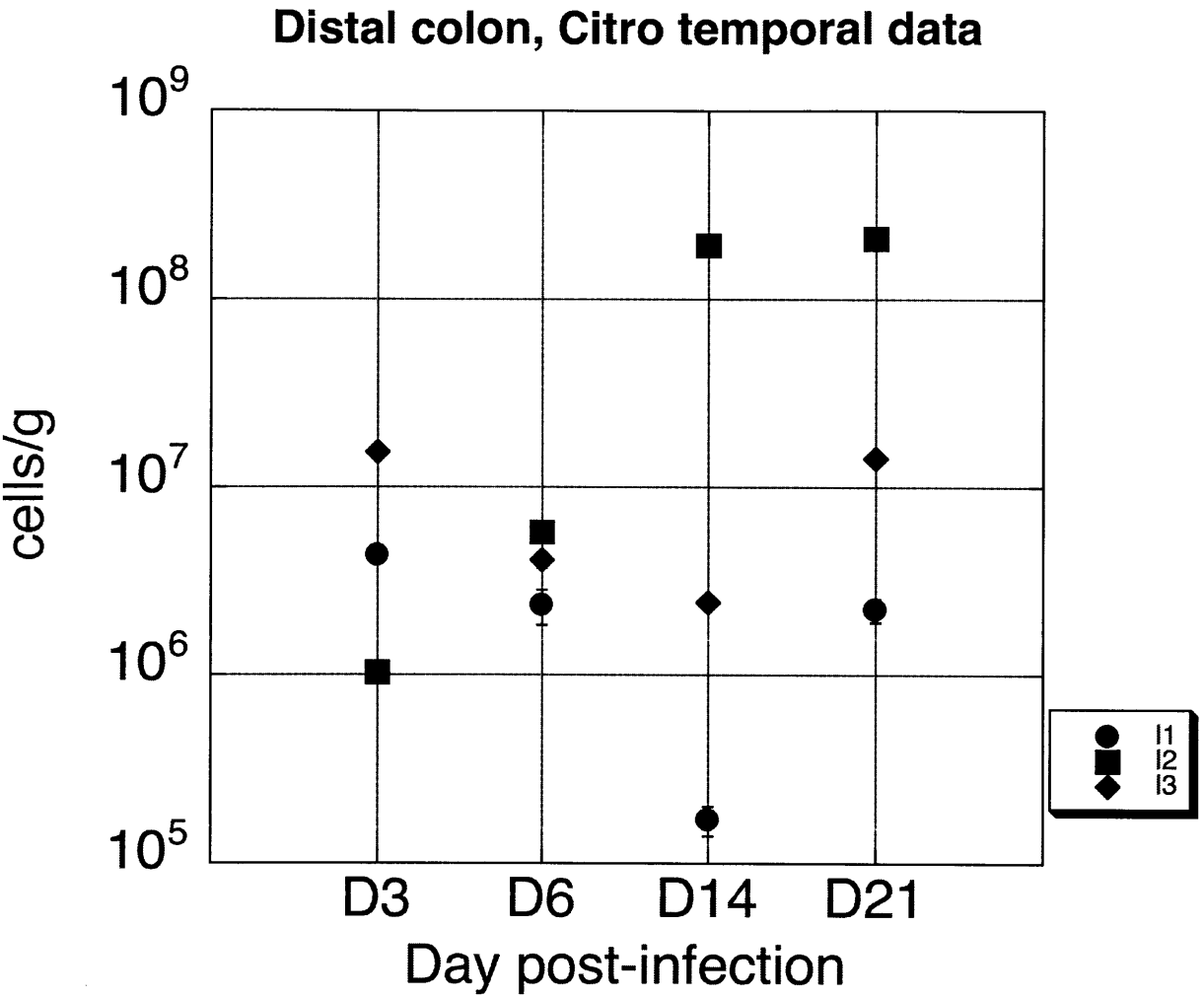
Distal colon, Citro temporal data
10^9
10^8
10^7
10^6
10^5
cells/g
D3
D6
D14
D21
Day post-infection
I1
I2
I3

Figure 1(C)

Controls

Infected

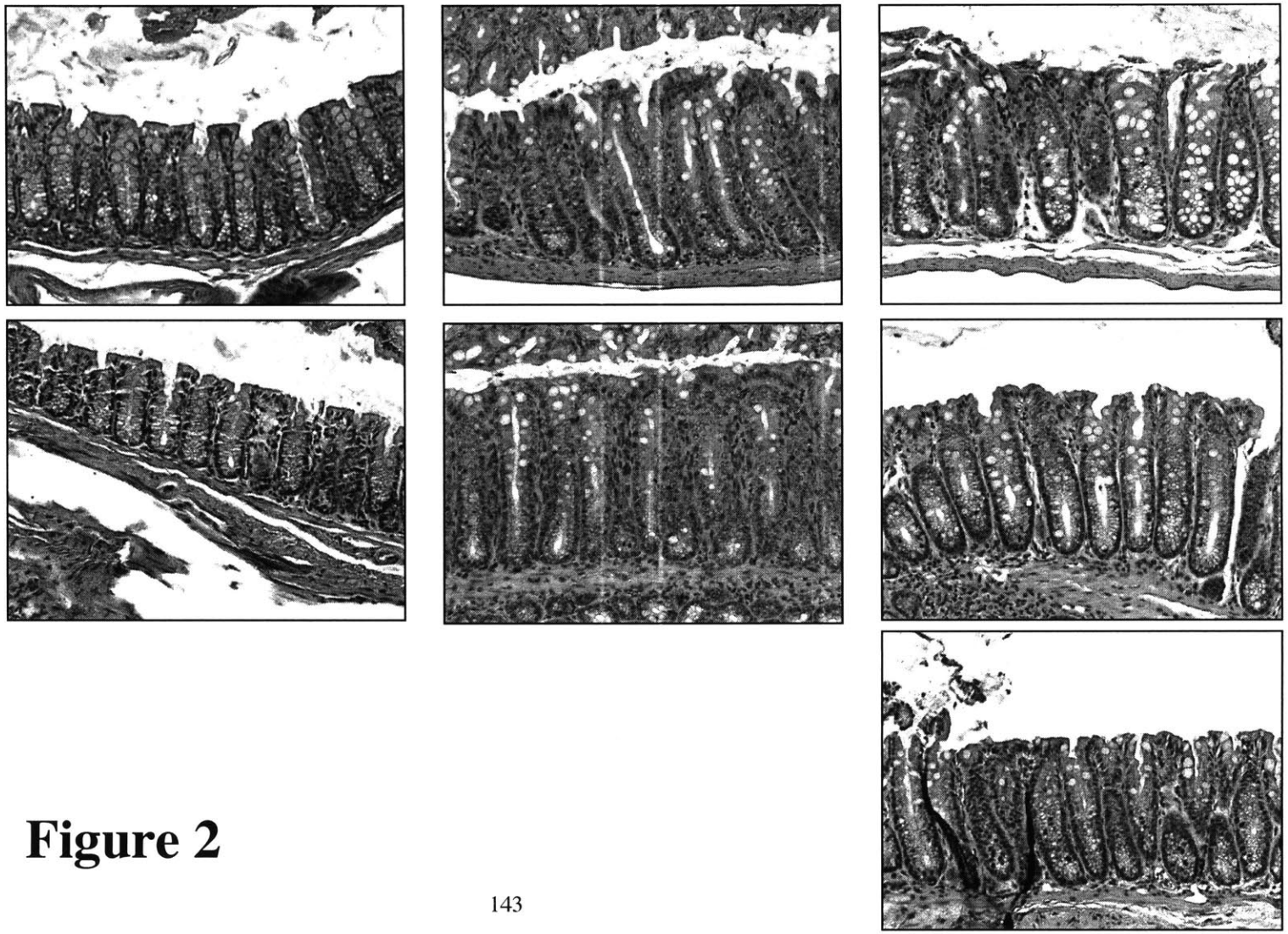

Figure 2

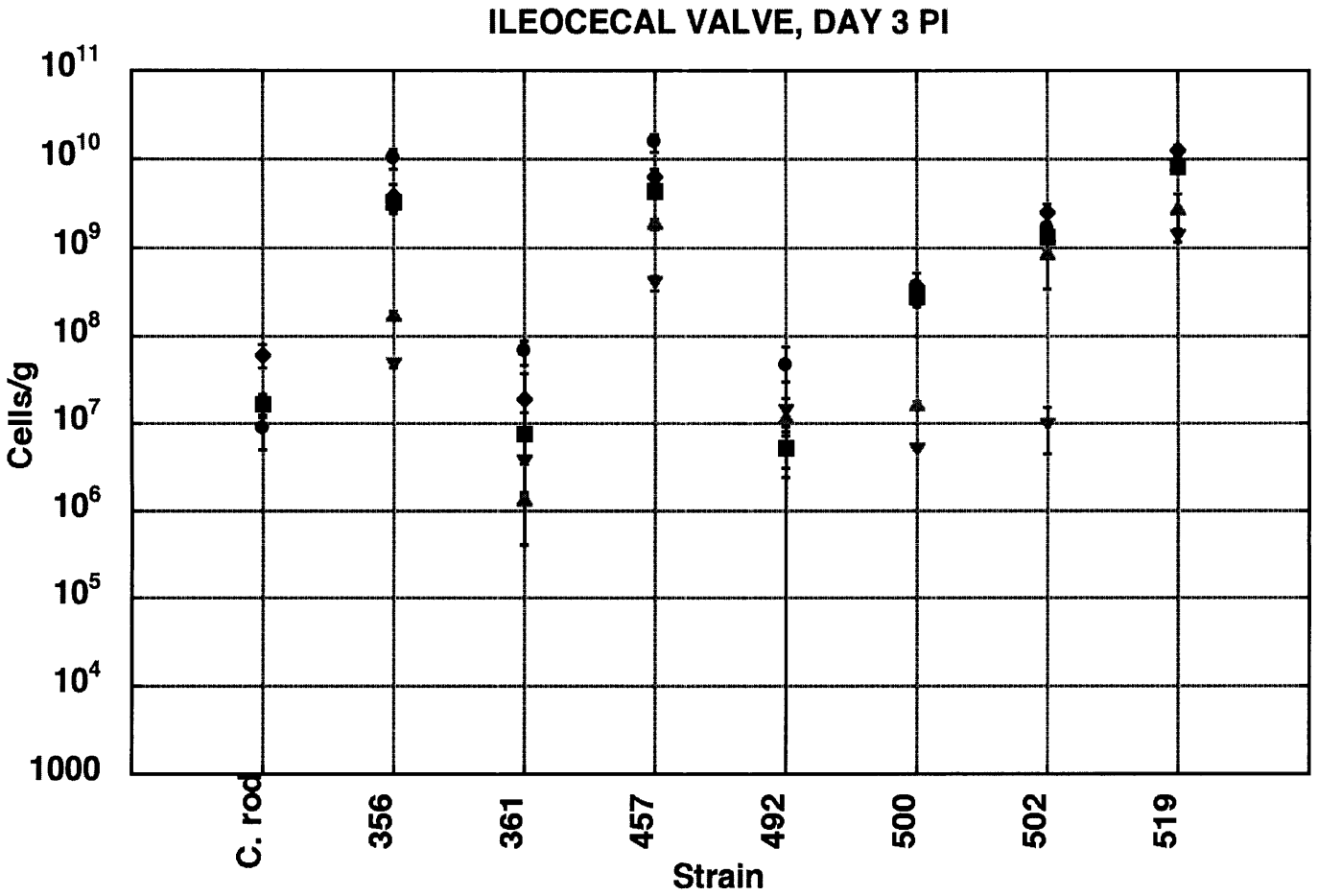
ILEOCECAL VALVE, DAY 3 PI
Cells/g
10^11
10^10
10^9
10^8
10^7
10^6
10^5
10^4
1000
C. rod
356
361
457
492
500
502
519
Strain

Figure 3

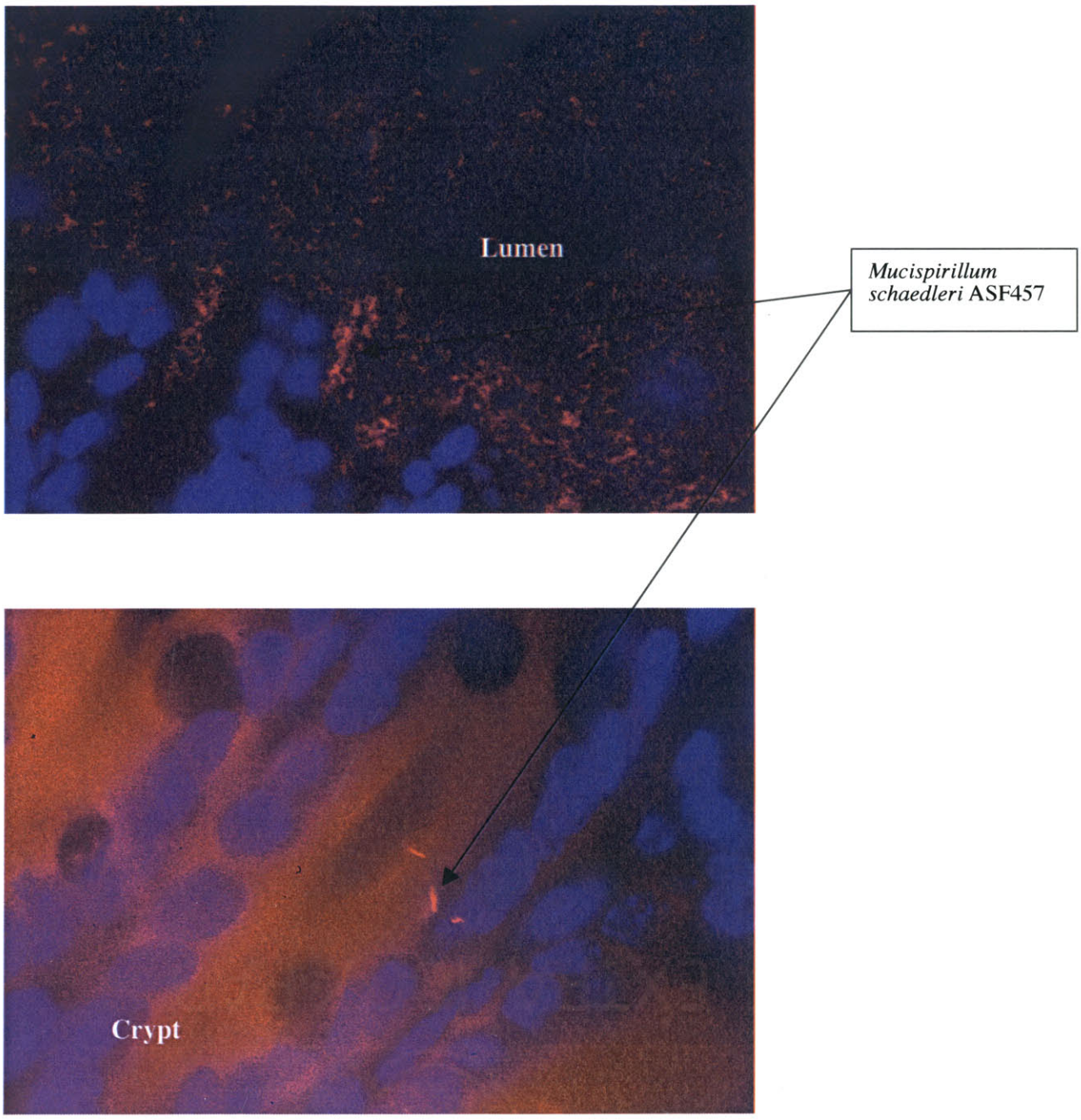

Figure 4 (A) & (B)

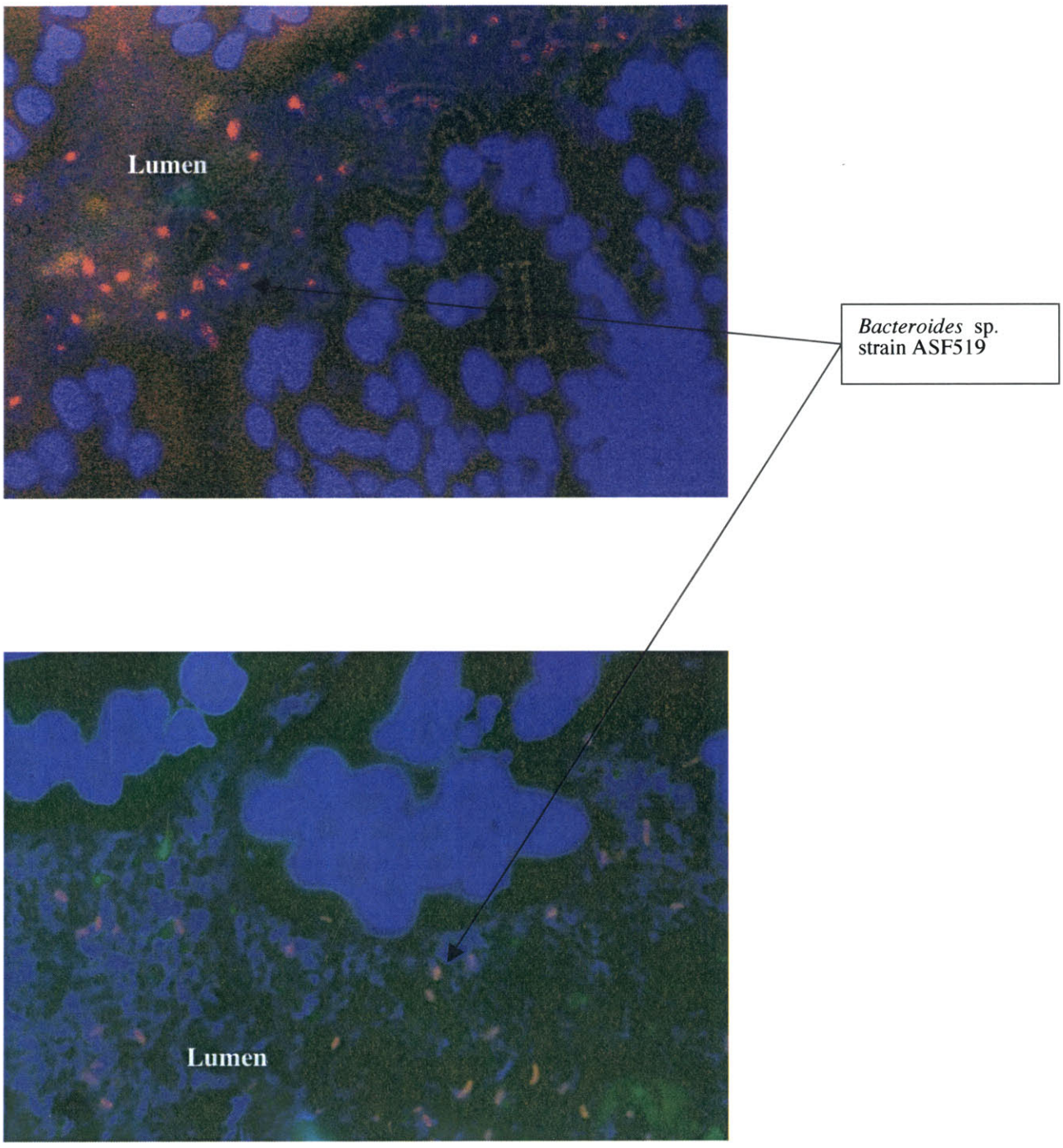

Figure 4 C& D

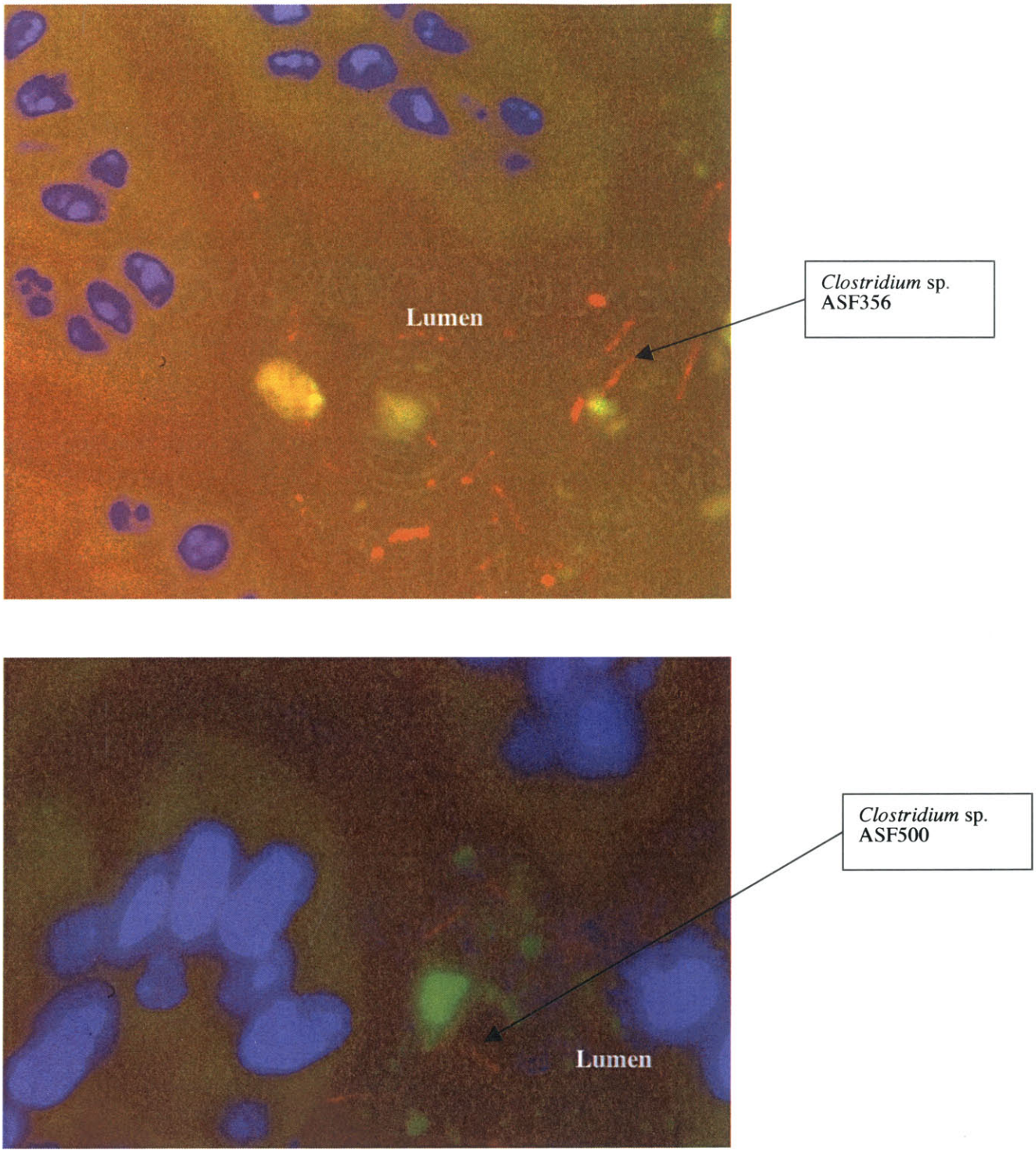

Figure 4 E&F

TABLE 1. Primer sequences and QPCR parameters[a] used in the study.

Strain name Species/group	Forward and reverse primers (concentration used in QPCR reaction, nM)
Clostridium sp. ASF356	356-144F CGGTGACTAATACCGCATACGG (100)
	356-538R CCTTGCCGCCTACTCTCCC (100)
Lactobacillus sp. ASF360	360-81F CTTCGGTGATGACGCTGG (300)
	360-189R GCAATAGCCATGCAGCTATTGTTG (200)
Lactobacillus murinus ASF361	361-278F GCAATGATGCGTAGCCGAAC (200)
	361-435R GCACTTTCTTCTCTAACAACAGGG (300)
Flexistipes gp. ASF457	457-130F CCGAAAGGTGAGCTAATGCCGG (100)
	457-219R GGGACGCGAGTCCATCTTTC (100)
Eubacterium plexicaudatum ASF492	492-57F CTGCGGAATTCCTTCGGGG (100)
	492-204R CCCATACCACCGGAGTTTTC (100)
Low-G+C-content gram positive gp. ASF500	500-183F GTCGCATGGCACTGGACATC (200)
	500-445R CCTCAGGTACCGTCACTTGCTTC (200)
Clostridium sp. ASF502	502-195F CGGTACCGCATGGTACAGAGG (200)
	502-600R CAATGCAATTCCGGGGTTGG (300)
Bacteroides sp. ASF519	519-834F CACAGTAAGCGGCACAGCG (200)
	519-1243R CCGCTCACACGGTAGCTG (200)
Citrobacter rodentium	espB-403F CTGCTTCTGCGAAGTCTGTCAA (300)
	espB-648R CAGCAGTAAAGCGACTTAACAGATT (300)

[a] $MgCl_2$concentration for ASF QPCR was 4 mM, as optimized in Sarma-Rupavtarm, Chapter 2. $MgCl_2$ concentration in *C. rodentium* QPCR mastermix was 3 mM.

Table 2. Probes used in the study

Name	Target	Sequence (5'-3')	Reference
EUB338	Eubacteria	GCTGCCTCCCGTAGGAGT	Amann, 1990
Bact 1080	*Bacteroides*	GCACTTAAGCCGACACCT	Dore, 1998
Lab 158	*Lactobacillus-Enterocc*	GGTATTAGCACCTGTTTCCA	Harmsen, 1999
Enter1432	Enterobacteriaceae	CTTTTGCAACCCACT	Sghir, 2000
ASF500	ASF500	CAATGTGGCCGGCCAACC	Schuppler, 2004
ASF356	ASF356	TCCACAGTAAAAGGCAGGTTGCC	This study
ASF457	ASF457	GGGACGCGAGTCCATCTTTC	This study

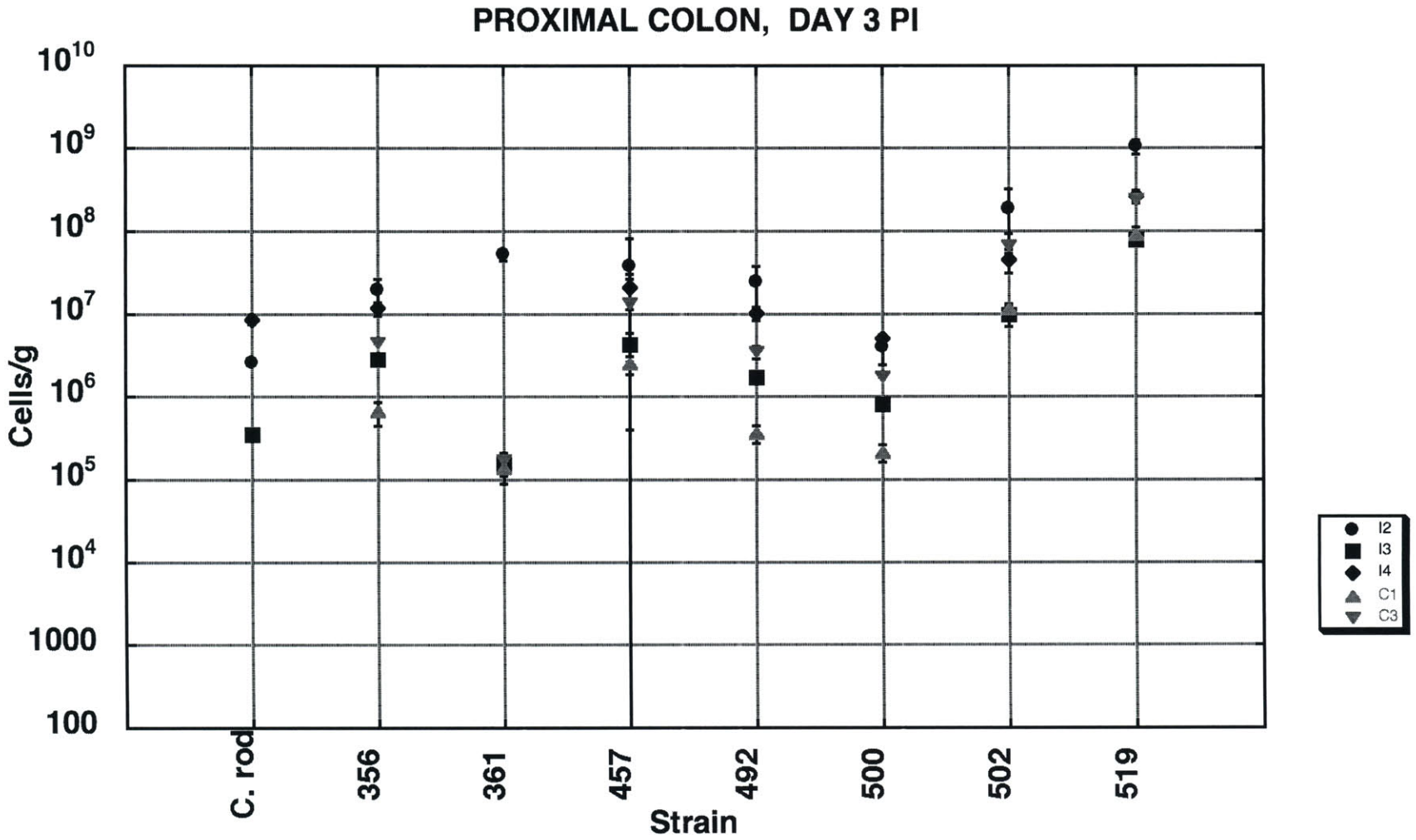
PROXIMAL COLON, DAY 3 PI
Cells/g
10^10
10^9
10^8
10^7
10^6
10^5
10^4
1000
100
C. rod
356
361
457
492
500
502
519
Strain
I2
I3
I4
C1
C3

FIGURE S1 (A)

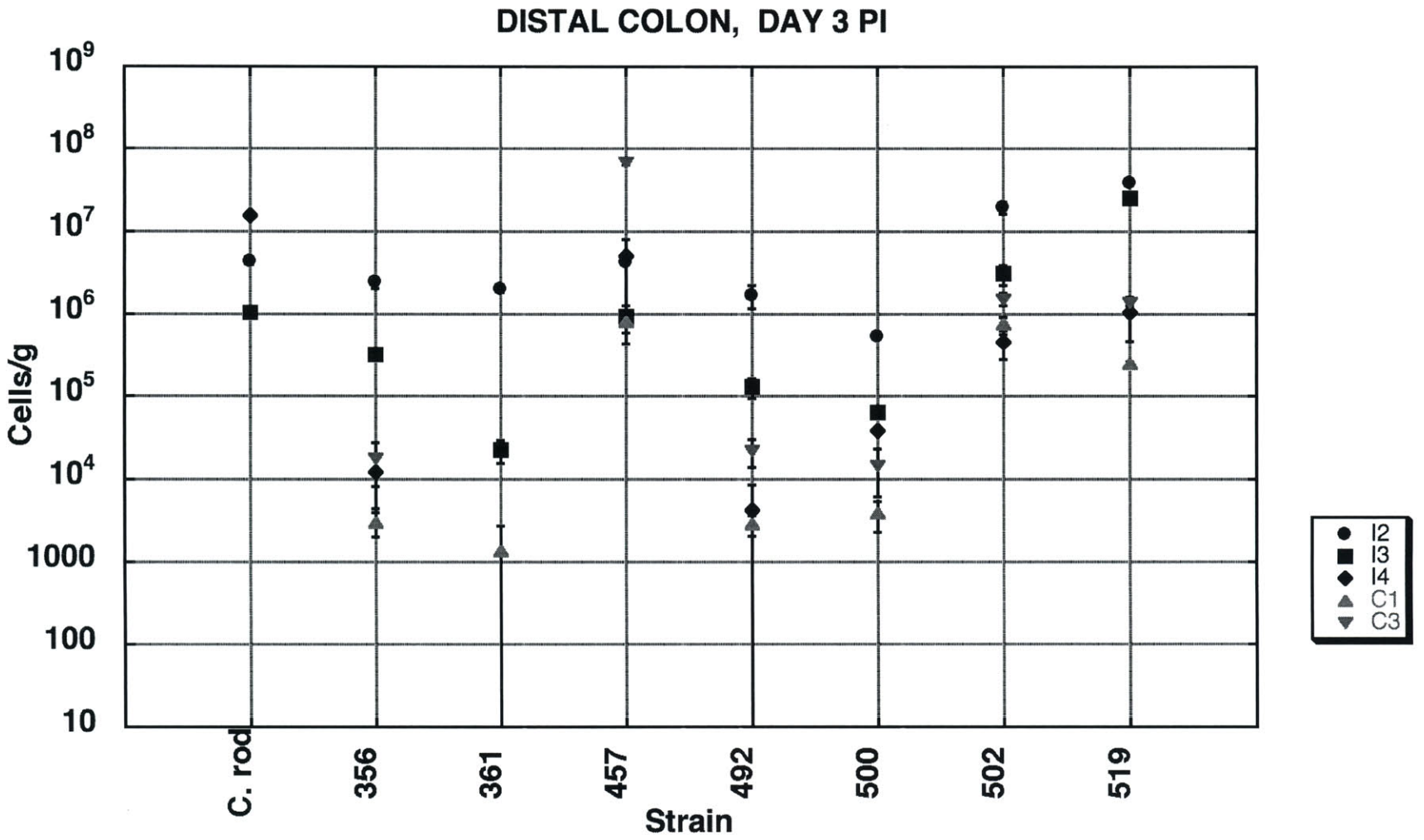
DISTAL COLON, DAY 3 PI
Cells/g
10^9
10^8
10^7
10^6
10^5
10^4
1000
100
10
C. rod
356
361
457
492
500
502
519
Strain
I2
I3
I4
C1
C3

FIGURE S1 (B)

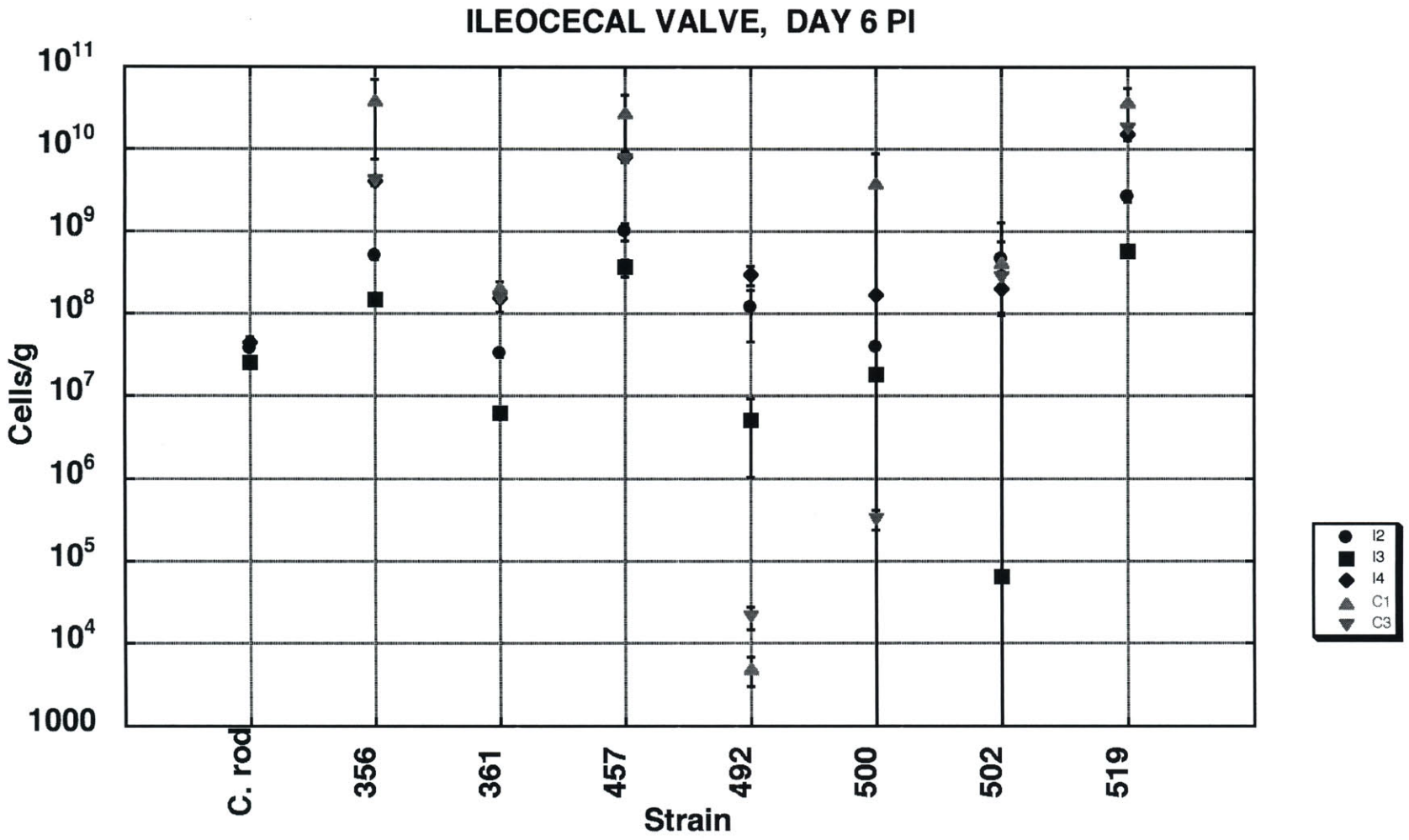
ILEOCECAL VALVE, DAY 6 PI
Cells/g
10^11
10^10
10^9
10^8
10^7
10^6
10^5
10^4
1000
C. rod
356
361
457
492
500
502
519
Strain
I2
I3
I4
C1
C3

FIGURE S2 (A)

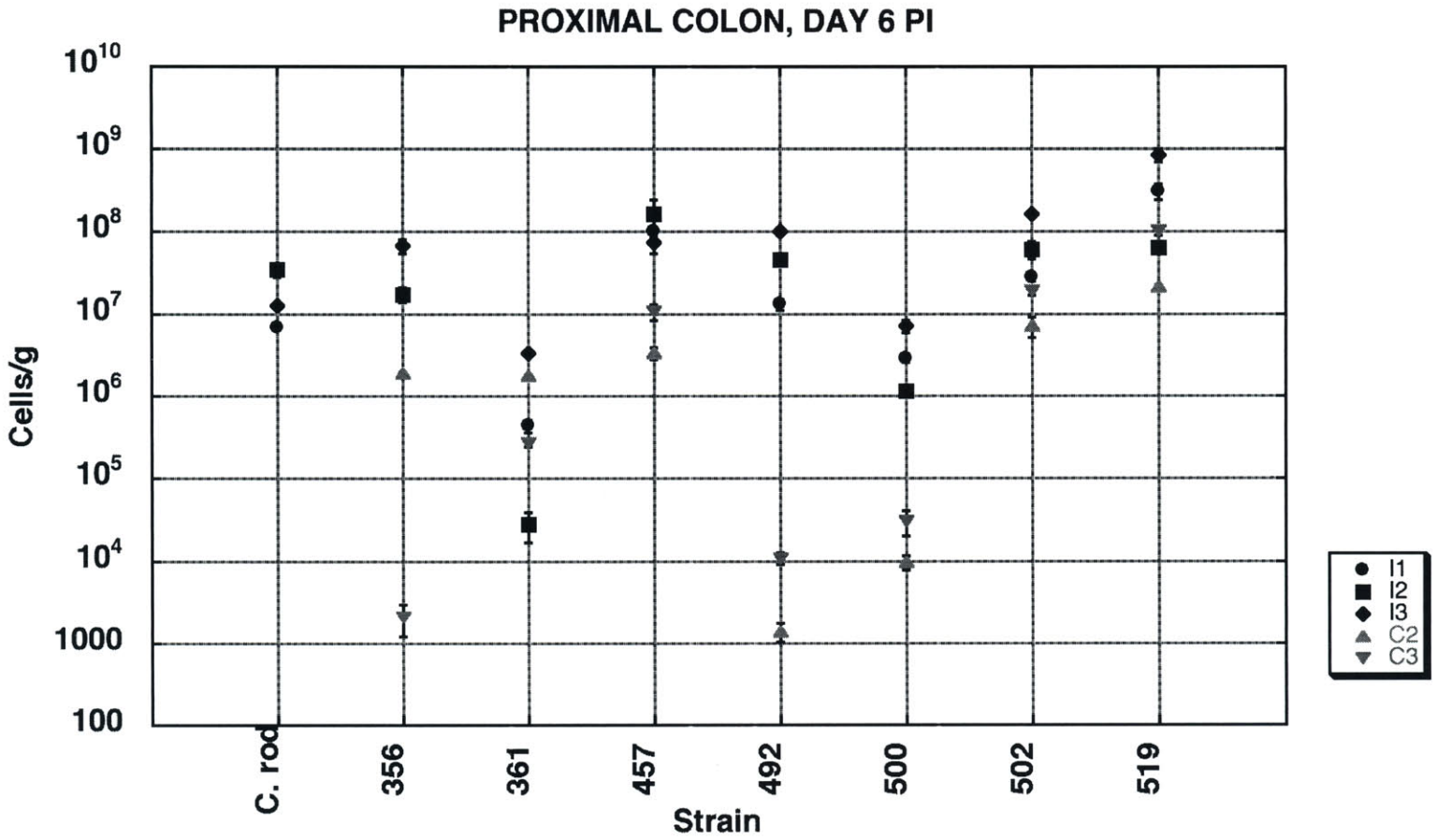
PROXIMAL COLON, DAY 6 PI
Cells/g
10^10
10^9
10^8
10^7
10^6
10^5
10^4
1000
100
C. rod
356
361
457
492
500
502
519
Strain
I1
I2
I3
C2
C3

FIGURE S2 (B)

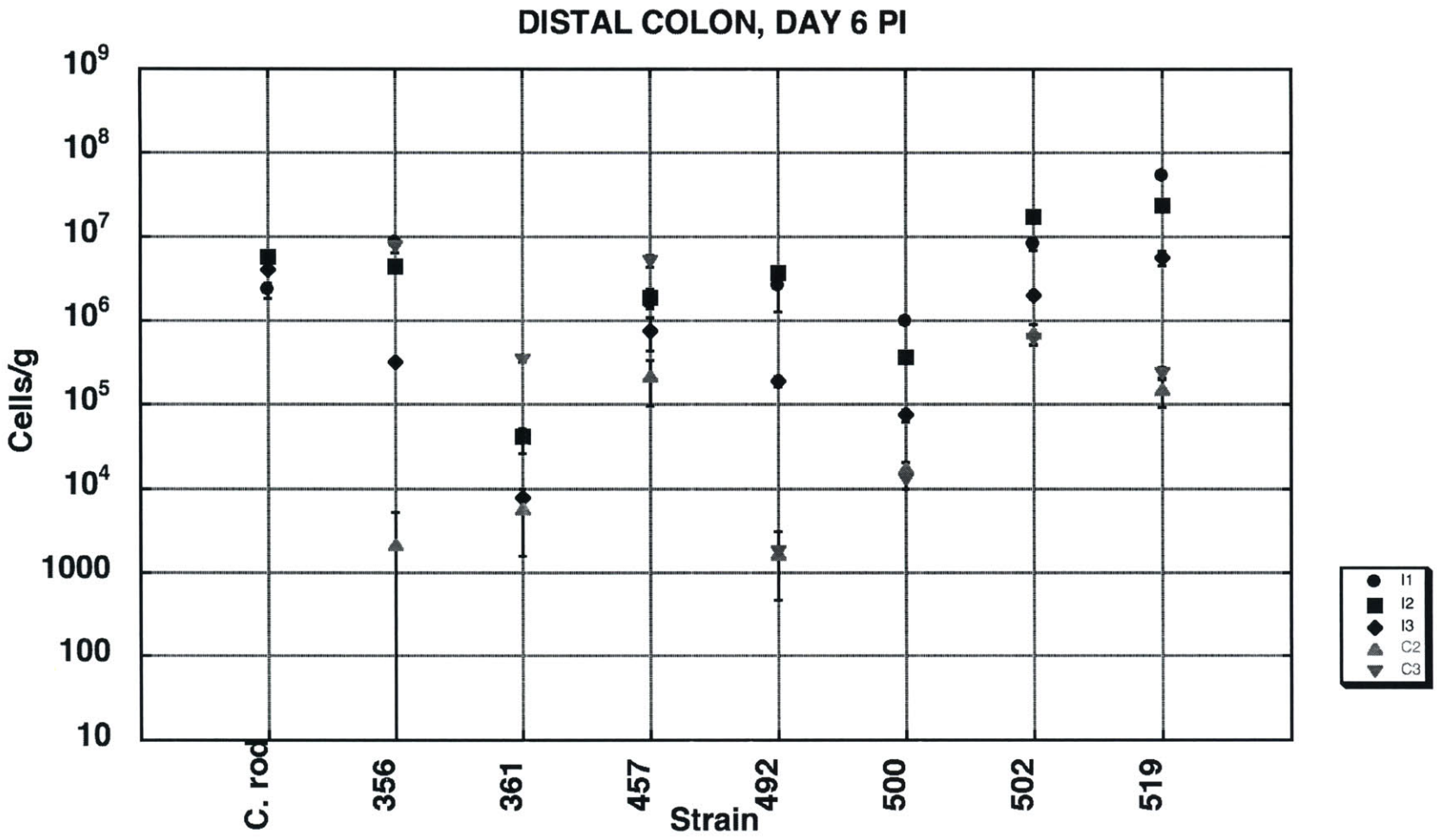
DISTAL COLON, DAY 6 PI
Cells/g
10^9
10^8
10^7
10^6
10^5
10^4
1000
100
10
C. rod
356
361
457
492
500
502
519
Strain
I1
I2
I3
C2
C3

FIGURE S2 (C)

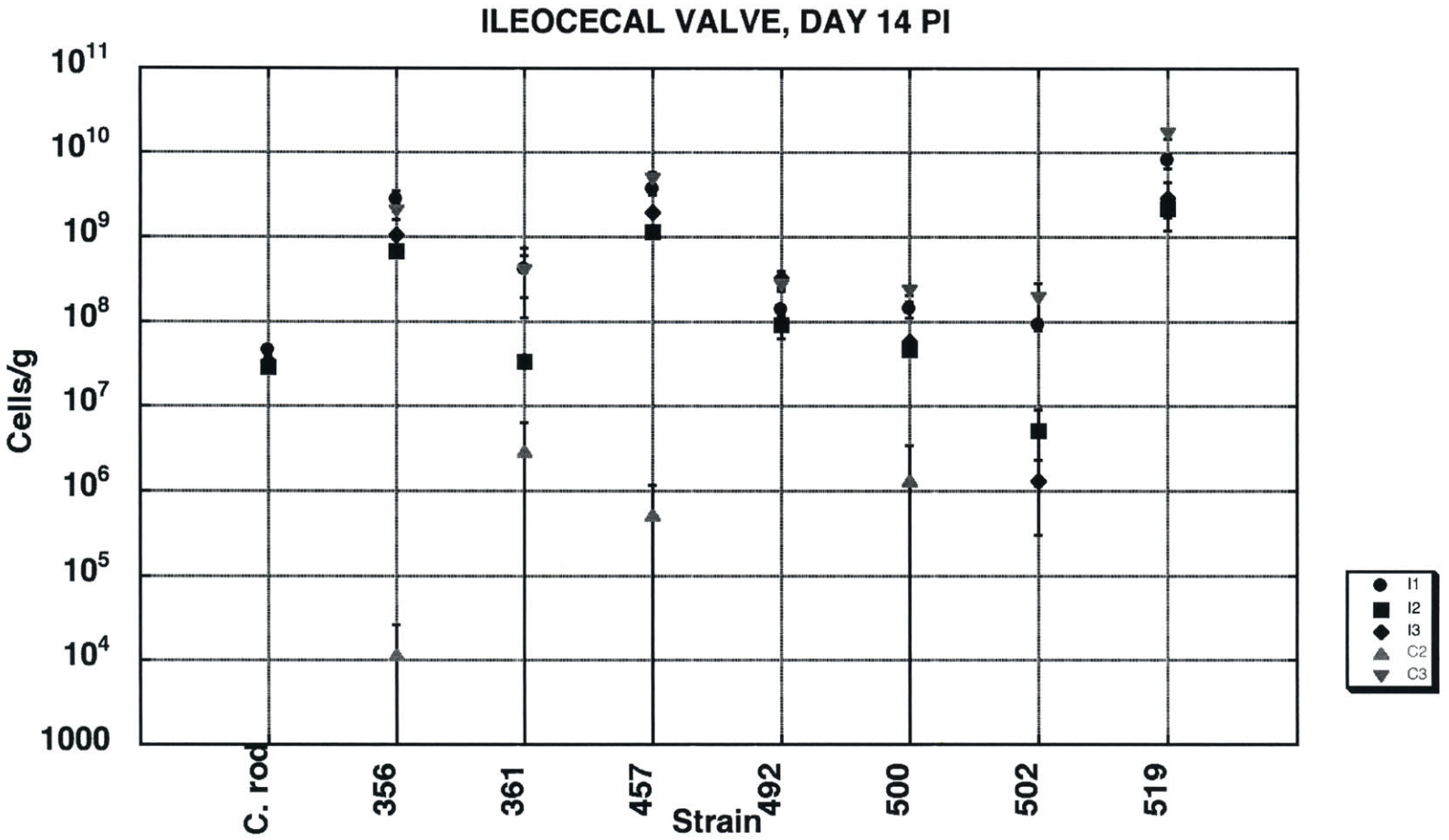
ILEOCECAL VALVE, DAY 14 PI
Cells/g
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
1000
C. rod
356
361
457
492
500
502
519
Strain
I1
I2
I3
C2
C3

FIGURE S3 (A)

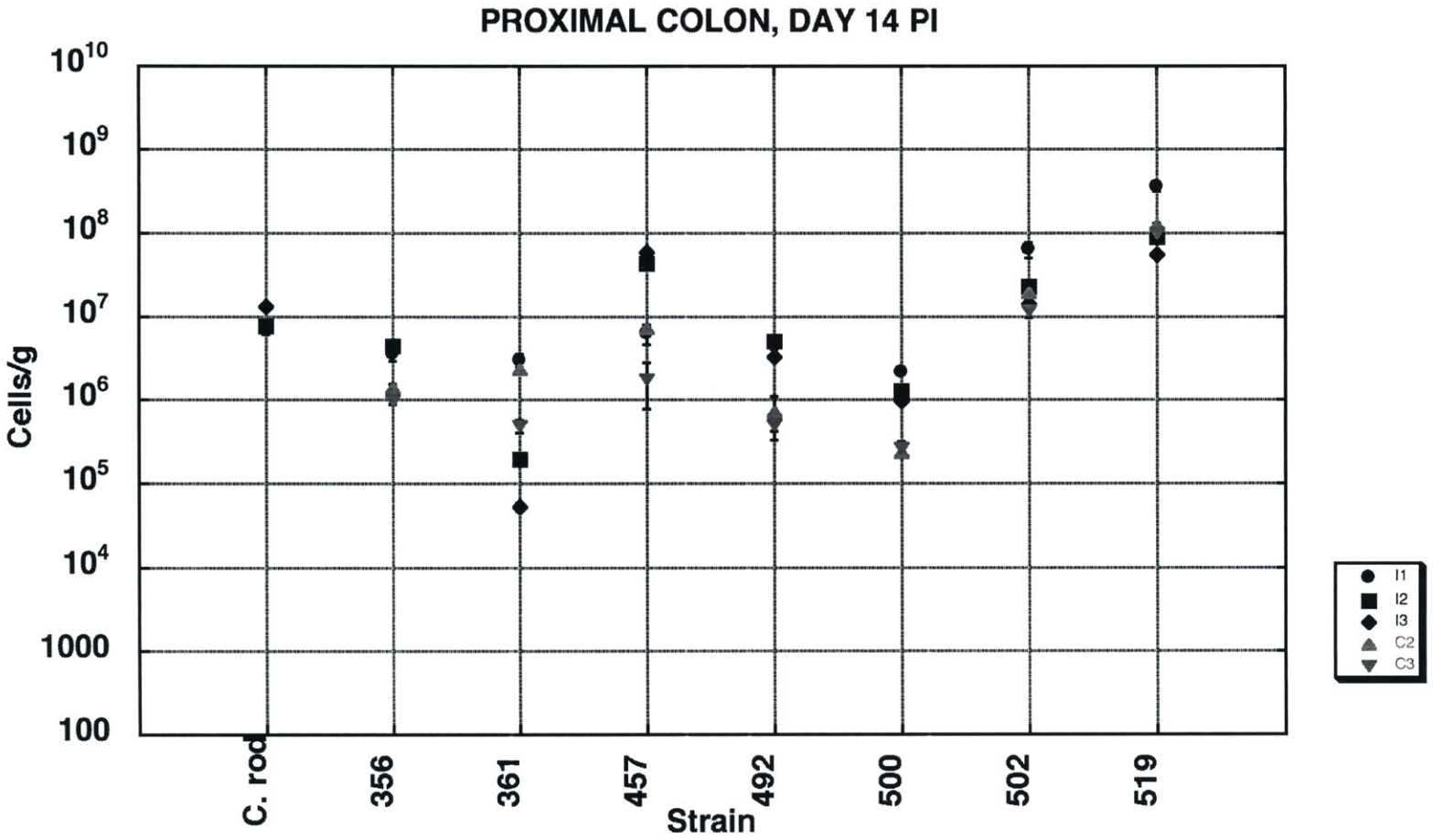
PROXIMAL COLON, DAY 14 PI
Cells/g
10^10
10^9
10^8
10^7
10^6
10^5
10^4
1000
100
C. rod
356
361
457
492
500
502
519
Strain
I1
I2
I3
C2
C3

FIGURE S3 (B)

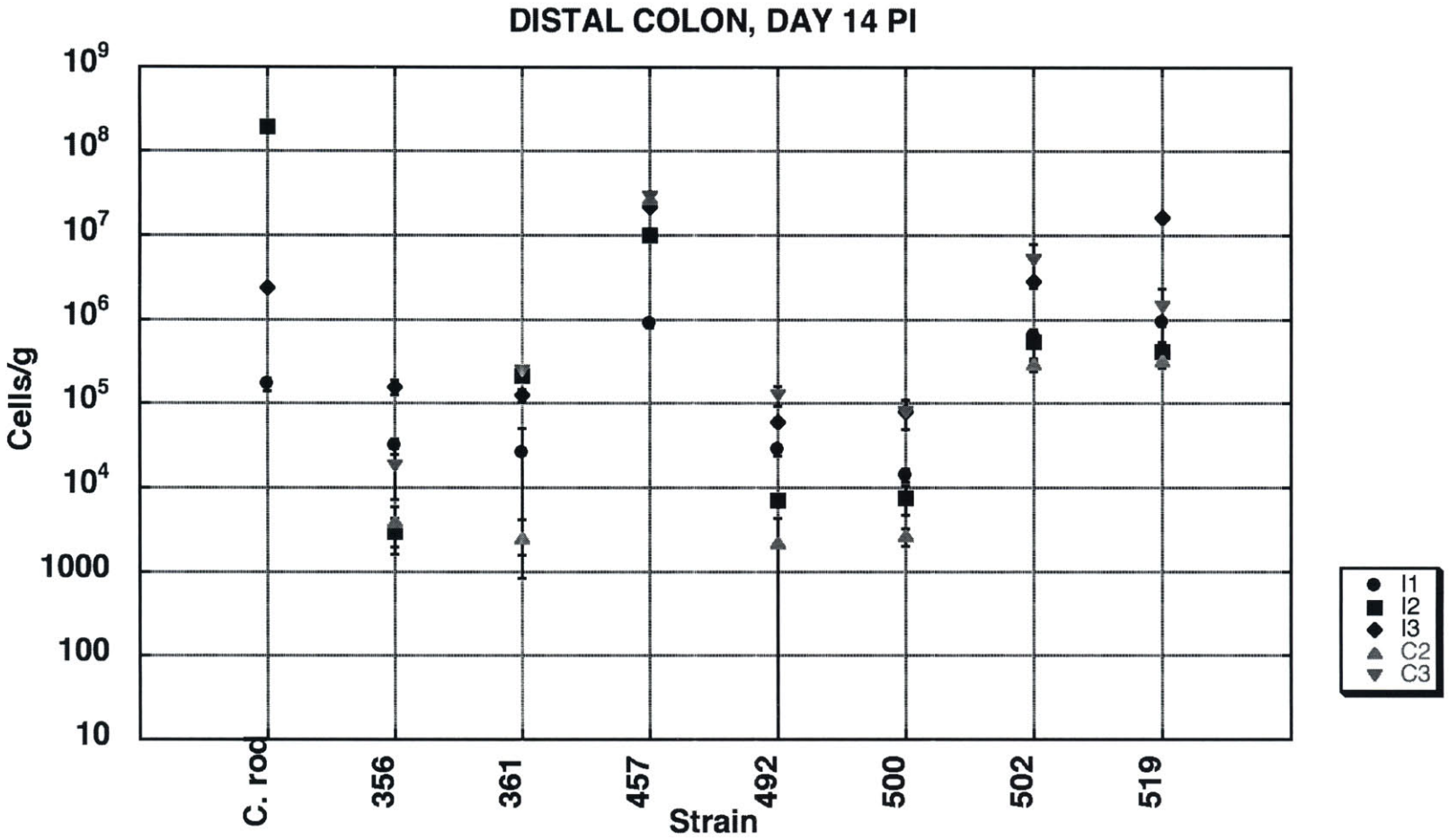
DISTAL COLON, DAY 14 PI
Cells/g
10^9
10^8
10^7
10^6
10^5
10^4
1000
100
10
C. rod
356
361
457
492
500
502
519
Strain
I1
I2
I3
C2
C3

FIGURE S3 (C)

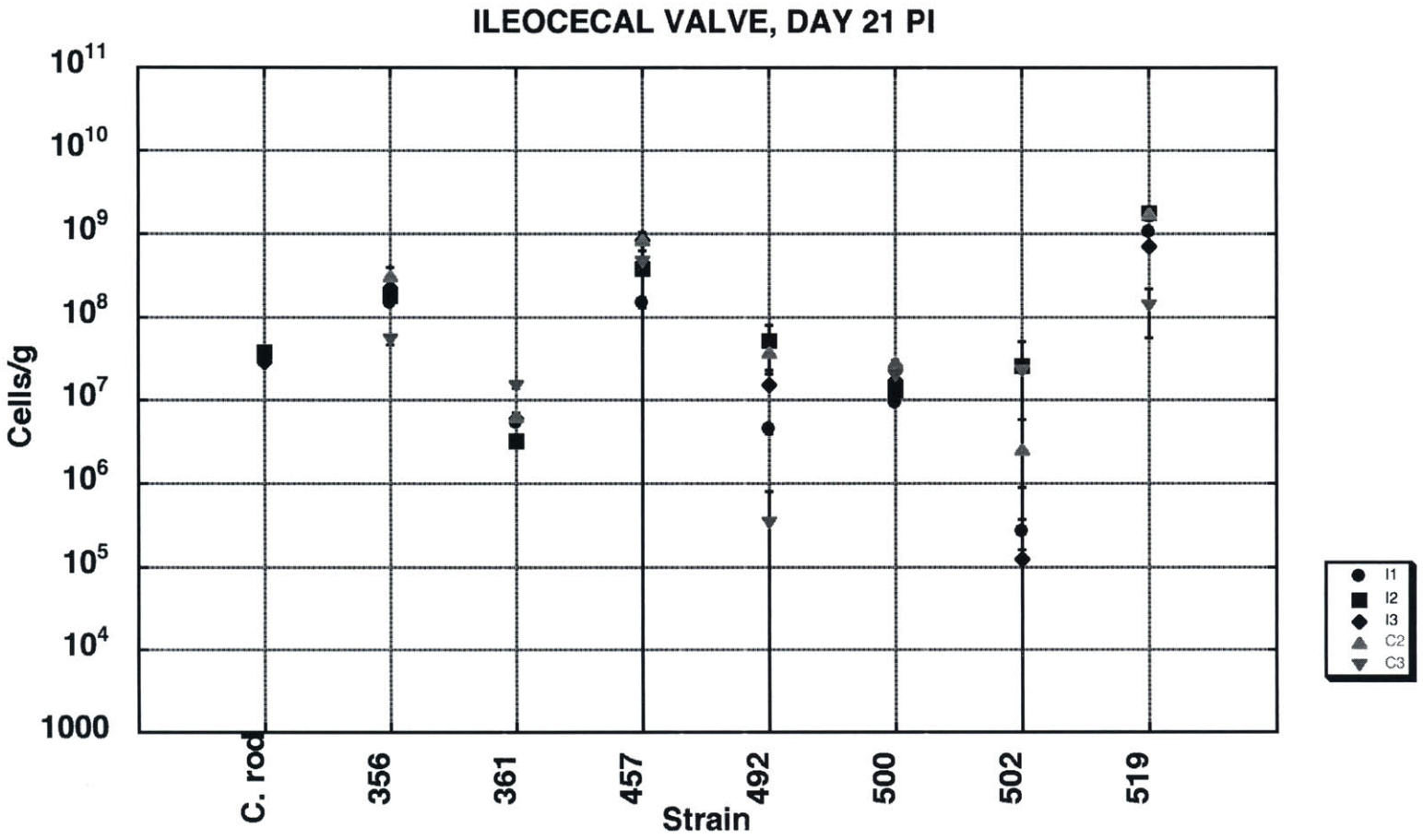
ILEOCECAL VALVE, DAY 21 PI
Cells/g
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
1000
C. rod
356
361
457
492
500
502
519
Strain
I1
I2
I3
C2
C3

FIGURE S4 (A)

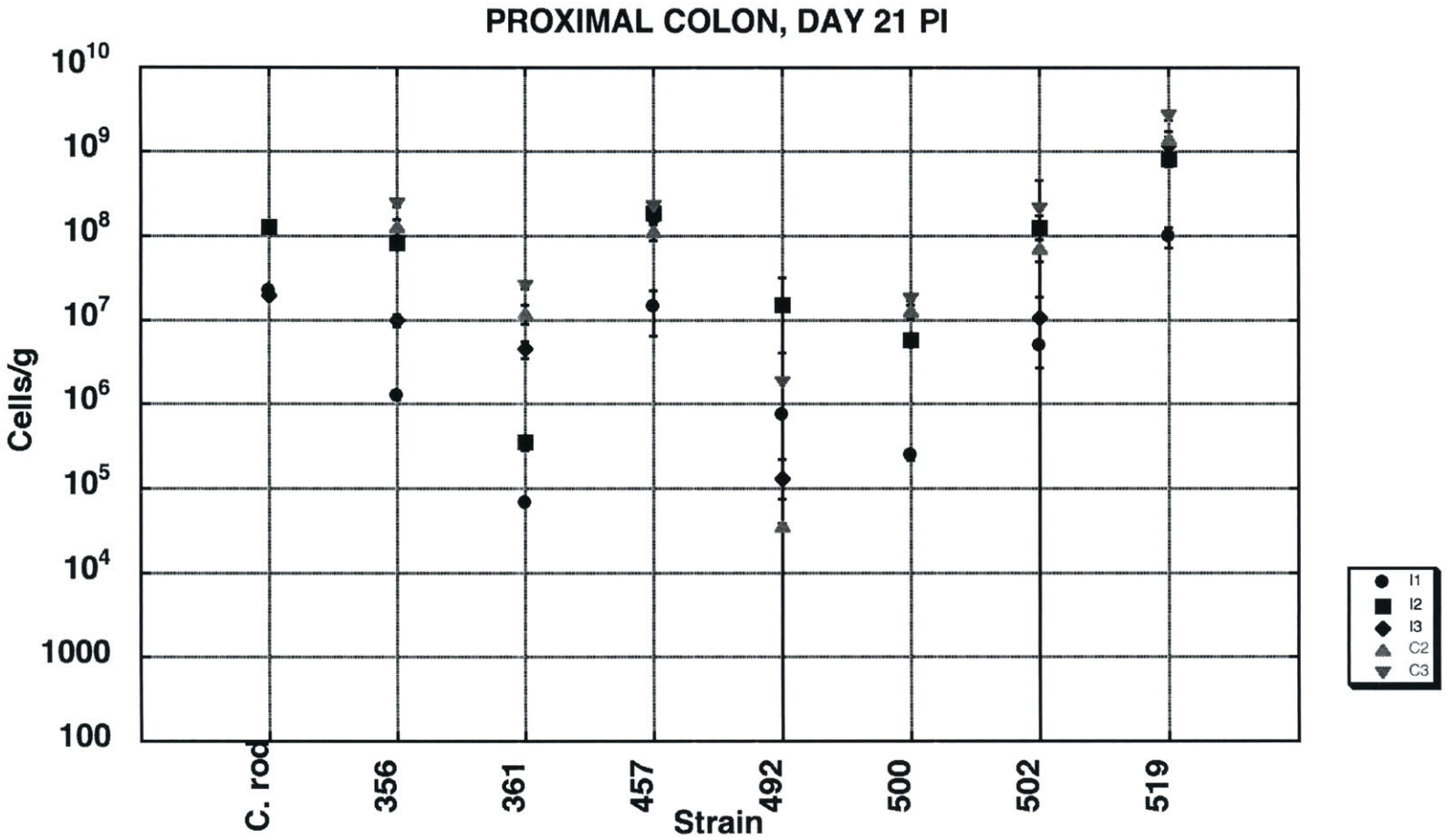
PROXIMAL COLON, DAY 21 PI
Cells/g
10^10
10^9
10^8
10^7
10^6
10^5
10^4
1000
100
C. rod
356
361
457
492
500
502
519
Strain
I1
I2
I3
C2
C3

FIGURE S4 (B)

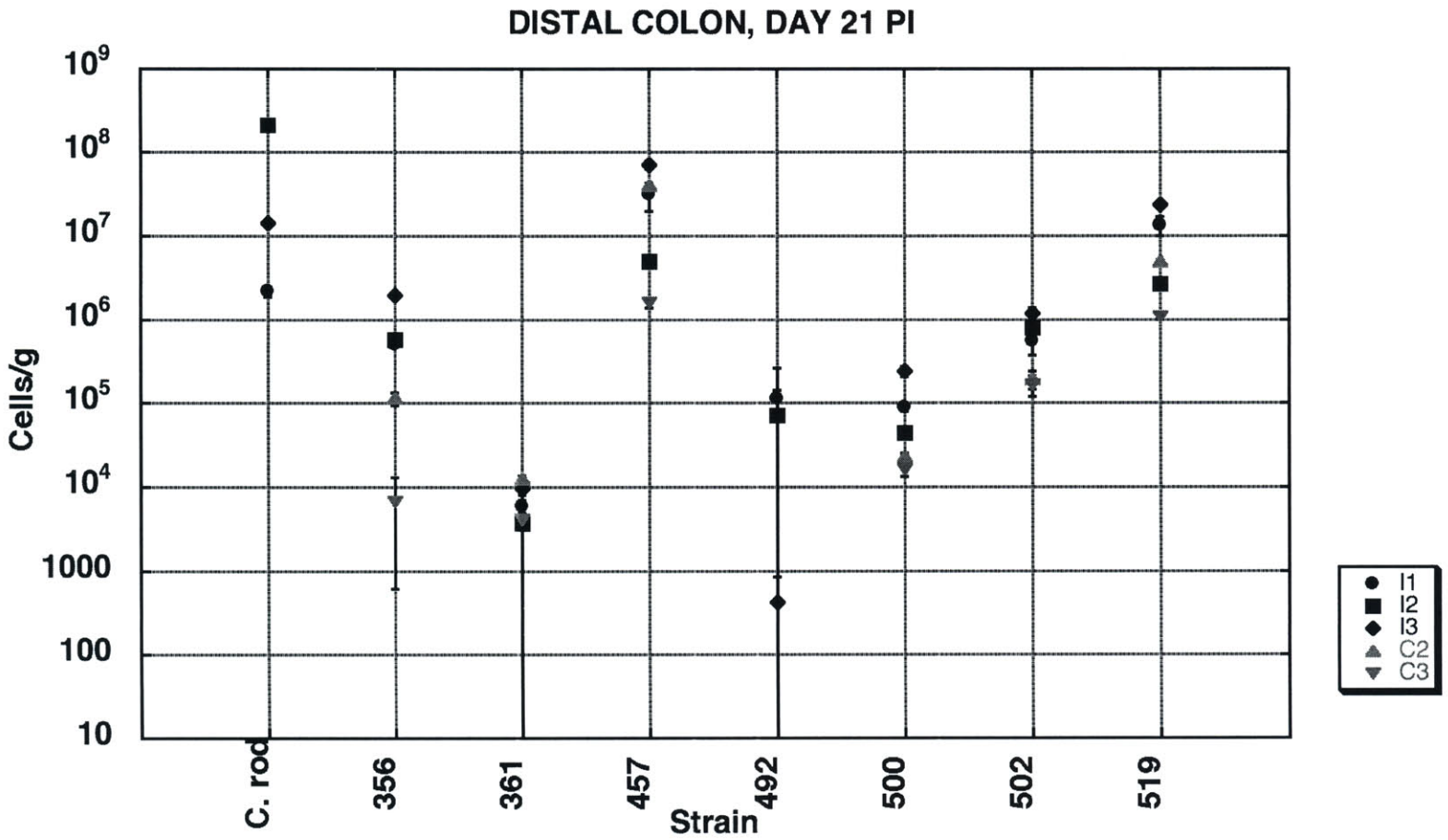
DISTAL COLON, DAY 21 PI
Cells/g
10^9
10^8
10^7
10^6
10^5
10^4
1000
100
10
C. rod
356
361
457
492
500
502
519
Strain
I1
I2
I3
C2
C3

FIGURE S4 (C)

SUMMARY

SUMMARY

This aim of this thesis is to understand aspects of the microbial ecology of the mammalian GI tract through a multi-species functional defined flora mouse model, the altered Schaedler flora (ASF). The overall goals of the thesis were to

A) establish a set of molecular biological tools targeting the ASF strains with which complex interactions in the GI tract could potentially be studied,

B) demonstrate the viability of the model by assessing the presence of the ASF strains in mice from commonly used vendors that have potentially acquired a flora of higher complexity and,

C) conduct the first in-depth study of the interactions between a functional multi-species GI microflora and a pathogen, at both a bulk population level and at a microscale spatial level.

The result of the first part of the thesis demonstrate that the ASF strains are present at high levels in the GI tract of mice, following a pattern of strain abundance that mirrors the oxygen tolerance of the strain and oxygen levels in the different regions of the GI tract. It was also shown that the fecal levels of ASF strains do not correlate well with the colonic levels, thus indicating that studies inferring colonic levels or relative abundance of bacteria from fecal abundances have to be interpreted cautiously. Results from the second part of thesis indicate that a majority of the ASF strains are indeed a stable part of the mouse GI ecosystem, even in the face of competition, and their presence and abundance further validates the use of the ASF mouse model to study aspects of gastrointestinal microbial ecology.

The third part of the thesis yielded several surprising results. Firstly, it was seen that infection by a colonic pathogen leads to an initial increase in the total levels of the ASF strains. Secondly, it was seen that the perturbation from an exogenous pathogen is minimal, and the ASF strain levels remain fairly stable through the course of an infection. While it was not possible to conclusively establish colocalization of the pathogen with the ASF strains, the spatial localization of the ASF strains did not change between across the luminal-mucosal cross-section. These results could indicate that interactions between a pathogen and the GI microflora may be a complex process, involving more than solely a competition for adhesion to the gut wall. The tools developed in this thesis and the results of the pathogen-microflora interaction study provide a basis for designing further studies to understand several aspects of GI microbial ecology.