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AN INVESTIGATION OF THE INTERACTIONS BETWEEN *ESCHERICHIA COLI*
AND THE INTESTINAL MICROBIOTA *IN VIVO* AND *IN VITRO*

BY

MATTHEW E MOKSZYCKI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

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2015

DOCTOR OF PHILOSOPHY DISSERTATION
OF
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ABSTRACT

We previously proposed that colonizing strains of *E. coli* likely occupy specific niches as minimal members of mixed biofilms formed primarily by anaerobic members of the gut flora. These “restaurants” formed by the different community members of the gut provide individual strains of *E. coli* with various unique binding pockets and locally provided mono- and disaccharides by breaking down polysaccharides which facultative anaerobes, such as *E. coli*, could not break down on their own. This allows strains of *E. coli* that cannot grow as efficiently on available sugars to colonize if they are able to occupy niches that other competing strains could not. For example, if the less efficient strain could bind better to specific anaerobic members or had a higher tolerance for bile salts it would be able to co-colonize with a strain that could grow better on available sugars as it would not be in direct competition to occupy the same niche. While the anaerobes in mixed biofilms provide a source of nutrients for minimal members such as *E. coli* through breakdown of large polysaccharides, the facultative anaerobes may improve the environment for strict anaerobes by reducing the concentration of oxygen within the biofilms. Oxygen diffuses from surrounding tissue into the intestines, oxygen from swallowed air is present in flatus, and at least one predominant anaerobe in the gut microbiome, *Bacteroides fragilis*, respire oxygen at low concentrations. As such, manuscript I looks at how colonization with specific strains of *E. coli* can affect the development of the intestinal microbiota. Five representative *E. coli* strains were used for this study: Nissle 1917, EDL933, MG1655, and two MG1655 mutants selected by the mouse gut (*envZ*_{P41L} and *flhDC*). While slight variances were observed between strains, these

were likely due to differences between hosts rather than the colonizing *E. coli* and no significant differences between the communities could be surmised.

In order to better understand the interactions between *E. coli* and the members of the intestinal microbiota, a novel *in vitro* method was developed. Manuscript II examines the development of the *in vitro* system as it was compared to the mouse model. This method was designed to be simple and inexpensive while providing an environment meant to replicate the natural habitat of these organisms: the mammalian gut. The current study examines the development of said *in vitro* model and its ability to mimic the mouse gut in terms of diversity of organisms, as well as its usefulness in examining the colonizing ability of competing *E. coli* strains. It is shown that, while still in development, this system is currently able to maintain diversity comparable to what is seen in mice. While the abundance of these diverse organisms is not necessarily at the levels seen in mice, the model is already an effective system for studying the microbial community of the gut in a controlled environment. Furthermore, the system is able to mimic certain colonization experiments of competing *E. coli* strains done in mice, and with improvements to bolster the growth of the diverse population could be used as a non-invasive method for studying gut microbes.

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PREFACE

This dissertation has been prepared in the Manuscript Format according to the guidelines of the Graduate School of the University of Rhode Island with the style presented in the American Society for Microbiology journal, Infection and Immunity. The dissertation includes an introduction and the following two manuscripts:

The first manuscript: “Analysis of how Different Strains of *Escherichia coli* Affect Development of the Intestinal Microbiota”

The second manuscript: “Developing an *In Vitro* Method for Studying the Interaction between *E. coli* and the Intestinal Microbiota”

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LITERATURE REVIEW

Introduction

The human intestinal tract is known to maintain a diverse population of microorganisms represented by both anaerobic and facultative anaerobic species (10). It is well known that commensal strains of *E. coli* colonize as a minority member of these communities as a facultative anaerobe (22). Commensal strains include organisms that naturally inhabit a host organism's body without causing harm, and in some cases even providing the host with a particular advantage (21). In contrast, a pathogenic strain is usually an invasive organism that may be of the same species, such as *E. coli* EDL933, but disease is caused rather than a harmless coexistence (21).

From ingestion to colonization

When a host ingests *E. coli* from the environment the *E. coli* must be able to pass the acid barrier of the stomach, as well as be able to attain the nutrients necessary to begin logarithmic growth. As stationary phase bacteria are acid-tolerant (14), as few as 10 *E. coli* cells can lead to transmission (24). In order to exit lag phase and begin logarithmic growth upon reaching the colon, *E. coli* must compete for nutrients with a dense community (15), be able to penetrate into the mucus layer (31), and grow rapidly enough to make up for the constant turnover of the mucus layer (37) all while avoiding host defenses (7, 29). When successful in colonizing the colon, some *E. coli* cells are still removed with the turnover of mucus. These cells are able to re-enter the environment where they can be ingested by the next host, and thus spread and continue to exist (34, 35).

Basic principles of colonization

According to Freter's nutrient-niche hypothesis, colonizing strains can coexist as long as they are able to outcompete one another on one or more sugars, or are able to bind to the intestinal wall. Thus if two strains (X and Y) are in direct competition and (X) can utilize all sugars better than (Y), then the less efficient strain (Y) cannot co-colonize (11). This hypothesis assumes that all available nutrients are homogenized, or equally mixed, throughout the intestine (15). As such, a diverse and stable community would hinder invading species and prevent them from colonizing. This ability to prevent invasion from outside species is called "colonization resistance" (41). Even with this in mind, *E. coli* strains can coexist due to diverse strategies for the utilization of limited nutrients within the colon.

Central metabolism and intestinal colonization

E. coli is a Gram-negative, prototrophic, facultative anaerobe. While *E. coli* grows best on sugars it cannot grow on larger polysaccharides, as it does not have the enzymes available to do so (12). It can also grow on substrates that feed into the TCA cycle, which require gluconeogenesis to metabolize. Other important central metabolism pathways in *E. coli* include the Embden-Meyerhof-Parnas glycolytic pathway (EMP), the pentose phosphate pathway (PP), the Entner-Duodoroff pathway (ED), and many fermentation pathways all of which are highly conserved (9).

Catabolic pathway diversity in *E. coli*

While the central metabolism genes are highly conserved across strains of *E. coli*, there is variation between strains in terms of the catabolic pathways that feed into these core central metabolism pathways (9, 32). In other words, some *E. coli* strains

have different preferences in terms of growth on sugars or other substrates. It has been shown that while *E. coli* strains have similar catabolic potential, the sugars each strain uses to support their colonization differs significantly (5, 8, 12, 20, 28, 30, 39).

Nutrient availability in the intestine

Mucus in the gut exists in two forms, the loose layer and the firm layer (19). The loose mucus layer is where most of the microbiota of the gut grow and form their biofilms, while the firm mucus layer resides closer to the epithelial layer of the intestines and prevents direct interaction of the commensal microbiota with host epithelium (19). The mucus itself consists of a large mixture of molecules, including but not limited to amino acids, large polysaccharides, mono- and disaccharides, and mucin (4). This mixture exists as a viscous liquid in which the microbes are suspended and in which form biofilms. This environment is mostly anaerobic, with minimal oxygen leaking from epithelial cells of the intestine (6, 16, 18, 23). Mucin is a glycoprotein with large polysaccharide strands with which the anaerobe populations of the gut can attach and form biofilms around (2). The biofilms formed by these anaerobes can break down the large polysaccharides of the mucus, and parts of the mucin, thus providing facultatives with usable mono- and disaccharides in bulk which would usually be limited (22, 33, 40). This gives facultatives, such as *E. coli*, a reason for joining as minimal members in these anaerobe-formed biofilms, and perhaps even promoting the growth of preferential anaerobes that provide specific mono- and disaccharides (18). The facultatives that join these mixed biofilms likely provide the anaerobes with protection from the minimal amount of oxygen that is released from the epithelial tissue of the gut.

Competition for limited nutrients in the intestine

In order to compete for limiting resources in the intestine, *E. coli* is able to expand their transcriptome in order to induce a broad set of gene systems used for carbon-source transport and catabolism (17). Additionally, it has been shown that glycogen stores are used for survival during times of carbon limitation and are also important for colonizing *E. coli* strains when colonizing the intestine (20). By expanding the gene systems used for carbon-source transport and catabolism, *E. coli* is able to use a diverse set of sugars and can use nutrients that are available which no other member of the microbiota has used. A specific *E. coli* strain can also outcompete other strains by growing faster on the available nutrients (13). Furthermore, if an *E. coli* strain is being outcompeted for its preferred nutrients it will activate other mechanisms in which it is no longer in direct competition (i.e. switching to use other available sugars or pathways such as gluconeogenesis) (3, 5, 28, 38). Finally, if a strain is able to enter a niche in which it forms a symbiotic relationship (i.e. in a mixed anaerobic biofilm) that provides its preferred sugar(s), it will be able to colonize away from direct competition with faster growing strains (1, 22).

Biofilms in the intestine

It has been shown that biofilms form in the mucus layers of the large intestines of healthy mammals (25, 26, 33, 40). Furthermore, biofilms containing *Bacteroides*, *Enterobacter*, and *Clostridia* species found in the intestine can form rapidly on mucin in continuous flow culture systems developed to mimic the human intestine (27), thus being able to develop faster than the flow of mucus (36). Recent studies of these bacterial biofilms in the intestines have shown that anaerobes form dense biofilms

with *E.coli* as a minority member (22). These biofilms form around intestinal mucin and break down large polysaccharides, such as fiber, that cannot usually be utilized by facultative anaerobes (22, 33, 40). Differences in the population of anaerobes within mixed biofilms would thus provide *E. coli* with various niches based on nutrients made available by the anaerobes preferred metabolic pathways.

The “restaurant” hypothesis

Our laboratory previously proposed an adjustment to the Freter’s hypothesis which states that the mono- and disaccharides that *E. coli* strains use for growth in the intestine are made available locally from polysaccharide degradation by the anaerobes in the mixed biofilms that the *E.coli* inhabit, rather than evenly mixed throughout the intestine. Furthermore, if one strain can bind better to the anaerobes in the mixed biofilms, the colonization ability of this strain would be improved. Recent studies of bacterial biofilms in the intestines have shown that anaerobes form dense biofilms with *E.coli* as a minority member (22). These biofilms form around intestinal mucin and break down large polysaccharides, such as fiber, that cannot usually be utilized by facultative anaerobes (22, 33, 40). Differences in the population of anaerobes within mixed biofilms would thus provide *E. coli* with various niches based on nutrients made available by the anaerobes preferred metabolic pathways.

While the anaerobes in mixed biofilms provide a source of nutrients for minimal members such as *E. coli* through breakdown of large polysaccharides, the facultative anaerobes may improve the environment for strict anaerobes by reducing the concentration of oxygen within the biofilms. Oxygen diffuses from surrounding tissue into the intestines, oxygen from swallowed air is present in flatus, and at least

one predominant anaerobe in the gut microbiome, *Bacteroides fragilis*, respire oxygen at low concentrations (6, 16, 18, 23). It has been shown that respiration in *E. coli* provides an important competitive edge when colonizing the intestine of streptomycin-treated mice. Mutants of ATP synthase or cytochrome *bd* oxidase, both necessary for respiration, showed a significant loss in colonizing ability when colonized against wild type of the same strain (18). Since *E. coli* uses oxygen as the final electron-acceptor in respiration, the oxygen concentration within the intestine will be lower in the presence of *E. coli*.

Conclusions

While *E. coli* is only a minimal member of a large and dense microbial community found within the gut, it is still able to colonize and compete with such diversity even when co-colonized with other strains of *E. coli*. It is likely that the relationships formed by colonizing *E. coli* strains within dense anaerobe-formed biofilms help develop unique niches not only for the *E. coli*, but also for the anaerobes that are in direct interaction with them. Understanding how such a minor member is able to overcome such complexity, as well as the effects on the community it causes, is key to identifying mechanisms utilized by commensal and pathogenic bacteria to invade and colonize new hosts. As such, this thesis aims to identify the effects of colonization by various strains of *E. coli* and the effects they have on the overall intestinal microbiota. Furthermore, the development of a novel *in vitro* system used to aid in further understanding these relationships is discussed.

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MANUSCRIPT I

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Title: Analysis of how Different Strains of *Escherichia coli* Affect Development of the Intestinal Microbiota

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MANUSCRIPT I

Analysis of how Different Strains of *Escherichia coli* Affect Development of the Intestinal Microbiota

Abstract

We previously proposed that colonizing strains of *E. coli* likely occupy specific niches as minimal members of mixed biofilms formed primarily by anaerobic members of the gut flora. These “restaurants” formed by the different community members of the gut provide individual strains of *E. coli* with various unique binding pockets and locally provide mono- and disaccharides. This allows strains of *E. coli* that cannot grow as efficiently on available sugars to colonize if they are able to occupy niches that other competing strains could not. For example, if the less efficient strain could bind better to specific anaerobic members or had a higher tolerance for bile salts it would be able to co-colonize with a strain that could grow better on available sugars as it would not be in direct competition to occupy the same niche. As such, this study looks at how colonization with specific strains of *E. coli* can affect the development of the intestinal microbiota. Five representative *E. coli* strains were used for this study: Nissle 1917, EDL933, MG1655, and two MG1655 mutants selected by the mouse gut (*envZ_{P41L}* and *flhDC*). While slight variances were observed between strains, these were likely due to differences between hosts rather than the colonizing *E. coli* and no significant differences between the communities could be surmised.

Introduction

The human intestinal tract is known to maintain a diverse population of microorganisms represented by both anaerobic and facultative anaerobic species (11, 12). It is well known that commensal strains of *E. coli* colonize as a minority member of these communities as a facultative anaerobe (27). Commensal strains include organisms that naturally inhabit a host organism's body without causing harm, and in some cases even providing the host with a particular advantage (25). In contrast, a pathogenic strain is usually an invasive organism that may be of the same species, such as *E. coli* EDL933, but disease is caused rather than a harmless coexistence (25).

To prevent infection by pathogens, probiotic strains that block or reduce the colonizing ability of pathogenic strains can be used, such as *E. coli* Nissle 1917 (25). Once colonized, a probiotic strain will fill the niche normally used by the pathogenic strain, preventing the pathogen from settling in. According to Freter's nutrient-niche hypothesis, colonizing strains can coexist as long as they are able to outcompete one another on one or more sugars, or are able to bind to the intestinal wall. Thus if two strains (X and Y) are in direct competition and (X) can utilize all sugars better than (Y), then the less efficient strain (Y) cannot co-colonize (15). This hypothesis assumes that all available nutrients are homogenized, or equally mixed, throughout the intestine (15).

In order to compare the colonization ability of strains, mice are often used as a model system. In order to test *E. coli* strains within animal models, the niches that are already filled by normal flora must be cleared. If this is not done, any attempts at colonization will fail, as the niches are filled by other facultative anaerobes (8). There

are currently two accepted models for testing the colonization ability of commensal strains in mice; the germ-free mouse model and the streptomycin treated mouse model (9, 24). In germ-free mice, no microbiota are present. This allows colonization to occur, but removes any relationships with anaerobes normally present as part of the microbiome (19). The streptomycin-treated mouse model does leave much of the population intact, while still clearing the facultative anaerobe population for colonization to occur. In this way the streptomycin-treated mouse model is superior, as relationships between anaerobes and the facultative anaerobes being tested are kept mostly intact (19).

E. coli strain MG1655 is a common laboratory strain used to test colonization ability. When colonized by said strain, the intestine selects for mutants with improved colonization ability. These include a non-motile strain with a deletion in the *flhDC* region, and a motile strain with a missense mutation in the *envZ* gene (16, 26, 27). The *flhDC* operon is responsible for making the FlhD₄C₂ complex, which activates transcription of class II flagellar genes and represses a number of metabolic genes, including those involving sugar catabolism (14, 43, 44). The deletion in the *flhDC* region removes the ability to produce this regulatory complex and has been shown to improve growth when compared to the parent MG1655 in vitro on cecal mucus and other available carbon sources normally found within the intestine (16, 26). The *envZ* gene codes for the transmembrane osmolarity sensor protein EnvZ, a histidine kinase that acts on the OmpR regulatory protein as both a kinase and a phosphatase. OmpR-phosphate regulates a number of genes including: *ompC*, *ompF*, the sRNAs *omrA* and *omrB*, and *flhDC* (13). The *envZ* missense mutant selected by the intestine, a P41L

mutant (ccg to tcg), reduces the phosphatase activity of EnvZ which results in a higher concentration of OmpR-phosphate (27). In vitro the *envZ* missense mutation, hereafter called *envZ*_{P41L}, results in a faster growth rate on several sugars relative to the parent MG1655 strain, and a slower growth rate relative to the *flhDC* deletion mutant on almost all sugars. However, when grown in vitro on galactose, the *envZ*_{P41L} strain is far superior to both the parent MG1655 and *flhDC* deletion strains (27).

The MG1655 *envZ*_{P41L} missense mutant strain was recently shown to have improved colonization ability against the parent MG1655 strain and colonized as well as the MG1655 *flhDC* deletion mutant (27). In accordance with Freter's hypothesis, the *envZ*_{P41L} and *flhDC* mutants should be able to co-colonize as both are able to grow well on different sugars and as such are not in direct competition. As expected, when both strains are simultaneously fed to mice in the same numbers (ie. 10⁵ CFU per mouse) these mutant strains grow up to 10⁹ CFU/gram of feces and co-colonize equally well thereafter. Also, when 10¹⁰ CFU of the *flhDC* mutant is fed to mice along with 10⁵ CFU of the *envZ*_{P41L} mutant, the *envZ*_{P41L} mutant is able to grow up in the intestine and almost reach the level of the *flhDC* mutant (21). This again agrees with Freter's hypothesis, as the strains are not in direct competition for sugars. However, when the 10⁵ CFU of the *flhDC* mutant is fed to mice along with 10¹⁰ CFU of *envZ*_{P41L} mutant, the *flhDC* mutant is unable to grow up against the *envZ*_{P41L} mutant (27). From these results, it can be inferred that there are at least two niches filled by *envZ*_{P41L} mutant with some selective force that prevents *flhDC* from colonizing one of these niches, and that *envZ*_{P41L} mutant is somehow able to prevent *flhDC* from growing up in the shared niche.

Our laboratory previously proposed an adjustment to the Freter's hypothesis which states that the mono- and disaccharides that *E. coli* strains use for growth in the intestine are made available locally from polysaccharide degradation by the anaerobes in the mixed biofilms that the *E. coli* inhabit, rather than evenly mixed throughout the intestine. Furthermore, if one strain can bind better to the anaerobes in the mixed biofilms, the colonization ability of this strain would be improved. Recent studies of bacterial biofilms in the intestines have shown that anaerobes form dense biofilms with *E. coli* as a minority member (27). These biofilms form around intestinal mucin and break down large polysaccharides, such as fiber, that cannot usually be utilized by facultative anaerobes (27, 40, 53). Differences in the population of anaerobes within mixed biofilms would thus provide *E. coli* with various niches based on nutrients made available by the anaerobes preferred metabolic pathways. If a specific mixed biofilm produced mostly galactose as a metabolic byproduct of polysaccharide breakdown, then *envZ*_{P41L} mutant would be provided with a significant competitive edge over the *flhDC* deletion mutant in that biofilm.

Due to the increased levels of OmpR-phosphate in the MG1655 *envZ*_{P41L} mutant, the surface of the cells is different from both the parent MG1655 and the MG1655 *flhDC* mutants. OmpR-phosphate acts to upregulate two small RNAs (sRNAs), *omrA* and *omrB*. These sRNAs are responsible for regulating outer membrane proteins by binding to the mRNA and preventing translation. One of the targets of these sRNAs is *cirA*, which produces a surface protein that binds colicin toxins produced by other bacteria (5). The MG1655 *envZ*_{P41L} mutant has shown increased resistance to colicin V, a toxin produced by *E. coli* strain F-18, which could be representative of its

resistance to other bacteriocins produced in certain mixed biofilms throughout the intestine (27). OmpR-phosphate also directly down regulates the production of the OmpF porin protein, which is known to transport bile salts into the cell. With this reduction in OmpF, less bile salts can move into the cell, and MG1655 *envZ*_{P41L} gains increased resistance to bile salts (27). The flagella of MG1655 *envZ*_{P41L} also remain intact allowing the cells to remain motile and adding yet another difference in the surface proteins of the MG1655 *envZ*_{P41L} mutant vs. the MG1655 *flhDC* mutant. These differences in outer surfaces could provide the MG1655 *envZ*_{P41L} mutant with an improved affinity towards members of the mixed biofilm niche where it is in direct competition with the MG1655 *flhDC* mutant. With better binding affinity the MG1655 *envZ*_{P41L} would have a lower washout rate than wildtype MG1655, allowing it to compete equally with the faster growth rate of the MG1655 *flhDC* mutant on most sugars. The improved resistance of MG1655 *envZ*_{P41L} to certain toxic substances in addition to improved binding affinity could increase the types of mixed biofilms with which it could associate.

While the anaerobes in mixed biofilms provide a source of nutrients for minimal members such as *E. coli* through breakdown of large polysaccharides, the facultative anaerobes may improve the environment for strict anaerobes by reducing the concentration of oxygen within the biofilms. Oxygen diffuses from surrounding tissue into the intestines, oxygen from swallowed air is present in flatus, and at least one predominant anaerobe in the gut microbiome, *Bacteroides fragilis*, respire oxygen at low concentrations (2, 18, 21, 28). It has been shown that respiration in *E. coli* provides an important competitive edge when colonizing the intestine of streptomycin-

treated mice. Mutants of ATP synthase or cytochrome *bd* oxidase, both necessary for respiration, showed a significant loss in colonizing ability when colonized against wild type of the same strain (21). Since *E. coli* uses oxygen as the final electron-acceptor in respiration, the oxygen concentration within the intestine will be lower in the presence of *E. coli*. Growth of anaerobes in close association with *E. coli* will be provided with a more preferential environment through depletion of oxygen, which is toxic to anaerobes. This will allow the anaerobes to provide a better niche for the *E. coli* through increased breakdown of large polysaccharides, leading to a mutualistic relationship in which both the anaerobes and *E. coli* in mixed biofilms benefit.

This study tested the hypothesis that the overall community structure of the gut may be influenced, and altered, by colonization with varying strains of *E. coli*. Based on the differing surface structures, binding affinities, and sugar preferences of the *E. coli* strains discussed thus far, each strain might prefer a different “restaurant,” which would then lead to the preferential growth of the anaerobe members of the specific niche filled by each *E. coli*.

Materials and Methods

Bacterial strains:

Bacterial strains used in this study are listed in Table 1. The original *E. coli* K-12 strain was obtained from a stool sample from a convalescing diphtheria patient in Palo Alto, CA, in 1922 (1). The sequenced *E. coli* MG1655 strain (CGSC 7740) was derived from the original K-12 strain, having only been cured of the temperate bacteriophage lambda and the F plasmid by means of UV light and acridine orange treatment (3). It has an *IS1* element in the *flhDC* promoter (14). *E. coli* Nissle 1917

was originally isolated during World War I from a soldier who escaped a severe outbreak of diarrhea (50). It has a beneficial effect on several types of intestinal disorders, is well tolerated by humans, and has been marketed as a probiotic remedy against intestinal disorders in several European countries since the 1920s (50). The allelic exchange method described by Datsenko and Wanner (10) was used to construct *E. coli* MG1655 Str^R Δ *flhDC::cam* (Table 1), 546-bp deletion encompassing region immediately downstream of ISI in regulatory region of *flhD* and into *flhD*. *E. coli* EDL933 is the prototype strain of enterohemorrhagic *E. coli* (EHEC) O157:H7 (42).

Media and growth conditions:

LB broth-Lennox (DIFCO) was prepared as directed (20g/L in water) and sterilized. The approximate formula of LB broth Lennox (DIFCO) is listed as follows: 10g/L tryptone, 5g/L yeast extract, 5g/L sodium chloride. Bottles containing 10 ml of LB broth Lennox (DIFCO) were inoculated with an individual strain of *E. coli* (Table 1) and allowed to grow for 18 h while shaking at 200rpm at 37°C. The overnight cultures grew to approximately 10⁹ CFU/ml, which were then diluted into 20% (w/v) sucrose to 10⁵ CFU/ml.

Colonization experiments:

The specifics of the streptomycin-treated mouse model used to compare the large intestine colonizing abilities of *E. coli* strains in mice have been described previously (34, 38, 54, 55, 56). Briefly, sets of three male CD-1 mice (5 to 8 weeks old) were given drinking water containing streptomycin sulfate (5 g/L) for 24 h to eliminate resident facultative bacteria (35). Following 18 h of starvation for food and water, the

mice were fed 1 ml of 20% (w/v) sucrose containing 10^5 CFU of LB broth Lennox (DIFCO) grown *E. coli* strains. After ingesting the bacterial suspension, both the food (Teklad mouse and rat diet; Harlan Laboratories, Madison, WI) and streptomycin-water were returned to the mice, and 1 g of feces was collected after 5 h, 24 h, and on odd-numbered days at the indicated times. Mice were housed individually in cages without bedding and were placed in clean cages at 24 h intervals. Individual fecal pellets were therefore no older than 24 h. Each fecal sample (1 gram) was homogenized in 10 ml of 1% Bacto-Tryptone (DIFCO), diluted in the same medium, and plated on Lactose MacConkey agar plates (DIFCO) with appropriate antibiotics. When appropriate, 1 ml of a fecal homogenate (which were sampled after the feces have settled) were centrifuged at $12,000 \times g$, resuspended in 100 μ l of 1% Bacto-Tryptone, and plated on a Latose MacConkey agar plate with appropriate antibiotics. This procedure increases the sensitivity of the assay from 10^2 CFU/gram of feces to 10 CFU/gram of feces. To distinguish the various *E. coli* strains in feces, dilutions were plated on lactose MacConkey agar containing streptomycin sulfate (100 μ g/ml), streptomycin sulfate (100 μ g/ml) and nalidixic acid (50 μ g/ml), streptomycin sulfate (100 μ g/ml) and rifampicin (50 μ g/ml), or streptomycin sulfate (100 μ g/ml) and chloramphenicol (30 μ g/ml). Streptomycin sulfate, chloramphenicol, rifampicin, and nalidixic acid were purchased from Sigma-Aldrich (St. Louis, MO). All plates were incubated for 18 to 24 h at 37°C prior to counting (27). Mice were colonized with a single representative strain of *E. coli* for 10 days prior to harvesting of cecum. The *E. coli* strains used were (see Table 1): an O157:H7 pathogenic strain (EDL933), the probiotic strain (Nissle 1917), a common laboratory strain (MG1655), and the two

mutants of MG1655 selected by the mouse intestine (*envZ* _{P41L} and *flhDC*). Also included were a set of mice inoculated with a placebo of uninoculated 20% sucrose.

Fluorescent in situ Hybridization (FISH):

In order to study how different strains of *E. coli* associate locally with members of the microbiota, we used Fluorescent In Situ Hybridization (FISH) on cross-sections of cecum from streptomycin-treated mice colonized with a single strain of *E. coli*. This was done in parallel with community analysis sequencing of bacterial populations in mucus samples obtained from the cecum of similarly treated mice to better understand how each strain of *E. coli* affects the development of the microbiome as a whole. The mice included an uninoculated control set (blank 20% sucrose) and mice inoculated with one of five representative strains of *E. coli* listed above. Each strain, as well as the control, was fed simultaneously to 6 mice (10^5 CFU/mouse), which were colonized for 10 days. On the tenth day, mice were killed via CO₂ and their ceca were harvested. Three of the mice from each set were used for FISH, while 3 of the mice from each set were used for community analysis sequencing of mucus samples.

The cecum of a mouse designated for FISH was cut into 1-2 cm sections, suspended in TissueTek® O.C.T compound (Sakura Finetek USA, Inc.) and snap frozen in 2-methylbutane suspended in liquid nitrogen. Sections were then stored at -80°C until ready for sectioning. For sectioning, the Vibratome UltraPro 5000 cryostat was used to cut sections at a thickness of 10µm, which were adhered to poly-L-lysine treated slides for visualization (LabScientific, Inc). Fixing of slides was done in 4% Paraformaldehyde for 1 hour at room temperature, followed by a wash in PBS for 10 min. Slides were allowed to dry overnight prior to hybridization. Fluorescent probes

were diluted in hybridization solution consisting of 10 ml of 1M Tris pH7.2, 18 ml of 5M NaCl, and 0.1 g of SDS in a total volume of 100 ml H₂O. All slides with sections were treated using the FISH technique used by S. Macfarlane et al. (31), with hybridization of *E.coli* specific (5'-/5Cy3/CACCGTAGTGCCTCGTCATCA-3') Cy-3 probe (red) and Eubacteria specific (5'-GCTGCCTCCCGTAGGAGT/36-FAM/-3') FitC probe (green) at concentrations of 5ng/μl and 25ng/μl, respectively. These probes were obtained from Integrated DNA Technologies (Coralville, IA). Briefly, 10μl of diluted probes in hybridization solution was pipetted to each slide, which were then covered with a HybriSlip hybridization cover (Life Technologies, Carlsbad, CA) and allowed to incubate in the dark for 2 h at 50°C. After 2 h the slides were dipped in a wash buffer, which is simply hybridization solution without SDS added, and then allowed to soak in the wash buffer for 20 min at 50°C. Slides were then rinsed with distilled water and allowed to dry overnight before viewing. Sections on poly-L-lysine treated slides were imaged using the Zeiss axioimager M2 imaging system with the Zeiss LSM 700 confocal microscope and corresponding lasers (Carl Zeiss, Oberkochen, Germany). The images acquired from the poly-L-lysine treated slides were used to observe the biofilms with which the individual *E. coli* strains associate. The poly-L-lysine treated slides were additionally treated with Vectashield (Vector Laboratories, Inc.), an antibleaching agent which helps prolong fluorescence for imaging. This involves placing a small drop of Vectashield on the hybridized slide and then covering with a coverslip. Allowing 30 min at room temperature for the Vectashield to dry made viewing easier, but was not absolutely necessary.

Isolation of cecal mucus and DNA in cecal mucus:

For each mouse cecum being used in sampling of microbiota in the mucus, mucus samples were scraped and collected into 5 ml of HEPES-Hanks buffer (pH7.2). Microbial DNA was isolated using a MO BIO PowerSoil™ DNA Isolation Kit.

Sequencing:

PCRs to prepare samples for sequencing were done similarly for all mucus scrapings. Primers were designed to match those used in a study of the human gut microbiome which targeted the V4 variable region of the 16S rRNA (57). These primers were obtained from Integrated DNA Technologies (Coralville, IA) and were as follows: Forward (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG A-3') and Reverse (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA G-3'). *Taq* DNA polymerase (New England BioLabs, Ipswich, MA.) was added to each PCR reaction, which consisted of 25µl reactions: 2.5µl 10x Standard *taq* Reaction Buffer (New England BioLabs Inc.), 0.5µl dNTP Solution Mix containing 10 mM of each dNTP (New England BioLabs Inc.), 0.5µl of a 100µM solution of each primer, 0.5µl of the aforementioned *taq* DNA polymerase (New England BioLabs), all added to 17.5µl of DEPC treated nuclease free sterile water (Fisher Scientific). Each PCR reaction began with an initial denaturation of 95°C for four min followed by a cycle of three steps repeated 35 times. These were as follows: denature at 95°C for 30 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s. The PCR ended with a final primer extension at 72°C for 4 min. PCR was done using an Eppendorf® Mastercycler 5331 Gradient PCR Thermocycler (Westbury, NY). PCR products were checked via gel

electrophoresis to be sure they contained one band of approximately 300 bp before submission to the Rhode Island Genomics and Sequencing Center (RIGSC).

Once submitted to the RIGSC, after amplicon submission, a second round of PCR (5 cycles) to attach Nextera (Illumina) indices and adapters was performed. Round 2 PCR reactions were cleaned with Agencourt Ampure XP (Beckman Coulter, Inc.), visualized by agarose gel electrophoresis, and selected samples were analyzed using the Agilent BioAnalyzer DNA1000 chip (Illumina). Qubit quantification was performed on all samples prior to pooling, and KAPA quantification was performed on the final pooled library prior to loading on the MiSeq flow cell. The samples were then run on the Illumina MiSeq (Illumina RTA 1.17.28) hi-throughput sequencer.

Sequencing analysis:

Data output from the MiSeq comes as raw fastq files, which were then classified using the published Mothur pipeline version 1.33 (49). Sequence processing was aligned, cleaned of chimeric sequences, and classified using the MiSeq SOP (standard operating procedure) made available by the Mothur authors (49).

Statistics

Means and standard deviations derived from the indicated samples were compared by an unpaired Student's *t* test (*P* values). A *P* value of >0.05 was interpreted as indicating no significant difference, and a *P* value of <0.05 was interpreted as indicating a significant difference.

Results

Comparing communities

The quantification of the level of colonization by each strain was measured by diluting and plating feces collected from colonized mice, as previously described (Fig. 1). Each *E. coli* strain was able to colonize mice at between 10^8 and 10^9 CFU/g feces. As the control mice were fed a 20% sucrose placebo (without the addition of any *E. coli*) strain, no *E. coli* colonies should have grown when plated on MacConkey agar without any antibiotics added. As expected, no colonies were seen. FISH images confirm that the strains exist in close proximity to other members of the gut microbiota as a minimal member in larger biofilms (Fig 2-7). It can also be seen in these images that the non-*E. coli* microbes are diverse in cell morphology.

Once the interaction between the *E. coli* strains and the members of the microbiota had been confirmed, as well as the diversity of the microbial community, the community structure of each individual strain was compared. This was done at the phylum and family levels. As seen in (Fig. 8), all colonized mice showed similar levels of Bacteroidetes, and Firmicutes. For the *E.coli* Nissle 1917 and *E. coli* EDL933 colonized mice, Deferribacteres seemed to be at lower levels than for mice colonized with the other *E. coli*. Upon comparing the Nissle 1917 and EDL933 colonized mice to mice colonized with all of the other *E. coli* strains through an unpaired students t test, it was shown that these low levels are only statistically relevant when compared to the control and conventional mice ($P < 0.0001$). Mice that were not colonized with any of the tested *E. coli* strains, i.e. the streptomycin treated controls and untreated conventional mice, showed very little Proteobacteria at this

level of classification. In contrast, the *E. coli* colonized mice showed much higher levels of Proteobacteria, with the exception of the MG1655 *envZ*_{P41L} and *flhDC* mutants. The *envZ*_{P41L} and *flhDC* mice showed lower levels of Proteobacteria when compared to the other treatments inoculated by *E. coli* strains, but only the *envZ*_{P41L} was statistically different in terms of Proteobacteria and only when compared to Nissle 1917 ($P < 0.05$). Even the Proteobacteria in the conventional and control mice were only statistically different from Nissle 1917 ($P < 0.05$).

It appears that mice colonized with all the *E. coli* strains display similar diversity at the family level (Fig. 9), with high levels of many of the same major members across all mice. Statistically relevant differences between the population of s24-7, a member of the Firmicutes phylum found in mice, is lower in Nissle 1917 samples when compared to MG1655 *envZ*_{P41L} samples ($P < 0.02$), streptomycin treated controls ($P < 0.01$), and the MG1655 *flhDC* mutant samples ($P < 0.002$). Deferribacteraceae is considerably lower in Nissle 1917 and EDL933 colonized mice when compared to control ($P < 0.0001$) and conventional mice ($P < 0.0001$). EDL933 colonized mice had the lowest level of Lachnospiraceae, which was only a statistically significant difference when compared to MG1655 *envZ*_{P41L} colonized mice ($P < 0.05$). Mice colonized with the MG1655 *envZ*_{P41L} and MG1655 *flhDC* mutants had lower levels of Enterobacteriaceae when compared to the mice colonized with the other *E. coli* strains, but only Nissle 1917 colonized mice were statistically higher and only when compared to mice colonized with the MG1655 *envZ*_{P41L} mutant ($P < 0.05$). The same was true for the control and conventional mice, with only Nissle 1917 colonized mice being statistically different in levels of Enterobacteriaceae ($P < 0.05$).

Increasing the sample set for Nissle 1917 and MG1655

From the initial MiSeq sample set it was concluded that no significant difference could be observed in the cecal mucus community structures of mice colonized with the 5 different *E. coli* strains. While certain differences did appear (i.e. s24-7 in Nissle 1917) this may have been due to the limited sample set (n=3), and so a second MiSeq run was performed. Rather than testing only a few additional mice for each strain, 2 strains were chosen so as a larger data set could be gathered for each: Nissle 1917 and MG1655. These strains were chosen as they are known to have different preferences in terms of growth on sugars, and are able to co-colonize the gut without being in direct competition (28).

An additional 11 mice colonized with MG1655 and 12 mice with Nissle 1917 were used. This would clarify if a larger sample size would allow visualization of changes in the overall microbial community caused by colonization with specific strains of *E. coli*. The relative abundance of major members of the gut microbiota was compared at the phylum and family level in order to ascertain if colonization by individual *E. coli* strains resulted in a percentage shifts of the major members, or if different major members appeared.

As is seen in the (Fig. 10), Nissle 1917 colonized mice have a higher relative abundance of Proteobacteria when compared to MG1655 ($P < 0.02$), and both Nissle 1917 and MG1655 fed mice have more Proteobacteria than the streptomycin treated control mice ($P < 0.0001$ and $P < 0.02$, respectively). This difference is seen again at the family level (Fig. 11) as represented by Enterobacteriaceae (a family in the Proteobacteria phylum) with Nissle 1917 colonized mice having more

Enterobacteriaceae than MG1655 colonized mice ($P < 0.02$), and both Nissle 1917 and MG1655 colonized mice having more Enterobacteriaceae than the streptomycin treated control mice ($P < 0.0001$ and $P < 0.02$, respectively). As the mice were treated with streptomycin in order to clear natural Proteobacteria from the niches that colonizing strains would inhabit, this difference in levels when compared to the streptomycin treated controls would be expected. As for the other members of the microbiota, there were no statistically significant differences between the individual *E. coli* treatments.

Discussion

In the present study, we examined the community structure of mice colonized with various strains of *E. coli* and their individual effects on the diversity of the cecal mucus population. It has been shown that *E. coli* inhabits the gut as a minimal member of mixed biofilms consisting of large anaerobe populations (27, Fig. 2-7), and while certain major members of the population may be influenced by colonization with specific strains of *E. coli*, more samples are likely necessary in order to elucidate any statistically relevant shift in a population. This is shown in comparing the Nissle 1917 data, which in the first sample set ($n=3$) showed changes in levels of s24-7 and Deferribacteriaceae, but increasing the sample size ($n=15$) debunks this finding as Deferribacteriaceae and s24-7 are present at a higher relative abundance (Fig. 9 vs Fig. 11). It is important to note however that the depth of sequencing is important for identifying key differences in these experiments, as the family level shows much greater differences than the phylum level. Future work would likely need to focus on

the family level, or if able to, the genus level of classification to elucidate key differences caused by the colonizing *E. coli* strains.

Deferribacteres is a relatively recently described lineage, but one genus of Deferribacteres, *Mucispirillum*, has been identified as existing in the mammalian gut and being able to degrade mucin (20, 48). Lachnospiraceae is a member of the Firmicutes that is able to produce butyric acid and has been previously linked to obesity (36). Ruminococcaceae, another Firmicutes, has been previously shown to exist in the gut, but is difficult to classify and is often a mistakenly identified Lachnospiraceae (30). Paenibacillaceae is a Firmicutes with species that exist as plant growth promoting rhizobacteria and may have been introduced through the feed given to mice (4). The s24-7 family is a member of the Bacteroidetes that has been found to inhabit the mouse gut, but little else has been described (17). Another bacteroidetes, Porphyromonadaceae, has been previously shown to occasionally inhabit the gut, but more often resides in oral cavities as a pathogen (39). Rikanellaceae is another Bacteroidetes that has a recently reclassified member, *Alistipes putredinis* which was previously classified as a *Bacteroides* and shows similarities with *Bacteroides fragilis* and members of *Porphyromonadaceae* (47). Bacteroidaceae contains the *Bacteroides* genus, which can use simple sugars when available, but use glycans as a main source of energy (33).

Variation in the microbial communities amongst different hosts is common, and even hosts from a common facility or litter may have differences amongst the populations that inhabit their guts (29, 37, 45). This is illustrated by the fact that mice used to test the same *E. coli* strain, and at the same time and under identical

conditions, show differences in their community structures. As seen in (Fig. 9), showing the relative abundance at the family level of an individual *E. coli* strain, the standard deviation is considerable for many of the organisms present. Some families aren't even present in some of the mice, but are in high enough number to be represented in another mouse. As such, it is possible that any small effect in colonization by an individual *E. coli* strain could be masked by this diversity amongst hosts.

In addition to variation amongst hosts, the microbiota also consists of a vast amount of members (between 500-1000 commensal strains [6, 7, 22, 41, 46, 51, 52]). This could further mask any alteration in the community structure induced by colonization with specific *E. coli* strains. If a specific organism were present as a major member of the microbiota in one mouse, but only a minor member (or not present at all) in another mouse, this would result in a large standard deviation for that organism. So, even if a colonizing *E. coli* had an effect on this specific organism in the population, it would look the same as if it were just an artifact of having different hosts. This is further complicated by the overall noise level of the gut, again comprising of 500-1000 different organisms.

While the diversity between mice, as well as the large and complicated community of the gut, may mask shifts in the populations caused by colonizing *E. coli* it is still likely that the overall composition of the gut is not affected by the colonization of a specific *E. coli* strain. However, this does not mean *E. coli* is not important within the community and could still have a more localized effect on members of the population. Direct cell to cell interactions between *E. coli* and

members of the microbiota that are within close proximity of one another may be more directly affected by these individual strains, as opposed to an obvious shift in large populations within the microbiome. Redundancy of gene programs and processes may allow the overall population to remain mostly unaffected by a minimal member missing from the population (19), but a more subtle effect may occur in localized niches. Further studies of direct cell to cell interactions may be necessary to elucidate how an individual *E. coli* strain can affect the communities of the mammalian gut, such as laser capture micro-dissections (LCM) (23). LCM coupled with FISH techniques would allow the targeting of fluorescently labeled *E. coli* cells within mixed biofilms to be captured and used for community analysis sequencing. This would allow only the members in close association with the *E. coli* to be studied, and remove much of the noise created by the overall communities of the gut.

In summary, the data presented here suggest that colonization with different strains of *E. coli* does not affect the overall composition of the microbial communities found within mouse cecal mucus. While increasing the sample size may give clarity to some minute effects that occur due to colonization with these individual strains, it is likely the overall noise level of the gut and the variation in the communities between hosts will mask these effects. Moving forward, it could prove useful to directly study the members that closely associate with the colonizing *E. coli* strains, through use of laser capture micro-dissections, in order to avoid the clutter of the dense microbial communities. Furthermore, focusing on deeper levels of classification could help elucidate differences in these populations.

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Table 1 – Bacterial strains

<u><i>E. coli</i> strain</u>	<u>Genotype/phenotype</u>	<u>Designation in the text</u>	<u>Source or reference</u>
MG1655 Str ^R	Spontaneous streptomycin resistant mutant of MG1655	MG1655	3
MG1655 Str ^R $\Delta flhD::cam$	546-bp deletion beginning immediately downstream of IS1 in the regulatory region of <i>flhD</i> and ending in <i>flhD</i> , streptomycin and chloramphenicol resistant	MG1655 <i>flhDC</i>	15, 23
MG1655 <i>envZ</i> P41L Str ^R	P41L <i>envZ</i> missense mutant of MG1655 Str ^R	MG1655 <i>envZ</i> _{P41L} mutant	24
Nissle 1917 Str ^R	Spontaneous streptomycin resistant mutant of Nissle 1917	Nissle 1917	22, 41
EDL933 Str ^R Rif ^R	Spontaneous streptomycin resistant mutant of EDL933	EDL933	35

Table 2 – Primers used

<u>Primer designation</u>	<u>Sequence</u>
of <i>E.coli</i> specific Cy-3 probe (red) used for FISH	5’-/5Cy3/CACCGTAGTGCCTCGTCATCA-3’
Eubacteria FitC probe (green) used for FISH	5’-GCTGCCTCCCGTAGGAGT/36-FAM/-3’
16S V4 forward used for community analysis sequencing	5’-TCG TCG GCA GCG TCA GAT GTG TAT AAG A-3’
16S V4 reverse used for community analysis sequencing	5’-GTC TCG TGG GCT CGG AGA TGT GTA TAA G-3’

Fig 1. Single strain colonizations of mice colonized with individual strains of *E. coli*. This shows that the individual *E. coli* strains are able to colonize the mice at similar levels.

Fig 2. FISH image of a cross-section of cecum collected from mice colonized with EDL933 for 10 days. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig 3. FISH image of a cross-section of cecum collected from mice colonized with Nissle 1917 for 10 days. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig 4. FISH image of a cross-section of cecum collected from mice colonized with MG1655 for 10 days. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig 5. FISH image of a cross-section of cecum collected from mice colonized with MG1655 *flhDC* for 10 days. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig. 6. FISH image of a cross-section of cecum collected from mice colonized with MG1655 *envZ*_{P41L} for 10 days. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig. 7 FISH image of a cross-section of cecum collected from mice treated with streptomycin but not colonized with any *E. coli*. Green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig. 8 Bar plot showing relative abundance of major phyla in mucus samples by treatment. In this set of experiments $n=3$. Error bars indicate standard error of the mean. EDL933 and Nissle 1917 colonized mice show lower levels of Deferribacteres when compared to the control and conventional mice ($P < 0.0001$). Only Nissle 1917 shows greater levels of Proteobacteria, and only when compared to MG1655 *envZ*_{P41L} and the control and conventional mice ($P < 0.05$).

Fig. 9 Bar plot showing relative abundance of major families in mucus samples by treatment. In this set of experiments $n=3$. Error bars indicate standard error of the mean. The level of s24-7 in the Nissle 1917 colonized mice was lower when compared to the MG1655 mutants (*envZ_{P41L}* $P < 0.02$ and *flhDC* $P < 0.002$) and the streptomycin treated control ($P < 0.01$). Deferribacteraceae was lower in Nissle 1917 and EDL933 colonized mice when compared to the control and conventional mice ($P < 0.0001$). EDL933 colonized mice had the lowest level of Lachnospiraceae, which was only a statistically significant difference when compared to MG1655 *envZ_{P41L}* colonized mice ($P < 0.05$). Enterobacteriaceae were lower in the *envZ_{P41L}*, control and conventional mice when compared to Nissle 1917 mice ($P < 0.05$).

Fig. 10 Bar plot showing relative abundance of major phyla in mucus samples by treatment. For Nissle1917 samples $n=15$. For MG1655 samples $n=14$. For streptomycin treated control samples $n=15$. Error bars indicate standard error of the mean. The only statistically significant differences were seen in the levels of Proteobacteria, with Nissle 1917 fed mice having more than MG1655 fed mice ($P < 0.02$), and both Nissle 1917 and MG1655 fed mice having more Proteobacteria than the streptomycin treated controls ($P < 0.0001$ and $P < 0.02$, respectively).

Fig. 11 Bar plot showing relative abundance of major families in mucus samples by treatment. For Nissle1917 samples $n=15$. For MG1655 samples $n=14$. For streptomycin treated control samples $n=15$. Error bars indicate standard error of the mean. Nissle 1917 colonized mice having more Enterobacteriaceae than MG1655 colonized mice ($P < 0.02$), and both Nissle 1917 and MG1655 colonized mice having more Enterobacteriaceae than the streptomycin treated control mice ($P < 0.0001$ and $P < 0.02$, respectively)

MANUSCRIPT II

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Title: Developing an *In Vitro* Method for Studying the Interaction between *E. coli* and the Intestinal Microbiota

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MANUSCRIPT II

Developing an *In Vitro* Method for Studying the Interaction between *E. coli* and the Intestinal Microbiota

Abstract

In order to better understand the interactions between *E. coli* and the members of the intestinal microbiota, a novel *in vitro* method was developed. This method was designed to be simple and inexpensive while providing an environment meant to replicate the natural habitat of these organisms: the mammalian gut. The current study examines the development of said *in vitro* model and its ability to mimic the mouse gut in terms of diversity of organisms, as well as its usefulness in examining the colonizing ability of competing *E. coli* strains. It is shown that, while still in development, this system is currently able to maintain diversity comparable to what is seen in mice. While the abundance of these diverse organisms is not necessarily at the levels seen in mice, the model is already an effective system for studying the microbial community of the gut in a controlled environment. Furthermore, the system is able to mimic certain colonization experiments of competing *E. coli* strains done in mice, and with improvements to bolster the growth of the diverse population could be used as a non-invasive method for studying gut microbes.

Introduction

While there are a diverse group of phyla represented within the mammalian gut, the microbiota is dominated by two: Firmicutes and Bacteroidetes (15, 30, 55). Facultative anaerobes, such as *E. coli*, are minimal yet important members of the gut community, and it has been shown that *E. coli* inhabits mixed biofilms formed by

anaerobes as a minority member (28). According to Freter's nutrient-niche hypothesis the community structure of the intestine is decided by nutrient availability, but also assumes that all available nutrients within the intestine are perfectly mixed (17). We previously proposed an adjustment to Freter's nutrient-niche hypothesis, the "Restaurant" hypothesis, which can be used to explain how the anaerobes in mixed biofilms inhabited by *E. coli* provide them with mono- and di-saccharides locally, rather than from a perfectly mixed pool available to all species (28). Furthermore, it was hypothesized that differences in the outer surface between strains of *E. coli* may have an effect on the binding affinity to the mixed biofilms and their consequent growth (28).

Mucus in the gut exists in two forms, the loose layer and the firm layer (24). The loose mucus layer is where most of the microbiota of the gut grow and form their biofilms, while the firm mucus layer resides closer to the epithelial layer of the intestines and prevents direct interaction of the commensal microbiota with host epithelium (24). The mucus itself consists of a large mixture of molecules, including but not limited to amino acids, large polysaccharides, mono- and disaccharides, and mucin (3). This mixture exists as a viscous liquid in which the microbes are suspended and in which form biofilms. This environment is mostly anaerobic, with minimal oxygen leaking from epithelial cells of the intestine (4, 20, 25, 29). Mucin is a glycoprotein with large polysaccharide strands with which the anaerobe populations of the gut can attach and form biofilms around (1). The biofilms formed by these anaerobes can break down the large polysaccharides of the mucus, and parts of the mucin, thus providing facultatives with usable mono- and disaccharides in bulk which

would usually be limited (28, 43, 56). This gives facultatives, such as *E. coli*, a reason for joining as minimal members in these anaerobe-formed biofilms, and perhaps even promoting the growth of preferential anaerobes that provide specific mono- and disaccharides (25). The facultatives that join these mixed biofilms likely provide the anaerobes with protection from the minimal amount of oxygen that is released from the epithelial tissue of the gut.

In vitro biofilm models allow the study of microbial communities as they form complex biofilm structures in a contained and controllable environment (14, 21, 61). Various biofilm models have been adapted to represent specific environments in order to best mimic biofilm formation *in vivo*. These systems are diverse in setup and function, ranging from flow displacement systems in which a constant flow of growth medium in and waste products out (4, 21), to cell-culture-based models which allow biofilms to develop on reconstituted human epithelia (50).

One system that is commonly used to study biofilms due to its simplicity is the microtiter plate based model (8, 26, 46, 59). The biofilms using this model are grown in microtiter well plates ranging from 6 to 96 well plates depending on necessity. This allows for many environmentally identical biofilms to be studied at once, or individual variables to be tested in each well (42). This system also allows for easy adjustment over time in order to view the effects of variation in the media or additives on biofilm development or maintenance (9, 23). However, a disadvantage of the microtiter plate based models is that the system does not take into account the flow through of certain environments such as the mouse gut (11, 47). This would mean that biofilms growing

in the wells of the microtiter plate would eventually deplete available nutrients while also accumulating waste (11).

Other systems take into account that the flow of new media into and old media out of the system is important. These flow models vary in design, with some developed to maintain a perfect mixture of nutrients and other more simplistic systems which allow the flow of the media to develop a natural gradient from one end of the flow to the other (21). The continuous flow stirred tank reactor (CFSTR), a system utilized by the CDC for biofilm studies, has an influx that matches the flow out of the system with constant stirring where the biofilms grow (21). Planktonic cells can be flushed out of the system by having the dilution rate of the media set faster than the doubling time of the organisms in the system, leaving behind the attached organisms in biofilms (21). The plug flow reactor (PFR) is similar in the control of media flow, but does not have a container of media with constant stirring. Instead, plugs seeded with biofilm forming organisms are locked in a row and media is allowed to flush past them. Thus the media will have a higher concentration of substrates earlier in the flow with a gradual decline in concentration further down the system (11, 21). While these systems are designed to mimic the natural flow of available substrates in certain environmental habitats, they also come with disadvantages. The continuous flow stirred tank reactor setup tends to be labor intensive, with specialized equipment that can be difficult to use or maintain. Both flow systems suffer from only being able to test a single biofilm forming community per setup or experiment, as is a similar issue with the media flowing into the system. The media required to maintain the system is

also a substantial amount, as the constant flow requires fresh media to be prepared based on the rate of flow (11).

In order to avoid some of the disadvantages of the previously described systems, a simple *in vitro* system was developed with the specific intention of mimicking bacterial growth in the mammalian gut. The developed gut system was designed to be simplistic in nature, making it accessible to other researchers interested in studying microbial communities in biofilms. The flow through of the natural gut was also considered, and the system was developed with a dilution system meant to mimic the diminishing and regrowth of the populations due to the constant flow of mucus (47). Additionally, due to the simple dilution of growth into fresh media, multiple variables could be tested on biofilms developed from a single source thus allowing the observation of multiple conditions on identical biofilm communities. The developed system is also small enough to be contained in a single microcentrifuge tube, which allows for multiple samples or experiments to be run in tandem.

Materials and Methods

Bacterial strains:

Bacterial strains used in this study are listed in Table 1. The original *E. coli* K-12 strain was obtained from a stool sample from a convalescing diphtheria patient in Palo Alto, CA, in 1922 (Bachman BJ, 1996). The sequenced *E. coli* MG1655 strain (CGSC 7740) was derived from the original K-12 strain, having only been cured of the temperate bacteriophage lambda and the F plasmid by means of UV light and acridine orange treatment (7). It has an *IS1* element in the *flhDC* promoter (16). *E. coli* Nissle 1917 was originally isolated during World War I from a soldier who escaped a severe

outbreak of diarrhea (53). It has a beneficial effect on several types of intestinal disorders, is well tolerated by humans, and has been marketed as a probiotic remedy against intestinal disorders in several European countries since the 1920s (53). *E. coli* EDL933 is the prototype strain of enterohemorrhagic *E. coli* (EHEC) O157:H7 (45). The allelic exchange method described by Datsenko and Wanner (datsenko wanner 2001) was used to construct *E. coli* EDL933 Str^R $\Delta ppsA$ $\Delta pckA::cam$ (Table 1), with deletions of 2,367 and 1,341 bp in the *ppsA* gene and the *pckA* gene, respectively. Phosphoenolpyruvate carboxykinase converts oxaloacetate to phosphoenolpyruvate and is encoded by the *E. coli pckA* gene (35). Phosphoenolpyruvate synthase is encoded by the *E. coli ppsA* gene and converts pyruvate to phosphoenolpyruvate (41). Since the conversion of TCA cycle intermediates to phosphoenolpyruvate is completely blocked in a *ppsA pckA* double mutant, it is unable to grow on gluconeogenic substrates “below” the step of phosphoenolpyruvate, but glycolytic pathways are not affected (18).

Media and growth conditions:

LB broth-Lennox (DIFCO) was prepared as directed (20 g/L in water) and sterilized. The approximate formula of LB broth Lennox (DIFCO) is listed as follows: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride. Bottles containing 10 ml of LB broth Lennox (DIFCO) were inoculated with an individual strain of *E. coli* (Table 1) and allowed to grow for 18 h while shaking at 200 rpm at 37°C. The overnight cultures grew to approximately 10⁹ CFU/ml, which were then diluted to 10⁵ CFU/ml in 20% (w/v) sucrose when intended for mouse colonization, or HEPES-Hanks buffer when diluted for addition into the *in vitro* system.

HEPES-Hanks buffer is 8 g/L NaCl, 0.4 g/L KCl, 0.185 g/L CaCl, 0.2 g/L MgSO₄, 0.5 g/L Na₂HPO₄, 0.35 g/L KH₂PO₄, and 2.6 g/L HEPES in distilled water. This solution was brought to pH 7.2 through addition of NaOH and filter sterilized (12). For the experiments in which agarose was added, agarose was prepared separately at 0.6% agarose (w/v) in distilled water and HEPES-Hanks buffer was prepared at twice the above listed concentrations. The agarose was prepared fresh daily, and, as autoclaving the agarose expels oxygen, provided further removal of oxygen from the samples. The agarose and HEPES-Hanks were then mixed 1:1.

For the *in vitro* gut model, cecal mucus samples were harvested from the *E. coli* colonized streptomycin-treated mice into 1 ml of HEPES-Hanks buffer (pH7.2) including a reducing agent (3 mM Cleland's reagent) to help prevent oxygen poisoning of the anaerobe population within the sample, as well as 500 ug/ml porcine intestinal mucin (Sigma). This sample was mixed thoroughly via pipetting, and then incubated in a BD anaerobic GasPak™ system (Becton, Dickinson and Company) overnight at 37°C. The following day, a 100µl sample of the overnight growth cecal mucus bacterial culture was used to inoculate a fresh 1 ml sample of HEPES-Hanks containing 2 mg/ml lyophilized mucus, 500µg/ml Porcine intestinal mucin (Sigma), 3 mM Cleland's reagent, and 0.3% agarose. The freshly inoculated 1 ml sample was labeled as Day 0 and incubated in a BD anaerobic GasPak™ system (Becton, Dickinson and Company) overnight at 37°C. This procedure was repeated for 7 days, using 100µl to inoculate a new 1 ml sample of fresh HEPES-Hanks containing 2 mg/ml lyophilized mucus, 500µg/ml Porcine intestinal mucin, 3 mM Cleland's reagent, and 0.3% agarose.

When using the *in vitro* method to compare colonization ability, slight alterations were made to accommodate multiple strains. Initial sampling remained the same, with pre-colonized mouse mucus being collected into 1 ml of HEPES-Hanks buffer (pH7.2) including a reducing agent (3 mM Cleland's reagent) to help prevent oxygen poisoning of the anaerobe population within the sample, as well as 500µg/ml porcine intestinal mucin (Sigma). This sample was mixed thoroughly via pipetting, and then incubated in a BD anaerobic GasPak™ system (Becton, Dickinson and Company) overnight at 37°C. On the same day as collection, strains to be used for competitions were grown overnight in LB at 37°C while shaking. The following day, a 10µl sample of the overnight culture was used to inoculate a fresh 1 ml sample of HEPES-Hanks buffer containing 2 mg/ml lyophilized mucus, 500µg/ml Porcine intestinal mucin (Sigma), 3mM Cleland's reagent, and 0.3% agarose. At this point, 10µl of the strains to be used for competition were diluted in at a final concentration of 10^2 - 10^3 CFU, having been diluted in HEPES-Hanks buffer from the overnight culture. 10µl of the freshly made sample was used for serial dilutions and plating on Lactose MacConkey agar (DIFCO) containing antibiotics as previously described (also described below in 'Colonization Experiments') to obtain counts of, and differentiate between strains of *E. coli* within the population.

Preparation of lyophilized cecal mucus

Cecal mucus was collected via scraping from 15 mice into 30 ml of HEPES-Hanks buffer. The mucus collections were then centrifuged using a Sorvall™ RC6 Plus (Thermo Scientific) at an rcf of 10,000 x g for 10 mins. The supernatant was collected without disturbing the pellet, at which point the collected supernatant was

frozen at -80°C and the pellet was discarded. Afterwards, the frozen supernatant was placed in a FreeZone Plus 12 Liter Cascade Console Freeze Dry System (LABCONCO) for 48 h to remove all liquid from the collected mucus. The lyophilized mucus was then stored at -20°C until needed. This was done for each *in vitro* experiment.

Colonization experiments:

The specifics of the streptomycin-treated mouse model used to compare the large intestine colonizing abilities of *E. coli* strains in mice have been described previously (34, 39, 56, 58, 60). Briefly, sets of three male CD-1 mice (5 to 8 weeks old) were given drinking water containing streptomycin sulfate (5 g/L) for 24 h to eliminate resident facultative bacteria (37). Following 18 h of starvation for food and water, the mice were fed 1 ml of 20% (w/v) sucrose containing 10^5 CFU, or 10^{10} , CFU of LB broth Lennox (DIFCO) grown *E. coli* strains. After ingesting the bacterial suspension, both the food (Teklad mouse and rat diet; Harlan Laboratories, Madison, WI) and streptomycin-water were returned to the mice, and 1 g of feces was collected after 5 h, 24 h, and on odd-numbered days at the indicated times. Mice were housed individually in cages without bedding and were placed in clean cages at 24-h intervals. Individual fecal pellets were therefore no older than 24 h. Each fecal sample (1 gram) was homogenized in 10 ml of 1% Bacto-Tryptone (DIFCO), diluted in the same medium, and plated on Lactose MacConkey agar plates (DIFCO) with appropriate antibiotics. When appropriate, 1 ml of a fecal homogenate (which were sampled after the feces settled) were centrifuged at 12,000 x g, resuspended in 100µl of 1% Bacto-Tryptone, and plated on a Lactose MacConkey agar plate (DIFCO) with appropriate

antibiotics. This procedure increases the sensitivity of the assay from 10^2 CFU/gram of feces to 10 CFU/gram of feces. To distinguish the various *E. coli* strains in feces, dilutions were plated on Lactose MacConkey agar (DIFCO) containing streptomycin sulfate (100 µg/ml), streptomycin sulfate (100 µg/ml) and nalidixic acid (50 µg/ml), streptomycin sulfate (100 µg/ml) and rifampicin (50 µg/ml), or streptomycin sulfate (100 µg/ml) and chloramphenicol (30 µg/ml). Streptomycin sulfate, chloramphenicol, rifampicin, and nalidixic acid were purchased from Sigma-Aldrich (St. Louis, MO). All plates were incubated for 18 to 24 h at 37°C prior to counting (28).

Fluorescent in situ Hybridization (FISH):

In order to observe how *E. coli* associate locally with members of the microbiota *in vivo*, we used Fluorescent In Situ Hybridization (FISH) on cross-sections of cecum from streptomycin-treated mice colonized with a single strain of *E. coli*. These observations were used to compare the similar interactions in the *in vitro* system. This was done in parallel with community analysis sequencing of bacterial populations in mucus samples obtained from the cecum of similarly treated mice in order to compare the diversity of the *in vivo* and *in vitro* models. The mice included an uninoculated control set (blank 20% sucrose) and mice inoculated with *E. coli* Nissle 1917. Samples of conventional and Nissle 1917 inoculated mice were done simultaneously for 6 mice, which were colonized for 10 days. On the tenth day, mice were killed via CO₂ and their ceca were harvested. Three of the mice from each set were used for FISH, while 3 of the mice from each set were used for community analysis sequencing of mucus samples. For *in vitro* samples, 10µl of the sample was

used to prepare slides for FISH and the remaining sample was used for community analysis sequencing.

The cecum of a mouse designated for FISH was cut into 1-2 cm sections, suspended in TissueTek® O.C.T compound (Sakura Finetek USA, Inc.) and snap frozen in 2-methylbutane suspended in liquid nitrogen. Sections were then stored at -80°C until ready for sectioning. For sectioning, the Vibratome UltraPro 5000 cryostat was used to cut sections at a thickness of 10µm, which were adhered to poly-L-lysine treated slides for visualization (LabScientific, Inc). Fixing of slides was done in 4% Paraformaldehyde for 1 hour at room temperature, followed by a wash in PBS for 10 min. Slides were allowed to dry overnight prior to hybridization. Fluorescent probes were diluted in hybridization solution consisting of 10ml of 1M Tris pH7.2, 18ml of 5M NaCl, and 0.1g of SDS in a total volume of 100ml H₂O. All slides with sections were treated using the FISH technique used by S. Macfarlane et al. (32), with hybridization of *E.coli* specific (5'-/5Cy3/CACCGTAGTGCCTCGTCATCA-3') Cy-3 probe (red) and Eubacteria specific (5'-GCTGCCTCCCGTAGGAGT/36-FAM/-3') FitC probe (green) at concentrations of 5ng/µl and 25ng/µl, respectively. These probes were obtained from Integrated DNA Technologies (Coralville, IA). Briefly, 10µl of diluted probes in hybridization solution was pipetted to each slide, which were then covered with a HybriSlip hybridization cover (Life Technologies, Carlsbad, CA) and allowed to incubate in the dark for 2 h at 50°C. After 2 h the slides were dipped in a wash buffer, which is simply hybridization solution without SDS added, and then allowed to soak in the wash buffer for 20 min at 50°C. Slides were then rinsed with distilled water and allowed to dry overnight before viewing. Sections on poly-L-

lysine treated slides were imaged using the Zeiss axioimager M2 imaging system with the Zeiss LSM 700 confocal microscope and corresponding lasers (Carl Zeiss, Oberkochen, Germany). The poly-L-lysine treated slides were additionally treated with Vectashield (Vector Laboratories, Inc.), an antibleaching agent which helps prolong fluorescence for imaging. This involves placing a small drop of Vectashield on the hybridized slide and then covering with a coverslip. Allowing 30 mins at room temperature for the Vectashield to dry made viewing easier, but was not absolutely necessary.

Isolation of cecal mucus and DNA in cecal mucus and in in vitro samples:

For each mouse cecum being used in sampling of microbiota in the mucus, mucus samples were scraped and collected into 5 ml of HEPES-Hanks buffer (pH7.2). The *in vitro* samples were collected directly from overnight growth into DNA isolation kits. Microbial DNA was isolated using a MO BIO PowerSoil™ DNA Isolation Kit.

Sequencing:

PCR reactions to prepare samples for sequencing were performed similarly for all mucus scrapings and *in vitro* samples. Primers were designed to match those used in a study of the human gut microbiome which targeted the V4 variable region of the 16S rRNA (62). These primers were obtained from Integrated DNA Technologies (Coralville, IA) and were as follows: Forward (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG A-3') and Reverse (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA G-3'). *Taq* DNA polymerase (New England BioLabs, Ipswich, MA.) was added to each PCR reaction, which consisted of 25µl reactions: 2.5µl 10x Standard *taq*

Reaction Buffer (New England BioLabs Inc.), 0.5µl dNTP Solution Mix containing 10 mM of each dNTP (New England BioLabs Inc.), 0.5µl of a 100µM solution of each primer, 0.5µl of the aforementioned *taq* DNA polymerase (New England BioLabs), all added to 17.5µl of DEPC treated nuclease free sterile water (Fisher Scientific). Each PCR reaction began with an initial denaturation of 95°C for four min followed by a cycle of three steps repeated 35 times. These were as follows: denature at 95°C for 30 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s. Each PCR reaction ended with a final primer extension at 72°C for 4 min. PCR was done using an Eppendorf® Mastercycler 5331 Gradient PCR Thermocycler (Westbury, NY). PCR products were checked via gel electrophoresis to be sure they contained one band of approximately 300 bp before submission to the Rhode Island Genomics and Sequencing Center (RIGSC).

Once submitted to the RIGSC, after amplicon submission, a second round of PCR (5 cycles) to attach Nextera (Illumina) indices and adapters was performed. Round 2 PCR reactions were cleaned with Agencourt Ampure XP (Beckman Coulter, Inc.), visualized by agarose gel electrophoresis, and selected samples were analyzed using the Agilent BioAnalyzer DNA1000 chip (Illumina). Qubit quantification was performed on all samples prior to pooling, and KAPA quantification was performed on the final pooled library prior to loading on the MiSeq flow cell. The samples were then run on the Illumina MiSeq (Illumina RTA 1.17.28) hi-throughput sequencer.

Sequencing analysis:

Data output from the MiSeq comes as raw fastq files, which were then classified using the published Mothur pipeline version 1.33 (52). Sequence processing

was aligned, cleaned of chimeric sequences, and classified using the MiSeq SOP (standard operating procedure) made available by the Mothur authors (52).

The in vitro system observations:

In order to visualize early microbial communities and how *E. coli* affects the growth of the anaerobes within mixed biofilms during early development in the *in vitro* system, FISH images were taken daily. 10µl of the overnight growth of the *in vitro* system was used to prepare and observe a slide using previously described FISH techniques. Another 10µl was used for serial dilutions and plating on Lactose MacConkey agar (DIFCO) containing antibiotics as previously described to obtain counts of *E. coli* within the population.

Generating crude rarefaction curves

Crude rarefaction curves were generated using the classifications generated through the Mothur pipeline (52) to group the sequences at the family level. This is done by randomly sampling from the families, with consideration that each family represents a unique member of the microbiota. A higher abundance of a specific family increases the likelihood that this specific family will be sampled, thus experiments with a majority of its members being represented by one or two families will have a lower measure of diversity when compared to those with abundance spread across more families. Actually having more unique families within an experiment will also increase the measure of diversity. Sampling was done from 100 picks to 5000 picks, with intervals of 100, and each was repeated 100 times.

Statistics

Means and standard deviations derived from the indicated samples were compared by an unpaired Student's *t* test (*P* values). A *P* value of > 0.05 was interpreted as indicating no significant difference, and a *P* value of < 0.05 was interpreted as indicating a significant difference.

Results

Initial experiments to develop the in vitro system

The *in vitro* model of the gut was designed to mimic the loose mucus layer found in the cecum of mice. To do this, the anaerobe populations needed to be protected from excessive oxygen stress. This was accomplished by adding 3mM of Cleland's reagent, a reducing agent that sequesters oxygen, thus providing the anaerobes with an oxygen free environment. In addition to this, 100µg/ml of mucin was added to provide nutrients and an attachment site for the anaerobes to begin forming biofilms. 2 mg/ml of mucus was also added to provide an additional source of nutrients. All of these additives were dissolved into HEPES-Hanks buffer at pH 7.2, a buffer used for gut microbes that lacks any source of carbon or nitrogen (12). In order to mimic the constant flow through and growth present in the gut, samples were diluted into fresh mixtures containing Cleland's, mucin, and mucus in HEPES-Hanks buffer every 24 hours. It was shown that while the microbial populations collected from the mouse gut could survive 1-2 days in this *in vitro* system (Fig. 1), a majority of the anaerobic population was lost after 3 days (Fig. 2). In addition to losing diversity and quantity of anaerobes, *E. coli* that was spiked into the system grew to be

a majority of the sample (Fig. 2). This contrasts what is seen in the natural environment inside mice, where *E. coli* is a minimal member of vast microbial biofilms (28).

Increasing mucin concentration to 500 µg/ml improves diversity beyond day 3

In an attempt to improve survivability of the anaerobe population, the mucin concentration was increased to 500 µg/ml. The increase of mucin concentration not only provided anaerobes with more binding sites for initiating biofilm formation and food in the form of large polysaccharide chains, but also visibly increased the viscosity of the solution. This increase in viscosity better mimicked the loose mucus of the gut, possibly providing the microbe populations with an environment more akin to what they prefer in their natural hosts. This 5-fold increase in mucin concentration improved the *in vitro* system to the point where the anaerobe populations survived well up to day 5. However, day 6 showed a steep loss of anaerobe populations and again showed the *E. coli* taking over as the majority (Fig. 3).

Adding agarose increases viscosity of solution and anaerobe survival

While increasing the concentration of mucin in the system increased the viscosity of the samples, they were still not quite as viscous as what is seen in the loose layer of mucus. To increase the viscosity further, 0.3% agarose was added to the solution. The specific amount of agarose to add was found to be the highest amount that could be added and still be feasibly pipetted. This addition of agarose created an environment that appeared to be similar in viscosity to mucus of the loose layer. As such, biofilms formed by the microbes of the gut would no longer settle to the bottom of the sample and could instead create networks throughout. The agarose may also

have provided additional protection from oxygen, as it would be more difficult for the oxygen to penetrate deep into the samples. The addition of agarose showed considerable improvement in the survivability of the anaerobe populations, with the populations remaining robust and diverse through day 6 (Fig. 4).

Precolonizing the mouse with E.coli prior to mucus collection further improves anaerobe survival in the in vitro system

In all previous experiments, *E. coli* was added after mucus samples were collected into the *in vitro* system. While the addition of agarose improved the longevity of anaerobe populations, *E. coli* growth was still higher than that seen in the mouse beyond day 6. The anaerobe populations also seemed to decline, albeit slowly, as time progressed beyond day 6. It was possible that this was due to *E. coli* being added to the cecal mucus preparations *in vitro* and not *in vivo*. In order to alleviate this, *E. coli* was fed to mice and allowed to colonize for 3 days prior to collection of the microbiota. This allowed the *E. coli* to form their relationships with their preferred anaerobes in the natural environment of these organisms. When *E. coli* was precolonized in mice prior to collection of the microbiota, anaerobe populations remained in high number and diversity through day 7 and beyond (Fig. 5, 6). Additionally, *E. coli* populations no longer grew to take over as majority members of the sample (Fig. 5, 6).

Testing the necessity of the individual additives in the in vitro system

Once the anaerobe populations were surviving beyond day 7 and the *E. coli* population was kept in check, it was necessary to test if all of the components within the *in vitro* system were actually involved in maintaining said populations. To

accomplish this, growth *in vitro* was tested after the removal of a single component in parallel with a sample containing every component. In samples *sans* Cleland's reagent or mucin the anaerobe populations were lost within 1-2 days and the *E. coli* population grew to 10^8 CFU/ml (Fig. 7). Without mucus, both the anaerobes and *E. coli* were lost (Fig. 8).

Can the concentration of mucus be adjusted in order to control the E. coli population?

With the *in vitro* system becoming more similar to the mouse gut, an attempt to control the *E. coli* population by adjusting the mucus concentration was tested. Several concentrations were tested: no mucus, 0.2mg/ml mucus, and 2mg/ml mucus (Fig. 8). While 10-fold less mucus (0.2mg/ml) showed no noticeable decline in the *E. coli* population, the anaerobe population suffered considerably (Fig. 8). Even without any added mucus the *E. coli* was able to maintain itself to some degree (1×10^7 CFU/ml) (Fig. 8), likely due to the mucin not being completely purified of mono- and disaccharides.

Comparing diversity of in vivo and in vitro samples

To better compare the diversity of the *in vitro* system to what is seen *in vivo*, samples were taken from the *in vitro* Nissle 1917 pre-fed experiments after 7 days and used for community analysis sequencing. The pre-fed experiments were those in which mice were first colonized with Nissle 1917 for 3 days prior to collecting into the *in vitro* system. Community analysis of the *in vitro* samples at day 7 shows some diversity amongst major members of the population at the family level (Fig. 9). A stacked bar plot was generated to compare the diversity in individual experiments

(Fig. 10). The *in vivo* and *in vitro* samples of Nissle 1917 have similar diversity with representation from the same set of families except Deferribacteraceae. However, the *in vitro* samples do have Deferribacteraceae at low levels, and are statistically similar when compared to the *in vivo* samples in terms of Deferribacteraceae using the student's t-test ($P > 0.05$). At the phylum level, Bacteroidetes and Firmicutes are comparable to what is seen *in vivo*, but Deferribacteres has little representation (Fig. 11, Fig. 12). Again, the difference in levels of Deferribacteres are not statistically significant between the *in vivo* and *in vitro* samples ($P > 0.05$).

In order to see if the overall population was similar in terms of overall diversity between the *in vitro* and the *in vivo* methods, a crude rarefaction curve was generated for select samples of each experimental type. When comparing 3 samples of *E. coli* Nissle 1917 *in vitro* to 3 samples of *E. coli* Nissle 1917 *in vivo* at the family level, both experimental types have similar levels of diversity (Fig. 13). This similarity in diversity when comparing the entire population, as opposed to the most abundant organisms within the population, suggests that the *in vitro* system is truly near the mouse gut in terms of community structure.

Using conventional mice for the in vitro system

While streptomycin treatment is important for testing the colonization ability of *E. coli*, cultivating the unaltered gut microbiome *in vitro* may be of more interest. As such, mice that were not treated with streptomycin (thus still containing the natural Proteobacteria) were killed and their mucus was collected for the *in vitro* system. Slides were prepared with FISH and images were taken for 7 days. Through day 7, *in vitro* samples showed diversity in cell morphology and *E. coli* labeled cells were seen

within mixed biofilms (Fig. 14). Community analysis of the samples at day 7 *in vitro* showed diversity amongst major members of the population at the family level (Fig. 15, Fig. 16), with members present within the *in vitro* system being comparable to those seen in *in vivo* samples, although differences between the *in vitro* and *in vivo* samples were seen in the Bacteroidaceae ($P < 0.0001$), Deferribacteraceae ($P < 0.001$), and Lachnospiraceae ($P < 0.001$). At the phylum level, Bacteroidetes were more abundant relative to Firmicutes in the conventional *in vitro* samples compared to what was seen in the *in vivo* samples, and Deferribacteres had little representation *in vitro* (Fig. 17, Fig. 18). The differences seen between each phylum; Bacteroidetes, Deferribacteres, and Firmicutes, are statistically significant ($P < 0.001$ for each). The loss of Firmicutes can be traced to the Lachnospiraceae family, which has a high abundance in the samples taken directly from mice but is considerably lower in the *in vitro* samples ($P < 0.01$) (Fig. 15).

In order to see if the overall population was similar in terms of overall diversity between the *in vitro* and the *in vivo* methods, a crude rarefaction curve was generated for select samples of each experimental type. When comparing 3 conventional *in vitro* samples to 3 conventional *in vivo* samples at the family level, both experimental types have similar levels of diversity (Fig. 19). It is also apparent that conventional samples are more diverse than Nissle 1917 samples, likely due to the treatment of streptomycin prior to colonization. This similarity in diversity when comparing the entire population, as opposed to the most abundant organisms within the population, suggests that the *in vitro* system is truly near the mouse gut in terms of community structure.

Testing the ability of pre-colonized strains to prevent later colonization with the same strain

When mice are pre-colonized with an *E. coli* strain, they are resistant to subsequent intestinal colonization by the same strain (2, 27). This is likely because the niches usually taken by these *E. coli* strains are already filled by the preexisting population. To test if this is true in the *in vitro* system, mice were pre-colonized with either Nissle 1917 or MG1655 and then collected into the *in vitro* system after 3 days of colonization. After 7 days in the *in vitro* system, the identical strain was spiked in at 10^3 CFU/ml (Nissle 1917 for Nissle 1917, and MG1655 for MG1655). As expected, pre-colonized Nissle 1917 prevented the late fed Nissle 1917 from growing up (Fig. 20), and pre-colonized MG1655 prevented late fed MG1655 from growing to higher numbers (Fig. 21).

Testing the colonization ability of Nissle vs MG1655 in vitro

Once the populations were maintained, it was important to test if the *in vitro* system was truly mimicking the mouse gut. To do this, competition between *E. coli* strains that had been previously tested in mouse colonization experiments were used. Mice were initially inoculated with the Nissle 1917 for 3 days, and then the microbiota was collected into the *in vitro* system. The populations were sustained for 7 days, and then MG1655 was spiked in at 10^5 CFU/ml. *In vivo*, mice fed Nissle 1917 early and MG1655 late show no direct competition between the strains, i.e. as Nissle 1917 and MG1655 can outcompete one another on one or more sugars, they are able to co-colonize *in vivo* without issue (17). *In vitro*, MG1655 was not able to co-colonize

with Nissle 1917, and dropped below detectable numbers 4 days after being spiked in (Fig. 22). However, the reverse showed more promising results. When MG1655 is fed early and Nissle 1917 fed late in mice, they are able to co-colonize as they are not in direct competition (17). This proved to be true *in vitro* as well, when MG1655 was fed early to mice before collection and Nissle 1917 was spiked in at day 7 *in vitro* (Fig. 23).

In the presence of E. coli Nissle 1917, E. coli EDL933 uses gluconeogenesis to colonize the in vitro system as it does in vivo.

In vivo, EDL933 uses gluconeogenesis when co-colonized with Nissle 1917 in order to maintain itself within the gut (51). This is known because when a mouse is pre-colonized with Nissle 1917 for 10 days and then fed 10^5 CFU each of EDL933 wildtype and EDL933 *ppsA pckA* (a mutant unable to grow on gluconeogenic nutrients “below” phosphoenolpyruvate) the mutant EDL933 is unable to compete and falls to undetectable numbers (51).

In order to test if the *in vitro* system mimicked the mouse gut, this experiment was repeated *in vitro*. To do this, Nissle 1917 was pre-colonized in mice at 10^5 CFU/ml for 10 days, and EDL933 and EDL933 *ppsA- pckA-* were spiked into the *in vitro* system at 10^3 CFU/ml one day after collection. EDL933 wildtype and the EDL933 *ppsA- pckA-* mutant were spiked at 10^3 CFU/ml because they were spiked into a fresh dilution of the microbiota, which would leave Nissle 1917 at approximately 10^7 CFU/ml. This would give a difference of 10^4 CFU/ml, the same difference when fed late to mice. When the *in vitro* experiment was performed with Nissle 1917 pre-colonized for 10 days after which the wildtype EDL933 and the

EDL933 *ppsA- pckA-* mutant were spiked into the *in vitro* system one day after collection, it mimicked the mouse experiments (Fig. 24), i.e Nissle 1917 colonized at approximately 10^8 CFU/ml, EDL933 wildtype maintained itself at approximately 10^4 CFU/ml, and EDL933 *ppsA- pckA-* fell below detectable numbers by day 4.

EDL933 vs. EDL933 ppsA-pckA-

In mice, EDL933 and EDL933 *ppsA-pckA-* can co-colonize equally well in the absence of Nissle 1917 (51), suggesting that in the absence of Nissle 1917, EDL933 is not forced to use gluconeogenic substrates in order to compete and thus the non-gluconeogenic mutant is not at a disadvantage. This was tested in the *in vitro* system by collecting mucus from streptomycin treated mice for collection into the *in vitro* solution, and EDL933 and EDL933 *ppsA- pckA-* were spiked into the *in vitro* system at 10^3 CFU/ml 1 day after collection. Initially, both strains were able to grow to similar counts at approximately 10^7 CFU/ml, but eventually the EDL933 *ppsA- pckA-* mutant began to drop. Unlike in mice, the EDL933 and EDL933 *ppsA- pckA-* were unable to co-colonize, with the EDL933 *ppsA- pckA-* mutant falling below detectable numbers after 10 days *in vitro* (Fig. 25).

Discussion

In this study, an *in vitro* method for cultivating the microbiota of the mammalian gut was developed. This method will allow us to study the relationship between *E. coli* and the rest of the gut microbiota with more control and accessibility than in *in vivo* models, such as the streptomycin treated mouse model. While other *in vitro* methods have been developed to study biofilms from various environments, this

novel method was designed specifically for the gut microbiota, and it was kept as simple and user friendly as possible.

During the development of this *in vitro* method, samples of growth from the *in vitro* system were submitted for community analysis sequencing of the microbial community. These samples were taken from day 7 *in vitro* samples of Nissle 1917 and another set of samples in which the cecal mucus of untreated mice (not colonized with *E. coli* or treated with streptomycin) were collected into the *in vitro* system (labeled “conventional”). The *in vitro* samples were compared to *in vivo* samples prepared similarly, by either pre-colonizing mice with Nissle 1917 for 10 days, at which point the mice were killed and mucus was collected, or *in vivo* samples that were collected from untreated (conventional) mice.

By comparing the Nissle 1917 *in vivo* and *in vitro* samples at the phylum level it can be seen that a similar spread of major members of the population can survive within the *in vitro* system, but a loss in the relative abundance of certain organisms occurs (Fig. 11, Fig. 12). While the abundance of Firmicutes and Bacteroidetes are comparable between the *in vivo* and *in vitro* methods, Deferribacteres seems to have trouble surviving the *in vitro* system. Deferribacteres is not completely lost, as it is present at lower levels in these samples but still exists.

At the family level, both Nissle 1917 *in vitro* and *in vivo* samples have representatives from the same families (Fig. 9, Fig. 10). Deferribacteriaceae has relatively low levels of representation in the *in vitro* system, but again is still present. However, the loss of relative abundance of these major members does not coincide

with an overall loss of diversity as shown by (Fig. 13) with both *in vivo* and *in vitro* samples of Nissle 1917 showing similar levels of diversity.

When comparing phyla of the conventional samples, *in vitro* to *in vivo*, a similar loss of Deferribacteres is seen (Fig. 17). Again, this loss is not a complete clearing of Deferribacteres but rather a loss in the relative abundance of the organism. The diversity of major members at the family level is much higher in the conventional samples (*in vitro* and *in vivo*), with a larger spread across various families than that seen in the Nissle 1917 samples (Fig. 15). Furthermore, the diversity of the individual conventional samples was much more robust than the Nissle 1917 samples both *in vivo* and *in vitro*, showing higher diversity in a crude rarefaction curve (Fig. 19). This shows that the conventional samples *in vitro* are truly diverse, and also mimic the *in vivo* conventional samples in level of diversity.

Deferribacteres is a relatively recently described lineage, but one genus of Deferribacteres, *Mucispirillum*, has been identified as existing in the mammalian gut and being able to degrade mucin (22, 49). Interestingly, Deferribacteres were lower in many of the *in vitro* samples, possibly due to the low concentration of mucin which *Mucispirillum* utilizes for growth. Lachnospiraceae is a member of the Firmicutes that is able to produce butyric acid and has been previously linked to obesity (36). Ruminococcaceae, another Firmicutes, has been previously shown to exist in the gut, but is difficult to classify and is often a mistakenly identified Lachnospiraceae (31). Paenibacillaceae is a Firmicutes with species that exist as plant growth promoting rhizobacteria and may have been introduced through the feed given to mice (5). The s24-7 family is a member of the Bacteroidetes that has been found to inhabit the

mouse gut, but little else has been described (19). Another bacteroidetes, Porphyromonadaceae, has been previously shown to occasionally inhabit the gut, but more often resides in oral cavities as a pathogen (40). Rikenellaceae is another Bacteroidetes that has a recently reclassified member, *Alistipes putredinis* which was previously classified as a *Bacteroides* and shows similarities with *Bacteroides fragilis* and members of Porphyromonadaceae (48). Bacteroidaceae contains the *Bacteroides* genus, which can use simple sugars when available, but use glycans as a main source of energy (33).

In order to show that the novel *in vitro* system could mimic the mouse in terms of colonization ability, a series of tests were done using the new *in vitro* system in parallel to those done with mice. Early attempts at comparing *in vivo* colonization experiments to those done *in vitro* proved that the system was not quite identical to the mouse, but similarities were present. When mice are pre-colonized with an *E. coli* strain, they are resistant to subsequent intestinal colonization by the same strain (2, 27). This was tested in the *in vitro* method with Nissle 1917 and MG1655, and this colonization resistance was also seen in the *in vitro* system (Fig. 20, Fig. 21). However, it was previously shown that colonization by a different strain would be possible, so long as the pre-fed strain and the new strain were not in direct competition for sugars (17). This was again tested *in vitro* using Nissle 1917 and MG1655. When MG1655 was pre-fed and Nissle 1917 was added late, Nissle 1917 was able to grow up with MG1655 as is seen in mice (Fig. 23). In contrast, when Nissle 1917 was pre-fed and MG1655 was added late, MG1655 was unable to grow up against Nissle 1917 and was eventually washed out of the system (Fig. 22). This may be due to the system

developing early with Nissle 1917, thus promoting an anaerobe population that is more preferential for the growth of Nissle 1917. If said population only produced mono- and disaccharides that resulted in Nissle 1917 and MG1655 growing in direct competition, with Nissle 1917 being able to grow faster on the supplied sugars, MG1655 would be unable to colonize and would eventually fall below detectable numbers. It is also possible that Nissle 1917 may use up all the nutrients *in vitro*, but not *in vivo*, as nutrients are likely more limited in the *in vitro* system. Another possibility is that the flow through of the gut is important for either directly keeping the population of Nissle 1917 in check, or for keeping certain key anaerobes in check that give Nissle 1917 a competitive edge over MG1655. This same effect could be carried out by the innate immune system of the gut. Considering that when MG1655 was pre-fed and Nissle 1917 was added late both were able to grow and colonize equally well, it is unlikely to be due to population control via flow or the innate immune system. It is important to note that as these experiments were done early in the development of the system, mice were only pre-colonized for 3 days prior to being moved into the *in vitro* system, and then allowed a further 7 days for the pre-colonized *E. coli* strain to settle before spiking in the competing strain. Perhaps allowing the pre-colonized strain to remain in mice for the full 10 days prior to collection into the *in vitro* system could improve these colonizations.

Another competition chosen was Nissle 1917 fed early against EDL 933 wildtype and EDL933 *ppsA*⁻ *pckA*⁻. EDL933 is an enterohemorrhagic *E. coli* (EHEC), and the mutant EDL933 *ppsA*⁻ *pckA*⁻ is unable to utilize gluconeogenic substrates (51). In mice, Nissle 1917 keeps EDL933 wildtype from colonizing higher

than 10^6 CFU/gfeces and EDL933 *ppsA*⁻ *pckA*⁻ is unable to colonize, falling below detectable numbers after only a few days (51). In the mice, late fed strains are fed 10 days after the pre-colonizing strain. To better mimic this, an experiment was done in which the pre-colonizing strain was allowed to remain in the mice for 10 days prior to collecting into the *in vitro* system. In this experiment, Nissle 1917 was fed to the mice while the EDL 933 and EDL 933 *ppsA*⁻ *pckA*⁻ strains were added later to the *in vitro* system. This experiment mimicked the mouse experiment, showing that the *in vitro* method was beginning to more mimic the mouse (Fig. 24).

A final experiment was done to see how closely the *in vitro* system mimicked the mouse gut in terms of colonization ability. In the absence of Nissle 1917, EDL933 and EDL933 *ppsA*⁻ *pckA*⁻ are able to colonize equally well when initially colonized at the same concentration (51). This was tested in the *in vitro* system by collecting mucus from streptomycin treated mice and spiking EDL933 and EDL933 *ppsA*⁻ *pckA*⁻ into the collection solution. While the experiment went well early on, with both strains growing to 10^7 CFU/ml, an eventual loss in EDL933 *ppsA*⁻ *pckA*⁻ shows that the system is not yet perfect (Fig. 25). It is likely that the lower concentration of mucin and mucus in the *in vitro* system results in less mono- and disaccharides being made available by the anaerobe population for the EDL933 and EDL933 *ppsA*⁻ *pckA*⁻ strains to grow without the use of gluconeogenic substrates. Through limitation of glycolytic substrates, this forced gluconeogenic shift would give the wildtype EDL933 a distinct advantage over EDL933 *ppsA*⁻ *pckA*⁻, as the mutant cannot use gluconeogenesis.

At this point the novel *in vitro* model designed to mimic the mouse gut is capable of cultivating the microbiota of the gut, but is only able to mimic certain colonization experiments of *E. coli* competitions. In terms of mimicking the diversity of the gut, only the relative abundance of certain members of the community is affected by growth in the *in vitro* system. This may be overcome by slight alterations to the system, such as an increase in the concentrations of mucin and/or mucus in order to provide more nutrients to these populations. This may also have an effect on the colonization experiments, as the key factor in these competitions is the availability of specific substrates within specific niches. Even continued streptomycin treatment within the *in vitro* system must be considered, as at this point streptomycin treatment stops once removed from the mouse. The streptomycin may be important for maintaining a clear niche for some of these competing strains to move into, thus resulting in the loss of a competitive advantage by not continuing streptomycin treatment within the *in vitro* system. Furthermore, the early competitions involving Nissle 1917 and MG1655 may be improved if the pre-colonizing strain is allowed to remain in mice for a full 10 days prior to collection. The innate host immune system may also be key in maintaining a balanced ecosystem within the gut (10, 13, 44, 54), and as such may be important for the development of available niches to colonizing *E. coli* strains. Thus, while the system is useful for observing the microbial community of the mouse gut and the interactions with a single colonizing *E. coli* strain, the novel *in vitro* gut model must be further improved in order to assess colonization ability of individual *E. coli* strains.

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Table 1. Bacterial strains

<u><i>E. coli</i> strain</u>	<u>Genotype/phenotype</u>	<u>Designation in text</u>	<u>Source or reference</u>
MG1655 Str ^R	Spontaneous streptomycin resistant mutant of MG1655	MG1655	6
Nissle 1917 Str ^R Nal ^R	Spontaneous naladixic acid resistant mutant of Nissle 1917 Str ^R	Nissle 1917	24, 44
EDL933 Str ^R Rif ^R	Spontaneous streptomycin resistant mutant of EDL933	EDL933	38
EDL933 Str ^R $\Delta ppsA \Delta pckA::cam$	Double deletion mutant of EDL933 Str ^r , carrying <i>cat</i> in the <i>pckA</i> deletion and retaining the pO157 virulence plasmid	EDL933 <i>ppsA</i> - <i>pckA</i> - mutant	42, 32

Table 2. Primers

<u>Primer designation</u>	<u>Sequence</u>
<i>E.coli</i> specific Cy-3 probe (red) used for FISH	5’-/5Cy3/CACCGTAGTGCCTCGTCATCA-3’
Eubacteria FitC probe (green) used for FISH	5’-GCTGCCTCCCGTAGGAGT/36-FAM/-3’
16S V4 forward used for community analysis sequencing	5’-TCG TCG GCA GCG TCA GAT GTG TAT AAG A-3’
16S V4 reverse used for community analysis sequencing	5’-GTC TCG TGG GCT CGG AGA TGT GTA TAA G-3’

Fig. 1 FISH images of the early *in vitro* system with (top) and without (bottom) *E. coli* taken from day 0 samples. Both populations are intact. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig. 2 FISH images of the early *in vitro* system with (top) and without (bottom) *E. coli* taken from day 3 samples. When *E. coli* is present, it grows out of proportion and takes over the system, and in both systems the rest of the population is mostly lost. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig. 3 FISH images of the *in vitro* system with mucin concentration increased to 500ug/ml taken from day 5 (top) and day 6 (bottom) samples. While increasing the mucin concentration improved the longevity of the system, most of the population is still lost beyond day 5. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig. 4 FISH images of the *in vitro* system after adding 0.3% agarose without *E. coli* taken from day 6 samples (top) and before adding agarose taken from day 6 samples (bottom). Even without the *E. coli* present, agarose has improved the survivability of the population within the *in vitro* system. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FITC-probe.

Fig. 5 FISH image taken from *in vitro* experiment in which mice were precolonized with *E. coli* for 3 days prior to collection into the *in vitro* system, taken from day 7 *in vitro* samples. The *E. coli* is no longer growing to take over the entire population, and the rest of the organisms are able to survive and form biofilm communities. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig. 6 Comparison of a FISH imaged biofilm of *in vivo* samples from a mouse colonized with Nissle 1917 for 10 days (left) and *in vitro* samples in which a mouse was precolonized with Nissle 1917 for 3 days prior to being collected into the *in vitro* system, taken from day 7 *in vitro* samples (right).

Fig. 7 FISH images taken from *in vitro* samples on day 7 in which either no mucin (above) or no Cleland's reagent (below) was added. These images show the importance of the mucin and Cleland's reagent in preserving the community within the *in vitro* system. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig. 8 7 FISH images taken from *in vitro* samples on day 7 in which either no mucus (above) or only 0.2 mg/ml mucus (below) was added. Decreasing the mucus concentration results in a loss of a majority of the population, with *E. coli* taking over as the majority of surviving cells. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig. 9 Bar plot showing relative abundance of major families in mice colonized with Nissle 1917 for 10 days (green) and *in vitro* samples in which mice were precolonized with *E. coli* for 3 days prior to collection into the *in vitro* system, taken from day 7 *in vitro* samples. While there seems to be a difference in terms of s24-7 and Deferribacteraceae, the differences are not statistically relevant and both mice and *in vitro* samples have similar levels of representation from the same major families. Error bars indicate standard error of the mean.

Fig. 10 Stacked bar plot showing relative abundance of Nissle 1917 *in vivo* (left) and *in vitro* (right) at the family level. The stacked bars are showing the same data as is seen in the relative abundance graphs, but separates the individual samples to better highlight the variation amongst hosts.

Fig. 11 Bar plot showing relative abundance of major phyla in mice colonized with Nissle 1917 for 10 days (green) and *in vitro* samples in which mice were precolonized with *E. coli* for 3 days prior to collection into the *in vitro* system, taken from day 7 *in vitro* samples. None of the differences seen are enough to be statistically significant ($P > 0.05$). Error bars indicate standard error of the mean.

Fig. 12 Stacked bar plot showing relative abundance of Nissle 1917 *in vivo* (left) and *in vitro* (right) at the phylum level. The stacked bars are showing the same data as is seen in the relative abundance graphs, but separates the individual samples to better highlight the variation amongst hosts.

Fig. 13 Crude rarefaction curves of Nissle 1917 samples used to compare richness of diversity *in vitro* (top) and *in vivo* (bottom).

Fig. 14 FISH image of an *in vitro* sample in which the cecal mucus of an untreated “conventional” mouse was collected into the *in vitro* system, taken from day 7 *in vitro* sample. Diversity in the cell morphologies can be seen amongst the organisms present. Red bacterial cells represent *E. coli* labeled by the Cy3-probe, while green bacterial cells represent all Eubacteria labeled by the FitC-probe.

Fig. 15 Bar plot showing relative abundance of major families in untreated “conventional” mice (white) and *in vitro* samples in which untreated “conventional” mice were collected into the *in vitro* system (blue), taken from day 7 *in vitro* samples. Error bars indicate standard error of the mean. Differences between the *in vitro* and *in vivo* samples were seen in the Bacteroidaceae ($P < 0.0001$), Deferribacteraceae ($P < 0.001$), and Lachnospiraceae ($P < 0.001$).

Fig. 16 Stacked bar plot showing relative abundance of conventional *in vivo* (left) and *in vitro* (right) at the family level. The stacked bars are showing the same data as is seen in the relative abundance graphs, but separates the individual samples to better highlight the variation amongst hosts.

Fig. 17 Bar plot showing relative abundance of major phyla in untreated mice (white) and *in vitro* samples in which untreated mice collected into the *in vitro* system (blue), taken from day 7 *in vitro* samples. Error bars indicate standard error of the mean. At the phylum level, Bacteroidetes were more abundant relative to Firmicutes in the conventional *in vitro* samples compared to what was seen in the *in vivo* samples, and Deferribacteres had little representation *in vitro*. The differences seen between each phylum; Bacteroidetes, Deferribacteres, and Firmicutes, are statistically significant ($P < 0.001$ for each).

Fig. 18 Stacked bar plot showing relative abundance of conventional *in vivo* (left) and *in vitro* (right) at the phylum level. The stacked bars are showing the same data as is seen in the relative abundance graphs, but separates the individual samples to better highlight the variation amongst hosts.

Fig. 19 Crude rarefaction curves of conventional samples used to compare richness of diversity *in vitro* (top) and *in vivo* (bottom).

Fig. 20 Competition experiments of Nissle 1917 early vs Nissle 1917 late: *in vivo* (top) and *in vitro* (bottom). This shows that colonization resistance is seen *in vitro* just as it is seen in mice.

Fig. 21 Competition experiments of MG1655 early vs MG1655 late: *in vivo* (top) and *in vitro* (bottom). This shows that colonization resistance is seen *in vitro* just as it is seen in mice.

Fig. 22 Competition experiments of Nissle 1917 early vs MG1655 late: *in vivo* (top) and *in vitro* (bottom). This shows that not all competitions are mimicked perfectly in the *in vitro* system, possibly due to limitation of nutrients.

Fig. 23 Competition experiments of MG1655 early vs Nissle 1917 late: *in vivo* (top) and *in vitro* (bottom). This shows that competitions in which MG1655 is fed early and Nissle 1917 is fed or spiked in late are similar in mice and *in vitro*.

Fig. 24 Competition experiments of Nissle 1917 early vs EDL933 wildtype and EDL933 *pckA-ppsA-* late: *in vivo* (top) and *in vitro* (bottom). Another competition in which the *in vitro* system mimics what is seen in mice, as the EDL933 gluconeogenic mutant cannot compete and falls out of the system while the wildtype is maintained.

Fig. 25 Competition experiments of EDL933 wildtype vs EDL933 *pckA-ppsA-*: *in vivo* (top) and *in vitro* (bottom). This competition shows that the *in vitro* system is unable to mimic the mouse perfectly as EDL933 does not switch to gluconeogenic substrates in mice that were not pre-colonized with Nissle 1917, but does switch in the *in vitro* system. Again likely due to limitation of nutrients.