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Interpersonal Gut Microbiome Variation Drives Susceptibility and Resistance to Cholera Infection

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The text of chapter two is a reprint of the material as it appears in "Interpersonal Gut Microbiome Variation Drives Susceptibility and Resistance to Cholera Infection" in Cell, 2020. Salma Alavi, Jonathan Mitchel, John Macbeth., Rui Lu, and Jennifer Cho performed the experiments. Ansel Hsiao directed and supervised the research which forms the basis for this dissertation.

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Dedication

For my loved ones, Liana and Amir Hossein.

ABSTRACT OF THE DISSERTATION

Interpersonal Gut Microbiome Variation Drives Susceptibility and Resistance to Cholera Infection

by

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Vibrio cholerae causes the diarrheal disease cholera that affects millions of people across the globe and causes over 100,000 deaths yearly. *V. cholerae* must out-compete the commensal gut microbial community (the gut microbiome) to establish itself in the gut and cause disease. The major functions of normal gut microbiota include nutrient metabolism, nutrient absorption, drug metabolism, and contribute to gut immunomodulation. *V. cholerae* is a gram-negative bacteria and a major human GI pathogen, despite improvements in treatment and understanding of the mechanisms of pathogenesis, cholera remains a significant global health burden.

We describe here that during infection with *V. cholerae*, individuals with different gut microbiome response differently. We used suckling and adult germfree mice models to introduce different gut communities and examine gut microbiome structure during *V. cholerae* infection.

Next, we showed that the community which includes strains from healthy human is able to decrease cholera infection. Then we identified *Vibrio*-antagonistic microbes across many different microbiome contexts by setting up combinatorial experiment.

Second, we explored how healthy community causes colonization resistance. We showed that this is mediated through the bile salt hydrolase enzyme activity which degrades the bile salt taurocholate that activates the expression of virulence genes.

In this dissertation, we used the transplantation of complete human fecal microbiomes and defined communities in both germfree adult mice and suckling antibiotic treated mice models to demonstrate that the variation in gut microbiome can result in different *V. cholerae* colonization outcomes.

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CHAPTER ONE: Introduction

Introduction

Gut microbiome

The mammalian gut is inhabited by a dense and diverse commensal microbial community, the gut microbiome. These microbes play important roles in many aspects of host biology, for example in host nutrition metabolism, drug metabolism, immunomodulation, and resistance to colonization by pathogenic organisms (Shreiner et al., 2015). This microbial community varies greatly from individual to individual in response to diet, microbial exposure, and numerous other factors such as using antibiotics (Stark and Lee, 1982) and develops with its human hosts in a process known as succession. Antibiotics have an important role on the gut microbiome because they can change the nutritional environment in the gut and transfer the resistance genes which results in increase of pathogenic strains in the gut. Milestones in gut microbiome development coincide with major transitions in diet, such as weaning and beginning of solid food consumption; diet is also a primary driver for microbial community structure in fully mature microbiota in adults (De et al., 2010). The composition of a mature gut microbiota is the two major phyla; Bacteroidetes and Firmicutes. The importance of the intestinal microbiota in human health is increasingly acknowledged, and there has been a surge in the number of published studies on the subject in recent years. Gut microbiota has a main role in beneficial factors which is associated with human health, it has been

shown that gut microbiota can also be the main reason for human diseases (Jandhyala et al., 2015).

A key role of the gut microbiome in human health is prevention of colonization and invasion by bacterial enteropathogens. The mechanisms of this colonization resistance are still poorly understood, but in the case of several major human diarrheal pathogens, resident bacteria provide colonization resistance by outcompeting invaders for niche space and resources and shaping the biochemical environment of the gut to disrupt virulence gene expression patterns in pathogens (Buffie and Pamer, 2013). Diarrheal diseases caused by pathogenic bacteria are a major threat to human health (Havelaar et al., 2009). However, we do not fully understand how microbiota structure is related to disease susceptibility, and how best to manipulate the microbiota to boost infection resistance.

There are different methods to study gut microbiota. Recently, thanks to Bioinformatics approaches and genome sequencing, researchers are able to study gut microbiome functions and its interaction with pathogens. The other way is stool collection and isolate the DNA from stool to study the gastrointestinal microorganisms (Jandhyala et al., 2015). Culture based techniques are the other approach to isolate and identify the gut microbiome but there are some limitations, it's difficult to study the multiple different colonies on the petri dish and it's also very time consuming (Jandhyala et al., 2015).

The importance of gut microbes to disease prevention can be demonstrated through the effects of microbiome disruption by antibiotic treatment. Antibiotic-

associated diarrhea occurs in 5%–25% of patients treated with antibiotics and causes thousands of deaths and billions of dollars of additional costs to the health care industry each year (Bergogne-Berezin, 2000). The general mechanism behind the pathogenesis of these diseases is well understood: broad-spectrum antibiotics deplete the commensal gastrointestinal (GI) microbiota and allow pathogens to colonize and proliferate (Högenauer et al., 1998). However, we are only beginning to describe the mechanisms by which normal gut microbiomes prevent and resolve infections. Gut dysbiosis can be defined as a diversion from the normal microbial ecosystem, and can be caused by poor diet, disease, and antibiotic usage in particular. The loss of key functions provided by the gut microbiome can shift the gastrointestinal environment into a state that is more susceptible to pathogenic bacteria.

Gut microbiome and *Vibrio cholerae*

Vibrio cholerae, the etiologic agent of the severe diarrheal disease cholera, is a major human bacterial GI pathogen (Ali et al., 2015). Although antibiotics and oral rehydration therapy (Ahmed et al., 2016) have been able to dramatically reduce the case fatality rate of cholera, it still imposes an extreme burden of morbidity, as the toxin responsible for diarrhea is internalized into host cells; diarrhea continues after the elimination of *V. cholerae* from the gut. A recent paper using gnotobiotic mice demonstrated that a healthy human gut microbiome was indeed resistant to colonization (Hsiao et al., 2014). A high-resolution time series analysis of the human gut microbiome during cholera, along with results from healthy children and adults, was used to identify

several commensal species associated with recovery from cholera diarrhea. By assembling model human gut microbiomes in germ-free mice resembling the gut microbiomes of healthy and recovering individuals, the authors were able to show that a complete healthy human gut flora is able to resist *V. cholerae* colonization. The authors further demonstrated that microbiome-mediated colonization resistance was strongly associated with a single commensal species, *Ruminococcus obeum*, which was able to reduce colonization by *V. cholerae* when co-colonized with the pathogen alone in germ-free mice (Hsiao et al., 2014).

The mechanism of *R. obeum*-mediated resistance to *V. cholerae* was shown to be through the production of an interspecies bacterial quorum sensing molecule, autoinducer-2 (AI-2), which prevented the proper activation of *V. cholerae* virulence genes during early infection. Many species of bacteria use quorum sensing, a mechanism involving the secretion and detection of diffusible signals known as autoinducers, to enable synchronized behaviors across a bacterial population. *V. cholerae* uses both a species-specific cholera autoinducer-I (CAI-I) and AI-2 to negatively regulate virulence (Chen et al., 2002, Miller et al., 2002). During a normal *V. cholerae* infection, autoinducer levels reach high density late in infection with the expansion of Vibrios in the gut, repressing virulence gene expression and promoting dissemination from the host. The production of microbiome-derived AI-2 short-circuits this process, limiting infection by interfering with virulence gene activation during early colonization. This represents the first in vivo evidence of natural AI-2 signaling between a pathogen and gut commensal microbes affecting pathogenesis. Previously, the importance of proper

regulation of virulence and quorum sensing was demonstrated by Duan, et al, who introduced into infant mice an Escherichia coli engineered to produce CAI-1, which increased survival of animals when infected with *V. cholerae* (Duan et al., 2010). The diversity of non-species-specific quorum sensing signals in complex gut microbiomes, and how these signals interact to modulate pathogen gene regulation, is only beginning to be explored.

What is increasingly clear is that not only is the presence of a gut microbiome important for colonization resistance against pathogens, but the composition of the gut microbiome is important to its ability to prevent infection. Thus, given the diversity of microbes from individual to individual, the gut microbiome may serve as a personalized target for therapeutic intervention against or prevention of infection by enteric pathogens.

The expansion of high-throughput sequencing has propelled research in the role of the gut microbiome structure in human and animal health. While most studies on the role of commensals in colonization resistance to pathogens to date have focused on animal communities, there is an increasing understanding of the species diversity of the gut microbiome across individuals healthy and diseased human contexts. Recent advances in anaerobic microbiological techniques to isolate previously unculturable human gut commensals (Greub, 2012, Lagier et al., 2016) has presented the possibility of identifying specific microbes in *human* gut microbiomes that mediate colonization resistance, and the use of gnotobiotic animals to establish human gut microbiomes in *in vivo* experimental systems is also becoming more common (Turnbaugh et al., 2009, McNulty et al., 2011). Moving forward, researchers may take advantage of a combination

of these techniques to delineate both microbial and molecular actors in microbiome-mediated colonization resistance, disease resolution, and vaccine responses. If successful, such work would allow for new opportunities to identify personal disease risk in individuals, and an avenue for prophylaxis against enteropathogens through manipulation of the host commensal bacterial community.

Animal models to study *V. cholerae*

To study the interaction between *V. cholerae* and gut microbiome and the molecular mechanisms that causes the severe diarrheal disease of cholera, we need a tractable animal model. Studies of pathogen-microbiome interactions are limited by a lack of suitable animal models, a problem that is particularly acute for cholera research, as the pathogenicity differs between many animal models and humans. Several animal model systems have been used to study cholera so far. These models include guinea pigs (Freter, 1956), Rabbits (Dutta and Habbu, 1955), gnotobiotic adult mice (Miller et al., 1972; Nygren et al., 2009), fruit flies (Blow et al., 2005) and zebrafish (Runft et al., 2013), each has its own advantages and disadvantages.

The first animal model which could cause cholera-like diarrhea and death (disease model) and was susceptible to *V. cholerae* was developed in infant rabbits (Dutta and Habbu, 1955). The infant rabbit model of cholera is a non-surgical small animal model to study cholera and mimics human cholera (Abel and Waldor, 2016) but one of the limitations of this model is that the attachment of *V. cholerae* to the epithelial cells is different from the human system as the villi and crypts in infant rabbits are not fully developed.

Adult rabbit model systems do not have this problem as they have more evolved small intestine, but they are surgical model and specialized equipment and training are required for this model (Abel and Waldor, 2016).

There is also an association between *V. cholerae* and insects based on environmental studies. Previous study shows that the fruit fly, *Drosophila melanogaster* is susceptible to oral *V. cholerae* (Blow et al., 2005). In this study, they report that the fly dies if the cholera toxin is present and if they receive K^+ channel blocker along with *V. cholerae,* they can survive the death. This might serve as a good animal model to study *V. cholerae* but their microbiome is very different from humans and they do not have adaptive cellular immune system. So, all of this make them not really reliable model to for *V. cholerae* studies.

One of the low-cost invertebrate infection host animal systems for *V. cholerae* is *Caenorhabditis elegans.* Studies have shown that the two secreted factors of *V. cholerae*, the protease PrtV and the hemolysin HlyA, can cause death in *C. elegans* (Cinar et al., 2010; Vaitkevicius et al., 2006). By using *C. elegans* as an infection model, it is shown that HapR regulates the novel bacterial cytotoxin (MakA) which is a virulence factor and its function depends on the flagellin (Dongre et al., 2018). All these can be evidence that we can study *V. cholerae* accessory toxins in a genetically tractable manner in *C. elegans* which makes it a unique model for *V. cholerae.*

The other model we want to talk about here is zebrafish, *Danio rario*. As *V. cholera* is a natural inhabitant of aquatic ecosystems, this model system can be an ideal model to study *V. cholerae*. The endemic areas for *V. cholerae* are in Asia which is the

natural habitat for zebrafish and can tell us they have been in close contact with each other. *V. cholerae* strains can successfully colonize in the intestine of zebrafish after the exposure to the pathogen and the colonization is as same as the colonization in mammals (Runft et al., 2013). Zebrafish is also an easy animal model to do imaging studies of infection diseases because they have transparent embryos. Their infection results in diarrhea and can transfer the infection to the other fish (Nag et al., 2020).

Mice models are the main animal models to study *V. cholerae* which are used in our studies. The conventionally-reared suckling mouse model is the most widely used animal models for *V. cholerae*, this model is inexpensive, readily available, and reproduces much of the virulence gene expression profile seen in humans, though it cannot replicate watery diarrhea (Olivier et al., 2009). There is a limitation for doing research on individual human microbiomes effects on *V. cholerae* infection because these mice are raised conventionally, also their microbiome can out-compete the introduced human microbiomes (Seedorf et al., 2014).

The existing animal models (germ-free model) for cholera are expensive, hard to breed and maintain, and have different virulence gene expression compared to humans (Olivier et al., 2007; Olivier et al., 2009). But as there are no external factors that can control the activity and structure of the microbiome, germ-free model is a useful model to study microbial-interactions (Shimamura et al., 1981) and is suitable for time-course experiments because it is possible to keep them in clean isolators with clean food and track microbial-interactions over time. Though germ-free model is imperfect, microbial-interactions are better examined in adult germ-free (Shimamura et al., 1981). With germ-

free mice model we can control what microbes are present and study the effects of different microbiome on pathogen colonization, however this mice model is not the disease model for *V. cholerae* and they have different virulence gene expression in regard to *V. cholerae* compared to humans and infant animal models (Olivier et al., 2007; Olivier et al., 2009). Genotobiotic (either adult or suckling) animals are expensive and need large systems for breeding which is hard for many laboratories. Even though germ-free mice model has some limitations, microbial-interactions are better assayed in this model.

To address these issues, we have set up the new model by modifying the suckling mouse model; replacing the mouse microbiome with human microbiomes through antibiotic (streptomycin) treatment followed by gavage of cultured human microbes. This model would greatly improve the throughput of studying interactions between pathogens like *V. cholerae* and commensal microbes. This will improve the accessibility of models for studying how gut microbiomes influence bacterial infections of the gut.

Our goal of this dissertation is investigating the role of human gut microbiome on cholera infection. The morbidity of cholera disease is so high and even after treating with antibiotics and reducing fatalities, morbidity is still present and leads to severe social and economic stresses for affected countries, and these effects mainly influence children (Ali et al., 2012). Early childhood diarrhea has been associated with impaired growth and cognitive function years after disease (Lorntz et al., 2006), meaning that the economic and social effects of cholera is magnified and extended far beyond seasonal epidemics, it is really important to explore the prevention of *V. cholerae*. So, we tested if the

composition of the human gut microbiome can represent potential susceptibility and prevention factors for *V. cholerae* infection.

In chapter two we show that disrupted microbiomes are susceptible to *V. cholerae* and transplanting the resistant communities can transfer the same effect. Then we identified commensals that has the strongest effect on enteropathogens across many community contexts, we stablished an experimental pipeline for randomization of microbiome members, and we showed that commensal species *Blautia obeum* can suppress virulence. Using complete human fecal micorbiomes in suckling antibiotic treated mice revealed that variation in *V. cholerae* infection outcomes is based on inter-personal gut microbiome composition.

Chapter three shows that how we developed an easily accessible suckling antibiotic treated mice model to study the interaction between gut microbiome and *V. cholerae*.

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CHAPTER TWO - Interpersonal Gut Microbiome Variation Drives Susceptibility and Resistance to Cholera Infection

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Summary

The gut microbiome is the resident microbial community of the gastrointestinal tract. This community is highly diverse, but how microbial diversity confers resistance or susceptibility to intestinal pathogens is poorly understood. Using transplantation of human microbiomes into several animal models of infection, we show that key microbiome species shape the chemical environment of the gut through the activity of the enzyme bile salt hydrolase. The activity of this enzyme reduced colonization by the major human diarrheal pathogen *Vibrio cholerae* by degrading the bile salt taurocholate that activates the expression of virulence genes. The absence of these functions and species permits increased infection loads on a personal microbiome-specific basis. These findings suggest new targets for individualized preventative strategies of *V. cholerae* infection through modulating the structure and function of the gut microbiome.

Introduction

Gastrointestinal infections represent a major global health concern. One major human diarrheal pathogen is *Vibrio cholerae*, the etiologic agent of the severe disease cholera that affects millions of people annually (Clemens et al., 2017). *V. cholerae* cycles between aquatic reservoirs and the small intestine, requiring the coordinated regulation of environmental fitness genes versus virulence genes including the attachment factor Toxin Co-regulated Pilus (TCP) and Cholera Toxin (CT) (Herrington et al., 1988; Miller et al., 1987). In this and other pathogens, regulation depends on the chemical state of the gut, shaped by the gut microbiome, the dense resident gut microbial community (Eckburg et al., 2005) that varies dramatically across host species and across individuals as a function of diet, geography, and environmental insults (Yatsunenko et al., 2012). In cholera-endemic areas, the gut microbiome is subject to mutually-reinforcing pressures: malnutrition, leading to reduced host infection resistance, repeated diarrhea, and poorly-controlled antimicrobial usage in an attempt to mitigate the resulting sequelae. Previous 16S ribosomal gene studies of the gut microbiome in these areas demonstrate that these factors are able to drive the gut microbiome into a characteristic dysbiotic state, dominated by Streptococci such as *Streptococcus salivarius*, Enterococci, and Enterobacteriaceae. This configuration has been shown to be inducible by malnutrition (Subramanian et al., 2014), and diarrhea irrespective of etiology, including enterotoxigenic *Escherichia coli, V. cholerae*, and rotavirus infection (David et al., 2015; Hsiao et al., 2014; Kieser et al., 2018).

Generalized model “healthy” microbial communities of the human gut have been shown to be resistant to *V. cholerae* infection (Hsiao et al., 2014), and associative metagenomic studies have examined how the microbiome differs between cholera patients and household contacts that did not exhibit disease symptoms (Midani et al., 2018). Yet few studies have mechanistically explored how interpersonal microbiome variation can drive pathogen susceptibility. Here we show that the post-malnutrition/post-diarrheal dysbiotic community is highly vulnerable to subsequent infection. Moving beyond a dichotomous "normal" versus "dysbiotic" comparison, we show that microbiome differences among healthy humans drive striking differences in susceptibility.

We show that fecal studies in animals and potentially humans may have limited utility for studies of community interactions with pathogens of the small intestine, and that microbiome-dependent infection susceptibility at the small intestine can be rescued by microbiome transplantation. In order to identify commensals that strongly interact with enteropathogens across many community contexts, we established an efficient unbiased experimental pipeline that revealed that the commensal species *Blautia obeum* can suppress virulence. We identify an enzymatic mechanism in *B. obeum* that degrades the host-produced virulence-inducing molecule taurocholate (TC), which *B. obeum* uses alongside other mechanisms (Hsiao et al., 2014) to suppress *V. cholerae* virulence gene activation and colonization.

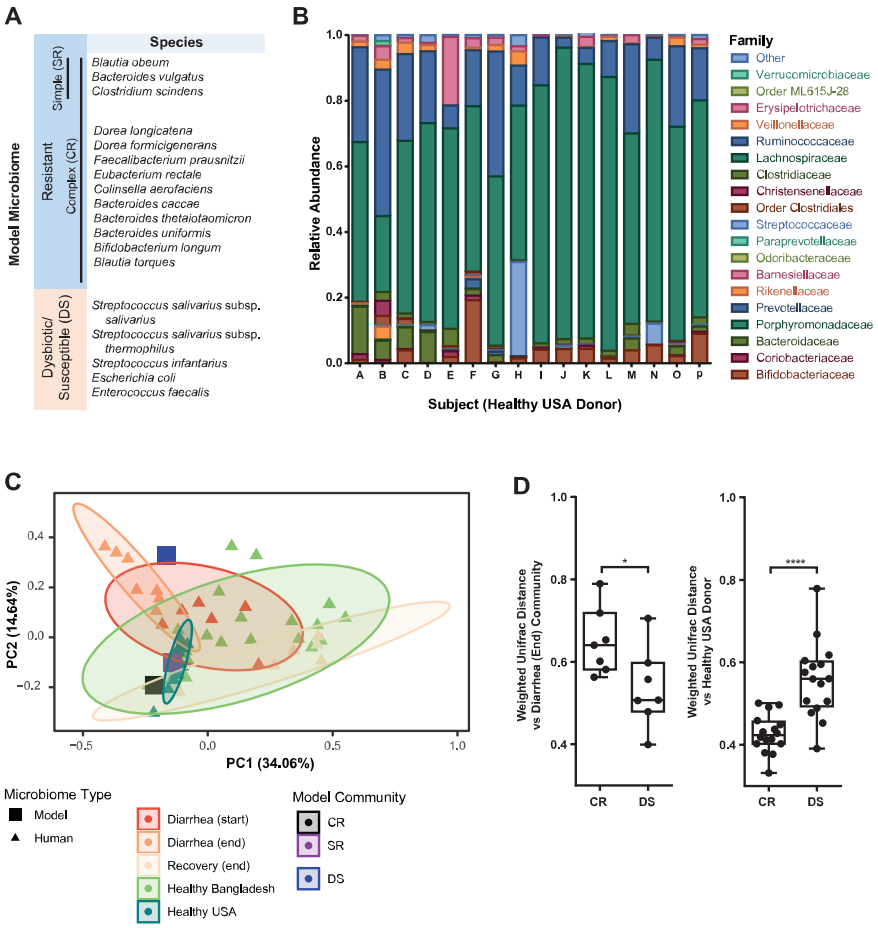

Figure 1. Model human gut microbiomes replicate structure of communities affected by diarrhea-induced dysbiosis.

Figure 1 shows: (A) Defined human gut communities. (B) Composition of healthy US human donor fecal microbiomes. (C) Principal coordinates analysis of defined and complete human gut microbiomes based on weighted UniFrac distance, % variance explained shown in parentheses. Ellipses show 95% confidence intervals. (D) Weighted UniFrac distance to indicated defined human model microbiomes of fecal samples from cholera patients at the end of diarrhea (left) and healthy human donors (right) *P<0.05,

****P<0.0001, Mann-Whitney U-test. Boxplots show inter-quartile range, whiskers minimum to maximum.

Results

Dysbiotic microbiomes are susceptible to *V. cholerae* colonization, and pathogen resistance can be rescued by microbiome transplantation

We took a two-pronged approach to study the effects of microbiome variation on pathogen resistance, both involving reconstituting human gut microbiomes in animal models of *V. cholerae* colonization and virulence. The first involved the construction of defined gut communities using cultured human isolates (Figure 1A), and the second involved studies with complete human fecal microbiomes (Figure 1B).

As the basis for designing defined model communities, we compared fecal microbiomes of a healthy adult volunteer cohort in the United States (Figure 1C, Table S2C) and previously published 16S ribosomal RNA gene sequencing of Bangladeshi adults (Hsiao et al., 2014; Subramanian et al., 2014). Previous studies in Bangladesh revealed that cholera drives the human gut microbial community to a highly dysbiotic, low-diversity state dominated by Streptococci, which recovers to a configuration similar to non-diarrheal individuals over the course of weeks after the cessation of acute disease (Hsiao et al., 2014). This has also been observed in other diarrheal infections such as enterotoxigenic *E. coli* and rotavirus (David et al., 2015; Kieser et al., 2018), and other gut pathologies such as severe malnutrition (Subramanian et al., 2014). Principal coordinates analysis (PCoA) of a human cohort from Bangladesh (Hsiao et al., 2014) displays the dysbiosis caused by cholera (Figure 1C, Diarrhea (start)), and community structure weeks after the cessation of diarrhea (Diarrhea (end) to Recovery (end)), when the microbiome becomes similar to that of individuals in the same area not suffering from

acute malnutrition or diarrhea (Subramanian et al., 2014). Interpersonal microbiome variation in Bangladesh was far higher than among healthy US individuals sampled as part of this study; indeed, some Bangladeshi "healthy" microbiomes closely resemble cholera-diarrheal communities. As infectious diarrhea and malnutrition are frequent in cholera endemic areas, we hypothesized that the distinctive dysbiotic microbiome structure observed during recovery from multiple sources of environmental insult to the gut may be a recurring window of vulnerability to cholera.

We then used human-derived isolates to assemble defined gut communities (Figure 1A) based on these metagenomic analyses. One model microbiome ("CR") was based on metagenomic surveys of healthy individuals, characterized by high taxonomic diversity but commonly including members of the genera Bacteroides, Clostridium, and Blautia (Arumugam et al., 2011; Qin et al., 2010; Yatsunenko et al., 2012). Another ("DS") model microbiome is characteristic of the dysbiotic state found in cholera-endemic areas, comprising Streptococci*, Enterococcus faecalis*, and *E. coli*. 16S sequencing analysis confirmed that the CR community is more similar to healthy human microbiomes than dysbiotic diarrheal microbiomes, while the DS model community was more similar to microbiomes at the conclusion of cholera (Figure 1C-D).

We grew bacterial species from each defined group in pure culture, and used culture optical density (OD_{600}) to introduce equivalent amounts of each member species with *V. cholerae* to germfree (GF) adult C57BL/6J mice by intra-gastric gavage. Overall microbial load during infection was equivalent as measured by quantitative PCR of 16S gene levels (Figure 2F). Mice that received the CR microbiome at infection were resistant

to *V. cholerae* colonization, compared to animals receiving DS microbes (Figure 2A-B). We observed these colonization phenotypes in both feces and in the medial and distal thirds of the small intestine. In prior studies in adult mice, small intestinal colonization by *V. cholerae* required antibiotics (Freter, 1955, 1956) and ketamine anesthesia (Olivier et al., 2009). Our results with human, as opposed to murine, gut bacteria suggest that microbiome differences across host species and inter-individual variation within host species both play key roles in determining pathogen susceptibility. Significantly, we could restore colonization resistance by mixing the CR and DS bacteria, suggesting that susceptibility is reversible through microbiome modification (Figure 2A-B). In "Mix" groups, where mice were administered a 1:1 mixture of CR and DS, *V. cholerae* levels were significantly less compared to that in DS mice, in fact dropping below the level of CR mice 2 days post infection.

We also observed increased colonization susceptibility of DS microbiomes when compared to a simplified model healthy microbiome ("SR") when GF mice were colonized with defined communities for 2 weeks prior to introduction of *V. cholerae* (Figure 2C). The SR community consisted of three species representing major phylogenetic lineages commonly found in the healthy human gut. To model an attempted microbiome restoration of a fully established and dense gut community, we also introduced DS microbes for 10 days, followed by a gavage of SR microbes 4 days prior to infection with *V. cholerae*. In this Mix group, pathogen colonization was strongly inhibited compared to DS-colonized mice, suggesting that microbiome modification

could be used to restore colonization resistance even to entrenched dysbiotic communities.

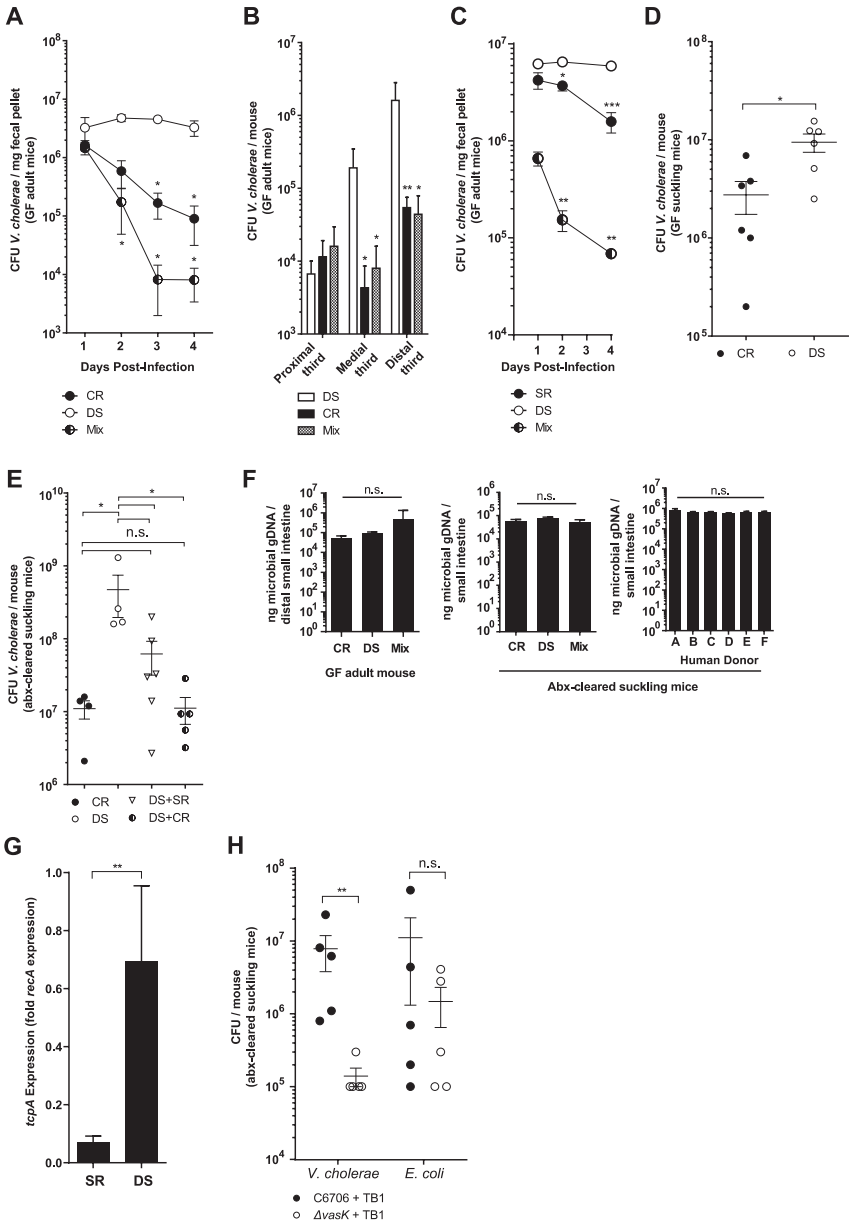

Figure 2. Gut microbiome composition contributes to *V. cholerae* infection resistance.

In Figure 2, we see *V. cholerae* colonization in germfree adult mice harboring defined model communities co-gavaged with V. cholerae in (A) feces and (B) small intestines 4 days post infection. (C) Fecal *V. cholerae* colonization in GF adult mice harboring defined communities for 2 weeks and then gavaged with *V. cholerae*. Mix: 10 days DS colonization, followed by a SR microbes 4 days prior to *V. cholerae* infection. (D) Intestinal *V. cholerae* colonization of GF suckling mice co-gavaged with model communities and *V. cholerae*. (E) Intestinal *V. cholerae* colonization of antibiotic-treated CD-1 suckling mice co-gavaged with indicated communities. (F) 16S gene abundance in small intestine. (G) Expression of *tcpA* in infected mice with model human microbiomes. (H) T6SS effects on small intestine colonization in antibiotic-treated CD-1 pups. * P<0.05, ** P<0.01, *** P<0.001 (Mann-Whitney *U*-test); n.s. not significant. Error bars represent mean ± SEM. *n*=6-12 animals for all experiments.

We then profiled gut microbiome structure during infection in feces and small intestines of gnotobiotic animals with different communities (Figures 3A-C, 3G). In these samples, the CR and DS communities were distinct and the CR community more similar to complete fecal microbiomes of healthy United States donors, while co-inoculation of CR and DS led to an intermediate final microbiome.

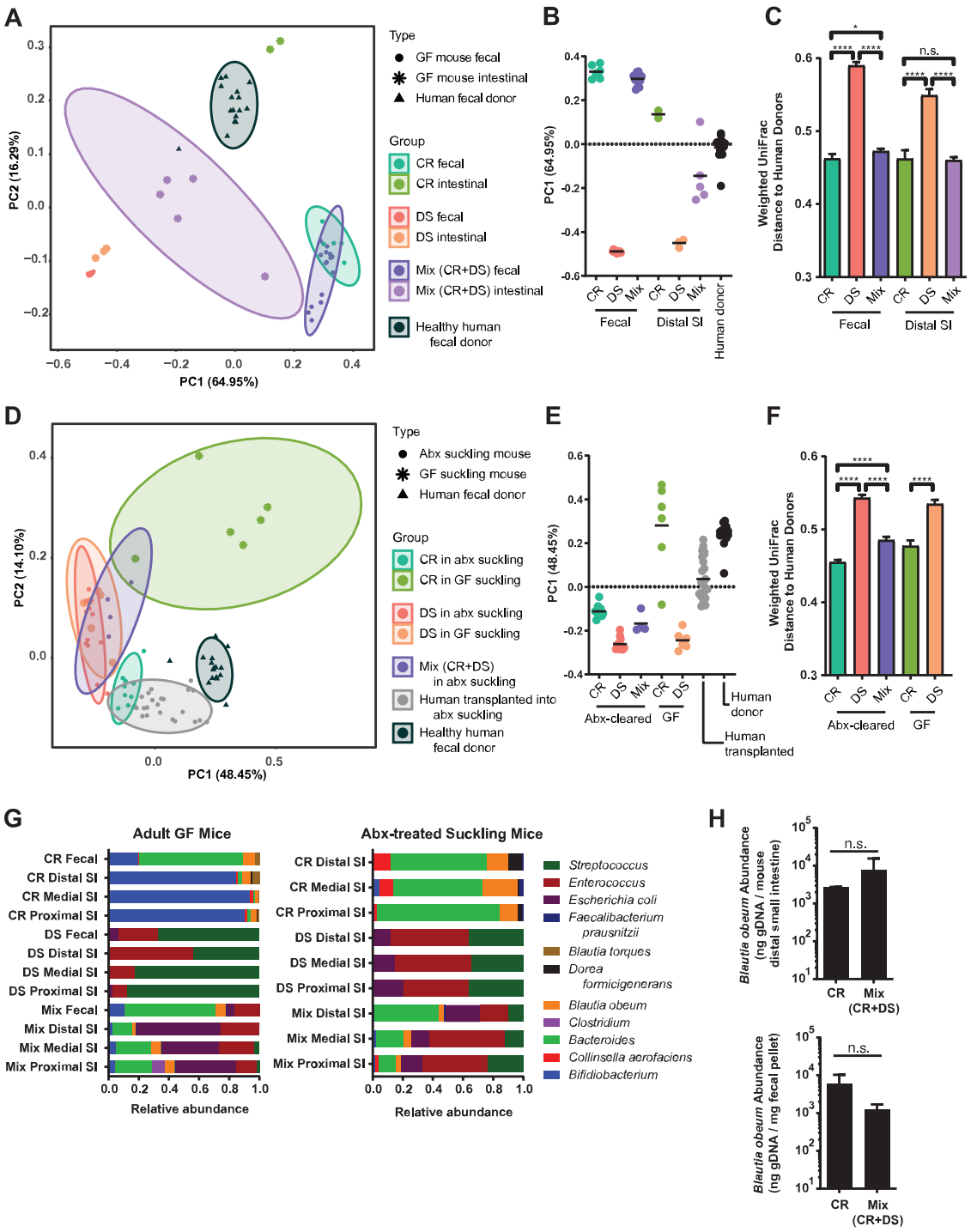

Figure 3. Addition of the CR model human microbiome to mice hosting DS microbes yields a community structure closer to complete fecal communities of healthy human volunteers.

Figure 3 displays: (A, D) Principal coordinates analysis (PCoA) of microbial community diversity based on weighted UniFrac distance, % variance explained shown in parentheses for each axis. Ellipses show 95% confidence intervals. (A) PCoA of fecal samples and distal third of small intestine of GF mice with model communities during *V. cholerae* infection compared to healthy US donor fecal samples, with (B) PC1 positions and (C) all pairwise weighted UniFrac distances to healthy US donor fecal samples. (D) PCoA of model communities and healthy human donor communities in suckling mice, with (E) PC1 positions and (F) all pairwise weighted UniFrac distances to healthy US donor fecal samples. (G) Microbiome structure during infection with *V. cholerae* and host reads filtered (left) and in antibiotic-treated suckling mice without *V. cholerae* (right). (H) *B. obeum* abundance in adult GF mice containing indicated microbiomes during *V. cholerae* infection (4d post infection). * $P<0.05$, **** $P<0.0001$; n.s. not significant (Mann-Whitney *U*-test). Error bars represent mean ± SEM.

Non-dysbiotic human microbiomes reduce virulence gene expression and colonization of *V. cholerae* in a suckling mouse model of infection

While gnotobiotic adult mice serve as a useful microbial-interaction model, we extended our studies to suckling mice, where the pathology and virulence gene expression observed during *V. cholerae* infection is closer to that of humans (Klose, 2000). First, we recapitulated the CR and DS resistance phenotypes in suckling GF C57BL/6J animals (Figure 2D). We then constructed a more accessible and scalable model of microbiome-pathogen interaction by clearing the native murine flora of CD-1 pups with streptomycin before introduction of human-associated species. Using this

system, we observed similar microbiome-dependent infection outcomes; competitive CR/DS transplantation yielded a dominant CR-like phenotype (Figure 2E), while CR and DS colonization load did not differ in non-*Vibrio*-infected animals (Figure 2F). This pattern was reflected in 16S sequencing data (Figure 3D-F, Table S3). During infection, pups receiving CR microbes had very different community structure (with *Vibrio* reads filtered) compared to animals with DS microbes, and animals receiving a mixed inoculum (CR+DS) closely resembled CR mice.

Total microbial diversity was not a dominant factor in infection resistance, as we restored colonization resistance in DS mice to almost full CR levels by transplanting only a small subset of CR species (SR). Expression levels of the key colonization factor *tcpA* were reduced ~9.7 fold in SR compared to DS animals (Figure 2G). We did not observe significant microbiome-dependent differences in cholera toxin gene expression, diarrhea, or fluid accumulation in these animals (Figure S1).

Recent studies have shown Type VI secretion system (T6SS) killing of murine commensal *E. coli* acts to drive increased virulence in infection of suckling mice (Zhao et al., 2018). As our DS model community contains *E. coli*, albeit a different strain, we tested the effects of T6SS on *V. cholerae* colonization and E. coli levels in our experimental system. A T6SS Δ*vasK* mutant was deficient for colonization compared to wild type *V. cholerae* in antibiotic-cleared suckling mice (Figure 2H). However, T6SS activity did not significantly alter levels of a co-inoculated streptomycin-resistant K12 *E. coli,* and we observed *E. coli* at comparable levels in DS and Mix (DS+CR) communities

in the small intestine (Figure 3G). These differences may be *E. coli* strain-specific, or due to the much higher levels of *V. cholerae* used in previous T6SS studies.

Together, our data suggested that the mechanism for improved colonization resistance of CR/SR microbes lay in T6SS-independent manipulation of *V. cholerae* virulence gene expression.

Inter-individual variation in pathogen colonization resistance of human gut microbiomes

Our microbiome transplantation system in suckling mice allowed us to screen numerous intact human fecal microbiomes collected from healthy adult volunteers without malnutrition or recent antibiotic usage or diarrhea for effects on *V. cholerae* (Figure 1B). These complete fecal communities were taxonomically similar to the CR, but not DS microbiomes in both original microbial content and community structure upon transplantation (Figures 3D-F). This was unsurprising, as the CR model community represented up to 73% of genus-level diversity by total relative abundance in these samples, while members of the DS community only represented <1% of the total (Table S4).

We normalized fecal slurries and transplanted these samples into antibiotic-treated suckling mice with *V. cholerae* (Figure 4), with dramatically different effects on *V. cholerae* colonization, even though these fecal communities colonized suckling animals at similar efficiencies and density (Figure 2F). We observed an approximately 1.5-$\log_{10}$ range of *V. cholerae* colonization depending on the human donor (Figure 4), suggesting wide variation in infection outcomes based on individual gut microbiome

structure. The higher basal microbiome diversity in Bangladesh (Figure 1C) suggests that interpersonal variations in infection resistance in endemic areas could be substantially higher.

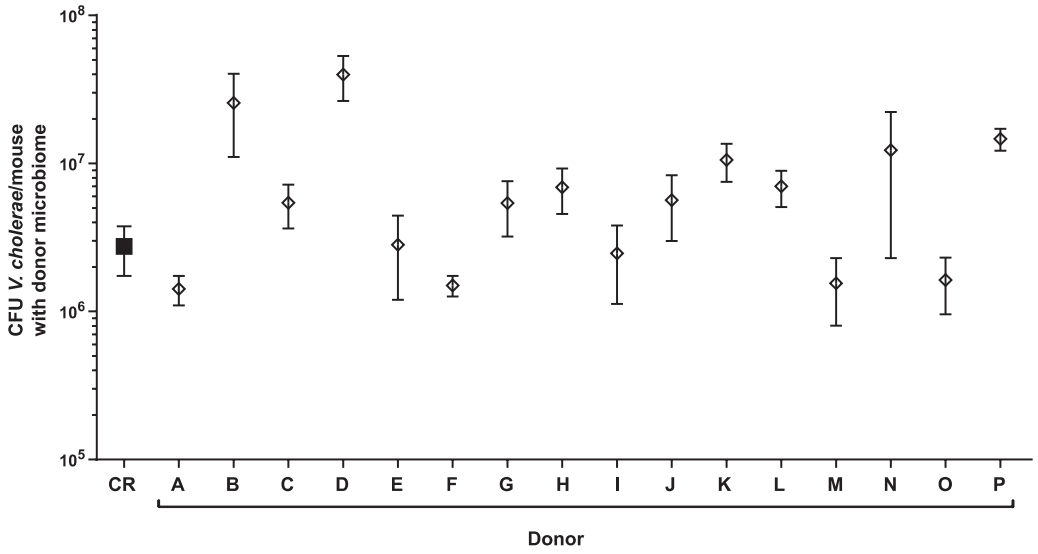

Figure 4. Gut microbiome composition contributes to inter-personal differences in *V. cholerae* infection resistance.

We showed Intestinal *V. cholerae* colonization of antibiotic-treated CD-1 pups colonized with complete fecal microbiomes from healthy US human volunteers in figure 4. *n*=5-7 animals for all experiments.

A pipeline for randomization of microbiome members identifies commensal species consistently correlated with *V. cholerae* infection outcome

We hypothesized that the CR species best able to colonize intestines with the DS community might be prophylactic for infection, since transplantation of CR microbes into

DS communities reduced *V. cholerae* colonization. Therefore, we examined gut microbiome structure during *V. cholerae* infection in GF animals with a mixed CR+DS community (Figures 3A, 3G). The CR community in the small intestine was quite distinct from that in feces, but all animals with this community were consistently colonized by Blautia and Bacteroides species. The DS community was consistent in feces and small intestine, and dominated by Streptococci. The lack of generalizability of fecal data to other gut compartments suggested that fecal sampling studies may mask important differences for pathogenesis in the proximal gastrointestinal tract.

In the small intestine, *B. obeum* maintained its relative abundance in gnotobiotic CR+DS and CR-only animals (Figure 3H), suggesting that it may play a role in CR infection resistance and in transmitting this phenotype to DS animals. However, these findings had potentially limited translational applicability given the basal inter-personal diversity in humans; the ability to displace one dysbiotic microbiome and resist *V. cholerae* colonization may not be representative across diverse individuals and microbial communities. To identify *Vibrio*-antagonistic microbes across many different microbiome contexts, we generated random, unique, 5-member combinations drawn from CR and DS strains (Figure 5A-B) and established OD_{600}-normalized mixtures of these bacteria in antibiotic-cleared suckling animals with and without *Vibrio* infection. We reasoned that species whose presence/absence or abundance consistently correlated against *V. cholerae* colonization in many different communities would be excellent putative targets for anti-*Vibrio* interventions.

We identified several species consistently associated with pathogen levels across multiple microbiome combinations. Higher levels of *B. obeum* were significantly associated with reduced *V. cholerae* colonization (Figure 5C), but did not directly correlate with *V. cholerae* abundance. This is consistent with the effects of the mixed CR/DS microbiome on infection; *B. obeum* consistently established in the DS small intestine, but high levels were not required to affect *V. cholerae*. That this effect was seen across numerous randomized communities suggests that the inhibitory activity of *B. obeum* on *V. cholerae* infection may be broadly generalizable across many gut microbiomes. Except for *B. obeum*, we found no statistically significant effects on *V. cholerae* colonization based on the presence or absence of an SR or CR species. In contrast, levels during infection of DS microbes (*Streptococcus, E. faecalis*, and *E. coli*) all positively and significantly correlated with higher *Vibrio* levels (Figure 5D). In mice with combinations with both *B. obeum* and *S. thermophilus*, *V. cholerae* colonization was comparable to mice with combinations including *B. obeum* but not *Streptococcus*, again supporting the observation that *B. obeum*'s effects on pathogenesis are dominant across diverse microbiomes (Figure 5C).

Since SR microbes largely recapitulated the colonization resistance of the full CR microbiome, we performed similar analyses looking for whether combinations of different SR microbes with *B. obeum* yielded lower *V. cholerae* colonization than when those species were present in isolation. We observed no statistically significant additive effects on *V. cholerae* colonization of adding either *Bacteroides vulgatus* or *Clostridium scindens* to *B. obeum* in defined communities (Figure S2).

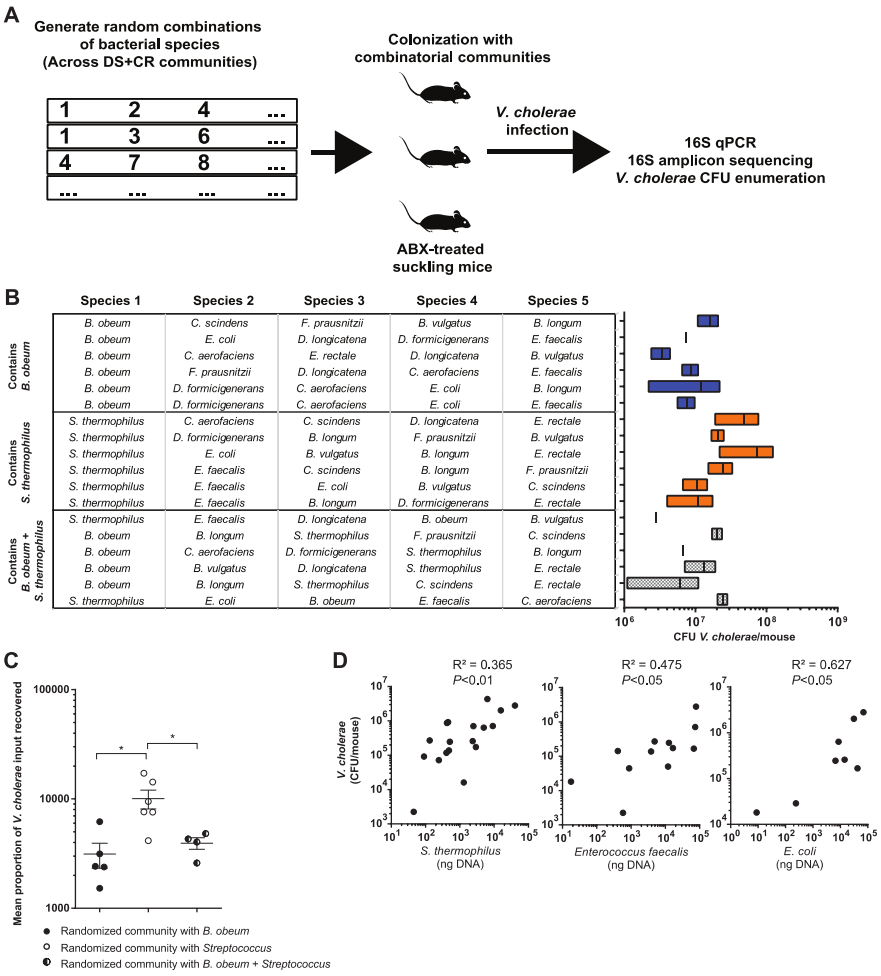

Figure 5. An unbiased combinatorial strategy for identifying commensal correlates of protection against and susceptibility to *V. cholerae* colonization.

Figure 5 shows: (A) Combinatorial strategy. (B) 5-member microbiome embodiments randomly generated using CR/DS members (left). Resulting *V. cholerae* infection of antibiotic-treated suckling mice containing defined microbiome embodiments are shown at right. (C) Mean *V. cholerae* colonization in suckling mice bearing communities containing *B. obeum* or *Streptococcus* species alone and in combination. Data normalized across experiments as fold CFU gavaged *V. cholerae* recovered after

infection. (D) Abundance of DS member species in randomized microbiomes correlated to resulting *V. cholerae* abundance after infection. Points represent mice receiving different microbiome embodiments. * $P<0.05$, ** $P<0.01$ (Mann-Whitney *U*-test); *n*.s. not significant. Error bars represent mean ± SEM.

Intestinal signals that induce *V. cholerae* virulence gene expression are depleted by *B. obeum*

Having identified a candidate driver of *V. cholerae* infection resistance, we began to search for a molecular mechanism for these effects. Numerous host and some commensal microbial cues regulate *V. cholerae* gene expression *in vivo* (Gupta and Chowdhury, 1997; Kovacikova et al., 2010; Yang et al., 2013). Prior studies have identified a role for a *B. obeum*-produced AI-2 autoinducer in down-regulating the expression of TCP biogenesis genes during infection (Hsiao et al., 2014). Consistent with this finding, we observed reduced *tcpA* expression during infection of mice with microbiomes containing *B. obeum* (Figure 2G).

In vivo conditions for virulence gene regulation can be mimicked ex vivo using microaerophilic/anaerobic growth of *V. cholerae* with intestinal tissue from mice (Yang et al., 2013). We took homogenates of suckling mouse intestine and incubated them anaerobically with a V. cholerae *lacZ*:P_{tcpA} *–sh ble* zeocin resistance reporter. As expected, intestinal homogenates induced *tcpA* expression, while pre-treatment of intestinal homogenates with *B. obeum* ablated *tcpA* induction (Figure 6A). As a control, we boiled intestinal homogenates that had been incubated with *B. obeum* cultures in order to remove AI-2, which is heat labile (Figure S3). Strikingly, homogenates incubated with

B. obeum remained unable to induce *tcpA* even after boiling, in contrast to boiled homogenates alone or homogenates incubated with *S. salivarius* and then boiled (Figure 6A). These data suggested that *B. obeum* can deplete virulence-activating signals within the gut as well as produce virulence-suppressing signals.

The bile salt taurocholate acts as a potent virulence gene activator, and is more efficiently degraded by commensal microbes in healthy but not dysbiotic communities

One abundant heat-resistant molecule present in the small intestine is bile. In humans and mice, bile acids are synthesized in the liver from cholesterol, and stored in the gallbladder. These primary bile acids are then secreted into the duodenum, where they, typically in their sodium salt form, act to aid in the emulsification and absorption of dietary fats. More than 95% of secreted bile acids are actively absorbed by the terminal ileum and sent back to the liver in a process known as enterohepatic circulation (Di Ciaula et al., 2017). Bile is a highly complex mixture, though bile acids dominate the dry weight of biliary bile (Muraca et al., 1991). Many prior studies have focused on crude extracts from varying sources, including ruminants, containing poorly defined mixtures of bile molecules. Human bile acids secreted into the small intestine are predominantly conjugated to taurine (taurocholic acid/sodium taurocholate) and glycine (glycocholic acid/sodium glycocholate), while taurine-conjugated forms predominate in mice (Li and Dawson, 2018; Sayin et al., 2013). Commensal microbial action is important for bile acid metabolism; bacterial enzymes (*bsh*, bile salt hydrolases) are able to remove the

conjugated amino acids from secreted bile acids in the small intestine, for example converting glycocholate (GC) and taurocholate (TC) to cholate/cholic acid (CA) (Jones et al., 2008; Ridlon et al., 2006; Song et al., 2019). Indeed, in GF mice, the bile pool in the small intestine is almost exclusively taurine-conjugated (Sayin et al., 2013).

Previous studies have shown that TC, one of the most abundant bile molecules in humans and mice, can induce *tcp* expression under anaerobic conditions through modulating the structure and activity of the upstream virulence activator TcpP (Yang et al., 2013). Similarly, we saw that TC activated P_{tccpA}, while CA was not an efficient inducer (Figure 6B). Intestinal homogenate effects on *tcpA* was bile-specific; pre-treatment of homogenates with the bile-sequestering resin cholestyramine ablated their ability to activate P_{tcpA} (Figure 6A).

We next screened the DS and CR species for their effects on TC. We incubated TC solutions at a physiologically relevant concentration with pure cultures of these microbes, heat treated and filter-sterilized the resulting supernatants, and measured their ability to induce P_{tcpA} (Figure 6C). We observed dramatic differences in the ability of these strains to affect TC virulence induction, with members of the CR/SR microbiomes in general being better able to prevent *tcp* activation. The ability to ablate TC-mediated induction of *tcp* expression varied at genus level, with *Blautia torques* unable to affect *tcp* induction by TC in comparison to *B. obeum*, and *Streptococcus infantarius* able to process TC in contrast to other DS Streptococci. Of SR species, *B. vulgatus*, but not *C. scindens*, showed efficient TC processing.

Since the SR community largely recapitulated the CR colonization resistance phenotype, and *B. vulgatus* demonstrated high activity against TC *in vitro*, we wanted to examine the relative contribution of *B. obeum* and *B. vulgatus* on TC levels in the distal small intestine. We colonized adult GF mice with CR members, or CR species without *B. obeum*, and measured TC and CA in the distal third of the small intestine 2 days post-colonization, compared to GF mice (Figure S4A). As expected given the absence of microbial *bsh,* GF distal small intestine showed a high TC/CA ratio, while the presence of CR microbes efficiently processed TC to CA, yielding low TC/CA ratios. Strikingly, the removal of *B. obeum* restored the TC/CA ratio in the distal small intestine to a level not statistically significantly different from GF animals, though there was a trend towards more TC in GF animals. This suggested that while other CR organisms can contribute to TC processing to CA, *B. obeum* is particularly well suited to manipulating the level of this bile acid in the distal small intestine. This agrees with our findings that *B. obeum* efficiently colonizes the small intestine (Figures 3, 5), and that the presence of *B. vulgatus* and *B. obeum* together does not significantly improve the ability of a microbiome to exclude *V. cholerae* (Figure S2).

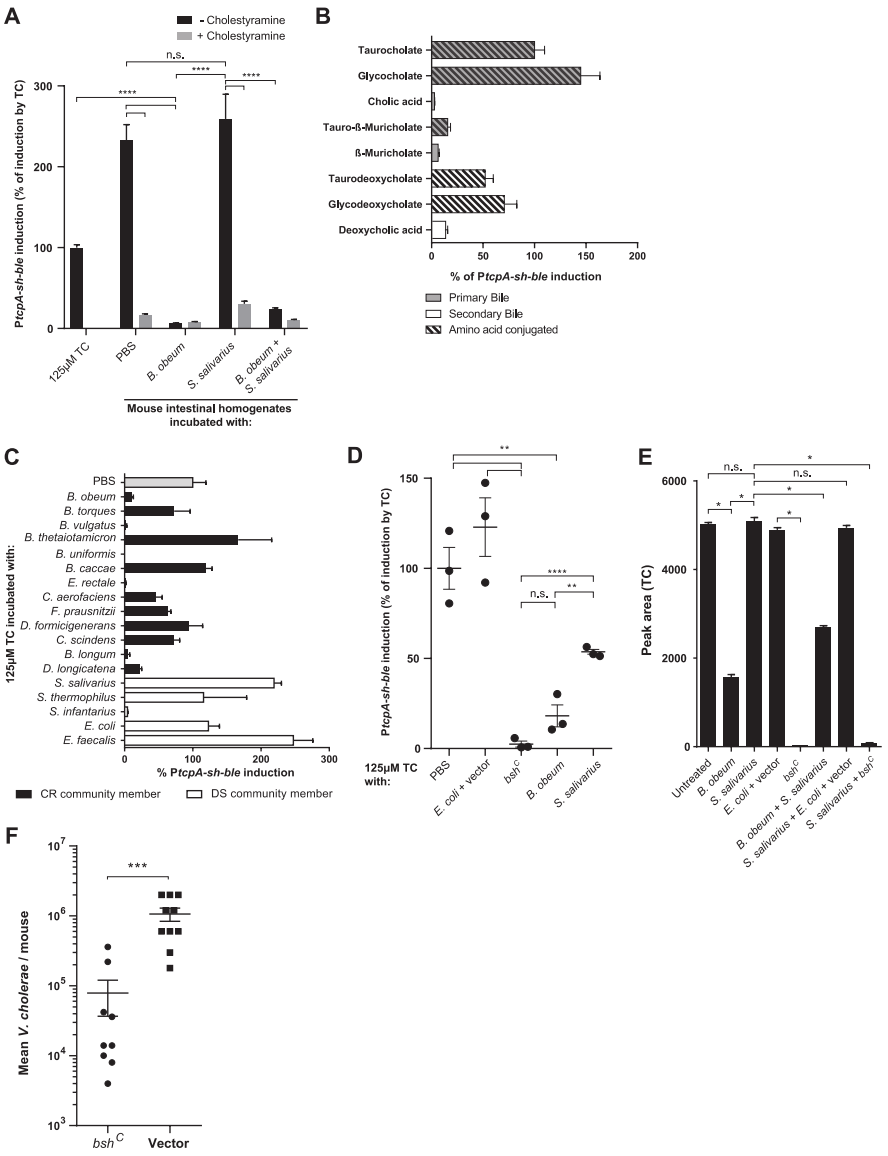

Figure 6. *B. obeum* exerts effects on *V. cholerae* colonization through degradation of the in vivo virulence gene activating signal taurocholate (TC).

Figure 6 shows P_{tcpA} activity normalized to *tcpA* induction by 125μM TC unless noted. (A) Modulation of *tcpA*-activating signals in suckling CD-1 mouse intestinal homogenates by pure cultures of *B. obeum* and *S. salivarius*, with heat treatment. (B) Bile effects on *tcp* gene expression *in vitro*. (C) Effects of CR and DS pure cultures on TC

activation of virulence *in vitro*. (D) Effects of *B. obeum bsh* enzyme expression on TC-mediated *tcp* activation *in vitro*. (E) Mass spectrometry measurement of TC in suckling CD-1 mouse intestines after incubation with pure cultures of indicated strains. (F) *V. cholerae* infection of suckling CD-1 mice after 1-day of colonization with indicated *E. coli* strains. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ (unpaired Student's t-test). Error bars represent mean ± SEM. n=3-10 for all experiments.

A bile salt hydrolase enzyme encoded by *B. obeum* is able to degrade virulence-activating signals in the gut

To determine the molecular basis for *B. obeum*'s effect on TC-dependent virulence induction, we examined genetic determinants of bile acid processing. The *B. obeum* genome encodes for a hypothetical choloylglycine hydrolase (EC 3.5.1.24). Such bile salt hydrolase (*bsh*) enzymes catalyze the removal of the conjugated amino acids of bile salts, for example the removal of taurine from TC to form CA.

Putative *bsh* genes are broadly distributed across gut microbial species, as the ability to survive the inhibitory effects of bile are extremely important for enteric commensals (De Smet et al., 1995). A recent study classed bacterial *bsh* genes into several phylotypes based on sequence similarity and showed that *bsh* phylotypes have variable and substrate-dependent deconjugation activity (Song et al., 2019). We binned predicted *bsh* genes in the CR and DS genomes into these phylotypes (Table S5). By sequence alignment and predicted structure, *B. obeum* encodes for predicted type 1 *bsh* enzymes, which are highly effective at deconjugating TC, GC, glycodeoxycholate, and taurodeoxycholate (Song et al., 2019), the strongest activators of *tcpA* expression in *V.*

cholerae (Figure S5). All DS members except *S. infantarius* lacked annotated *bsh* genes, while many CR species encoded *bsh* homologs. While *S. infantarius* showed high activity against TC in vitro, despite bearing *bsh* homologs to phylotypes with poor predicted TC activity, we observed no statistically significant difference in effects on *V. cholerae* colonization compared to *S. salivarius* (Figure S4B), suggesting that there may be differences in *in vivo* regulation of these enzymes in *S. infantarius*. Conversely, *B. torques*, despite encoding several putative *bsh* genes, was not able to prevent *tcpA* induction by TC. *B. vulgatus* was an efficient TC processor in vitro, but despite encoding 3 putative *bsh* genes could not drive significantly lower levels of TC to CA processing in the distal small intestine in the absence of *B. obeum* (Figure S4A), further suggesting that enzyme expression or function may diverge in *in vitro* vs *in vivo*.

V. cholerae also encodes a putative *bsh* enzyme (VCA0877). However, this is a predicted phylotype 6 *bsh*, which has poor activity against TC but higher activity against rarer bile acids that do not participate in regulation of virulence but may be bacteriostatic *in vivo* (Table S5). *V. cholerae* cannot prevent TC-mediated *tcp* activation in vitro, suggesting that *V. cholerae* has limited *bsh* activity against TC in comparison to *B. obeum* (Figure S4C).

We constitutively expressed the *B. obeum bsh RUMOBE_000028* by cloning this locus downstream of a constitutive $P_{Ltet\text{-}O1}$ promoter in *E. coli*, generating strain bsh^{C}. This bsh^{C} strain efficiently reduced levels of TC and *tcp* activation compared to the isogenic vector control in both pure TC solutions (Figure 6D) and intestinal homogenates (Figure 6E). Significantly, given the dominant effect of *B. obeum* on Vibrio resistance,

pure cultures of either *B. obeum* or bsh^C reduce *tcp* activation by TC (Figure 6D) and TC levels in intestinal homogenates (Figure 6E) in the presence of *S. salivarius*. The activity of this *B. obeum* enzyme is able to affect *V. cholerae in vivo*, as *V. cholerae* is unable to colonize mice gavaged with bsh^C as effectively as mice with the vector control (Figure 6F).

Bile salt hydrolase levels in human gut microbial communities are correlated to *V. cholerae* infection outcome

To determine the distribution of *bsh* phylotypes in human gut microbiomes predicted to be dysbiotic or healthy, we re-examined an existing deeply-sequenced shotgun metagenomic dataset of human cholera patients in Bangladesh (Table S2D) (David et al., 2015). Importantly, data was available from patients at presentation at clinic for cholera ("Diarrhea (d0)") without any prior antibiotic usage, and from patients that received oral antibiotics as part of the standard of care for cholera ("Diarrhea + abx"). These data showed that *bsh* levels were already affected by diarrhea prior to any clinical intervention. We found that several *bsh* phylotypes followed diarrhea-dependent patterns in comparison to a healthy Bangladeshi cohort. Phylotypes 1, 3, 4, and 5 were significantly depleted in dysbiotic samples compared to healthy controls (Figure 7A, S6). Of these, phylotypes 1, 3, and 4 were shown to be highly active against TC ((Song et al., 2019), summarized in Table S5). These data suggested that a characteristic of healthy human microbiomes that may modulate *V. cholerae* susceptibility is their ability to deconjugate TC into non virulence-inducing forms.

We then assayed whether complete healthy US fecal communities were differentially able to convert TC to non-*tcp*-activating forms. We took the first six healthy US donors and anaerobically cultured bacteria from their fecal samples *in vitro*, and were able to recover species representing 66-99% relative abundance of the original sample (Table S4C). We inoculated these complex fecal specimens in media and used standardized amounts of the resulting mixed cultures to treat TC solutions, which we then used for virulence reporter assays. Strikingly, we observed that microbiomes (subjects B, D) that allowed higher *V. cholerae* levels when transplanted in suckling animals were also unable to completely remove TC from solution after 24 hours, while communities exhibiting stronger colonization resistance (A, C and E) reduced TC to undetectable levels (Figure 7B).

Since species in genus Blautia demonstrated differences in *bsh* activity in vitro, as well as association with cholera patients and uninfected family members (Midani et al., 2018), we assayed for the level of the *bsh* gene of *B. obeum* specifically in total DNA extracted from human fecal samples by real-time PCR. This also served as a function-specific measure of *B. obeum* abundance in these complex fecal mixtures. We found that communities associated with higher *V. cholerae* colonization had lower levels of *B. obeum bsh* (Figure 7C), suggesting that *B. obeum* abundance and specifically the presence of the *bsh* activity is associated with resistance to *V. cholerae* infection.

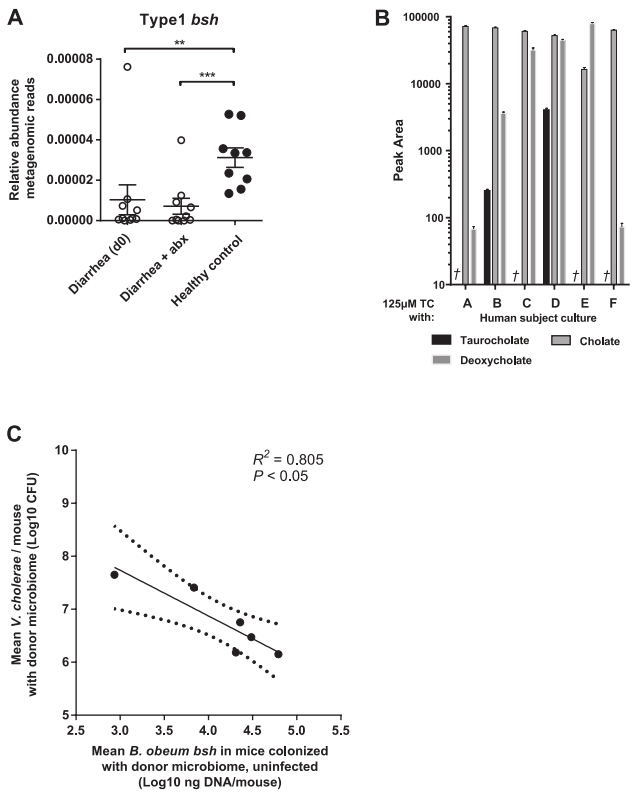

Figure 7. Levels of *B. obeum bsh* enzymes in human samples correlate to infection outcome, and can independently modulate *V. cholerae* colonization.

In figure 7, we see (A) Levels of phylotype 1 *bsh* enzymes in metagenomic sequencing of fecal microbiomes of cholera patients pre- ("Diarrhea (d0)") and post-antibiotic ("Diarrhea + abx") treatment, compared to healthy adults in Bangladesh. (B) Mass spectrometry measurement of bile levels in 125μM solutions of TC incubated with indicated cultured human fecal communities *in vitro*, †: TC not detected. (C) *B. obeum bsh* levels in intestines of antibiotic-cleared suckling CD-1 mice colonized with complex human fecal samples without *V. cholerae*, compared to *V. cholerae* colonization of

antibiotic-cleared suckling animals bearing human donor microbiomes. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (Mann-Whitney U-test). Error bars represent mean ± SEM.

Discussion

A role for gastrointestinal microbes in resistance to enteropathogens such as *V. cholerae* has been recognized for some time (Freter, 1955, 1956). However, this colonization resistance has often been examined in dichotomous terms, either germfree or conventional, or "normal" and damaged by specific factors such as antibiotics. Our results suggest that beyond extreme fluctuations in structure, such as those due to diarrhea and severe malnutrition, diversity even within otherwise "healthy" human populations can serve as predictive markers for infection resistance.

A key difficulty in identifying taxa that can drive infection susceptibility is the complexity of animal gut microbiomes, compounded by dramatic differences in the taxonomic diversity across host species (Seedorf et al., 2014). The limited taxonomic and functional resolution of many commonly-employed metagenomic techniques is also a barrier for converting observations in large population studies to mechanistic insights on microbial effects on pathogenesis and other phenotypes. Findings in this study and others (Hsiao et al., 2014), in which single genes encoded by specific microbiome members are able to affect *V. cholerae* colonization in isolation from other functions, suggest that correlative studies have distinct limits in their abilities to provide mechanistic insights to microbiome-pathogen interactions. For instance, a recent sequencing-based study that sampled gut microbes of cholera patients and healthy household contacts identified microbes from the same genus as associated with both individuals with cholera and individuals with putative exposure but non-progression to disease (Midani et al., 2018).

Thus, genus-, and possibly even species-, level data may be insufficient to identify clear targets for future mechanistic studies in the absence of experimental manipulations.

Recent developments in high-throughput sequencing, anaerobic microbiology, and gnotobiotic animal systems have allowed for mechanistic studies of the interaction of human commensals and human pathogens in animal models of colonization and disease. Specific target taxa identified by multi-omics approaches can be established in animals, with species and gene content defined before introduction of pathogens. These types of approaches allowed us to identify *B. obeum* as a key member of the human gut microbiome that drove infection resistance, and microbial interactions with bile acids as a driver of virulence gene regulation in *V. cholerae* and a mechanism of protection against infection.

We hypothesize that microbial bile metabolism most affects *V. cholerae* pathogenesis during early infection. *V. cholerae* tightly regulates gene expression in response to host environmental signals such as bile. Bile acids are thought to stabilize the structure of the key virulence regulator TcpP (Yang et al., 2013), which drives the activation of *toxT* transcription. ToxT then causes full induction of *tcp* and cholera toxin, with TCP-dependent colonization thought to begin prior to toxin expression (Lee et al., 1999). Following colonization, the activity of bile becomes less clear. Some studies have demonstrated that the unsaturated fatty acids in bile are able to modulate the binding of ToxT to target promoters, reducing CT and TCP expression (Plecha and Withey, 2015). The deconjugated bile salt sodium deoxycholate promotes interaction between the virulence activators ToxR and ToxS and subsequent activity (Midgett et al., 2017).

However, ToxRS likely does not directly activate *toxT*, but rather acts to boost the activity of TcpP at the *toxT* promoter (Krukonis et al., 2000). Both conjugated and deconjugated bile acids are also able to induce ToxT-independent activation of cholera toxin dependent on ToxRS (Hung and Mekalanos, 2005).

Our results suggest a model where at the initial point of colonization in the small intestine, *tcp* gene expression and thus TCP biogenesis is determined by the balance of bile acids that is modulated by commensal microbes with differential *bsh* activity. Differences in early *tcp* gene expression and thus colonization may have disproportionate impact on the progression of *V. cholerae* infections; variation in microbiome *bsh* activity may help determine whether infection proceeds to fulminant diarrhea, or low or temporary colonization that leads to mild or asymptomatic infections that are common in cholera endemic areas (King et al., 2008). Once severe diarrhea has begun, the commensal community and existing lumenal bile is depleted, and any future bile secretion is predominantly conjugated primary species that stimulate virulence.

While *B. obeum* bile modification is an important regulator of *V. cholerae* colonization, there may be additive effects on pathogen behavior of multiple community members, and through other mechanisms. Removal of *B. obeum* was sufficient to raise TC levels in the mouse distal small intestine, but while constitutive expression of *B. obeum bsh* yielded a 1 log drop in *V. cholerae* colonization, the full CR microbiome yielded almost 2 log fold differences in colonization compared to DS microbiomes. In *V. cholerae*, virulence gene expression is negatively regulated by several different quorum sensing systems involving the sensing of specific autoinducers (Jung et al., 2015). Prior

studies identified *B. obeum*-produced autoinducer AI-2 as a suppressor of *tcpA*, functioning through a pathway bypassing the canonical AI-2 receptor LuxP and involving the regulator VqmA (Hsiao et al., 2014). VqmA has been shown to activate the master quorum sensing regulator HapR, which leads to repression of *tcpP* (Liu et al., 2006; Zhu et al., 2002). Thus, AI-2 expression and *bsh* activity by *B. obeum* may synergize to reduce the level and activity of TcpP during infection. Additional studies will be required to determine how these two inputs exert their effects on regulation of virulence determinants of *V. cholerae* in vivo. Non-*B. obeum* CR members may also impact *V. cholerae* colonization, both at the level of virulence regulation and possibly metabolic competition for colonization niches. Host diet may play a role in colonization; bile is secreted from the gallbladder in response to food ingestion, and this varies by the fat content of the meal (Marciani et al., 2013). Diet is also a potent driver of microbiome structure (Faith et al., 2011), but the effect of different dietary compositions on driving the microbiome to infection resistant or susceptible states has not been well studied. Ingestion of food has been shown to dramatically reduce the infectious dose of *V. cholerae* due to buffering of stomach pH, but also possibly by raising the levels of virulence-activating conjugated bile acids secreted into the small intestine.

Taken together, our results suggest that variation in human gut microbiomes can be a significant contributor to *V. cholerae* infection risk, and that this can be modulated through introduction of a human gut commensal with multiple molecular effects on *V. cholerae*, able to affect levels of both virulence-activating and virulence-suppressing

signals at the site of infection. This suggests that targeted microbiome modification can be a promising prophylactic target against cholera.

Star Methods

Experimental Model and Subject Details

Human studies

All human samples were part of a study approved by the UCR Institutional Review Board and followed NIH guidelines. We collected intact fecal samples from a cohort of healthy adult volunteers at the University of California, Riverside. Inclusion criteria were: 1) age between 18 and 40 years, 2) must be able to provide signed and dated informed consent, 3) must be willing and able to provide stool specimen. Exclusion criteria were: 1) systemic antibiotic usage (oral, intramuscular, or intravenous) in the 2 weeks prior to sampling; 2) acute disease at time of enrollment (presence of moderate or severe illness with or without fever); 3) diarrhea (liquid or very loose stools not associated with a change in diet) in the 2 weeks prior to sampling; 4) active uncontrolled GI disorders or diseases including Inflammatory bowel disease (IBD), ulcerative colitis, Crohn's disease, or indeterminate colitis, persistent, infectious gastroenteritis, colitis, or gastritis, and chronic constipation; 5) Major surgery of the GI tract, excluding cholecystectomy and appendectomy, but including major bowel resection at any time. Age inclusion criteria were chosen to avoid age-related microbiome differences, which are strongest in early life (Yatsunenko et al., 2012). Fecal samples were collected aseptically from each person at UCR and immediately preserved at −80°C until processing for DNA extraction, culturing, and animal colonization. Stocks of fecal slurries for subsequent experiments were prepared by resuspending samples at 1:3

weight/volume in sterile reduced PBS and adding sterile glycerol to a final concentration of 25% volume/volume.

Animal studies

All animal experiments used protocols approved by the Institutional Animal Care and Use Committee of the University of California, Riverside (UCR) and followed NIH guidelines. All CD-1 suckling animals were purchased from Charles River Laboratories. Suckling and adult germfree C57BL/6J mice were reared at the UCR gnotobiotic facility. No distinction was made between male and female animals for bacterial studies. Adult animals were used at >3 weeks of age. Germfree suckling mice used at 5-6 days of age. Animals were checked for signs of moribund condition prior to use in experiments, and used for one experimental procedure only. Adult animals were co-housed in cages without mixing sex. Male mice were separated except in cases of littermates.

For the antibiotic-cleared suckling mouse model, 4-day old suckling CD-1 animals were fasted for 1.5 hours, then orally dosed with ~1mg/g body weight streptomycin using 30-gauge plastic tubing, after which the animals were placed with a lactating dam for 1 day. After 24 hours, mice received microbial communities with *V. cholerae* in a maximum gavage volume of 50µl. At 18 hours post-infection, animals were sacrificed, and relevant sections of intestinal tissue dissected and homogenized for CFU numeration and nucleic acid extraction.

Germ-free C57BL/6J mice were bred and maintained in plastic gnotobiotic isolators at University of California, Riverside. Mice were fed an autoclaved, low-fat plant polysaccharide-rich mouse chow (Lab Diet 5K52) and were 5–8 weeks old at time

of gavage. Bacterial cultures were prepared as described above. Mice were fasted for 30 minutes prior to introduction of bacteria, and stomach pH was buffered by intra-gastric gavage of 100μL 1M NaHCO3, followed by gavage with 150uL of balanced defined microbial libraries. Fecal samples were collected across the course of the experiment. Mice were sacrificed 4 days post gavage and small intestine collected and cut to three equal (proximal, medial, distal) sections by length. Samples were homogenized and used for CFU enumeration of bacteria on LB agar containing 200 μg/mL streptomycin.

For establishing defined microbiomes prior to V. cholerae infection, germ-free C57BL/6J mice were maintained as mentioned above and used at 6-13 weeks of age. Mice were fasted and given NaHCO3 as previously described and either given 150 μL of the simple resistant (SR), or dysbiotic (DS) communities. For the Mix group, mice were initially given the DS microbiome embodiment. 10 days after microbiome introduction and 4 days prior to *V. cholerae* infection, the SR community was introduced into the Mix group animals by gavage. 2 weeks after human commensal colonization, each group was infected with ~5 x 10^9 CFU V. cholerae O1 El Tor C6706. Fecal samples were suspended in 500 μL of PBS and homogenized using a bead beater (BioSpec) at 1,400 RPM for 30 seconds. CFU enumeration of *V. cholerae* was done on LB agar containing 200 μg/mL streptomycin.

We used the antibiotic-treated infant mouse model described above to determine which members of the healthy human microbiome contribute most to resistance to *V. cholerae*. We made 18 random combinations of human gut microbiome strains and introduced them to suckling mice along with *V. cholerae*. Each combination included five

unique strains, and each gavage contained the equivalent total microbial mass of 300µl of OD_{600}=0.4 culture, divided evenly across all constituent strains. After introducing human microbiome and *V. cholerae* to suckling mice, *V. cholerae* levels in homogenized intestines were determined by plating on selective agar. The absolute abundance of each species was determined with a combination of 16S rRNA gene qPCR and 16S rRNA sequencing.

Bacterial strains and growth conditions

All human gut commensal strains used are listed in Table S1. Unless otherwise noted, human gut strains were propagated in LYHBHI liquid medium (BHI supplemented to 5g/L yeast extract, 5mg/L hemin, 1mg/mL cellobiose, 1mg/mL maltose and 0.5mg/mL cysteine-HCl). Cultures were then propagated in a Coy anaerobic chamber (5% H2, 20% CO2, balance N2) or aerobically at 37oC.

All *V. cholerae* strains were derived from the C6706 El Tor pandemic isolate, including the *lacZ:*P_{tcpA}*:sh-Ble* zeocin resistance reporter strain (Liu et al., 2008), and propagated in LB media with appropriate antibiotics at 37°C. To construct a strain resistant to kanamycin, the plasmid pZE21 was cloned into *V. cholerae* C6706 (C6706-KM^R) and propagated in LB with kanamycin sulfate (Fisher Scientific, 50µg/ml) and streptomycin sulfate salt (100µg/ml). *Vibrio harveyi* BB170 was propagated in LM medium (Bassler et al., 1994) aerobically at 37°C.

To construct bsh^C, a strain constitutively expressing a bile salt hydrolase found in *B. obeum*, the *RUMOBE_00028* locus was amplified from *B. obeum* genomic DNA (primers: 5'-GTCGACGGTATCGATAATGCTTATGTGTACAGCTGC-3' and 5'-

GCAGGAATTCGATATCACTAATTCTGAAAATGAATCTGC-3'). All cloning and amplification primers are listed in Table S6. This amplicon was then cloned downstream of the constitutive PLtet-O1 promoter of plasmid pZE21 through digestion of the vector backbone with HindIII followed by Gibson assembly (New England Biolabs). The resulting plasmid was then introduced by electroporation into E. coli DH5αλpir to generate bsh^C. Strains were propagated aerobically in LB with kanamycin (50µg/ml) at 37°C.

A strain overexpressing the AI-2 signal of *B. obeum* (*BW30045_RO_AI-2*) was constructed by constitutively expressing the *B. obeum luxS* AI-2 synthase into *E. coli BW30045* (Δ*luxS*). The *B. obeum luxS* coding region (from genome position 33,305-33,784) was codon-optimized for expression in *E. coli*, placed downstream of the $P_{Ltet-O-1}$ constitutive promoter sequence derived from the plasmid vector pZE21 vector, and the construct cloned into vector pMK using the GeneArt Subcloning & Express Cloning Service (ThermoFisher). This expression construct was then amplified and inserted into the *endA* gene of the *E. coli* genome using primers (forward: 5'-CCAAAACAGCTTTCGCTACGTTGCTGGCTCGTTTTAACACGGAGTAAGTGTTAGAAAAATTCATCCAGCA-3', reverse: 5'-GGTTGTACGCGTGGGGTAGGGGTTAACAAAAAGAATCCCGCTAGTGTAGGCGGGCAGTGAAAGGAAGGCC-3').

We used natural transformation (Dalia et al., 2014) to construct a Δ*vasK V. cholerae.* Fragments of flanking genomic DNA upstream (forward: 5'-GAACTTTCGTCACGTAAGTC-3', reverse: 5'-

GTCGACGGATCCCCGGAATCATGAATTGTGTCCTTGTTTAC-3') and downstream (forward: 5'-GAACTTTCGTCACGTAAGTC-3', reverse: 5'-GTCGACGGATCCCCGGAATCATGAATTGTGTCCTTGTTTAC-3') of *vasK* and an antibiotic resistant gene cassette (forward: 5'-ATTCCGGGGATCCGTCGAC-3', reverse: 5'-TGTAGGCTGGAGCTGCTTC-3') were amplified from *V. cholerae* genomic DNA. Amplicons were then joined by overlapping PCR and introduced into C6706 via natural transformation. Resistant colonies were then selected on trimethoprim-containing agar (10ug/ml) and insertion confirmed via PCR.

Method Details

16S library preparation

For DNA from human fecal samples, ~200 mg (average wet weight) fecal sample was suspended in 600µl PBS. For mouse intestinal samples, tissues were dissected, homogenized in 5mL PBS, and 500µl of the homogenate used for DNA extraction. ~500µl 0.1mm glass beads (BioSpec), 210µl SDS %20, and 500µl neutral phenol:chloroform:isoamyl-alcohol (24:24:1, Fisher Scientific) were added to each sample, and samples were lysed by bead-beating followed by ethanol precipitation (Hsiao et al., 2014).

The V4 variable region of bacterial 16S ribosomal RNA genes was amplified in 25µl total volume reactions comprising 1µl of extracted DNA as template, 10µl Platinum Hot Start PCR Master Mix (ThermoFisher), 13µl PCR-grade water and 0.5µl of forward and reverse primers (10µM). Cycling conditions were 94°C for 3 min, followed by 33

cycles (94°C for 45 sec, 50°C for 60 sec, 72°C for 90 sec), and 72°C for 10 min. An equal amount of each amplicon (~240ng) was pooled into libraries, which were then purified using QIAquick PCR purification columns (Qiagen) and subjected to sequencing using the Illumina MiSeq platform. Paired-end 150nt reads were assembled, de-multiplexed, rarefied to >900 reads per sample, and analyzed using the QIIME 1.9.1 software package (Caporaso et al., 2010). Sequencing run results are summarized in Table S2A and S3.

Human gut microbiome 16S meta-analysis

For the analysis of bacterial composition between the human gut microbial communities and our artificial communities, the sequencing data of the V4 region of the 16S rRNA gene from published studies and samples collected for this study. For the different phases of *V. cholerae* infection, we used the first and last time points of diarrhea, and the last time of recovery (Hsiao et al., 2014). The last time point of fecal samples collected from parents of malnourished Bangladesh children were selected as the healthy adult Bangladesh control (Subramanian et al., 2014). See Table S2C for sequence accession numbers. Defined community inputs were designed on the basis that all the strains in the specific community are evenly distributed (CR: 1000 reads/species; SR: 3000 reads/species; DS: 2000 reads/species). All of the sequencing data were collected together and analyzed using QIIME as described above.

Metagenomic analysis of *bsh* phylotypes

The protein sequences of the eight representative BSH were obtained from Song et al., 2019 (Song et al., 2019). Deep metagenomic sequencing data was obtained from

David et al (David et al., 2015) (see Table S2D for sequence accession numbers). The microbial community DNA was aligned to the protein sequences using blastx. For queries that hit multiple protein sequences, the one that had the highest hit score was selected. The relative abundance of each type of the BSHs = the BSH reads count / (total reads count – human reads count). Host reads were determined by mapping metagenomic DNA to *Homo sapiens* reference genome (assembly GRCh38.p13) using HISAT2 (Kim et al., 2015).

Preparation of bacteria for animal studies

Each human gut bacterium was cultured from glycerol stocks in LYHBHI media for 48 hours at 37°C, and then diluted (1:50) in fresh LYHBHI media. After growth for an additional 48 hours, cultures were normalized for density by OD_{600}. For inoculation into suckling mice, the equivalent of a total of 300µl of 0.4 OD_{600} culture divided evenly across strains by community was pooled, pelleted by centrifugation, and resuspended in fresh LYHBHI. Each mouse received this mass of bacterial cells in a maximum gavage volume of 50µl per pup. In mice containing multiple defined communities, normalized mixtures were prepared so that 300µl of OD_{600}=0.4 equivalent of each community was represented in the final gavage. In mice receiving *V. cholerae*, the total resuspension volume of commensal strains was 25µl, with the remaining 25µl containing $1x10^4$-$1x10^5$ CFU *V. cholerae* in PBS.

Microbial levels in human fecal slurries were estimated via real-time PCR using universal 16S primers as described below, and samples were normalized to so that each

suckling animal received slurries containing the equivalent of ~20μg of microbial genomic DNA.

Measurement of fluid accumulation

Suckling mice were treated as described above. The fluid accumulation ratio percentage was determined as: [weight of intestines (Large and Small) / mouse body weight] x 100.

Assessment of T6SS killing by *V. cholerae in vivo*

CD-1 suckling animals were gavaged with antibiotics as described above. 3-5 day old infant mice were orally inoculated with total of ~$1x10^9$ CFU E. coli TB1 (*lacZ*-) and ~$1x10^4$ CFU *V. cholerae* (*lacZ*+) together in 50μl LB. Animals were sacrificed after 16 hr of infection. Mouse intestines were dissected and homogenized in 5 ml of PBS, and 10μl of the homogenate used for enumeration of bacteria via serial dilution on LB agar containing streptomycin and X-gal.

Quantitative real-time PCR

Total bacterial load in fecal samples and intestinal homogenates was determined by using real-time quantitative PCR. Reactions comprised 2 μL of extracted DNA (200ng/reaction) as template, 12.5 μL SYBRGreen Master Mix (BioRad), 10 μL PCR-grade water, and 0.25 μL of forward and reverse primers at 10μM (forward: 5'-CTCCTACGGGAGGCAGCAG-3', reverse: 5'-TTACCGCGG CTGCTGGCAC-3'). Cycle conditions were 95°C for 3 min, followed by 39 cycles (95°C for 10 sec, 55°C for 30 sec, 95°C for 10 sec, 65°C for 5 sec, 95°C for 5 sec).

Levels of the *B. obeum bsh* enzyme (RUMOBE_00028) were determined by real-time PCR as described above, using the primers 5'-GCGATCAGATTACGATCACTC-3' and 5'-GCCATGCCAACACCTTTTTC-3'. 200ng of purified DNA from intestinal homogenates of CD-1 mice colonized with complex human fecal samples were used as template for each reaction.

Levels of *tcpA* and *ctxA* expression in antibiotic-cleared CD-1 suckling animals containing SR and DS microbiomes were measured using real-time PCR (*tcpA*: primers 5'-GAAGAAGTTTGTAAAAGAAGAACACG-3' and 5'-CGCTGAGACCACACCCATA-3', *ctxA*: primers 5'-CACTAAGTGGGCACTTCTCA-3' and 5'-TGATCATGCAAGAGGAACTCA-3'), with *recA* (primers 5'-ATTGAAGGCGAAATGGGCGATAG-3' and 5'-TACACATACAGTTGGATTGCTTGAGG-3') as a control. Templates were generated by Trizol (Ambion) extraction of total RNA from intestines of SR and DS-colonized mice infected with V. cholerae, followed by cDNA synthesis with the SuperScript IV First-Strand Synthesis System (Invitrogen) following manufacturers' instructions. Real-time PCR was performed using conditions 95°C for 3 min, followed by 39 cycles (95°C for 10 sec, 55°C for 30 sec, 95°C for 10 sec, 65°C for 5 sec, 95°C for 5 sec).

Culturing of complex human fecal communities

Fecal slurries of complex human fecal samples were prepared as described above, and spread on LYH-BHI agar and incubated aerobically and anaerobically at 37°C. All colonies recovered were gathered by scraping, and DNA extracted and 16S rRNA genes amplified for sequencing as described.

AI-2 heat-stability assay

Cultures of *BW31145_RO_AI-2*, BB170 and C6706 were grown overnight. *BW31145_RO_AI-2* was subcultured 1:100 into 12ml of LB and grown in a shaker at 37oC for until $OD_{600} \approx 0.22$, centrifuged, and the supernatant filter sterilized. Aliquots of supernatant were then heated at 100°C for 30 minutes and cooled to room temperature. AI-2 activity was assessed using the BB170 bioassay (Bassler et al., 1994). Briefly, overnight cultures of reporter strain BB170 in LM medium were diluted at 1:1000 in AB medium, and 10µl of cell-free supernatant or heat-treated cell-free supernatant were then added to 90µl of BB170 dilution. Luminescence and OD_{600} of each sample were measured immediately and after ~3.5hrs growth at 30°C with agitation.

***In vitro* bile-dependent *tcp* induction**

P_{tcpA}-*sh-ble* was grown as overnight culture, diluted 1:1000 in fresh LB, and grown for ~2 hours at 37°C. Each reaction was prepared in 40µl 0.5X pH 8.5 LB medium, with sodium taurocholate hydrate (TC, Sigma-Aldrich), sodium glycocholate hydrate (Sigma Aldrich), cholic acid (CA, Alfa Aesar), sodium taurodeoxycholate hydrate (Sigma-Aldrich), sodium glycodeoxycholate (Sigma Aldrich), deoxycholic acid (DCA, MB Biomedicals), tauro-β-muricholic acid (Steraloids Inc.), or β-muricholic acid (Steraloids Inc.) added to a final concentration of 125µM. 2µl of reporter strain subculture was then added, and samples incubated anaerobically at 37°C for 4hrs. 2µl of each reaction was then added to 200µl of 0.5X LB pH 8.5 +/-10µg/ml of zeocin (Sigma Aldrich), incubated for 30 minutes aerobically at 37°C with agitation, and then serially diluted and plated onto LB agar plates with streptomycin to determine survival rates.

Induction represents percentage of P_{tcpA}-*sh-ble* reporter cells surviving treatment with zeocin after incubation with indicated samples under anaerobic conditions, defined as (zeocin-treated sample survival/average of no-zeocin controls)*100. Where noted, survival rates were normalized to that induced by 125μM taurocholate.

BSH structure comparisons

The potential 3D models of unknown structure were produced by Phyre2 (Kelley et al., 2015), based on amino acid sequence alignment to known protein structure. The 3D structure comparison and root-mean-square distance (RMSD) per-column spatial variation among structures were calculated using UCSF developed Chimera (V1.14) (Pettersen et al., 2004).

Commensal effects on *tcp*-activating signals

Commensal isolate cultures were grown for 48 hrs, and then subcultured at 3:100 for 48hrs. Growth was measured by OD_{600} and cultures normalized to 1.5mL of OD_{600}=0.4 culture. bsh^c and the corresponding vector strain were grown overnight in LB with kanamycin, subcultured at 1:100 for 24hr, and normalized as above. All cultures were clarified, the aqueous layer removed, and the pellets resuspended in sterile PBS with TC to a final concentration of 125uM. Cultures grown with antibiotics were washed one additional time with 1 volume of PBS prior to addition of TC. Samples were then incubated anaerobically for 24hr at 37oC followed by heat treatment at 100oC for 30 minutes, cooled to room temperature, centrifuged and the supernatant filter-sterilized with a 0.22μm filter. These samples were then used to induce P_{tcpA}-*sh-ble*, and percent survival following zeocin treatment determined as described above.

Removal of *tcp* activating signals in the gut was assayed as above, replacing pure TC solution in PBS with homogenate. Tissues were collected from 5-6-day-old CD-1 suckling animals in 2.5ml sterile H_2O, disrupted with a tissue homogenizer, pooled, and centrifuged to clear tissue debris. The resulting aqueous layer was heat-treated and filter sterilized as described above. The resulting sample was desiccated using a Savant Integrated speedvac system (Fisher Scientific) and resuspended in one-fifth volume of sterile water. Four volumes of acetonitrile (Sigma Aldrich) were then added and sample was incubated at room temperature for 20 minutes for deproteinization (Humbert et al., 2012). Samples were clarified, with the aqueous layer filter sterilized, desiccated and resuspended in one-fifth original volume sterile H_2O as described above. To sequester bile salts, 12.5mg of cholestyramine (Sigma-Aldrich) was added to 0.5ml of de-proteinized sample, and the mixture incubated at 1 hour at 37oC with agitation followed by passage through a 3kDa protein concentrator (Pierce PES Protein Concentrators).

Human complex fecal sample TC processing was assayed in vitro. 100µl of fecal slurries in glycerol prepared as described above was inoculated into 5ml LYBHI and incubated anaerobically for 2 days at 37°C. Cells were then pelleted, normalized to ~OD_{600} =0.4 in 1.5ml sterile PBS with 125µM TC, and incubated anaerobically at 37°C for 24hrs. Supernatants were then collected via centrifugation, heat-treated and filter sterilized as described above, and submitted for mass spectroscopy (see below).

The effects of B. *obeum bsh* on *V. cholerae* colonization was assayed by introducing bsh^C and the isogenic vector control into suckling animals prior to infection with Vibrio. 4-day old CD-1 suckling mice were treated with 50µl of 75mg/mL

kanamycin, then returned to lactating dams. Overnight cultures of *bsh*^{C} and vector strains were normalized to the equivalent 300µl of OD_{600}=0.4 culture, and cells pelleted and resuspended in fresh LYHBHI. 50µl of this was then introduced via intra-gastric gavage into antibiotic-treated suckling mice that had been fasted for 1.5 hours. Pups were then returned to a lactating dam. After 1 day of pre-infection colonization with *E. coli*, animals were gavaged with *V. cholerae* as described above.

Quantification of bile salts

All standards (TC, CA, and DCA) were submitted as 10mM solutions. LC-MS analysis of bile acids was performed on a Synapt G2-Si quadrupole time-of-flight mass spectrometer (Waters) coupled to an I-class UPLC system (Waters). Separations were carried out on a CSH phenyl-hexyl column (2.1 x 100mm, 1.7µM) (Waters). The mobile phases were (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The flow rate was 250 µL/min and the column was held at 40° C. The gradient was as follows: 0min, 1% B; 1min, 1% B; 8min, 40% B; 13min, 58.8% B; 13.5min, 100% B; 15.5min, 100% B; 16min, 1% B; 18min, 1% B. Flow rate was ramped to 600 µL/min at 13.5 min to speed up column flushing and re-equilibration.

The MS was operated in positive ion mode (50 to 1200 m/z) with a 100ms scan time. Source and desolvation temperatures were 150° C and 600° C, respectively. Desolvation gas was set to 1100L/hr and cone gas to 150L/hr. All gases were nitrogen except the collision gas, which was argon. Capillary voltage was 1kV. Injection volume was 1µl for all samples. The identity of bile acids in samples was confirmed by mass, retention time, and MS/MS as compared to authentic standards. Samples were analyzed

in random order and injected in duplicate. Leucine enkephalin was infused and used for mass correction. Data processing (peak integration) was performed using QuanLynx software (Waters). Accuracy of peak integrations was checked manually.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical tests were performed in the GraphPad Prism software package. Results are representative of two independent experiments. Statistical parameters for studies are reported in relevant figure legends and tables.

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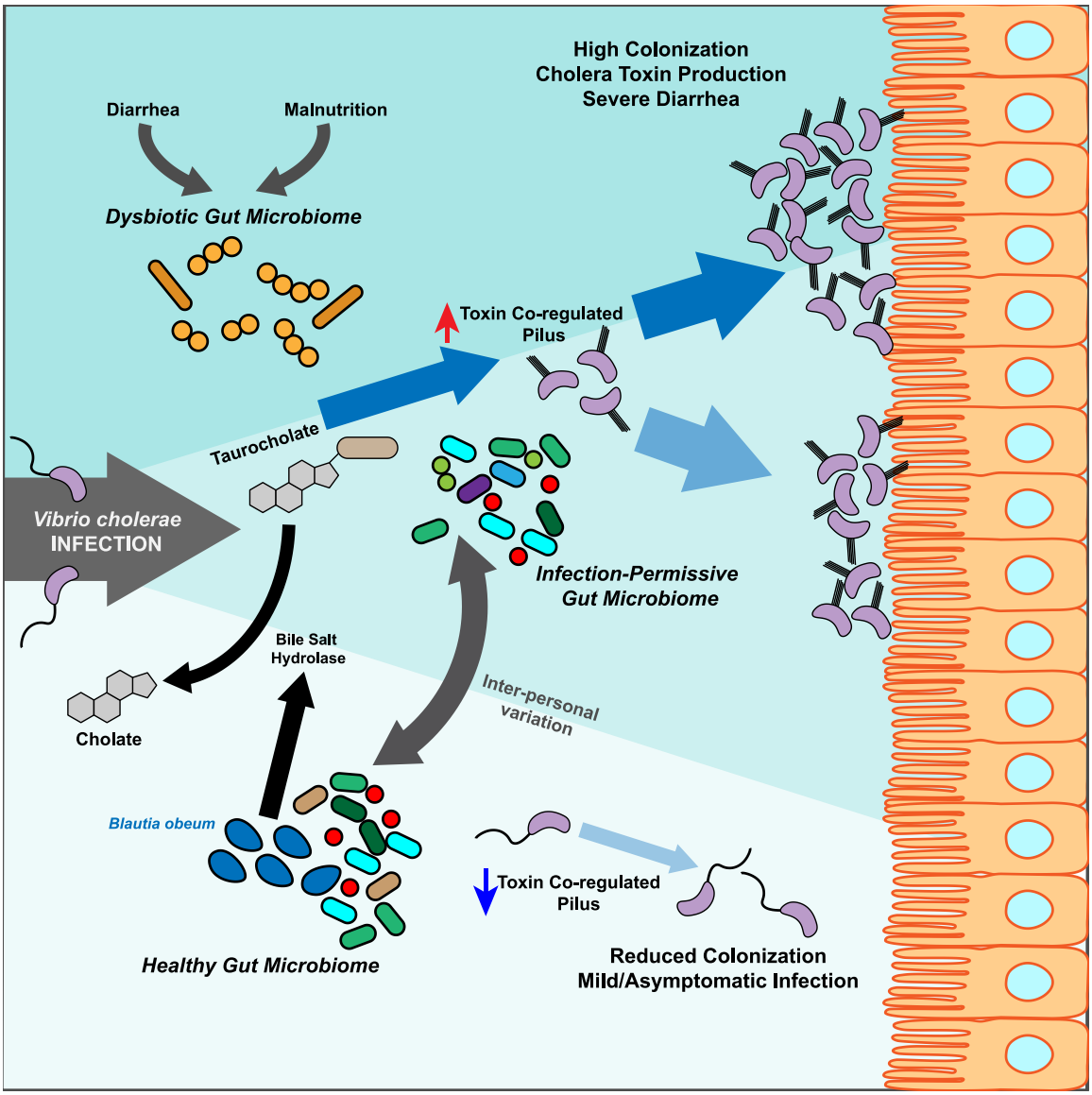

Figure 8. Graphical abstract of chapter 1.

CHAPTER THREE - Protocol for microbiome transplantation in suckling mice during *Vibrio cholerae* infection to study commensal-pathogen interactions

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Summary

The gut microbiome plays an important role in the exclusion of pathogens, and thus infection outcomes. Microbiome-pathogen interaction studies are complicated by a lack of tractable animal models and differences in animal model versus human microbiomes. We have adapted the suckling mouse model of infection of the human pathogen *Vibrio cholerae* to clear murine microbes and establish human-associated gut microbes during infection. Our method allows for the easy examination of the contribution of different human microbial communities to enteropathogenesis.

Before you Begin

The gut microbiome participates in numerous host-related phenotypes, including susceptibility to pathogens. The substantial differences between human and native animal model microbiomes complicate studies of microbiome-pathogen interactions (Seedorf et al., 2014). Conventionally-reared animals without modification are highly refractory to *V. cholerae* colonization (Olivier et al., 2009), while existing germ-free animal models for cholera are expensive and difficult to breed and maintain. Here, we describe an easily accessible animal model for studying interactions between human gut microbiome and *V. cholerae* in the context of infection. We have adapted the popular suckling mouse model of *V. cholerae* infection (Klose, 2000) through pre-infection treatment with the antibiotic streptomycin to substantially reduce existing mouse microbes, followed by the establishment of human-associated gut microbes to examine microbiome-pathogen

interactions during infection. Animals can be inoculated with complete human fecal specimens or with defined mixtures of pure bacterial cultures.

Option 1: Prepare Defined Communities of Bacteria for Gavage

Timing: 1-2 hours, ~ 72 hours incubation, 30 minutes next three days, ~48 hours incubation, 1-2 hours next two days

Note: Systems for generating anaerobic conditions may vary. We performed experiments using a Coy vinyl anaerobic chamber with internal incubator cabinet and atmosphere of 7% H2, 20% CO2, and balance N2. The chamber employs a palladium catalyst reacting with H2 to remove atmospheric oxygen by conversion to water. Typical O_2 concentrations are <10 ppm.

1. Ensure that there is sufficient deoxygenated media for experiment available. At least 3 days before the start of the experiment, place sterilized reagents, and autoclaved glass vials and crimp-top sealing caps with polyurethane septa, into anaerobic chamber with loose caps to allow for deoxygenation/pre-reduction.
2. Prepare media and reagents. Refer to "Materials and Equipment" for component list of LYH-BHI media. Add all components except hemin to a glass beaker. Stir at 52 ^{o}C and add hemin until dissolved. When the solution is clear, sterilize by filtration through 0.22 μm filter. Filter-sterilize 50% vol/vol glycerol and place into anaerobic chamber.

3. Prepare glycerol stocks from pure culture of anaerobic human isolates. Streak to single colony of suitable agar growth medium and pick one isolated colony for inoculation into 5mL liquid broth. For anaerobic strains, culture at 37 ºC in anaerobic chamber until late logarithmic phase (varies by species). Mix 1:1 vol/vol culture with filter-sterilized and deoxygenated 50% glycerol, mix by pipetting, and aliquot 300 µl in 2 ml glass vials with crimp-top caps with polyurethane septa. Place crimp-seal caps on vials with forceps sterilized with 70% vol/vol ethanol and crimp to seal. Cycle from anaerobic chamber and freeze immediately at -80ºC.
4. To start cultures for colonization, thaw the glycerol stock cultures of the bacterial strains in anaerobic chamber at room temperature (20-22 ºC). Stocks of aerobic strains can be thawed outside the chamber. Remove culture by syringe from thawed glycerol stock into 5 ml LYH-BHI media. Incubate for 3 days at 37ºC.

   CRITICAL: If the strain is a strict anaerobe, incubate and prepare cultures and stocks under anaerobic conditions.

   Note: Culture preparation for colonization studies starts 5 days before the start of the experiment. Preparation of complex human fecal samples can start on the same day of the start of the experiment.
5. After 3 days (2 days before the start of the experiment), dilute the cultures (1:50) in fresh LYH-BHI media. Incubate at 30ºC for 48 hours.
6. After 2 days (on the day of the experiment), measure the optical density (OD_{600}) of the culture. Use at least 100 µl of the culture for measurement.

7. Normalize cultures for density by OD_{600}. The equivalent of a total of 300 µl of 0.4 OD_{600} culture should be divided evenly across strains by community. For example, a gavage of a 10-member defined community will contain the equivalent number of cells in 30 µl of 0.4 OD_{600} culture of each member strain.
8. Mix the calculated amount for each strain together, centrifuge for 2 minutes at 9000 x g to pellet. Resuspend combined cell pellet in fresh LYH-BHI media to a maximum of 50 µl per suckling mouse

   CRITICAL: Pelleting of anaerobic strains should be performed in an anaerobic chamber. For mixed anaerobic/aerobic strains, pellet aerobic strains in normal atmosphere, and transfer cell pellets to the anaerobic chamber to resuspend in fresh deoxygenated LYH-BHI media.

   Note: Amount of LYH-BHI to resuspend cell pellets varies based on number of animals to be gavaged. Each mouse receives a maximum gavage volume of 50 µl. Mice not receiving *V. cholerae* at time of gavage can receive a community mixture of 50 µl. For co-infections, the cells of the community mixture should be resuspended in 25 µl final volume, with the remaining 25 µl being the *V. cholerae* inoculum, generally $1x10^4$ - $1x10^5$ CFU *V. cholerae* in LB media.

   Note: More than one gavage may be required if the first is not successful. In this case, gavages can be attempted while the mouse is still slowed from first exposure to isoflurane. If the mouse does not appear well or the color of the skin has changed, place back in the incubator and try to gavage again after 15-30 minutes.

9. Prepare *Vibrio cholerae* inoculum. One day before the start of colonization experiment, inoculate *V. cholerae* from glycerol stock in 3 ml LB media and incubate at 37oC for 18 hours On the day of the experiment dilute the culture (1:100 vol/vol) in fresh LB in 125 ml flask and incubate at 37oC for 3 hours with 250 rpm agitation on shaking incubator until the OD_{600} reaches ~ 0.3. Dilute culture (1:10) in fresh LB. Pellet by centrifugation, and transfer cell pellet into anaerobic chamber and resuspend in 1 ml deoxygenated LYH-BHI. Dilute 1:1000 of this for gavage. Each mouse receives 25µl of *Vibrio cholerae* inoculum.

   CRITICAL: When preparing gavages for a large number of mice, some of the inoculum will remain in the needle and syringe. To ensure that sufficient inoculum is available, consider preparing 2 times more inoculum than calculated as necessary for the number of mice to be gavaged.

Option 2: Prepare Complex Human Fecal Samples for Gavage

Timing: 4-5 hours (after sample collection), 1-2 hours on the day of the experiment

10. Human fecal samples are collected separately from each donor in specimen collection containers of choice (e.g. Fisherbrand Commode Specimen Collection System). After collection, stool samples can be processed immediately or frozen at -80ºC (for up to 6 months) for later processing.

    Note: Pooled samples would not be appropriate for the determination of inter-individual variation in pathogen-microbiome interactions.

11. To begin sample processing, transfer fecal specimens into anaerobic chamber on ice. Remove 2-3 cm piece with chisel sterilized with 70% vol/vol ethanol and weigh. Add 1:3 weight/volume of sterile deoxygenated PBS containing 0.1% wt/vol cysteine. Vortex for 5 minutes to resuspend.
12. After resuspending in PBS, pass sample through the sterile 70 µm cell strainer to remove large particulates.
13. Add sterile deoxygenated 50% weight/volume sterile glycerol to a final concentration of 25% vol/vol glycerol. Aliquot into 500µl into 2ml glass vial (large opening crimp-top). Sterilize tips of tweezers with 70% vol/vol ethanol and use to place 11 mm crimp seal red rubber septa (cap). Take care to avoid contact with the interior of the cap. Crimp cap to seal. Freeze stocks immediately at -80ºC.
14. Quantify the bacteria in each sample by quantitative real-time PCR measurement of bacteria 16S DNA. Normalize fecal slurries to be representative of 16S levels in 300 µl of OD=0.4 culture of defined community described above. Primers: 5'-CTCCTACGGGAGGCAGCAG-3', 5'-TTACCGCGGCTGCTGGCAC-3'.
    Note: For detailed protocol of quantitative real-time PCR, please refer to (Alavi et al., 2020).
15. On the day of the experiment, thaw sufficient prepared stocks in anaerobic chamber. Centrifuge the calculated amount for 2 minutes at 9000 x g to pellet. Resuspend cell pellet in fresh LYH-BHI media. LYH-BHI volume depends on the number of mice to be gavaged. See above after step 8.

16. If you have any aerobic strains, prepare as explained above, take the pellet to the anaerobic chamber and resuspend in calculated amount of fresh LYH-BHI or suitable media.

Reagent	**Concentration**	**Amount (for 1L)**
LYH-BHI media		
BHI broth (granulated)	37 g/L	37 g
Yeast Extract	5 g/L	5 g
Hemin	5 mg/L	5 mg
Cellobiose	1 g/L	1 g
Maltose	1 g/L	1 g
Cysteine	0.5 g/L	0.5 g
ddH_2O	-	to 1 L
LB media		
NaCl	5 g/L	5 g
Tryptone	10 g/L	10 g
Yeast Extract	5 g/L	5 g
ddH_2O	-	to 1 L
Bacto-agar (for plates)	15 g/L	15 g

Table 1. Materials and equipment

Step-by-Step Method Details

Preparation of Suckling Mice for Gavage

CRITICAL: SPF mice need to be pre-treated with oral antibiotics for 24hrs prior to introduction of bacteria. Gavage procedures for antibiotics and bacteria cultures or human fecal specimens are identical.

Note: All 30ºC steps are in incubator with open water pan to maintain humidity.

Timing: 1 hour on Day 0

1. Mice should be 4 days old at time of antibiotic administration. CD-1 suckling animals were purchased from Charles River Laboratories. Germfree suckling animals were obtained from the UCR gnotobiotic animal facility. Similar results have been obtained with C57/BL6 background suckling animals, and specific mouse backgrounds may be more appropriate depending on the pathogen of interest.
2. Fast infants (separate them from the lactating dam) for 1.5 hours before gavage. Place pups in 50ml plastic beaker lined with paper-towel or Kimwipe in 30ºC incubator with open water pan to maintain humidity.
3. During fasting period, prepare materials needed for gavage including isoflurane, 30-gauge plastic tubing, glycerol, 30G x 1 needles, and 1ml syringe.
4. Target antibiotic dosing is 1 mg/g body weight streptomycin. Based on the average weight of 4 days old suckling mice, concentration of streptomycin in 50 µl gavage would be 75 mg/ml in ddH_2O. Gavage procedure for streptomycin is identical to that described in steps 6-12.

Preparation of Bacterial Gavage

Timing: 2 hours on each Day 1 and Day 2

5. Place the 50 ml beaker containing fasted animals in 500 ml plastic jar (wide-mouth polypropylene jar with lid) lined with tissue paper or Kimwipe (Fig 9A).
6. Take a representative animal, place the plastic tubing to be used for gavage parallel to their body, and mark on the tube using permanent marker the distance from the tubing end represented by the snout to the stomach measurement (Fig 9B). During gavage, tubing will need to be inserted until this mark reaches the snout of the animal to ensure intra-gastric inoculation (Fig 10).
7. Absorb 150 µl of isoflurane into tissue paper in jar.

   CRITICAL: This prevents direct exposure of skin to isoflurane, which can cause pain/discomfort.

   CRITICAL: Work inside chemical fume hood when working with isoflurane.
8. Place suckling mouse to be inoculated in small interior container (Fig 9C), re-place the lid on the outer container, and wait 30 seconds (Fig 9D).

   Note: For anaesthetization of 2 mice, use ~300 µl isoflurane.

   CRITICAL: Be careful not to tighten the lid of the outer container.
9. Take the bacterial mixtures (either complete or cultured microbiomes with *V. cholerae*, see Steps 8-9 in Before you Begin) that have already been prepared by syringe from the vial. Place the tubing on the needle using forceps sterilized with 70% vol/vol ethanol and dip the marked area of the tubing in sterile 50% glycerol to lubricate tubing.

10. Carefully insert tubing into the mouth of the suckling mouse, aligning the trajectory of the tubing to match the esophagus (Fig 10).
11. Once the mark on tubing reaches the snout, inject 50 µl of the inoculum into stomach. For example of complete gavage procedure.
12. After each gavage, place mouse back in 30°C incubator.
13. After 15 minutes to disperse any residual isoflurane, place infants with dam.
14. The next day, after preparing the cultures for gavage, fast the suckling mice in 30°C for 1.5 hours.
15. Repeat the gavage with bacteria cultures/human fecal samples.
16. Place inoculated mice separated from dam in 30°C incubator for 15-16 hours.

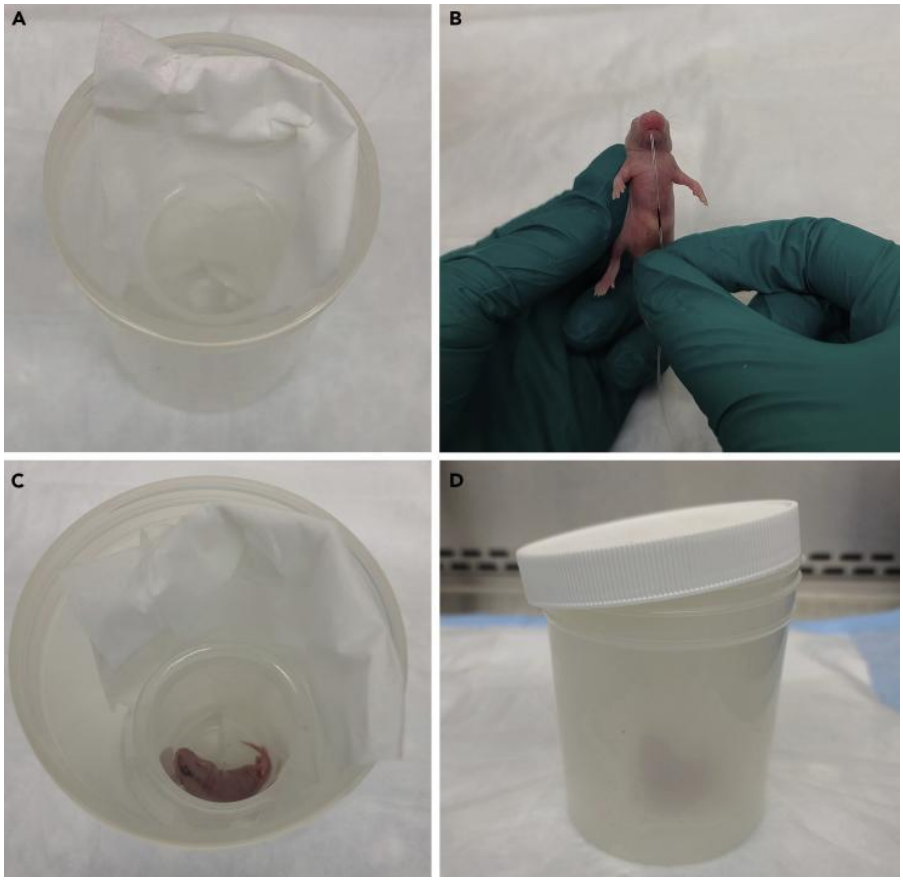

Figure 9. Preparing the container setup and mice for gavage.

In figure 9, we can see (A) A large container is lined with paper to absorb isoflurane. (B) Measure and mark gavage tube length needed to reach the stomach for intra-gastric gavage. (C) Place 1-2 pups in small container, and place inside large container to isolate from isoflurane. (D) After absorbing the tissue in the outer container with isoflurane, place lid loosely and wait 30 seconds to anaesthetize pups.

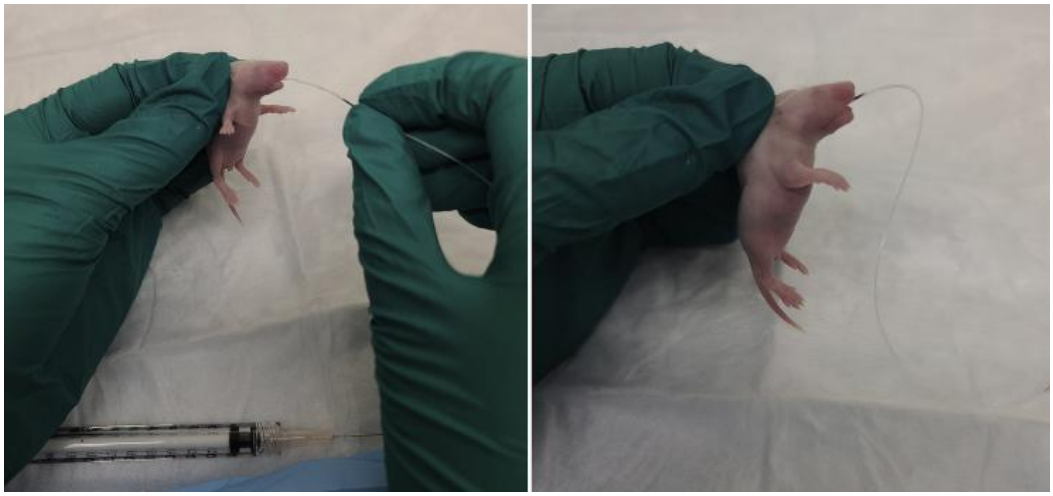

Figure 10. Gavage of pups

Mouse Dissection and Intestinal Homogenization

Timing: 4-5 hours

17. Anesthetize the mice using the two-container method as described above for gavage, but add 1 ml isoflurane and close the outer lid tightly.
18. After 4-5 minutes, remove mice from container. Perform cervical dislocation to euthanize, and prepare for dissection.

    CRITICAL: Disinfect all the forceps and scissors by ethanol (let them dry by air).

Figure 11. Mouse dissection.

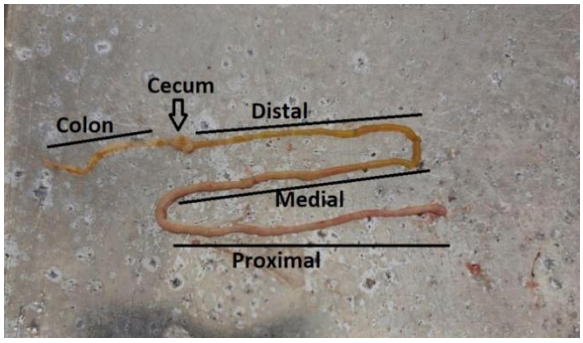

Figure 12. The small intestine is spread out to three equal sections based on length.

19. Spray 70% vol/vol ethanol on the surface of the body and allow it to air dry.
20. Make initial incision in the abdominal wall with dissection scissors (Fig 11). Continue excision laterally, taking care not to cut the intestine. Gently cut fascia binding the intestines so that they can be removed intact.
21. Once the intestines are moved out of the body cavity, cut at the proximal end where duodenum joins the stomach and at the distal end of the ileum.

22. Place the small intestine on the clean dissection tray. Divide the small intestine to three equal sections by length (Fig 12).
23. Place each section in 5 ml sterile PBS.
24. Use tissue homogenizer to fully break the tissue.
    a. Initially pulse the homogenizer in sterile water for 30s.
    b. Move to 100% vol/vol ethanol, and repeat.
    c. Pulse in third container of sterile water, and then homogenize tissue at full power for 60s.

    Note: Repeat these steps (a, b, and c) after each homogenization.
25. To extract DNA from the homogenate sample.
    a. Transfer 500 µl of homogenate to 2 ml screw-cap tube with ~500 µl 0.1mm zirconia/silica beads (autoclaved).
    b. Add 210 µl 20% wt/vol SDS.
    c. Add 500 µl phenol:chloroform:isoamyl alcohol (25:24:1).
    d. Bead beat on high speed for 2 minutes to disrupt tissue and lyse cells.
    e. Centrifuge 16000 x g at 4ºC for 15 minutes.
    f. Remove aqueous phase into new tube.
    g. Add 500µl phenol:chloroform:isoamyl alcohol, mix by inversion for 15 seconds.
    h. Centrifuge 16000 x g at 4ºC for 15 minutes.
    i. Remove aqueous phase into new tube.

j. Add 600µl of -20ºC isopropanol, 60µl (1/10 volume) 3M sodium acetate and mix by inversion.

k. Place in -20ºC for 16hrs or -80ºC for 1hr.

l. Centrifuge at 16000 x g for 30 minutes at 4ºC.

m. Decant supernatant.

n. Add 500 µl 100% ethanol.

o. Decant supernatant.

p. Air-dry at 37ºC for 8-10 minutes.

q. Suspend pellet in 50 µl water by pipetting.

26. For CFU enumeration of *V. cholerae* in homogenate:

a. Add 10 µl of intestinal homogenate to 90 µl sterile PBS.

b. Serially dilute 10-fold until 10^{-4} dilution.

c. Plate 50µl of the 10^{-3} and 10^{-4} dilution on the LB agar containing 200 mg/mL streptomycin.

d. Incubate plates at 30oC 16-18hrs and count colonies to determine colonization level of *V. cholerae* (see Fig. 13).

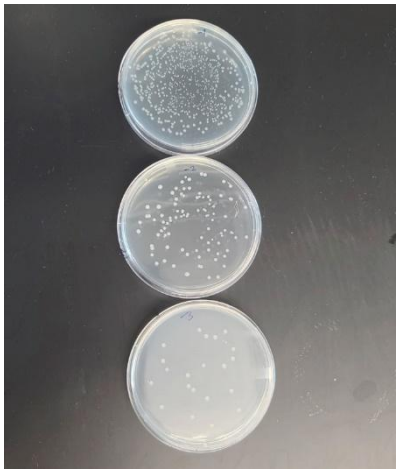

Figure 13. CFU enumeration of *V. cholerae* by serial dilution and plating.

Expected Outcomes

In this antibiotic-cleared mice model, the mice microbiome is ablated, allowing for the establishment of added human-associated bacteria in the mouse gut. The level of depletion can be quantified by extraction of DNA from intestinal homogenates followed by quantitative real-time PCR for microbial 16S genes using universal primers (Alavi et al., 2020). For a gavage of $1x10^4$-$1x10^5$ CFU of *V. cholerae*, a colonization level of 10^6-10^8 CFU / small intestine is expected after overnight infection depending on the defined community used. The level of colonization for mice also receiving complex human fecal specimens range from $1.5x10^6$-$4x10^7$ CFU.

Limitations

The method described here is only suitable for short term colonization experiments, as suckling mice cannot survive without long term access to lactating dam. Replacing colonized pups with dam introduces differences in available nutrients and thus

potentially different microbiome configurations, as diet is a potent driver of microbiome structure (Charbonneau et al., 2016). Non-dietary milk components are also able to directly interact with *V. cholerae* during infection in mice, potentially confounding microbiome-dependent results (Hsiao et al., 2006). Long-term colonization also risks cannibalization of sick pups before microbial data can be obtained. Previous studies involving continuous colonization with pups also have required substantially larger and more frequent administrations of *V. cholerae* to maintain colonization (Mao et al., 2018).

At the indicated doses of *V. cholerae*, the majority of microbes recovered after overnight infection are pathogens. For examination of microbiome-specific transcriptional or metabolomic activity during infection in vivo, the dosing of *V. cholerae* will need to be reduced. We also recommend the inclusion of uninfected control groups to examine *in vivo* commensal activity and viability. Intestinal samples from such animals can be used for 16S rRNA amplicon sequencing to determine the similarity of the transplanted community to the original fecal specimen.

The microbiomes endemic to individual animal facilities and backgrounds may differ, along with the presence of potentially streptomycin-resistant commensal organisms. Different antibiotic regimens may be required to ablate the murine microbiome prior to transplantation of human fecal specimens.

For the preparation of defined communities containing microbes requiring specific growth conditions, LYH-BHI may not be appropriate.

Troubleshooting

Problem 1:

High levels of variability in colonization per mouse or failed colonization (step 11, Step-by-Step).

Potential Solution:

Pups can regurgitate the gavage. To determine if the gavage is retained, add ~1 µl of food dye (filter sterilized) to the gavage preparation. The inoculum will then be just visible in the stomach of pups under the skin.

Problem 2:

Cannibalization of pups by dam after colonization (step 16).

Potential Solution:

After gavage, make certain to disperse residual isoflurane before putting pups back with the dam. This can be accomplished by maintaining them for at least 15-20 minutes in incubator after gavage to make sure.

Problem 3:

Mouse injury can happen during the gavage. Mouse deaths can happen from improper gavage (step 11, Step-by-Step).

Potential Solution:

Suckling mice should be checked frequently in the hour after gavage to ensure that they are moving. Attrition due to gavage can lead to the loss of some animals, which should be accounted for in the number of animals available at the start of the experiment.

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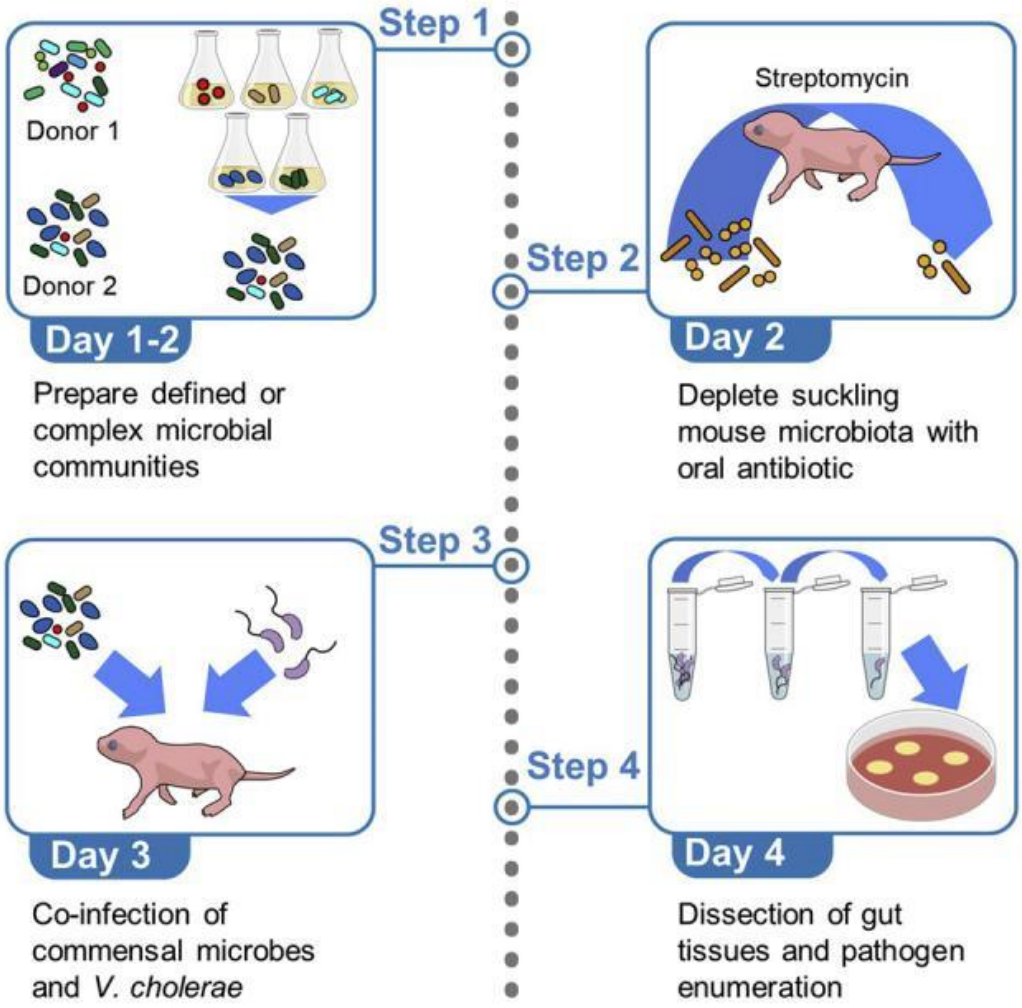

Figure 14. Graphical abstract of chapter 2.

CHAPTER FOUR- Conclusion

Conclusion

Previously, it has been proved that gut microbiome can confer colonization resistance in animal models, but whether human microbiomes contribute to this process and if so which components of those microbiomes mediate the largest effect has been little studied.

The work outlined in chapter two of this dissertation presents our finding that microbial community of gastrointestinal tract (gut microbiome) plays a key role in *V. cholerae* infection outcome. By transplanting of defined gut communities which we constructed and also complete human fecal microbiome into several animal models of infection and colonization, we showed that different gut microbiomes from different healthy individuals can cause strong differences in infection resistance.

Our complex resistant community model contains 13 bacterial species that much more closely resembled a normal healthy adult microbiome. The main character of our dysbiotic microbiome is low diversity of commensal bacteria and high levels of Streptococci and Enterobacteria which is caused by different environmental insults such as malnourishment and diarrhea by different enteric pathogens. Then we used these communities and our human fecal microbiome in suckling mice system which have cleared native murin flora by using streptomycin. Mice bearing the complex resistant model showed high resistancy of infection, co-inoculation of both models also recapitulated the resiatnt phenotype which means microbiome-dependent infection resistance can be restored through co-transplantation.

By transplanting complete human fecal microbiome to our suckling mice system, we showed that there is a range of *V. cholerae* colonization depending on the human donor, this data suggests wide variation in infection outcomes based on individual gut microbiome structure.

Previous studies have shown that taurocholate can induce TCP (toxin co-regulated pilus). To investigate the mechanism by how complex resistant community inhibits infection, we screened the effects of DS and CR communities on TC. We showed that CR members do better in inactivating *tcp*. Then we showed that CR community causes colonization resistance through the bile salt hydrolase enzyme activity which deconjugates TC, the strongest activators of *tcpA* expression in *V. cholerae*.

At the end of chapter two, we used realtime PCR to measure the level of the *bsh* gene of *B. obeum* in total DNA extracted from human fecal samples. We found that communities associated with higher *V. cholerae* infection had lower levels of *B. obeum bsh.*

In chapter three, we showed the new mice model which was developed for this dissertation. We showed how to set up an inexpensive and easily accessible suckling mouse model for studying interactions between microbiome and *V. cholerae*. We have modified the suckling mouse model by treating them with streptomycin to ablate existing microbes which can allow for the establishment of human gut commensals in the infant mouse.

In this body of work, we investigated the effect of variant human gut microbiome on *V. cholerae* infection risk. We showed that interpersonal human gut microbiome variation confers variable infection resistance, and this is mediated through the effects of both virulence-activating and virulence-suppressing signals at the site of infection.

Our studies also focus on developing new models and tools to identify members of the microbiome that are important for resisting infection. We used new approaches in antibiotic-treated infant mice and gnotobiotic animal models to show that the composition of the gut microbiome can contribute to resist colonization by *V. cholerae*. We were able to identify which members of healthy microbiomes are best able to mediate colonization resistance, and how these species affect microbiome composition and *V. cholerae* pathogenesis. These species may serve as potential probiotics or targets of prebiotic interventions to promote prevention of *V. cholerae* infection. This data suggests that manipulation of gut microbiome through these species may serve as a prophylactic intervention against *V. cholerae.*

While our studies contribute a lot to the field of host-pathogen interaction and can provide deep knowledge in how to prevent cholera infection by altering gut microbiome composition which helps lots of people who live in cholera endemic areas, still more research is needed to study the other factors can impact the gut microbiome structure such as diet. The question of how and what diet can drive the microbiome to resistant or susceptible structure has not been well answered. Future research should investigate the relationship between gut commensals and gut nutrient sources and how it can affect *V.*

cholerae. It is really important to better understand these factors as diet also can have a role in colonization trough bile secretion.

CHAPTER FIVE- Appendix

Unpublished Data

Vibrio cholerae colonization in the rest of healthy US human volunteers

As we mentioned before in chapter two, 30 human fecal samples were collected. We screened different human microbiomes by transplanting into our microbiome transplantation system in antibiotic treated suckling mice with *V. cholerae*. The colonization results from sixteen of these healthy humans were shown in figure 4 in chapter two. The colonization results of the other fourteen human fecal samples are shown below.

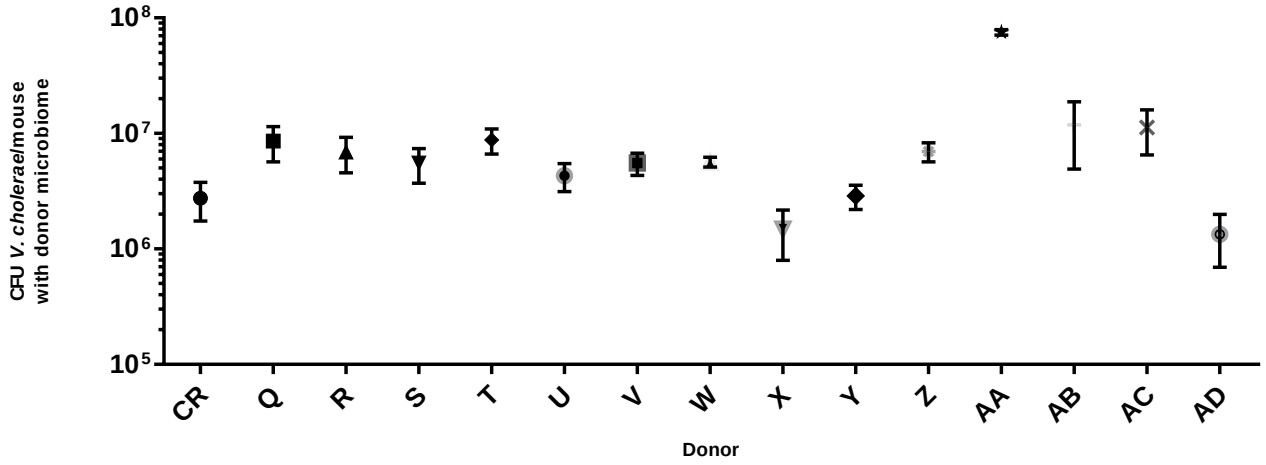

Figure 15. Intestinal *V. cholerae* colonization of CD-1 pups with fecal microbiome from donors.

GALT Project

Isolation of single colonies from human fecal sample

Human gut microbiota has been associated with health and diseases. However, most of the bacteria that reside in human gut microbiome are unculturable. Although, metagenomics studies have contributed a lot to the field, but numbers of sequences have been generated that could not been assigned to a known microorganism (Lagier et al., 2016). One method that has been improved over the time, is anaerobic culturing which has helped to isolate previously unculturable gut commensals (Greub, 2012). Previous studies have shown microbial culturomics which is a high throughput multiple culture conditions to study gut microbiome commensals. Lagier et al. have used the combination of multiple culture conditions such as MALDI–TOF (matrix-assisted laser desorption/ionization–time of flight) to grow the colonies and 16S rRNA sequencing to identify the growing colonies. Lau et al. have showed 66 culture conditions to detect higher bacterial diversity, they used 33 media and incubated the plates anaerobically and aerobically.

Our goal for this project is isolating single colonies from the human fecal samples that were collected. First, we plated the fecal sample A on LYHBHI agar plate. Then we scraped off all the colonies from the plate and performed 16S MiSeq sequencing on. For isolating single colony, we had to do it manually in the lab by picking every single colony. So, we decided to send it to GALT company.

GALT company uses a Prospector which can increase the speed of isolation (compare to manual colony picking we used to do in our lab) and decreases required time

for picking the colonies. Also, it can reduce contamination and increase number of colonies. Human fecal sample A was sent to GALT and they gave us frozen (in LYHBHI media) isolates in 96 well plates.

16S MiSeq sequencing was performed on all of them, then we selected 62 wells to perform 16S full length Sanger sequencing on.

Methods

1- Dilute fecal sample in reduced PBS.
2- Make stocks and store at -80^{o}C.
3- Send one the stocks to GALT company.
4- GALT sends the isolates back in 96 wells plates, frozen in LYHBHI in glycerol. They send two copies of each. Store them at -80^{o}C.
5- Transfer one of the plates (one from each set) to the anaerobic chamber, let it thaw in the chamber on ice.
6- Inoculate 40 ul of stock into 260 ul fresh media.
7- After 2 days, they should have grown. Make 2 stocks of each plate. which means make two 96 wells plates of each plate as the stocks. Take 100 ul of the culture into 100 ul glycerol. Store at -80^{o}C.
8- Take 1 ul of the same plate that was used to make stock to do 16S MiSeq illumine sequencing.
9- Perform the 16S MiSeq on all the plates.
10- After analyzing the data and figuring out what specie the wells are, do 16S full length Sanger sequencing.

11- In order to do Sanger, scrape the surface of the still-frozen stock on dry ice in the anaerobic chamber. Dump it to the fresh media.

12- When the cultures are grown, can continue to Sanger sequencing.

13- Make stocks of the desired species you found out from Sanger sequencing, can use the culture in the tube (that was used for Sanger) to make stocks.

Results

We got 54% relative abundance of the human sample 1 from prospector. The rarest species (*Eggerthella lenta*) isolated 0.1% of the sample which we couldn't get it by manual picking in the lab.

For sanger sequencing, selected wells were inoculated in LYHBHI media and on blood agar plates. PCR was done on the ones that grew and then they were sent out for sanger sequencing. The strains that we got the full hit and same in both sequencing methods were saved as stocks at -80. You can see the comparison between manual plate scrape in the lab and prospector from GALT company for species-level and genus-level in table 2.

A. Species-level recovery

Species detected	Original Sample	Plate Scrape (Illumina 16S MiSeq)	GALT (Single isolate)
Faecalibacterium prausnitzii	23%	0.1%	+
Eggerthella lenta	0.1%	-	+
Blautia obeum	4%	-	+

B. Genus-lever recovery

Genera detected	Original Sample	Plate Scrape (Illumina 16S MiSeq)	GALT (Single isolate)
Clostridium	0.2%	-	+

Table 2. Comparison between plate scrape and prospector. A: Species-level recovery, B: Genus-level recovery.

Results from 16S MiSeq sequencing of sample A, aerobic, and anaerobic plate scrape are summarized in table 3. This table is a supplementary table from chapter 2.

A. Species-level recovery

Human Subject	Species detected in whole sample	%Detected after Anaerobic culturing	%Detected after Aerobic culturing
A	47	40%	6%

B. Genus-level recovery

Human Subject	Genera detected in whole sample	%Detected after Anaerobic culturing	%Detected after Aerobic culturing
A	25	52%	12%

Table 3. Recovery of microbial diversity from complex human fecal microbiomes by culturing. This is related to figures 4 and 7. A: Species-level recovery, B: Genus-level recovery.

After doing both 16S V4 region illumine sequencing and 16S full-length sanger sequencing on the plates from GALT, we obtained these isolates (result was the same in both sequencing methods):

Blautia obeum

Bacteroides uniformis

Collinsella aerofaciens

Bifidobacterium longum

Eggerthella lenta

Dorea formicigenerans

Bacteroides fragilis

Bifidobacterium pseudocatenulatum.

Limitations

They are some species did not grow well by isolation technique. For example, *Parabacteroides distasonis* and *Ruminococcus gnavus* did not grow at all either in LYHBHI media or on blood agar plate. Some of *Faecalibacterium prausnitzii* and *Bifidobacterium* were the strains did not grow well as well.

According to a previous study (Lau et al., 2016) there are culture enriched conditions that we can capture the diverse human gut microbiota. One media that they report is useful to culture *Lachnospiraceae* isolates is BHI/1% inulin agar which includes:

BHI + vitamin K and 1 % propionic acid, 0.2× BHI or M9 minimal media (BD) + one of 1 g/L inulin, 0.5 g/L pectin, 0.5 g/L cellulose, 0.5 g/L mucin, or 0.5 g/L starch.

It has been shown that inulin can increase growth of butyrate-producing bacteria. BEEF is another media that shows increase in growth of *Lachnospiraceae* isolates which includes the genera of *Blautia, Ruminococcus, Dorea, Eubacterium,* and *Clostridium*. So, we can capture most of the isolates from the human samples.

The other media which can increase the growth of *Faecalibacterium prausnitzii* is Reinforced Clostridial which includes:

Tryptose (10.0 g)

Beef Extract (10.0 g)

Yeast Extract (3.0 g)

Dextrose (5.0 g)

NaCl (5.0 g)

Soluble Starch (1.0 g)

L-Cysteine HCl (0.5 g)

Sodium Acetate (3.0 g)

Resazurin (0.025%) (4 ml)

DI Water (1000 ml)

For doing 16S MiSeq on the plates from GALT, we had to do three runs which is more time consuming. They sent us 12 plates back and every four plates were done in one run as we have four plates barcodes. To improve this, we need a better barcoding system. If we could do it in one run instead of three runs, that would be much better. In future, we can use dual barcodes, then for each read we have two combinations of barcodes or we can use forward primers that have a barcode, so it would be faster.

We want to have defined communities from complete human samples that we collected to be able to test our defined community in our animal model systems. Having defined communities from our human samples let us put everything in as a defined community, or we can take them one put at the time or group at a time. Currently the designed communities are based on guesses of the 16S sequencing. So, it is really important to have an easy way of generating these libraries where we know everything goes in and detect the bacterial community from our complete human fecal samples. I mentioned the challenges we currently have, the future plan is making all these different

media in liquid and agar plates and try to grow the isolates on. By doing these enriched media, we might be able to get most of the species from the human gut microbiota. By having the defined community from our complete human fecal microbiome, we are able to test different species, single or in combination with others, in response to *V. cholerae* and can find new targets to hinder pathogenesis.

Chapter 2 Supplemental Figures

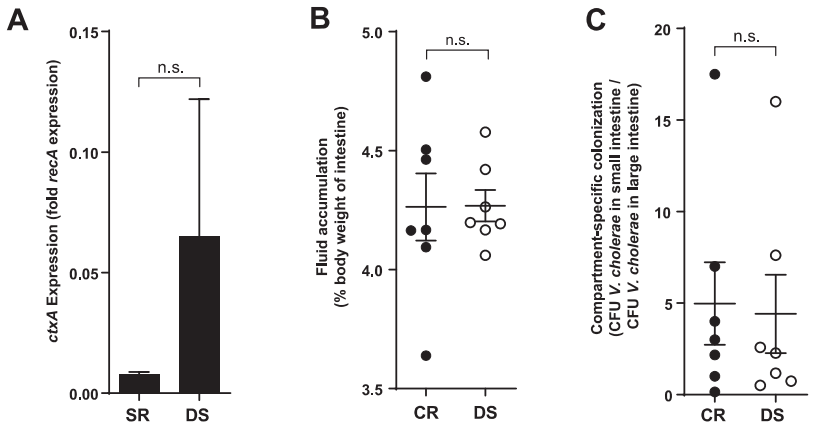

Figure 16. *V. cholerae* pathology and gut distribution in different microbiome contexts, Related to Figure 2.

All experiments are in antibiotic-cleared suckling CD-1 mice. (A) Expression of *ctxA* in intestinal tissues of infected mice containing defined model human microbiomes. (B) Fluid accumulation in intestines of infected mice containing defined model human microbiomes. (C) Distribution of *V. cholerae* in infected mice containing defined model human microbiomes. n.s. not significant (Mann-Whitney *U*-test).

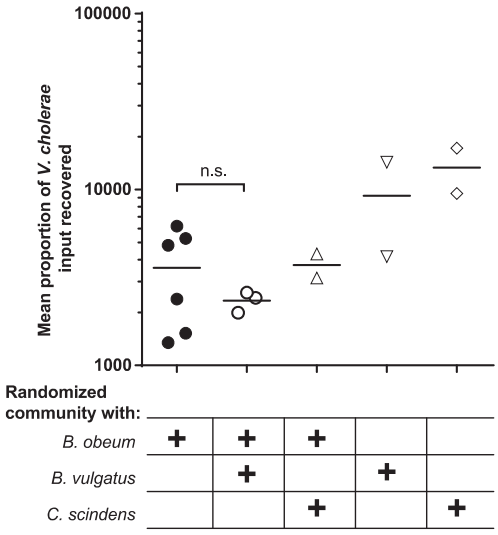

Figure 17. Mean *V. cholerae* colonization in antibiotic-cleared suckling CD-1 mice bearing communities containing *B. obeum* in combinations of SR species, Related to Figure 2.

Normalized colonization across experiments reported as fold CFU *V. cholerae* gavaged recovered after infection. n.s. not significant (Mann-Whitney *U*-test).

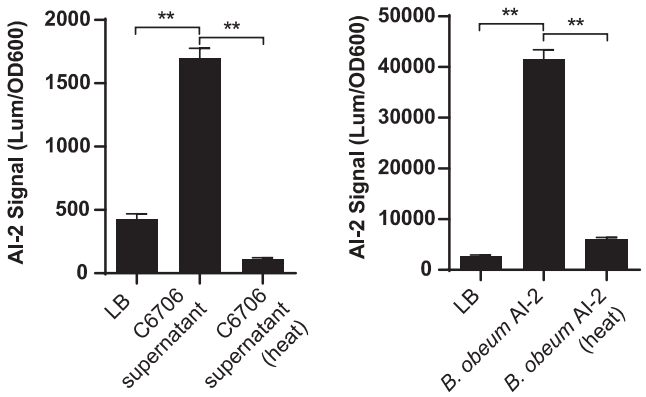

Figure 18. Induction of BB170 AI-2 reporter by indicated cell-free supernatants, Related to Figure 6. ** P<0.01, (Mann-Whitney U-test).

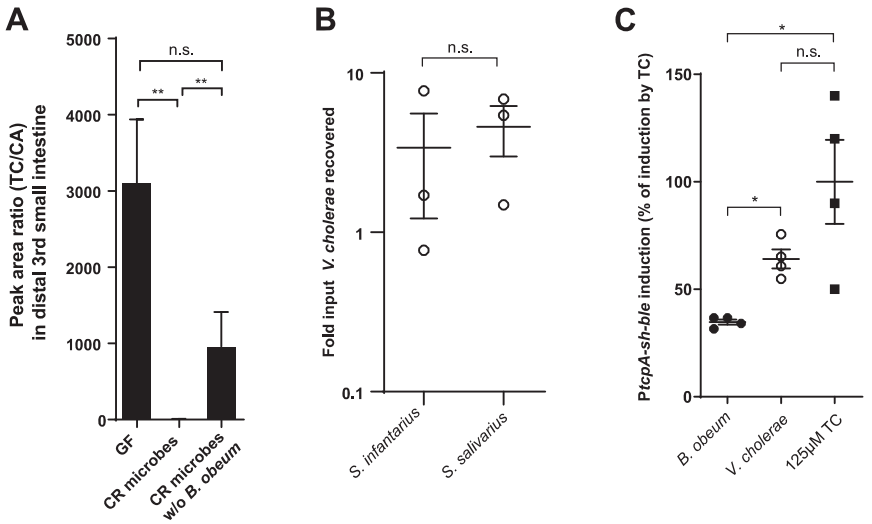

Figure 19. Contribution of different microbial species to TC levels, Related to Figure 6.

(A) Mass spectrometry measurement of taurocholate (TC) to cholic acid (CA) ratio in distal third of small intestine of adult germfree C57BL/6J mice 2 days after colonization with pure cultures of indicated strains. (B) Amount of *V. cholerae* recovered during co-infection of suckling CD-1 mice with either *S. infantarius* or *S. salivarius*, normalized to input CFU *V. cholerae*. (C) Ability of *B. obeum* and *V. cholerae* to interfere with TC activation of virulence in reporter *V. cholerae* in vitro after 5 hours incubation, normalized to induction by 125μM TC. * $P<0.05$, ** $P<0.01$ (Mann-Whitney *U*-test), n.s. not-significant.

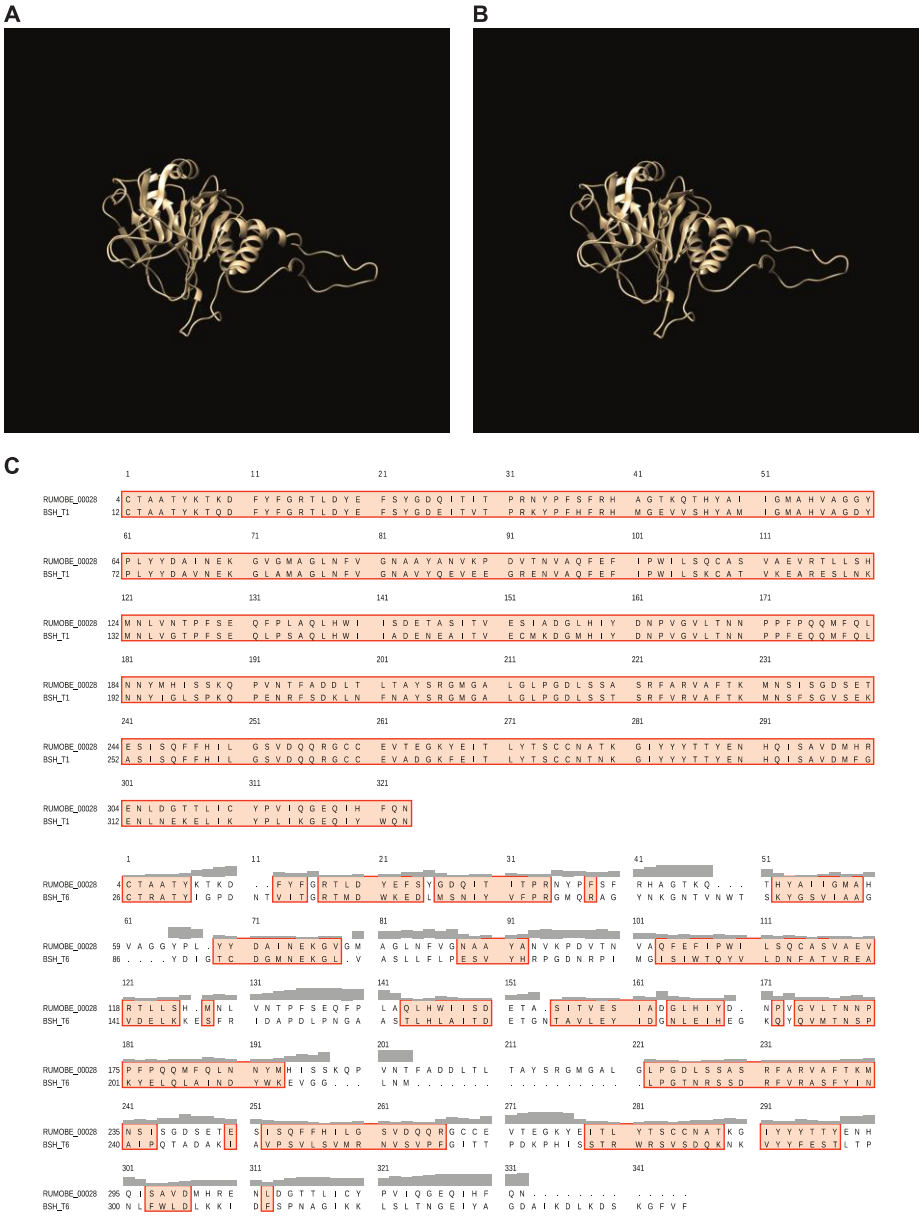

Figure 20. Comparison of bile salt hydrolases, Related to Figure 6.

(A) Predicted structure of *B. obeum* bile salt hydrolase RUMOBE_00028. (B) Predicted structure of consensus phylotype 1 BSH. (C). Amino acid alignment of

RUMOBE_00028, phylotype 1 BSH, and phylotype 6 BSH using Chimera. Header in gray shows the spatial variation per column. Colored boxes shows structural similarity between regions, with coloring of one-letter code amino acids using Clustal X, dependent on both residue type and the pattern of conservation across aligned sequences.

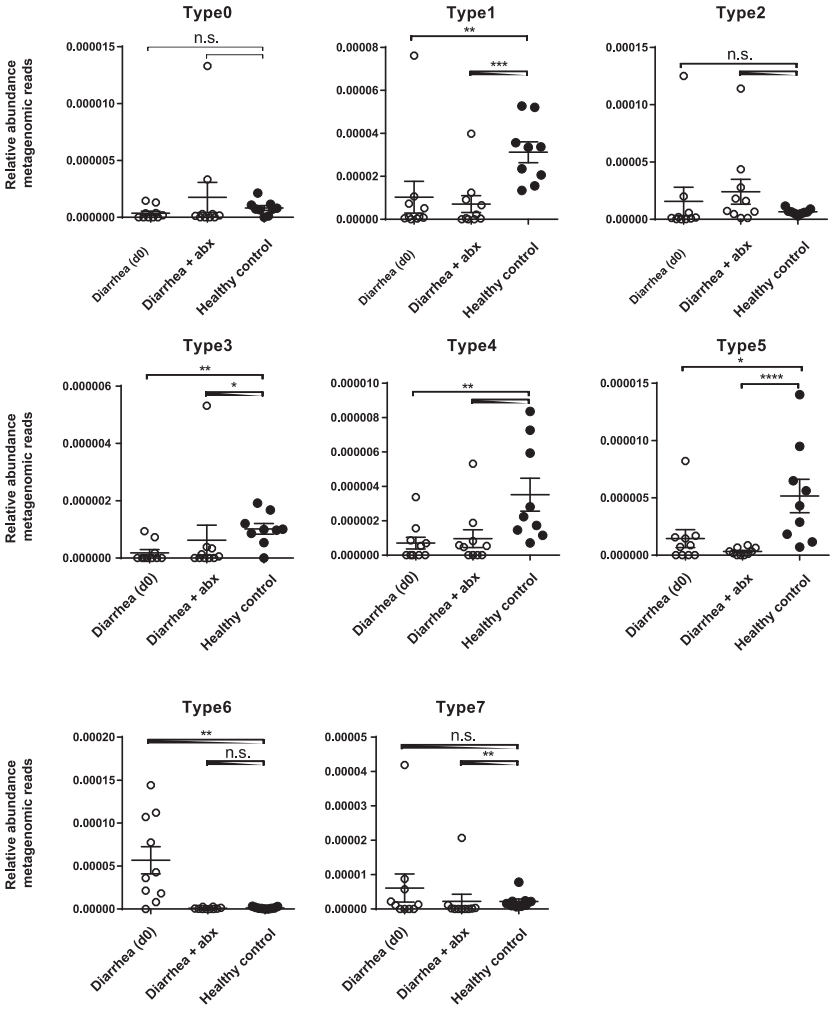

Figure 21. Levels of different phylotypes of microbial *bsh* enzymes in metagenomic sequencing of fecal microbiomes of cholera patients pre (d0) and post- (+abx) antibiotic treatment, compared to healthy individuals in Bangladesh, Related to Figure 7.

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