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Modulation of *Vibrio cholerae* Virulence Pathway through Microbiome Metabolism of Bile Acids

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Dedication

To my parents for fostering my love in science and being there for me even in the rough times.

To my fiancée Elizabeth Deyett for encouraging me to further my education, supporting me though this endeavor and being my rock.

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Table of Contents

List of Acronyms

Adenosine diphosphate (ADP)
Adenosine monophosphate (AMP)
Adenosine triphosphate (ATP)
Alpha-muricholic acid (α-MCA)
American Type Culture Collection (ATCC)
Beta-muricholic acid (β-MCA)
Bile salt hydrolase (BSH)
Blautia obeum (B. obeum)
Carbon (C)
Chenodeoxycholic acid (CDCA)
Chlorine (Cl-)
Cholera toxin (CT)
Cholera toxin phage (CTXφ)
Cholic acid (CA)
Cyclic adenosine monophosphate (cAMP)
Cytochrome P450, family 27, subfamily A, polypeptide 1 (CYP27A1)
Cytochrome P450, family 7, subfamily A, polypeptide 1 (CYP7A1)
Cytochrome P450, family 8, subfamily B, polypeptide 1 (CYP8B1)
Deoxycholic acid (DCA)
Deoxyribonucleic acid (DNA)
Escherichia coli (E. coli)
Farnesoid X receptor (FXR)
Glycochenodeoxycholic acid (GCDCA)
Glycocholate (GC)
Glycocholic acid (GCA)
Glycodeoxycholate (GDC)
Glycodeoxycholic acid (GDCA)
Hydroxysteroid dehydrogenase (HSDH)
Hyodeoxycholic acid (HDCA)
Kyoto Encyclopedia of Genes and Genomes (KEGG)
Liter (L)
Lithocholic acid (LCA)
Lysogeny broth (LB)
Microliter (ul)
Micromolar (uM)
Mililiter (ml)
Molar (M)
Murideoxycholic acid (MDCA)
National Center for Biotechnology Information (NCBI)
Nicotinamide adenine dinucleotide (NAD+)
Nicotinamide adenine dinucleotide phosphate (NADP+)
Nitrogen (N)

Optical density (OD)
Polymerase chain reaction (PCR)
Quantitative Real-Time PCR(qPCR)
Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH)
Reduced nicotinamide adenine diphosphate (NADH)
Ribosomal ribonucleic acid (rRNA)
Sodium (Na+)
Streptococcus salivarius Subsp. salivarius (S. salivarius)
Tauro-beta-muricholic acid (Tβ-MCA)
Taurochenodeoxycholic acid (TCDCA)
Taurocholate (TC)
Taurocholic Acid (TCA)
Taurodeoxycholate (TDC)
Taurodeoxycholic acid (TGDCA)
Toxin co-regulated pilus (TCP)
Universal Protein Resource (UNIPROT)
Ursodeoxycholic acid (UDCA)
Vibrio cholerae (V. cholerae)
Water (H_2O)
World Health Organization (WHO)

List of Tables

List of Figures

INTRODUCTION

Vibrio cholerae

Background

V. cholerae is a gram-negative bacillus responsible for the severe human diarrheal disease cholera, affecting millions annually worldwide (*1*). *V. cholerae* naturally reside in marine environments including brackish water, and in the environment is often found complexed with marine organisms such as plankton or shellfish (*2*). In human populations, infection occurs from the ingestion of contaminated food or water, often caused by poor sanitation infrastructure (*2, 3*). Once inside the host, *V. cholerae* preferentially colonizes the distal small intestine, where it expresses several virulence factors in order to establish infection, including the cholera toxin (CT) responsible for the watery diarrhea characteristic of cholera, and the toxin-coregulated pilus (TCP) required for colonization of the epithelium (*4*).

It is uncertain as to when *V. cholerae* became a human pathogen. Descriptions of sicknesses that strongly resemble cholera are described by Hippocrates (~460-377 B.C.E) as well as inscriptions found in India dating back to the era of Alexander the Great (356-323 B.C.E.) (*5, 6*).The first written documentation of the disease dates to 15th century India as European observers began arriving in the country (*5*). The first global pandemic of cholera occurred in 1817, originating from the Ganges river in India (*7*). This was followed by six subsequent cholera pandemics, with the seventh pandemic continuing up to the present day (*8*). *V. cholerae* was first identified as the etiologic agent of cholera by Filippo Pacini in studies of an outbreak in 1854 (*9*). Of the seven recorded pandemics, the

first six were caused by the Classical biotype of *V. cholerae,* while the ongoing seventh pandemic has been due to the newer El Tor biotype originating in South Asia in 1960 (*8*). While like Classical *V. cholerae* part of the O1 serogroup, El Tor biotype strains are characterized by lower toxin production and virulence. As a result El Tor biotype strains establish a less severe infection, leading to increased numbers of infected individuals and higher asymptomatic carriage (*2*). Today, infections caused by Classical strains are rarely reported.

The study of cholera has been an important driver of the fields of microbiology and our understanding of infectious diseases more broadly. For example, during the third pandemic, the efforts of John Snow to trace an outbreak of cholera in London contributed to the founding of the field of epidemiology (*8*).

Symptoms and Treatment

The symptoms between Classical and El Tor infections are similar, with El Tor typically leading to milder infections (*10*). Once infected, symptoms can take up to five days to develop. The hallmark symptom of this disease is severe watery diarrhea (up to 18 liters a day). This diarrhea has a distinctive “rice water” appearance caused by the presence of numerous *Vibrio* cells and host mucus (*3, 8*). Other accompanying symptoms include vomiting, muscle cramps and irritability as well as signs of dehydration such as loss of skin elasticity, dry mucus membranes, sunken eyes, reduced urine, and in severe cases lethargy (*11, 12*). If left untreated, the infection can cause acidosis, shock, and circulatory collapse within hours (*8*).

The principle treatment for a cholera infection is rehydration therapy which drops the mortality rate under one percent (*8, 12*). For severe cases, antibiotics including azithromycin and doxycycline for adults and erythromycin or azithromycin for children and pregnant women are used in conjunction with oral rehydration (*12*). Reports also indicate that zinc administration can reduce infection duration and severity (*10*).

Several vaccines on the market for *V. cholerae* have been approved by World Health Organization (WHO), including Dukoral, Shanchol, Euvichol and Vaxchora (*13*). Dukoral uses heat and formalin inactivated *V. cholerae* as well as the non-enzymatic B subunit of CT and provides a 3-4-year immunity in endemic and industrialized countries with approximately fifty percent efficacy. Shanchol also includes heat and formalin inactivated *V. cholerae* as well as the CT B subunit but grants five-year immunity and only provides protection to endemic populations with approximately sixty-five percent efficacy. Euvichol has similar efficacy to Shanchol except for the vaccine uses only heat and formalin inactivated *V. cholerae*. The most recent commercially available vaccines is Vaxchora, a live-attenuated vaccine that provides immunity for at least 6 months using a recombinant *V. cholerae* with a deletion for the genes encoding the CT enzymatic subunit and hemolysin A (*14*). Despite the availability of effective treatment options and some prophylactics, the morbidity of cholera is still high affecting up to 4 million annually and 143,000 deaths, and vaccines have demonstrated highly variable efficacy and typically short periods of provided immunity, highlighting the need for preventative care and alternative treatments (*7*).

Virulence Factors

Successful *V. cholerae* infection of mammalian hosts depends on a carefully regulated cascade of gene regulation, culminating in the production of CT, and the primarily colonization factor TCP (*15, 16*). The presence of CT was first proposed by Robert Koch in 1884, but it was not until 1959 that it was shown that cell-free culture filtrates caused diarrhea (*17, 18*). This work then led to CT being purified a decade later (*19*). CT consists of a single A subunit and five B subunit that are released by infectious *V. cholerae* cells through a Type II secretion system (*18, 19*). The two genes encoding these subunits, *ctxA* and *ctxB* respectively, are carried on the lysogenic vibriophage CTXφ. Infection with this phage can render previously non-infectious *Vibrio* strains toxogenic and dangerous (*20*). Before secretion, five CtxB subunits form a pentamer around a single CtxA monomer in the periplasm to form a single holotoxin (*21*). Once outside the cell, each of the monomers of the CtxB can bind with the glycan portion of the glycolipid GM1 receptor on epithelial cells lining the intestinal lumen. This interaction causes the cell to endocytose the toxin, whereupon the B subunit complex creates an opening in the endosome for the A subunit (A1) to enter the endoplasmic reticulum. A1 then unfolds, hijacking the cellular machinery allowing for export of misfolded proteins into the cytoplasm for proteasomal degradation (*22*). A1 then folds and causes G-protein ADP-ribosylation resulting in the constitutive activation of adenylate cyclase, increasing the levels of cAMP. cAMP, in turn, activates a cystic fibrosis transmembrane conductance regulator, leading to the constitutive expression of the transporter resulting in Cl^- leaving the cell. This leads to Na^+ loss to maintain

electroneutrality, prompting H_2O loss and resulting in cholera's distinctive watery diarrhea (*23*). This diarrhea may then help in elimination of other bacterial competition through purging of the gut microbiota and allows for *V. cholerae* to disseminate into the environment later in the infection.

While CT is required for diarrhea, *V. cholerae* uses TCP in order promote bacterial cell-cell interactions, microcolony formation, and attachment to the host epithelium cells, as well as providing receptors for the CTX phage (*24, 25*). It has been shown that the expression of *tcp*A, the pilin subunit of TCP, is induced an hour after inoculation into infant mice, while *ctx*A, the enzymatic subunit of CT, transcription occurred at four hours and that this was dependent on the prior expression of TCP. TCP has been shown to be absolutely required for infection of humans and mice (*16, 25*). TCP is a Type IV bundle forming pilus made of many monomers of the pilin TcpA, and the genes involved in its biogenesis, encoded in the *tcp* operon, are transcriptionally co-regulated with the *ctxAB* genes encoding CT (*25*). The TCP biogenesis apparatus can also act as a type IV secretion system, leading to the secretion of the soluble virulence factor TcpF which has been shown to aid in colonization; Δ*tcpF* mutants demonstrated a decreased colonization in suckling mouse intestines compared to WT (*26*). These pili, along with exopolysaccharides and proteins, help form a biofilm protecting the cells from host immunity, environmental factors and other microbial species (*27*).

The initial activation of virulence genes in *V. cholerae in vivo* is a complex process involving several coordinately-expressed regulators and environmental stimuli. The cell surface complexes ToxRS and TcpPH can respond to environmental signals,

such as oxygen concentration and bile acids to time the expression of downstream virulence regulators. ToxRS is able to directly activate CT gene expression, and the combined action of ToxRS and TcpPH can stimulate the expression of the master virulence regulator ToxT, which can then activate the *tcp* operon and *ctx* gene expression (*28-31*). The stability of both ToxRS and TcpPH are tied to bile acids in the host. Bile is secreted into the proximal small intestine with a large portion being reabsorbed in the distal small intestine where *V. cholerae* colonizes, and thus serve as an effective environmental stimuli of virulence activation in the preferred site of *V. cholerae* colonization (*16, 25, 32*). ToxS and TcpH are thought to stabilize ToxR and TcpP respectively, as mutations of these genes showed an increase in proteolytic activity (*30, 33*). It has been reported that proteolysis of ToxR was blocked by the introduction of deoxycholate (DC), while TC forms a disulfide bridge to form between two TcpP monomers leading to downstream signaling and virulence activation (*31, 34*). This disulfide bridge also serves to protect TcpP from degradation independent of TcpH (*35*).

Bile Acids

Synthesis and Regulation

Bile acids are molecules in the body derived from cholesterol, synthesized in the liver, and stored and secreted by the gall bladder into the small intestine. In the small intestines, bile acids play an important role in the emulsification and absorption of dietary fats (*36*). There are two pathways for bile acid synthesis, the classical or neutral pathway and the acidic or alternative pathway; together, both pathways utilize seventeen enzymes

(*32*, *36*, *37*). The classical pathway occurs in the liver and accounts for ninety to ninety-five percent of all bile acid synthesis (*36*). Synthesis starts when cholesterol interacts with the enzyme CYP7A1, with the end result being either cholic acid (CA) or chenodeoxycholic acid (CDCA), which are known as primary bile acids (see Figure 1).

Name	Chemical Structure	Molecular formula	Hydroxylated Backbone Configuration	Primary/ Secondary	Human/ Murine Bile Acid
Cholic Acid (CA)		$C_{24}H_{40}O_5$	C3α, C7α, C12α	Primary	Both
Chenodeoxycholic Acid (CDCA)		$C_{24}H_{40}O_4$	C3α, C7α	Primary	Both
α-Muricholic Acid (α-MCA)		$C_{24}H_{40}O_5$	C3α, C6β, C7α	Primary	Murine
β-Muricholic acid (β-MCA)		$C_{24}H_{40}O_5$	C3α, C6β, C7β	Primary	Murine
Deoxycholic Acid (DCA)		$C_{24}H_{40}O_4$	C3α, C12α	Secondary, derived from Cholic Acid	Both

Figure 1: Structure and characteristics of bile acids

Name	Chemical Structure	Molecular formula	Hydroxylated Backbone Configuration	Primary/ Secondary	Human/ Murine Bile Acid
Lithocholic Acid (LCA)		$C_{24}H_{40}O_3$	C3α	Secondary, Derived from Chenodeoxycholic Acid	Both
Ursodeoxycholic Acid (UDCA)		$C_{24}H_{40}O_4$	C3α, C7β	Secondary, Derived from Chenodeoxycholic Acid	Both
Murideoxycholic Acid (MDCA)		$C_{24}H_{40}O_4$	C3α, C6β	Secondary, Derived from α/ β-Murcholic Acid and Lithocholic acid	Murine, Trace Amounts in Humans
Hyodeoxycholic acid (HDCA)		$C_{24}H_{40}O_4$	C3α, C6α	Secondary, Derived from Muricholic Acid and Lithocholic Acid	Murine, Trace Amounts in humans

Figure 1 continued: Structure and characteristics of bile acids

The determinative step of bile acid synthesis is the action of enzyme CYP8B1, acting as a 12α-hydroxylase by adding a hydroxyl group to C12 of the steroid ring. If the intermediate molecule has the hydroxyl group added, then it will become CA, if not, the molecule will become CDCA. The acidic pathway occurs in many different types of tissues in the body, with synthesis initiated by one of three enzymes. CYP27A1 is found mitochondria of many tissue types, CH25H are found in the endoplasmic reticulum, and CYP46A1 is found in the brain (*37, 38*). It is thought that this pathway is used to control lipid homeostasis (*38*). This pathway produces oxysterol intermediates, which are transported to the liver with the end product being CDCA (*37*). The last step is conjugation of the carboxyl group of a bile acid to an amino acid, either glycine or taurine in humans, done in a ratio of 3:1 glycine to taurine respectively (*36*). However, diet can affect this ratio; for example, high taurine diets can lead to an increase in taurine conjugated bile acids (*36, 37*) (see figure 2). Bile acid conjugation serves several roles. First, since bile acids are hydrophobic, amino acid conjugation makes a portion of the molecule hydrophilic, as well as increasing the solubility of the molecule at physiological pH (*37*). Second, conjugation prevents cleavage by pancreatic peptidases that are released into the lumen at the same point as bile. Third, conjugation decreases passive reabsorption, which allows bile acids to act on food longer. Ultimately, ~95% of the bile acids in the gastrointestinal tract is reabsorbed to be reused. Approximately 200-600 mg of new bile acids are synthesized by the liver daily, which is roughly equivalent to the 5% lost in feces (*37*).

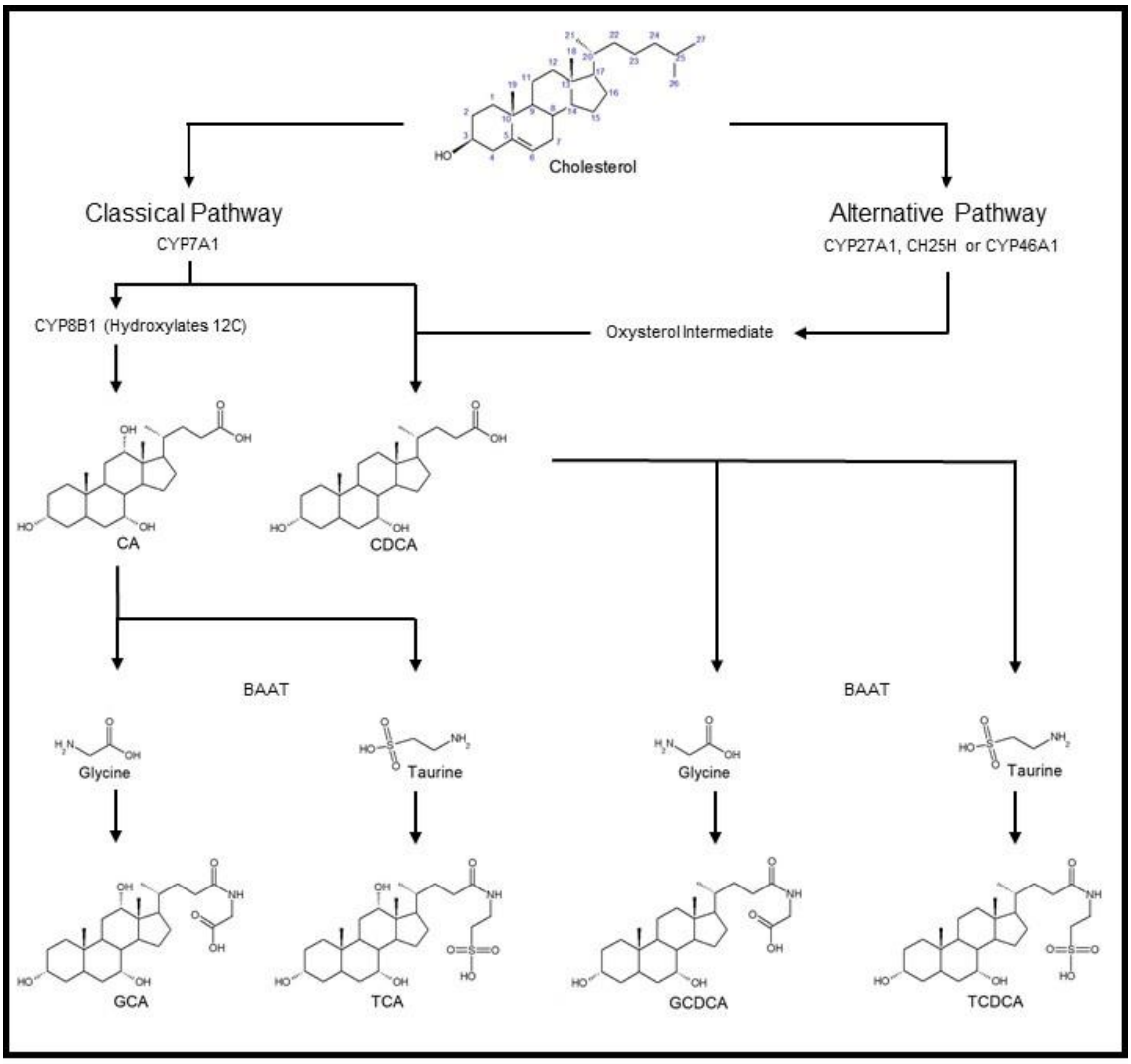

Figure 2: Bile acid biosynthesis. Classical bile biosynthesis is initiated by CYP7A1 hydroxylation of C7. Alternative biosynthesis is initiated by CYP27A1, CH25H or CYP46A1 via hydroxylation of C24, 25, or 27 respectively; an oxysterol intermediate that is converted to CDCA when translocated to the liver. CYP8B1 hydroxylates C12 to the sterol intermediate, the resulting bile acid is CA. Bile Acid: Amino-acid Transferase (BAAT) conjugates glycine or taurine to the carboxyl group of synthesized bile acids. The resulting conjugated bile acids Glycocholic acid (GCA), Taurocholic acid (TCA), Glycochenodeoxycholic acid (GCDCA) and Taurochenodeoxycholic (TCDCA) leaves then the hepatocyte. Figure created in ChemSketch. Chemical structures based on Structures found on PubChem.com and open source material.

The concentration of bile acids effect the regulation of their own synthesis. This was initially suggested when creation of biliary fistulas resulted in an increase in bile acid synthesis. The ability to return bile acid synthesis to a normal level with infusions of bile acids into the duodenum was indicative of a negative feedback loop or feedback inhibition (*39-41*). Studies in rats observed increased CYP7A1 activity in animals where the luminal bile was reduced by the administration of the bile sequestering cholestyramine. The cholestyramine effect was reversed when additional bile acids were fed to the animals (*37*). It has also been shown that CYP7A1 expression can be stimulated by cholesterol in rats. Furthermore, bile acids can bind to the nuclear receptor FXR and indirectly inhibit CYP7A1 synthesis (*37*). It has also been shown that glucose can increase CPY7A1 gene expression in diabetic patients while insulin inversely regulates bile acid synthesis. Diabetic patients saw higher levels of bile acids bile acid levels when in a state of hyperglycemia that decreased with insulin treatment (*42, 43*).

Secretion and Enterohepatic Circulation

Once bile acids have been conjugated, they are brought to the gallbladder to be concentrated and stored until needed. After a meal, the release of the hormone cholecystokinin by the intestinal epithelium causes the gallbladder to release bile into the small intestine (*44*). As bile acids continue down the alimentary canal, they undergo chemical changes resulting from interactions with the native microbial flora including deconjugation, dehydroxylation and hydroxylation. Deconjugation occurs when the bond

between the bile acid and the conjugated amino acid are broken. Dehydroxylation occurs when a hydroxyl group is removed from the steroid backbone creating secondary bile acids, while hydroxylation involves epimerizing hydroxyl groups from an *alpha* (α) to a *beta* (β) position or vice versa. (*36*).

Primary bile acids are reabsorbed by the distal ileum as bile acids pass through the small intestine, while secondary bile acids are passively absorbed in the large intestine, though not as efficiently as primary variants (Table 1). Absorbed bile acids then are bound by plasma proteins and shunted back to the liver. Once in the liver, both primary and secondary bile acids are conjugated and secreted once again. The exception is LCA, which undergoes sulfation at the 3-hydroxyl position (*36*). This may be to remove LCA from the system as sulfated LCA as it is not easily reabsorbed. As a result, the bile acid pool is highly diverse (Table 1, Figure 3).

Source	CA	CDCA	DCA	UDCA	LCA	Other
Gallbladder	35%	35%	25%	2%	1%	2%
Feces	2%	2%	34%	2%	29%	31%

Table 1: Composition of bile acids in the gallbladder and feces of healthy individuals, demonstrating bacterial bile acid metabolism though the gastrointestinal tract. Percentages derived from Ridlon *et. al*. 2006

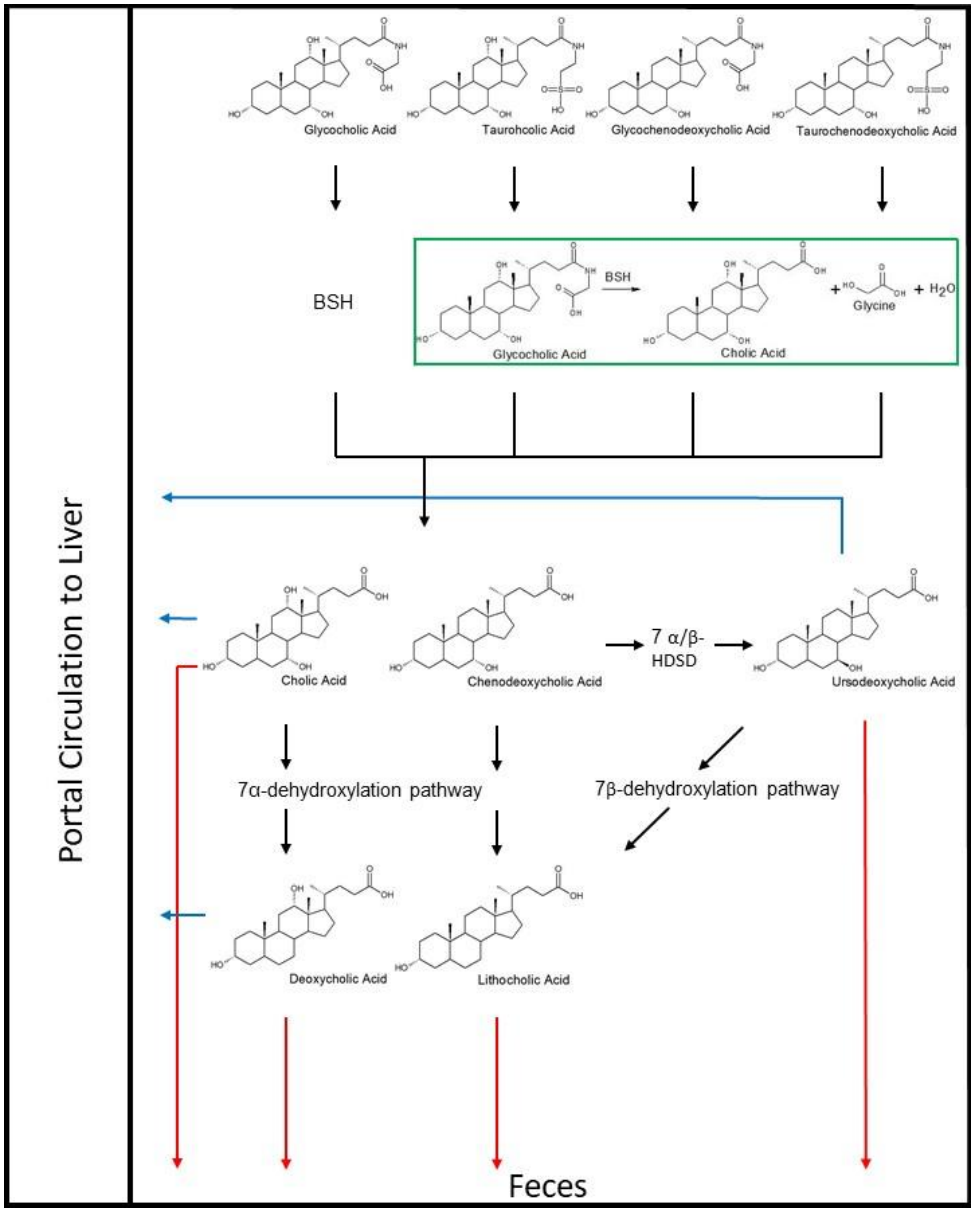

Figure 3: Bile acid metabolism and circulation. In the small intestine bile acids are deconjugated by BSH. CA and CDCA metabolized through the 7α-dehydroxylation pathway resulting in DCA and LCA respectively. CDCA is also metabolized by 7α/β- HDSD enzymes resulting in UDCA. UDCA is metabolized into LCA by the 7β-dehydroxylation pathway. These bile acids then reabsorbed into the portal circulation and transported to the liver or excreted through feces. Black arrow: bile acid metabolism, Blue arrow: portal circulation, Red arrow: excretion into feces, Green box: BSH deconjugation. Figure created in ChemSketch. Chemical structures based on Structures found on PubChem.com and open source material.

Function

Bile acids/salts have many functions in the body. The most well-known of these is solubilizing fats and vitamins, making these molecules available for the body to absorb. This is accomplished by the formation of bile acid micelles with fat soluble vitamins, cholesterol, monoglycerides, and fatty acids, acting as a solvent vehicle for absorption (*32*, *36*). These micelles are spherical and act in a similar manner as phospholipids in cell membranes; the amino acid portion of the bile salt, the hydrophilic portion, is exposed to the lumen while the hydrophobic molecules are contained within the micelle (*36*).

It has also been shown that bile acids act as an antimicrobial agent in the alimentary canal. This was first hypothesized after studies showed increased bacterial growth with bile duct ligation or in people with cirrhotic livers that had decreased bile acid production (*45*). Further studies have proposed that bile acids accomplish this in various ways. Conjugated bile acids, specifically GDCA, decreased the pH of microbial cells by causing an influx of protons, forcing cells to put energy into balancing intracellular pH (*46*). Another study suggested that bile acids caused damage to the cellular membrane/wall as bacteria exposed to CA and DCA demonstrated signs of intracellular leakage through visible changes in cellular morphology, cell survival assays, and levels of intracellular material (*47*). This effect is supported by the fact that bile acids possess detergent qualities. It is possible that bile acids can also induce DNA damage as *Escherichia coli* exposed to bile acids activated the promoter for *dinD*, a gene that is induced by DNA damage though the SOS response (*48*). Bile acids also serve as ligands

for nuclear receptors, including FXR. Not only is it involved with the negative feedback loop of bile acid synthesis, FXR also plays a role in immunity as a regulator for iNOS and IL-18, as well as in other pathways including lipid metabolism, glucose metabolism and bile acid transport (*49, 50*).

Bacterial Effects on Bile Acids

Bacteria have several methods to modulate and metabolize bile acids and decrease the bacteriostatic and bactericidal effects of primary/conjugated bile acids. A major pathway for bacterial interaction with bile acids is the action of the enzyme bile salt hydrolase (BSH, choloylglycine hydrolase) produced by commensal microbes in the gut and which serves to remove the conjugated amino acid on bile acid molecules. Depending on the type, this enzyme can be intracellular or extracellular (*51*). Homologs of this gene can be found in many species residing in the small intestine, with each enzyme varying in its regulation, subunit size, and function (*36*). BSH also shows specificity to the different conjugated bile acids; *Bacteroides thetaiotamicron VPI 5482* is unable to deconjugate CA conjugates, but was able to deconjugate CDCA, UDCA, DCA and LCA conjugates at different levels (*52*). The presence and function of these BSH enzymes can vary at the strain level within the same bacterial species (see Table 2 and Figure 5 and 6 for comparison of *bsh* in representative bacterial community). Some species host multiple *bsh* genes encoding BSH enzymes; for example *Blautia obeum* (*B. obeum)* has two genes, *RUMOBE_00028* and *RUMOBE_ 03454*, that have been identified through bioinformatic analyses as encoding BSH (*53*). In *Clostridium*

perfringens, a strain with its initial *bsh* deleted (Δ*bsh*) still retained partial deconjugation activity, indicating the presence of more than one homolog (*54*).

The most notable microbial dehydroxylation pathway is 7-α/β dehydroxylation, which is restricted to deconjugated primary bile acids. This intracellular process utilizes several enzymes to convert CA to DCA and CDCA to LCA using 7-α dehydroxylation pathway and UDCA to LCA by 7-β dehydroxylation. A well-studied bacterial species that metabolizes bile acids in this way is *Clostridium scindens*, containing a *bai* operon coding for the proteins required for dehydroxylation (*36*). Dehydroxylation of bile acids may serve several purposes in bacteria; it is theorized that bacteria can use bile acids as an electron acceptor/carrier ($NADP^+ \rightleftarrows NADPH$, $NAD^+ \rightleftarrows NADH$ and $ATP \rightarrow AMP$). It is also possible that this confers a competitive advantage over other bacteria, since DCA and LCA are more inhibitory to sensitive microbes (*36*).

Hydroxylation is a reversable process using two enzymes α and β HSDH (hydroxysteroid dehydrogenase). these enzymes act on either the C3, C7 or C12 hydroxyl groups on bile acids by oxidation and epimerization. It has been demonstrated that this process can occur either within a single species or shared among two (*36*). The most notable are 7-α/β HSDH act on CDCA to make UDCA, a less toxic bile acid as shown in a study comparing growth of *Clostridium absonum*; the bacteria grew on plates with 1 mM of UDCA but could not grow with the same concentration of CDCA (*36, 55*). See Figure 1 for bile acid properties and Figure 3 for a summarized bile acid metabolism pathway.

Rodent Models

Mice are a common animal model used not just microbiome related studies, but in studies to understand bile acid synthesis and mechanistic function. It is important to note that studies in rodents present a different bile acid pool to microbes than in humans, both at the level of synthesis and the relative chemical composition of the final bile acid mix. In rats, fasting may stimulate bile acid synthesis as evidenced by the induction of CYP7A1 expression (*37*). The acidic pathway plays a larger role on bile acid synthesis in mice than humans. When comparing CYP7A1 KO mice, their bile acid pool was 25% that of WT mice when both were on a normal mouse/rat diet. In CYP7A1 deficient humans, however, the bile acid pool is 5-10% that of healthy individuals (*56, 57*). People with CYP7A1 deficiency experience hyperlipidemic phenotype while CYP7A1 KO mice experience normal lipid phenotype (*56*). On a regular diet of 75 g of fat and 500 mg of cholesterol CYP7A1 deficient humans demonstrated more sterols and less fatty acids absorption through fecal analysis while CYP7A1 KO mice have been shown to absorb <5% of dietary cholesterol (*56, 58*). It is important to note that that mouse and human diets are quite different, and that diet on its own within either organismal system can affect bile acid pools of these organisms. The differences also extend to sex, as female mice demonstrate a larger bile acid pool than male, a pattern that is opposite than what is seen in humans (*59*). Mice also can alter a bile acids structure after synthesis by rehydroxylating the 7C of secondary bile acids and hydroxylating CDCA into α and β muricholic acid (MCA) (*36, 60*).

Rodent-human differences also extend to the composition of the final bile. In addition to having CA and CDCA as major primary bile acids, mice also have MCA, with α-MCA and β-MCA being the predominant bile acids (*56, 60*). MCA is a 6-hydroxylated bile acid having different forms depending on the steriochemistry of the hydroxyl group on C7, with the most common being α and β, the latter being the more prevalent of the two (*60-62*). Murideoxycholic acid (MDCA) and hyodeoxycholic acid (HDCA) are also part of the mouse secondary bile acid pool with only trace amounts found in humans, where the C7 hydroxyl group has been dehydroxylated (*60*). Both secondary bile acids differ in the C6 hydroxyl group, with one being in the alpha position and the other in the beta (*60, 63-66*). HDCA is formed in two different ways; the first is derived from MCA and is strictly microbial where it can go through an additional epimerization of the 6C hydroxyl group. LCA can also be metabolized through liver and microbial enzymes (*67*). Bile acids in the murine bile pool are almost exclusively conjugated to taurine (>95%) in contrast to the glycine/taurine conjugated mixture found in humans, with sulfation of LCA occurring only in humans (*60, 68*). Rodent bile acids have also been shown to be less hydrophobic than human bile acids (*69*).

Despite these differences, mice are useful and an important model for *in vivo* infection and bile manipulation studies. The anatomy and function of the mouse intestinal tract is similar to humans, including both digestion and immunity (*70*). This similarity also goes to the genetic level, with humans and mice having 90% of their genes in common (*71*). Mice can be controlled for genetic variability; inbred mice lines can be produced and reduce complications from having a larger gene pool. In addition, mice can

be genetically manipulated to knock-in, -out or -down genes of interest. Further, mouse diets can be specifically controlled for content and quantity. It is also possible to rear mice in germ-free conditions, removing microbial variability and allowing for the controlled introduction of specific microbial communities to assay for their effects on the host (*68*). Finally, the generation time is also much shorter with a gestation time around 21 days and mice reaching maturity between 3-6 months old (*72*). Mice are specifically used for bile acid studies because there are multiple models available such as CYP7A1 KO lines and humanized liver mouse models (*56, 57, 73*).

There are infant and adult models currently used for *V. cholerae* infection models, each with advantages and disadvantages. In suckling animals, *V. cholerae* colonization is dependent on production of TCP and CT, as in humans, however they do not produce the large volumes of diarrhea seen in humans (*25, 74*). The opposite is true for adults, where conventional mice are resistant to colonization of *V. cholerae*. Adult mice only become susceptible when they are treated with antibiotics or are reared germ-free, but TCP is not needed for *V. cholerae* colonization (*74-76*). Thus, adult germfree animals can serve largely as a colonization, rather than a virulence, model for *V. cholerae*.

Effects of Bile Acids on *V. cholerae*

Bile has different effects on *V. cholerae* in the gut. Like other bacteria, bile acids can have a toxic effect, requiring *V. cholerae* to develop defense mechanisms. One of the most important defense mechanisms is biofilm formation; in the presence of bile acids and crude bile, *V. cholerae* increases the formation of biofilm through the expression of

VpsR (*77*). However, certain bile acids have shown to be integral in virulence activation. This was demonstrated by Yang *et. al*. who that taurocholate (TC) was able to activate virulence gene expression, including TCP and CT production. They were able to show that the bile acid acted directly on TcpP, which normally is inactive in a monomer form. Under anaerobic/microaerophilic conditions characteristic of the GI tract, TCA acts on TcpP by promoting the dimerization via disulfide bridge formation between the C_{207} residues of each monomer, resulting in an activated TcpP dimer that activates ToxT expression leading to full virulence gene activation (*31*). A direct effect of bile acids on ToxR also had been demonstrated, with ToxR acting on the activation of *ctxAB* and *tcp* independently of ToxT (*28, 34*). These differences in responses to bile acids may be related to the fact that the strains used in these studies were of the El Tor and the Classical biotype respectively. It has also been shown that bile has had the opposite effect on virulence expression, more specifically crude ox bile (*28, 78*). However, oxen are not known for being susceptible to cholera infection and they are not used as a cholera animal infection model. The complexity of bile acid compositions in the GI tract may also contribute to differences in regulation. One study compared three commercially available ox gall products, showing conjugated bile acids had different ratios of taurine and glycine when compared to human bile and the overall abundance of bile acids were CA, DCA, and CDCA respectively compared to CA=CDCA and DCA respectively (*36, 79*). It is possible that these ratios have an effect on virulence including a larger percentage of a secondary bile acids, which are considered more cytotoxic than primary bile acids. It should be noted that there are other components of bile that have been

shown to affect virulence expression, specifically, unsaturated fatty acids were shown to affect ToxT by affecting the proteins ability to bind to DNA (*80*). These compounds illustrate the complexity of virulence expression for *V. cholerae* in the small intestine. It should also be noted that *V. cholerae* has a predicted *bsh* gene (NC_002506.1), however its expression, regulation, cellular localization, and substrate specificity have not been determined. As of the time of this thesis, we are not aware of any publications that address this, and it is unknown as to what affect this has on *V. cholerae* virulence cascade. It is possible that the enzyme may not have an effect on virulence activation if it is an intracellular enzyme; the current model for TCA acting on TcpP is an interaction occurring in the periplasmic space of the cell (*31*). If deconjugation occurs intracellularly, it is possible that this process is protecting DNA damage and not affecting the pool of TCA required for virulence activation.

The Gut Microbiome and Bile Acids

The microbiome is a complex system within the human GI tract, encompassing thousands of different species. This community is not homogenous, and the taxa represented can vary dramatically across individuals, ethnicities and human populations, though communities are more similar between closely related individuals and within closer geographic areas influenced by the food consumed in those areas (*7, 42, 81-83*). Even though these populations differ, it has been shown that the metabolic pathways of the community remain similar, highlighting the fact that different bacteria can inhabit the same niche/perform similar roles (*84*).

Microbial communities do not remain stagnant and they are ever-changing as a result of both internal and external factors. Gastrointestinal infections, such as diarrheal diseases, can dramatically but transiently change the composition of the gut microbiome, making the microbiome less diverse and abundant, and shifting adult gut microbiomes closer in structure to that of infants (*85*). Diet is another strong driver of the structure and abundance of the microbial community in the gut. These changed are seen in malnourished individuals, those who consume high fat diets (HFD) and in studies that modified ingredients in the diet (*86-88*). Particularly, HFD have been shown to shift phyla in the community to one that has a larger abundance of Firmicutes and less Bacteroides (*87*). Moreover, consumption of high-fat diets in obese participants caused an increase in total and secondary bile acids (*76*). Feeding rats CA caused an increased the abundance of Firmicutes and the decrease of Bacteroidetes when compared to the control (*89*). It is possible then that the high fat diet results in an increase in bile acids secreted into the small intestine, resulting in increased microbial deconjugation and the increase in free bile acids driving the change to a microbiome including a larger abundance of firmicutes.

Hundreds of species contain *bsh* or putative *bsh* genes in the gut microbiome. These genes vary when comparing sequence homology, and are classified into eight phylotypes (*36, 90*) BSH enzymes from each phylotype were tested for their docking and deconjugating ability on primary and secondary conjugated bile acids resulting in different deconjugation percentages among phylotypes demonstrating that gut bacteria likely have different effects on the bile acid pool (*90*). As noted above, alteration of the

bile acid pool is not restricted to *bsh* activity, as bacteria can metabolize primary bile acids into secondary through dehydroxylation and/or hydroxylation as bile passes through the alimentary canal. Therefore, the microbiome is important for shaping the bile acid pool and is readily observed by the complex array of bile acids detected in feces (*36*).

My Research

My research focuses on the microbiome can downregulate virulence activation in *V. cholerae,* specifically through deconjugation of bile acids. We designed complex resistant and dysbiotic microbial communities, using bacterial strains selected to represent the human gut microbiome under homeostatic and dysbiotic conditions in cholera endemic areas (*42, 81, 85, 86, 91, 92*). Malnutrition affects the composition of the gut microbiome, as seen with children with severe acute malnutrition where the diversity of the microbial community was reduced compared to healthy children (*91*). Diarrheal diseases also have been shown to decrease the diversity and increase the proportion of streptococcal species, including S*treptococcus salivarius* (*S. salivarius*), as well as enterococci and enterobacteriaceae (*86*). Studies following cholera infections found bacterial communities during an infection to be similar to malnourished and/or diarrheal states, but the communities shifted toward healthy adult-like communities as individuals recovered (*85*). These healthy adult microbiomes the genera *Dorea, Bacteroides, Collinsella* and *Blautia* are abundant and are correlated or strongly associated with disease recovery from a *V. cholerae* infection, while high levels of streptococcal species were associated with diarrhea (*85*). Moreover, *B. obeum* was shown to decrease *V.*

cholerae colonization in germ-free mice. Out of the bacteria tested *in vitro*, *B. obeum* and *S. salivarius* were identified as species that can and cannot deconjugate TC respectively. In studies performed by Salmasadat Alavi, these communities were introduced into suckling mice alongside *V. cholerae.* Salmasadat Alavi also performed studies in germ-free mice using these communities to look at their effect on *V. cholerae* colonization. Both studies saw *B. obeum* having a negative effect on *V. cholerae colonization*, while *S. salivarius* did not. These results coupled with studies with similar associations with these bacteria led me to incubate these two species in infant mouse intestinal homogenate, showing a decrease in *tcp* activation compared to control with further testing revealing that this effect was not due to AI-2 but by modification to an inducer present in the homogenate (*79, 80*). By testing with cholestyramine and measuring bile acid levels by mass spectrometry, we determine that the bile acid TC was involved with *tcp* induction and this signal was being lost due to *B. obeium* possessing a *bsh* gene. We looked for *bsh* homologs in the microbial community by *in vitro* deconjugation of TC and database searches. By performing several database searches for *bsh* homologs in the genomes of gut commensals in our representative communities, we identified species that deconjugated TC and found two loci in *B. obeum* as putative encoders of BSH enzymes. These findings for *Blautia obeum* agree with a study looking at the taxonomic profile of *bsh* genes found in BSH-T1 and BSH-T7 phylotypes were seen to be mainly distributed in genera including *Blautia*. BSH-T1 had the highest relative abundance of BSH's and had one of the higher specific activity to bile acids including TC, while BSH-T7 was had one of the lowest relative abundance and specific activity (*90*). One of these genes,

RUMOBE_00028, was cloned into a plasmid constitutive expression vector to enable the expression of this enzyme in a non-bile acid deconjugating *E. coli* strain. Using this transgenic *E. coli*, we found in both *in vitro* and *in vivo* experiments that *B. obeum bsh* genes were active and able to degrade virulence-gene activating bile acids in mouse tissues. We also performed quantitative real-time PCR (qPCR) for RUMOBE_00028 on bacterial DNA derived from complete fecal specimens of healthy human donors. When comparing to *V. cholerae* infection experiments, qPCR of the *B. obeum bsh* enzymes in these samples were predictive of the ability of these fecal communities to restrict *V. cholerae* colonization when compared to colonization studies in infected animals. Together, these results show that *V. cholerae* virulence activation is significantly affected the structure and BSH activity of human gut microbiomes, which forms a risk factor for susceptibility to this important human pathogen.

METHODS

Ethics Statement

All animal experiments used protocols approved by the Institutional Animal Care and Use Committee of the University of California, Riverside (UCR). All human samples were part of a study approved by the UCR Institutional Review Board. Both animal and human studies followed NIH guidelines.

Animal Experiments

All CD-1 suckling and adult animals were purchased from Charles River Laboratories.

Bacterial Strains and Growth Conditions

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 at 37ºC either in a Coy anaerobic chamber (5% H_2, 20% CO_2, balance N_2), under microaerophilic conditions with Campy BD GasPak^{TM}, or aerobically. All *V. cholerae* strains were derived from the C6706 El Tor pandemic isolate, including the *lacZ::*P_{tcp}*A:sh Ble* zeocin resistance reporter strain, and propagated in LB media with streptomycin (100µg/ml) 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 aerobically at 37°C.

To construct a strain constitutively expressing *bsh*, RUMOBE_00028 was amplified from *B. obeum* genomic DNA using primers 5'-GTCGACGGTATCGATAATGCTTATGTGTACAGCTGC-3' and 5'-GCAGGAATTCGATATCACTAATTCTGAAAATGAATCTGC-3'. This amplicon was then cloned downstream of the constitutive $P_{Ltet-O1}$ promoter of plasmid pZE21 through digestion of the vector backbone with HindIII followed by Gibson assembly (NEB). The resulting plasmid was electroporated into *E. coli* strain DH5*α*λpir 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 electroporating into *E. coli BW30045* (*ΔluxS*) a plasmid constitutively expressing the *B. obeum luxS* AI-2 synthase. The *B. obeum luxS* coding region (from genome Q9KUG4, 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, with the construct cloned into vector pMK using the GeneArt Subcloning & Express Cloning Service (ThermoFisher). Strains were propagated aerobically in LB with kanamycin (50μg/ml) at 37°C.

Human Study Design and Sample Collection

We collected intact fecal samples from six healthy adult volunteers in University of California Riverside using an IRB-approved protocol. 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 2 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. Fecal samples were collected aseptically from each person at UCR and immediately preserved at −80°C until processing for DNA extraction 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.

Preparation of Human Fecal Samples for Inoculation into Mice

Human fecal slurries were cultured from glycerol stocks in LYHBHI media for 24 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 total of 300µl of OD_{600}=0.4 were pelleted by centrifugation and resuspended in fresh LYHBHI. Each mouse received this mass of bacterial cells in a

maximum gavage volume of 25µl with the remaining 25µl containing ~1x10^4 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 16S amplicon.

Antibiotic Clearance of Murine Microflora in Suckling Mice

Four-day old suckling CD-1 mice were fasted for 1.5 hours, then orally dosed with ~1mg/g body weight streptomycin or 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 eighteen hours post-infection, animals were sacrificed, and their intestines homogenized for CFU numeration and DNA extraction.

For mice being pre-colonized with bacteria, four-day old suckling CD-1 mice received a ~1/g body weight oral gavage of kanamycin. Infants were colonized 24 hours later using overnight cultures of *bsh*^{C} and vector strains grown in LB with kanamycin (50µg/ml at 37°C then normalized for density by OD_{600}. For inoculation into suckling mice, the equivalent total of 300µl of OD_{600}=0.4 culture was pelleted by centrifugation and resuspended in fresh LB with kanamycin (50 µg/ml). 50µl of this was then introduced via intra gastric gavage into 5-day old CD-1 suckling mice that had been fasted for 1.5 hours. Pups were then returned to a lactating dam. 24 hours after pre-infection colonization, mice were co-gavaged with 50µl of *bsh*^{C} or the isogenic vector

and C6706-*KM*^{R}. For C6706-*KM*^{R}, the total resuspension volume of 50µl containing ~1x10^{4} CFU *V. cholerae* in LB with kanamycin (50µg/ml); the same CFU of *bsh*^{C} and vector strains were gavaged as the previous day. Suckling mice were fasted overnight during infection. After eighteen hours post introduction of *V. cholerae*, animals were sacrificed, and their intestines homogenized for CFU numeration.

Purification of Bile Acids from Intestinal Homogenate

Intestines were collected from six-day-old CD-1 suckling mice fasting for eighteen hours, homogenized in sterile H_2O (n*2.5ml), pooled, and centrifuged to clear tissue debris. The resulting aqueous suspension was treated at 100^{o}C for thirty minutes and sterilized with a 0.22µM filter. 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 vortex and incubated at room temperature for twenty minutes for deproteinization (*93*). Samples were then clarified, with the aqueous layer filter sterilized, desiccated and resuspended in one-fifth original volume sterile H_2O as described above.

Processing of *tcp*-Activating Signals by Commensal Bacteria

Commensal isolate cultures were grown for 2 days then sub-cultured at 3:100 for 2 days. 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, sub-cultured at 1:100 for 24hr, and normalized as above. Cells were pelleted,

the supernatant removed, and cells resuspended in sterile PBS with TC to a final concentration of 125uM for *in vitro* assays; for *ex vivo* assays cells were resuspended in purified homogenate. Cultures grown with antibiotics were washed an additional time with 1 volume of PBS prior to addition of TC. Samples were then incubated anaerobically for 24 hours at 37ºC followed by heat treatment at 100ºC for thirty minutes. Samples were then cooled to room temperature, centrifuged and the aqueous layer filter sterilized with a 0.22µm filter. To sequester bile salts, 12.5mg of cholestyramine resin (Sigma-Aldrich) was added to 0.5ml of de-protonated sample, and the mixture incubated at 1 hour at 37ºC with agitation followed by passage through a 3kDa protein concentrator (Pierce PES Protein Concentrators).

Bile-dependent Induction of *tcp* Gene Expression

PtcpA-sh Ble was grown as overnight culture, diluted 1:1000 in fresh LB with streptomycin (100µg/ml), and grown for ~2 hours at 37°C. Each reaction was prepared in 40µl of 0.5X LB pH 8.5 medium containing homogenate incubated with bacteria as described above or pure solutions of bile salts. TC (Sigma-Aldrich), GC (Sigma Aldrich), CA (Alfa Aesar), Taurine (Acros Organics), Glycine (Fisher Scientific), Taurine + CA, Glycine + CA, TDC (Sigma-Aldrich), TCDCA (Cayman Chemical), GCDCA (FrontierScientific), CDCA (Sigma Aldrich), GDC (Sigma Aldrich) DCA (MB Biomedicals), Taurine + DCA, LCA (Sigma Aldrich), UDCA (Alfa Aesar), Tβ-MCA (Steraloids Inc.) and β-MCA (Steraloids Inc.) were added to a final concentration of 125 µM in sterile H_2O (for a list of bile acid names, see Figure 1).

2µl of reporter strain subculture was then added, and samples incubated anaerobically at 37°C for four hours. 2µl of each reaction was then added to 200µl of 0.5X LB pH 8.5 +/-10µg/ml of zeocin (Sigma Aldrich), incubated for thirty minutes aerobically at 37°C with agitation. Samples were then serially diluted and plated onto streptomycin (200µg/ml) LB agar plates to determine survival rates, defined as (zeocin-treated sample CFU/average CFU of no zeocin control) *100.

Autoinducer-2 Heat Stability Assay

Cultures of *BW30045_RO_AI-2*, *BB170* and *C6706* were grown overnight. *BW30045_RO_AI-2* was sub-cultured at 1:100 into 12ml of LB and grown in a shaker at 37^{o}C until $OD_{600} \approx 0.22$, centrifuged, and the supernatant filter sterilized. For heat treatment, aliquots of supernatant were treated at 100^{o}C for thirty minutes and cooled to room temperature. AI-2 activity was assessed using the *BB170* bioassay (*94*). Briefly, overnight cultures of reporter strain *BB170* in LM medium was diluted at 1:1000 into AB medium. 10µl of supernatant or heat-treated supernatant were then added to 90µl of *BB170* dilution. Luminescence and OD_{600} of each sample were measured immediately, and at 3.5hrs, with growth at 30^{o}C aerobically with agitation.

Identification of Bile Salt Hydrolase Homologs

Using the Kyoto Encyclopedia of Genes and Genomes (KEGG), the Universal Protein resource (UniProt) and the National Center of Biotechnology Information (NCBI) protein database, we were able to identify *bsh* homologs and compared amino acid

sequences between species using NCBI blastn and blastp respectively. Percent identity matrices were constructed using results of pairwise comparison between species using Clustal Omega and MUSCLE provided through European Molecular Biology Laboratory.

Quantification of Bile acids with Mass Spectrometry

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 100 mm, 1.7 µM) (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 were held at 40° C. The gradient was as follows: 0 min, 1% B; 1 min, 1% B; 8 min, 40% B; 13 min, 58.8% B; 13.5 min, 100% B; 15.5 min, 100% B; 16 min, 1% B; 18 min, 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 100 ms scan time. Source and desolvation temperatures were 150° C and 600° C, respectively. Desolvation gas was set to 1100 L/hr and cone gas to 150 L/hr. All gases were nitrogen except the collision gas, which was argon. Capillary voltage was 1 kV. 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.

In vitro Processing of Bile by Human Complex Fecal Communities

The ability of a complex human donor fecal community to process taurocholate was assayed *in vitro*. 100µl of fecal slurries in glycerol prepared were inoculated into 5ml LYBHI and incubated anaerobically for 2 days at 37°C. Cells were then pelleted, normalized to ~OD600 =0.4 in 1.5ml sterile PBS with 125µM TC and incubated anaerobically at 37°C for 24hrs with a TC control. Supernatants were then collected via centrifugation and heat treated at 100°C for thirty minutes then sterilized with a 0.22µm filter then submitted for Liquid Chromatography Mass Spectrometry.

Quantitative Real-Time PCR

Levels of the *B. obeum bsh* enzyme (*RUMOBE_00028*) were determined by real-time PCR. Reactions comprised 2 µl of extracted DNA as template, 12.5 µl SYBR Green Master Mix (BioRad), 10 µl PCR-grade water, and 0.25 µl of forward and reverse primers at 10µM (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. Cycle conditions were 95°C for 3 min, followed by 39 cycles (95°C for 10sec, 55°C for 30 sec, 95°C for 10 sec, 65°C for 5 sec, 95°C for 5 sec). Average Cq results were used to compare to *in vivo V. cholerae* colony counts.

Total bacterial load in fecal samples were determined by quantitative real-time PCR as described above, using the primers forward: 5'-CTCCTACGGGAGGCAGCAG-3' and reverse: 5'-TTACCGCGGCTGCTGGCAC-3'.

RESULTS

Complex Resistant Microbiomes Suppresses *V. cholerae* Colonization

How the microbiome affects *V. cholerae* colonization in the gut is the central question of this thesis. Studies show that pressures such as diarrhea and malnutrition decrease the diversity of the gut microbiome and increase the abundance of specific species including Streptococci, enterococci and enterobacteria, creating a dysbiotic microbiome (*42, 79, 80, 91-93*). Healthy microbiomes have also been profiled, and increases in *Dorea*, *Bacteroides*, *Collinsella* and *Blautia*, which are characteristic of healthy individuals, have been associated with recovery from cholera and malnutrition (*85, 91*). Based on these studies, we designed a complex resistant (CR) and a dysbiotic (DS) microbiome. The CR community encompassed thirteen diverse species based on 16S rRNA that are commonly associated with healthy adult-associated microbiomes. The DS contained five species based on diarrhea-associated microbiomes and represented less taxonomic diversity (see Table 2). In studies performed by Salmasadat Alavi in the Hsiao laboratory, these communities were introduced into antibiotic cleared suckling mice by gastric gavage alongside *V. cholerae* infection, with their intestines collected and homogenized eighteen hours post-infection. Results showed that the presence of CR communities statistically significantly and dramatically lowered *V. cholerae* colonization compared to DS communities. In addition, co-administration of DS and CR microbiomes (or just a subset of the CR community) showed a rescue of the CR colonization resistance phenotype (data not shown), suggesting that the metabolic or enzymatic activity of CR

and SR microbes are able to compensate or suppress the activity of DS microbes as they pertain to affecting *V. cholerae* infection.

Another study from Salmasadat Alavi involved collecting fecal samples from healthy individuals that were prepared and stored in -80^{o}C in 25% volume/volume glycerol. These slurries were cultured and introduced into antibiotic cleared suckling mice by gastric gavage with *V. cholerae*, with their intestines collected and homogenized eighteen hours post-infection. Results demonstrated each community had different effects on *V. cholerae* colonization with the CR community being having the greatest effect on ablating pathogen colonization (data not shown), emphasizing the complex and individual-specific interaction between pathogen and the host microbiome.

Bacterium	Community	Strain Name	NCBI Taxonomy ID
Bacteroides caccae	CR	ATCC 43185	411901
Bacteroides thetaiotamicron	CR	VPI-5482	226186
Bacteroides uniformis	CR	ATCC 8492	411479
Bacteroides vulgatus	SR, CR	ATCC 8482	435590
Bifidobacterium longum subsp. Longum	CR	DSM 20219	565042
Blautia obeum	SR, CR	ATCC 29174	411459
Blautia torques	CR	ATCC 27756	411460
Clostridium scindens	SR, CR	ATCC 35704	411468
Collinsella aerofaciens	CR	ATCC 25986	411903
Dorea formicigenerans	CR	ATCC 27755	411461
Dorea longicatena	CR	DSM 13814	411462
Eubacterium rectale	CR	ATCC 33656	515619
Faecalibacterium prausnitzii	CR	DSM 17677	411483
Enterococcus faecalis	DS	OG1RF	474186
Escherichia coli	DS	DH5α	668369
Streptococcus infantarius subsp. *infantarius*	DS	ATCC BAA-102	471872
Streptococcus salivarius subsp. *salivarius*	DS	ATCC 13419	1304
Streptococcus salivarius subsp. *thermophilus*	DS	DSM 20617	1091038

Table 2: Human gut commensal strains, SR= Simple resistant, CR= complex resistant, DS= dysbiotic

An *ex vivo* Model of *tcp* Induction

V. cholerae in vivo virulence expression, and thus colonization, is regulated by numerous environmental cues, which can be mimicked *in vitro* using specific culture conditions such as microaerophilic/anaerobic growth with the addition of bile acids such as TC (*28-31, 33, 34*). To determine the relative contribution of bile acids and other *in vivo* compounds in virulence activation, the intestines of suckling mouse were processed via homogenization, heat treatment and deproteinization and assayed for their ability to activate a *tcp* transcriptional reporter. This reporter strain contained a P_{tcpA} *–sh ble* construct inserted into the *lacZ* locus of *V. cholerae* C6706; activation of *tcp* genes in this strain confers resistance to the bactericidal antibiotic zeocin. Due to the age of the infants and the amount of material required, samples were concentrated before downstream processing. To determine the effect homogenate had on *tcp* induction, P_{tcpA} *–sh ble* was incubated anaerobically for four hours with purified intestinal homogenate from suckling mice, and then subjected to zeocin for thirty minutes. Surviving *V. cholerae* were then enumerated by serial dilution and plating on LB agar with streptomycin. As has been reported previously(*31*), a major component of the virulence-inducing activity of intestinal homogenates was due to bile acids; intestinal homogenates demonstrated *tcp* induction levels similar to that of pure 125μM TC solution (Figure 4A).

To determine bacterial effects on induction, purified intestinal homogenate was incubated with either *B. obeum, S. salivarius* or a mixture of the two; *B. obeum* was selected due the strong association the bacteria has to *V. cholerae* recovery and decreasing colonization in germ-free mice (*79*), while *S. salivarius* has been shown to

increase in diarrheal diseases, making these two strains a subset of the CR and DS communities (*79, 80*). The bacteria were grown anaerobically for two days then pelleted and resuspended in purified homogenate to a concentration ~0.4 OD_{600} for twenty-four hours. Samples were then heat treated for 30 minutes at 100^{o}C, sterilized using a 0.22 µM filter and used for a *tcp* induction assay. Compared to *S. salivarius*, *B. obeum* was able to significantly lower induction of homogenate compared to the TC control and *S. salivarius* alone (Figure 4B). These results suggest that *B. obeum* is affecting levels of this activating signal in intestinal homogenates (*31*).

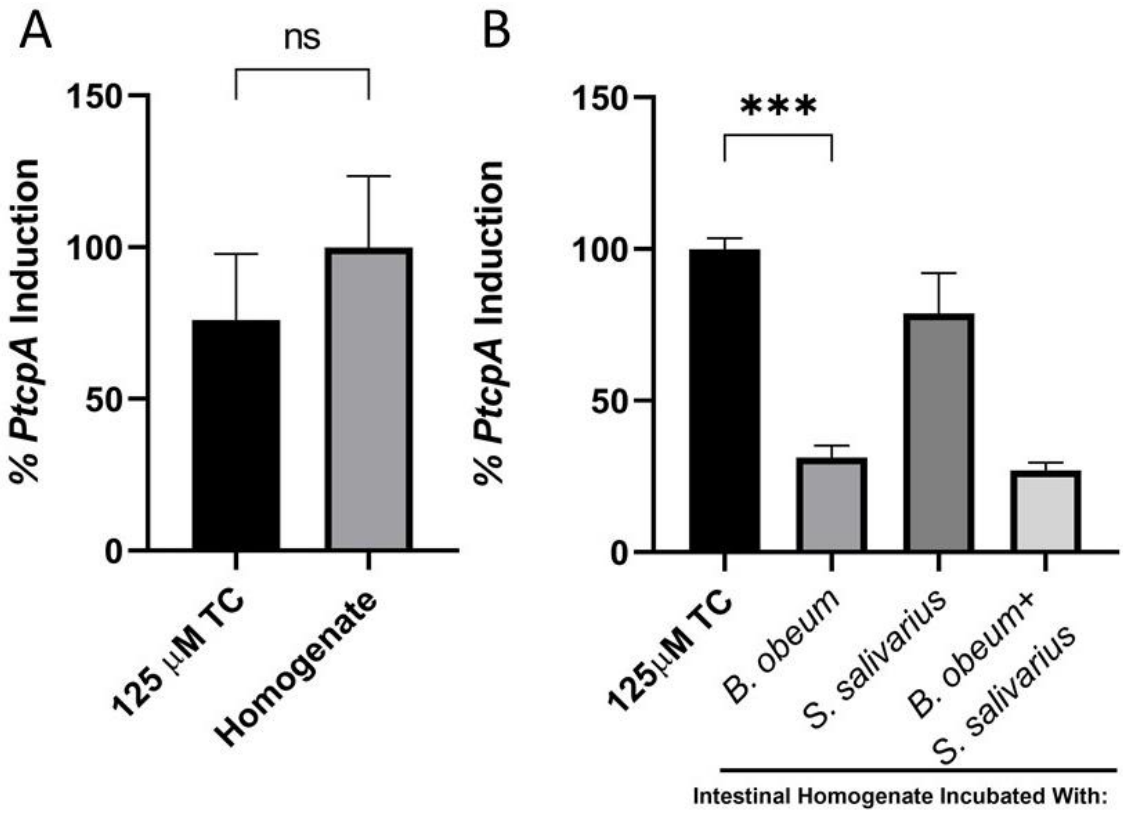

Figure 4: *tcpA*-activating signals in TC solution and suckling CD-1 mouse homogenate (A) and modulation of *tcpA*-activating signals in homogenate by pure cultures of *B. obeum* and *S. salivarius* (B). P*tcpA-sh ble* induction results were normalized to PBS with 125μM TC control. Error bars calculated using ±SEM based on unpaired t-test. ***P-value< 0.001

B. obeum Effects on Intestinal Homogenate Activation of *tcp* is not due to AI-2 Activity

B. obeum has also been shown to suppress *V. cholerae* virulence expression through the production of AI-2 quorum sensing signals (*79*). To determine if these signals were solely responsible for the suppression of P_{tcpA} *–sh ble* induction by intestinal homogenates and TC, we examined the ability of heat treatment to differentially affect virulence-regulatory signals. Bile acids have previously been demonstrated to be heat-resistant; heat treatment did not affect the ability of TC to interact with TcpP (*31*). To determine if AI-2 molecules made by *B. obeum* were heat labile, supernatants were collected from stationary cultures of *V. cholerae* and *BW3114_RO_AI-2*, which is an *E. coli* strain mutated for *luxS*, the AI-2 synthase, and which instead constitutively expresses the AI-2 synthase enzyme of *B. obeum*. Supernatants were collected, heat treated for thirty minutes at 100^{o}C and sterilized using a 0.22 µM filter. To assay for AI-2 activity, we used the *Vibrio harveyi* strain BB170, which contains an intact *lux* operon responsible for bioluminescence in the presence of AI-2. When BB170 was grown in the presence of heat-treated supernatants, heat-treated samples induced very little luminescence in comparison to non-heat-treated supernatants (Figure 5). These results indicate that treating samples with heat is enough to remove any AI-2 signal from solution and that virulent expression is affected by other means.

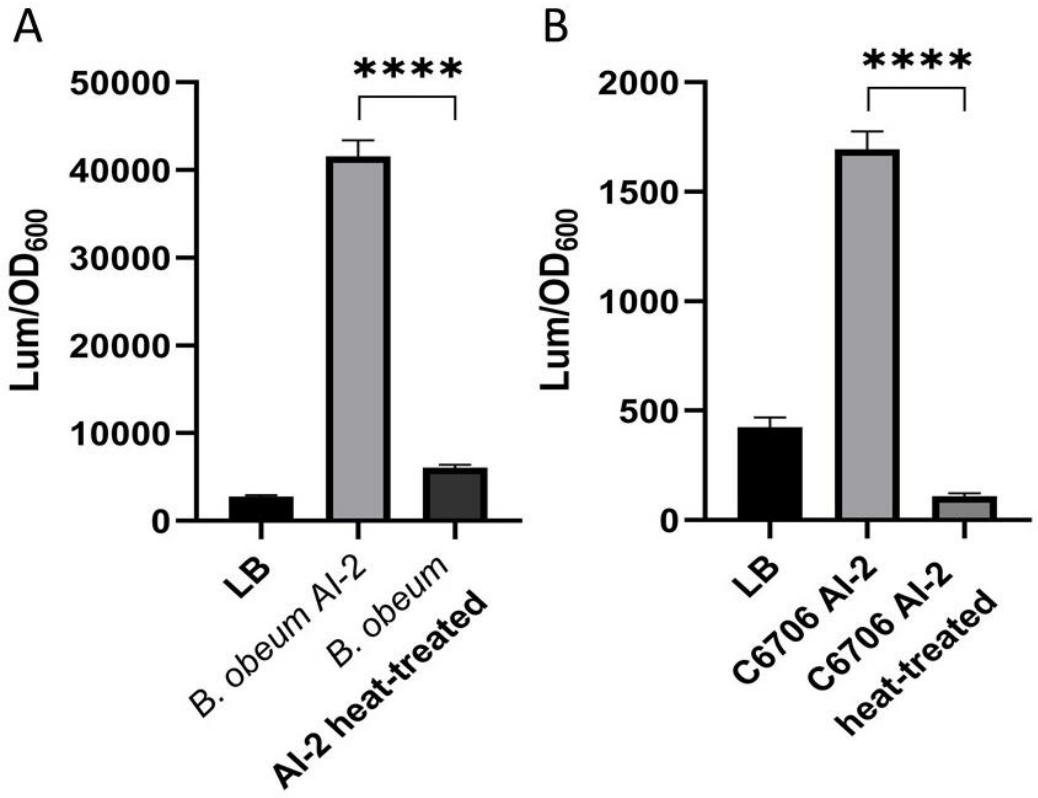

Figure 5: Induction of *BB170* AI-2 reporter by *B. obeum* (A) and C6706 (B) with and without heat treatment. Induction measured by luminescence/OD_{600}. Error bars calculated using SEM Significance based on unpaired t-test. ****P-value< 0.0001

Bile Sequestration Does Not Affect *B. obeum's* Effect on *V. cholerae* Virulence Activation

Previous work indicates that a key activating signal in intestinal homogenates are bile acids (*31*). To confirm if bile acids, which are highly heat stable, were causing *V. cholerae* virulence expression, infant mouse intestinal homogenate was once again treated with *B. obeum* and *S. salivarius* with the addition of cholestyramine (*31*). A solution with 125 μM of taurocholate TC was used as a positive control. Cholestyramine is a resin that binds to bile acids resulting in sequestering the steroid derivative and preventing interaction with the environment. It is used as a medication to treat hyperlipidemia; with the increased loss of bile acids through feces the liver increases bile acid synthesis and resulting in reduced cholesterol levels (*37, 39*). Pure TCA and mouse intestinal homogenates without bacterial treatment strongly induced *tcp* transcription. Again, incubation of homogenates with *B. obeum* ablated *tcp* induction and *S. salivarius* had no effect on homogenates' ability to induce virulence genes. Bile sequestration largely eliminated the ability of intestinal homogenates to activate *tcp*, confirming the role of bile acids in virulence induction in this system. Significantly, *S. salivarius* treated homogenates incubated with cholestyramine were largely unable to induce virulence, suggesting that the ability of these organisms to affect bile acid levels is a key driver in virulence activation by host intestinal signals. There was also no difference observed in virulence inducing capability of *B. obeum* treated homogenates incubated with and without cholestyramine (Figure 6A). These sample were analyzed by LC-MS to determine the level of dehydroxylation bile acids in the homogenate by the UCR

metabolomics core, using pure solutions of TC and CA as standards. Measuring peak area, the relative abundance of *B. obeum* and *B. obeum* + *S. salivarius* treated homogenate had significantly lower levels of TC compared to *S. salivarius* and untreated homogenate. Interestingly, the opposite was true for CA (data not shown). These results indicate that *V. cholerae tcp* expression is dependent on the bile acids in the mouse homogenate and these signals are being affected by *B. obeum* though BSH activity (Figure 6B).

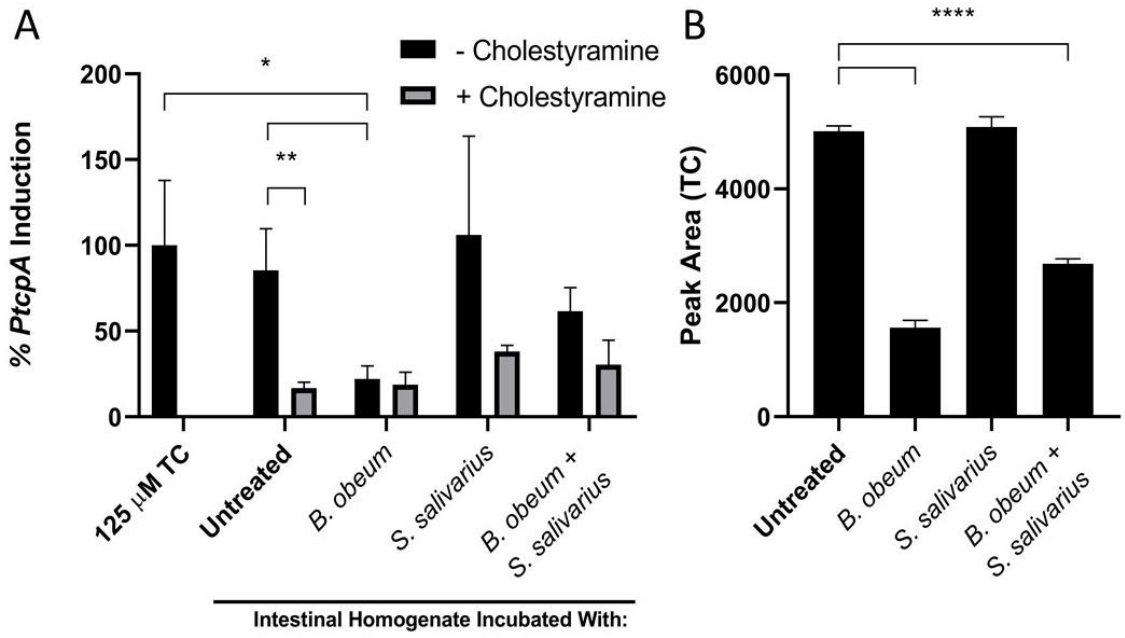

Figure 6: Modulation of *tcpA*-activating signals in suckling CD-1 mouse homogenates by pure cultures of *B. obeum* and S. *salivarius* as seen by P*tcpA-sh ble* induction (A) and peak area of TC by LC-MS (B). P*tcpA-sh ble* induction results were normalized to PBS with 125μM TC control Note TC Sample was not treated with cholestyramine. Peak area describes the relative abundance of bile acid per group. Error bars calculated using ±SEM based on unpaired t-test. *P-value< 0.05, **P-value<0.01, ****P-value<0.0001

bsh In the Model Microbiomes

The remainder of the bacterial strains in our model communities were investigated for their ability to affect TC induced virulence gene expression by incubating 125 μM of TC with pure cultures of these species for twenty-four hours then were heat treated and filter sterilized, and measuring how the resulting cell-free supernatants were able to induce a *tcp* expression in *PtcpA-sh-ble*. Dramatic differences in the ability these strains to affect TC induction of virulence as measured by survival after zeocin treatment were observed, with members of the CR microbiome in general being better able to prevent *tcp* activation. This varied at the genus level, with *Blautia torques* unable to affect *tcp* induction by TC in comparison to *B. obeum*, and *Streptococcus infantarius* showing TC effects in comparison to the rest of the *Streptococcus* species in the DS community (Figure 7). Levels of *tcp* induction were higher in groups including *E. faecalis* and *S. salivarius*. Although currently unknown, it is possible that these species boost virulence induction by some mechanism.

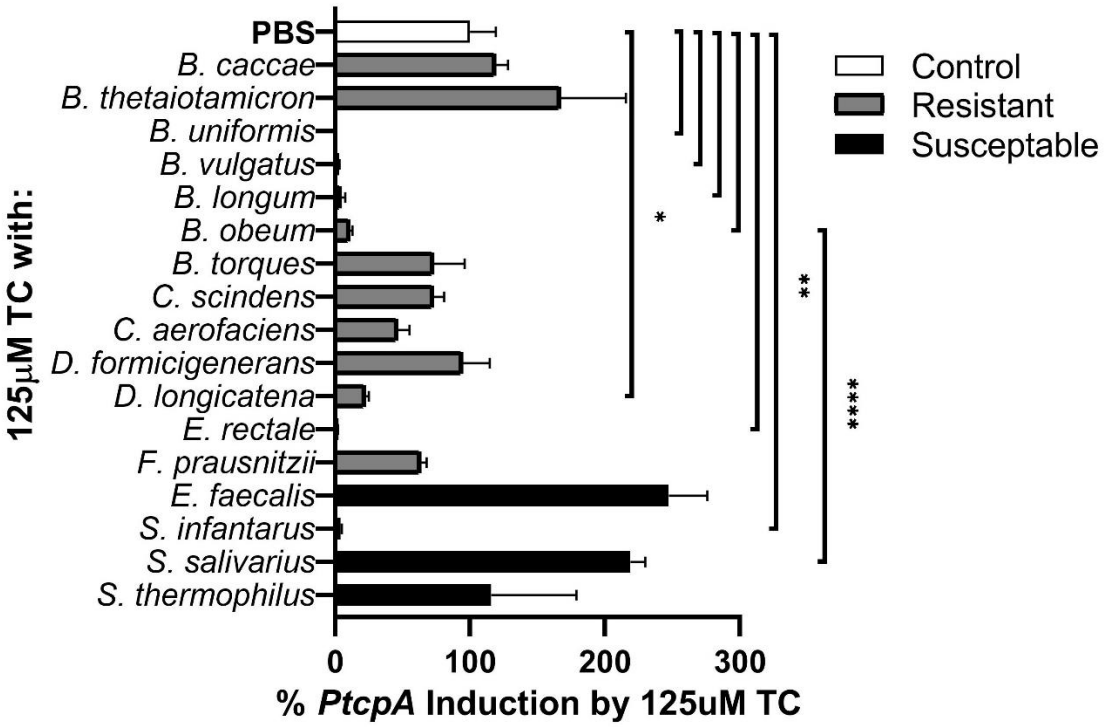

Figure 7: Ability of CR and DS-member pure cultures to interfere with TC activation of P*tcpA* in *V. cholerae in vitro*. P*tcpA-sh ble* induction results were normalized to PBS with 125μM TC control Error bars calculated using ±SEM based on unpaired t-test. *P-value< 0.05, **P-value< 0.01, ****P-value< 0.0001.

To search for *bsh* homologs in our community we searched the KEGG, UniProt and the NCBI protein databases using the EC designation 3.5.1.24 and bacterial species. Homologs were identified within the CR and DS microbial communities, with putative *bsh* genes in thirteen of the species of which six had multiple copies. *B. obeum genes* CK5_10630 and CK5_18830 were identified from the genome of the strain A2-162. When compared to homologs in the *B. obeum* ATCC 29174 used in our described experiments, there are mild difference in amino acid sequences with percent identity ranging between 80-90%, showing a higher similarity compared to other genes in the community (Table 3 and Figure 8). There were bacteria in the communities that demonstrated no effect on *V. cholerae tcp* expression, such as *Bacteroides thetaiotamicron,* have *bsh* homologs based on our database search. This is in congruence with previous metagenomic analysis of amino acid similarity to a reference BSH ranging from 25-100% identity, as well as reports in *bsh* differences in amino acid sequence and preference in bile acid deconjugation leading to the assignment of genes into eight phylotypes (*52, 53, 86*). Together, these results suggest that there are bacteria in our microbial community have *bsh* that may affect other conjugated bile acids rather than TC.

Species	Annotation	Genome	Locus
Bacteroides caccae	Linear amide C-N hydrolase, choloylglycine hydrolase family protein	AAVM02000007.1	*BACCAC_03140*
Bacteroides thetaiotamicron	Choloylglycine hydrolase	NC_004663.1	*BT_2086*
Bacteroides thetaiotamicron	Choloylglycine hydrolase	NC_004663.1	*BT_1259*
Bacteroides uniformis	Linear amide C-N hydrolase, choloylglycine hydrolase family protein	AAYH02000045.1	*BACUNI_02933*
Bacteroides uniformis	Linear amide C-N hydrolase, choloylglycine hydrolase family protein	AAYH02000035.1	*BACUNI_00572*
Bacteroides vulgatus	Putative choloylglycine hydrolase	CP000139.1	*BVU_2699*
Bacteroides vulgatus	Choloylglycine hydrolase	CP000139.1	*BVU_3993*
Bacteroides vulgatus	Putative choloylglycine hydrolase	CP000139.1	*BVU_1032*
Bifidobacterium longum subsp. *longum*	Choloylglycine hydrolase	AP010888.1	*BLLJ_0817*
Blautia obeum	Choloylglycine hydrolase	AAVO02000001.1	*RUMOBE_00028*
Blautia obeum	Llinear amide C-N hydrolase, choloylglycine hydrolase family protein	AAVO02000021.1	*RUMOBE_03454*
Blautia obeum	Penicillin V acylase and related amidases choloylglycine hydrolase	FP929054.1	*CK5_10630*
Blautia obeum	Penicillin V acylase and related amidases choloylglycine hydrolase	FP929054.1	*CK5_18830*
Blautia torques	Choloylglycine hydrolase	CYYD01000003.1	*ERS852577_00637*
Blautia torques	Choloylglycine hydrolase	CZBJ01000034.1	*ERS852530_03853*
Blautia torques	Choloylglycine hydrolase	CYYD01000049.1	*ERS852577_05096*
Collinsella aerofaciens	Penicillin V acylase and related amidases choloylglycine hydrolase	AAVN02000002.1	*COLAER_00574*
Collinsella aerofaciens	Choloylglycine hydrolase	CP024160.1	*CSV91_00430*
Collinsella aerofaciens	Penicillin acylase, choloylglycine hydrolase	CP024160.1	*CSV91_05910*
Dorea longicatena	Linear amide C-N hydrolase, choloylglycine hydrolase family	AAXB02000007.1	*DORLON_01571*
Eubacterium rectale	Choloylglycine hydrolase	CP001107.1	*EUBREC_2835*
Faecalibacterium prausnitzii	Linear amide C-N hydrolase, choloylglycine hydrolase family protein	ACOP02000008.1	*FAEPRAA2165_00537*
Enterococcus faecalis	Putative penicillin amidase, choloylglycine hydrolase	CP002621.1	*OG1RF_12285*
Streptococcus infantarius	Linear amide C-N hydrolase, choloylglycine hydrolase family protein	ABJK02000021.1	*STRINF_01499*

Table 3. Bile salt hydrolases found in genomes of defined community members.

Species	Locus	*BVU_1032*	*CSV91_05910*	*BT_1259*	*BACCAC_03140*	*BT_2086*	*BACUNI_00572*	*BVU_3993*	*OG1RF_12285*	*BACUNI_02933*	*BVU_2699*	*STRINF_01499*	*ERS852530_03853*	*FAEPRAA2165_00537*	*ERS852577_00637*	*EUBREC_2835*	*CK5_10630*	*RUMOBE_03454*	*DORLON_01571*	*ERS852577_05096*	*RUMOBE_00028*	*CK5_18830*	*BLLJ_0817*	*COLAER_00574*	*CSV91_00430*
Bacteroides vulgatus	*BVU_1032*	100	19	19	17	17	17	17	15	19	20	16	18	16	16	16	15	15	15	15	16	16	18	18	17
Collinsella aerofaciens	*CSV91_05910*	19	100	25	24	24	23	23	18	16	18	20	23	22	21	21	22	21	21	21	21	22	20	18	18
Bacteroides thetaiotamicron	*BT_1259*	19	25	100	53	54	53	52	21	27	26	20	21	19	22	22	20	19	19	19	21	21	23	20	20
Bacteroides caccae	*BACCAC_03140*	17	24	53	100	94	84	85	24	27	28	18	24	22	24	24	22	21	21	21	21	22	22	19	18
Bacteroides thetaiotamicron	*BT_2086*	17	24	54	94	100	84	85	23	27	27	18	23	21	23	23	21	20	20	20	20	22	21	19	18
Bacteroides uniformis	*BACUNI_00572*	17	23	53	84	84	100	84	24	28	26	19	24	21	23	23	23	21	21	21	22	23	22	19	19
Bacteroides vulgatus	*BVU_3993*	17	23	52	85	85	84	100	24	28	27	19	24	21	24	24	23	21	21	21	21	22	22	19	19
Enterococcus faecalis	*OG1RF_12285*	15	18	21	24	23	24	24	100	26	25	22	23	21	25	25	22	23	23	23	23	22	27	24	24
Bacteroides uniformis	*BACUNI_02933*	19	16	27	27	27	28	28	26	100	58	28	30	29	27	27	28	28	28	28	26	28	29	26	26
Bacteroides vulgatus	*BVU_2699*	20	18	26	28	27	26	27	25	58	100	28	27	27	27	27	28	28	28	28	26	28	29	25	26
Streptococcus infantarius	*STRINF_01499*	16	20	20	18	18	19	19	22	28	28	100	57	59	59	59	58	59	59	59	60	59	36	32	32
Blautia torques	*ERS852530_03853*	18	23	21	24	23	24	24	23	30	27	57	100	65	68	68	71	69	69	69	68	69	38	33	32
Faecalibacterium prausnitzii	*FAEPRAA2165_00537*	16	22	19	22	21	21	21	21	29	27	59	65	100	74	73	70	71	71	71	72	69	40	36	36
Blautia torques	*ERS852577_00637*	16	21	22	24	23	23	24	25	27	27	59	68	74	100	99	70	73	73	73	75	73	40	36	35
Eubacterium rectale	*EUBREC_2835*	16	21	22	24	23	23	24	25	27	27	59	68	73	99	100	70	73	73	73	74	73	39	36	35
Blautia obeum	*CK5_10630*	15	22	20	22	21	23	23	22	28	28	58	71	70	70	70	100	90	90	89	81	83	40	37	36
Blautia obeum	*RUMOBE_03454*	15	21	19	21	20	21	21	23	28	28	59	69	71	73	73	90	100	100	100	82	83	39	37	37
Dorea longicatena	*DORLON_01571*	15	21	19	21	20	21	21	23	28	28	59	69	71	73	73	90	100	100	100	82	83	39	37	37
Blautia torques	*ERS852577_05096*	15	21	19	21	20	21	21	23	28	28	59	69	71	73	73	89	100	100	100	82	83	39	37	37
Blautia obeum	*RUMOBE_00028*	16	21	21	21	20	22	21	23	26	26	60	68	72	75	74	81	82	82	82	100	87	38	36	35
Blautia obeum	*CK5_18830*	16	22	21	22	22	23	22	22	28	28	59	69	69	73	73	83	83	83	83	87	100	38	36	36
Bifidobacterium longum subsp. *longum*	*BLLJ_0817*	18	20	23	22	21	22	22	27	29	29	36	38	40	40	39	40	39	39	39	38	38	100	51	51
Collinsella aerofaciens	*COLAER_00574*	18	18	20	19	19	19	19	24	26	25	32	33	36	36	36	37	37	37	37	36	36	51	100	98
Collinsella aerofaciens	*CSV91_00430*	17	18	20	18	18	19	19	24	26	26	32	32	36	35	35	36	37	37	37	35	36	51	98	100

100%
81-99%
61-80%
41-60%
21-40%
0-20%

Figure 8: Percent identity matrix. pairwise comparison of amino acid sequences in *bsh* homologs created by clustal2.1 using online web source www. ebi.ac.uk (tools> multiple sequence alignment>Clustal Omega.

A Putative *B. obeum bsh* Ablates *tcp* Activation by TC and Intestinal Homogenates

Based on our findings, we wanted to determine if the *bsh* gene alone was enough to negatively affect *V. cholerae tcp* activation. We constitutively expressed, *RUMOBE_00028* from *B. obeum* to be cloned and expressed in *E. coli* DH5αλpir, which does not process TCA (*53*). The gene from *B. obeum* was amplified from purified genomic DNA by PCR and cloned downstream of a constitutive $P_{Ltet\text{-}O\text{-}1}$ promoter on plasmid pZE21 in *E. coli* DH5αλpir to generate strain bsh^C. The DH5αλpir with an empty vector did not degrade TC in infant homogenate as measured by Liquid chromatography mass spectrometry and *in vitro PtcpA-sh-ble* induction (see Figure 9A and 9B). Incubation of homogenate with bsh^C significantly ablated the *tcp* incubation of homogenate duction compared to the isogenic vector control (see Figure 11B), suggesting *RUMOBE_00028* alone can alter *V. cholerae* virulence activation though deconjugation of TC.

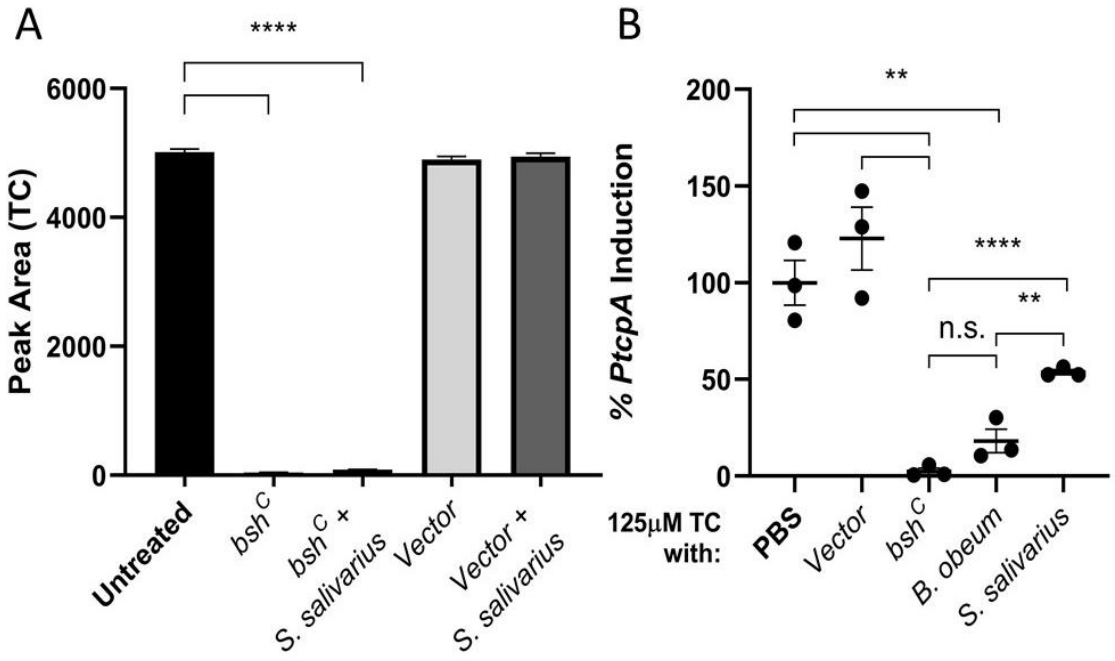

Figure 9: Modulation of *tcpA*-activating signals in suckling CD-1 mouse homogenates by pure cultures of *bsh*^{C}, vector and S. *salivarius* as seen by peak area of TC by LC-MS (A) and *in vitro* P*tcpA-sh ble* induction (B). P*tcpA-sh ble* induction results were normalized to PBS with 125μM TC control. Peak area describes the relative abundance of bile acid per group. Error bars calculated using SEM Significance based on unpaired t-test. *P-value< 0.05, **P-value<0.01, ****P-value<0.0001

RUMOBE_00028 Alone is Sufficient to Reduce *V. cholerae* Colonization

To determine if *RUMOBE_00028* was sufficient to affect *V. cholerae* colonization, five-day old kanamycin treated infant mice were colonized with bsh^C or the vector strain by gastric gavage. After twenty-four hours, these mice were gavaged with a co-culture of either bsh^C or the vector strain with and C6706 containing a kanamycin resistant plasmid by gastric gavage and left fasting. Eighteen hours after, small intestines were removed and homogenized. *V. cholerae* was enumerated by serial dilution and plating on streptomycin LB agar. There was a significant decrease in *V. cholerae* levels in bsh^C colonized animals, indicating that deconjugation by *RUMOBE_00028* alone can decrease *V. cholerae* colonization (see Figure 10).

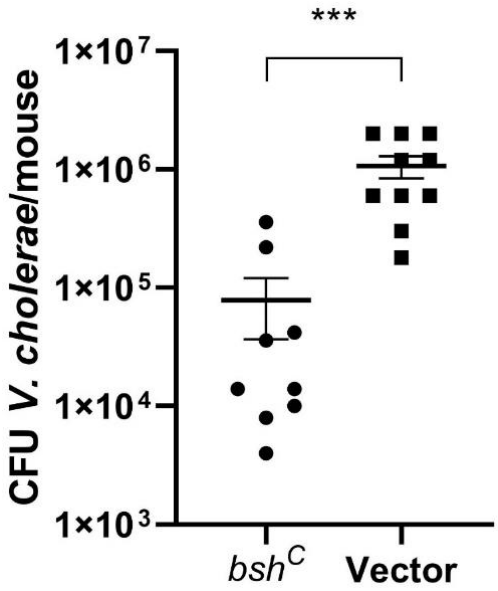

Figure 10: Effects on *V. cholerae* infection of suckling CD-1 mice by 1-day pre-colonization with indicated *E. coli* strains. Error bars calculated using ±SEM based on unpaired t-test.***P-value< 0.001

Interpersonal Differences in *B. obeum bsh* Levels in Complex Human Fecal Microbiomes Explain Differences in Resistance to *V. cholerae* Infection

Since *Blautia* demonstrated intra-genus differences in BSH activity, as well as association with cholera in human populations, we assayed for the level of the *B. obeum bsh* gene by real-time PCR in total DNA extracted from human fecal samples provided by healthy donors that did not have: (a) an acute disease or uncontrolled GI disorders or diseases, (b) any antibiotic usage or diarrhea two weeks prior to sample collection and (c) did not have major surgery of the GI tract (*79*). We found that communities associated with higher *V. cholerae* colonization had lower levels of *RUMOBE_00028* (Figure 12). These results mimic the bile acid levels seen in Figure 11, again highlighting the trend among *V. cholerae* CFU of mice colonized with donor bacteria.

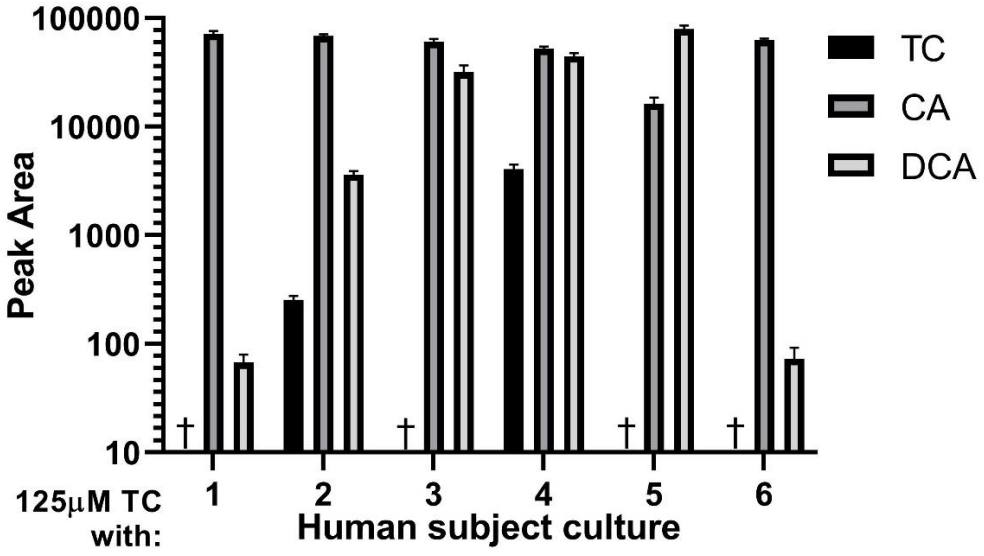

Figure 11: Result of incubation of TC solution with cultured samples of human fecal communities, †: TC not detected in sample. Peak area describes the relative abundance of bile acid per group.

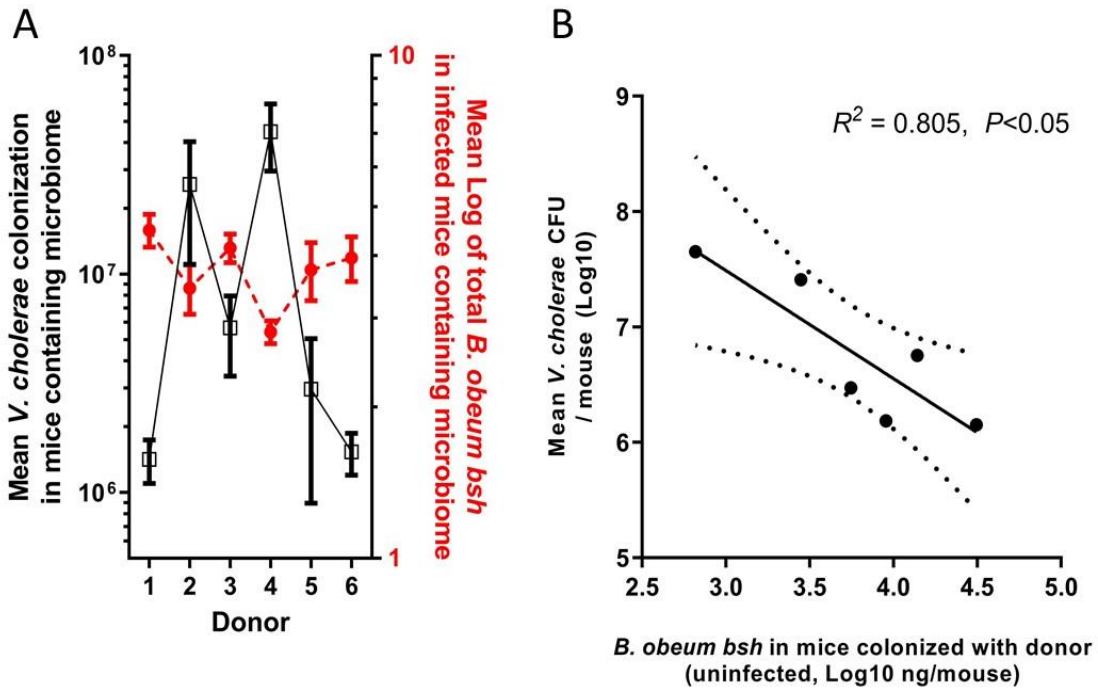

Figure 12: (A) Real-time PCR quantification of *B. obeum bsh* in DNA extracted from mice colonized with indicated complex human fecal samples. (B) shows resulting *V. cholerae* CFU (mean log per group) after infection of animals containing indicated complex human fecal sample. *V. cholerae* CFU provided by Salmasadat Alavi. Error bars calculated using ±SEM.

To determine whether these findings corresponded to functional degradation of TC, bacterial colonies were grown from donor fecal samples and then incubated *in vitro* as described above followed by heat treatment, filter sterilization and submitted for LC-MS. Levels of TC tended to be elevated in groups with increased *V. cholerae* counts when tested *in vivo*. DC was also found in each group, indicating that there were species that contained an intact 7α-dehydroxylation pathway. This is expected as 16S revealed bacteria that belong to *Clostridium* cluster XIVa were present and most 7-dehydroxylation bacteria are come from this culture (*95*) (data not shown).

Variation in Bile Acids Effect on *V. cholerae* Virulence

Previous studies that looked at bile acids activating *V. cholerae* virulence expression looked at a subset of total bile acids found in bile (*31, 33*). Therefore, we again tested *tcp* induction using our reporter assay and screened a panel including primary bile acids, individual amino acids, conjugated forms, secondary bile acids and several murine derived forms. When tested, amino acid alone or adding taurine or glycine to deconjugated bile acids was not enough for *tcp* induction, highlighting the need for conjugation (see Figure 15A). Except for TCDCA, we saw human primary conjugates induce higher virulence gene expression in *V. cholerae* compared to secondary conjugates and unconjugated bile acids. This highlights the need for conjugated bile acids for *V. cholerae* to properly express *tcp*. Interestingly, GCDCA had the highest induction when compared to other bile acids (see Figure 15B). It is important to note that glycine conjugates resulted in higher percent survival compared to taurine and that murine

derived bile acids showed low induction. It is possible that this is due to humans having a 3:1 ratio of glycine to taurine conjugates with the disease evolving to be a human pathogen, utilizing bile acids found in this environment (*37*).

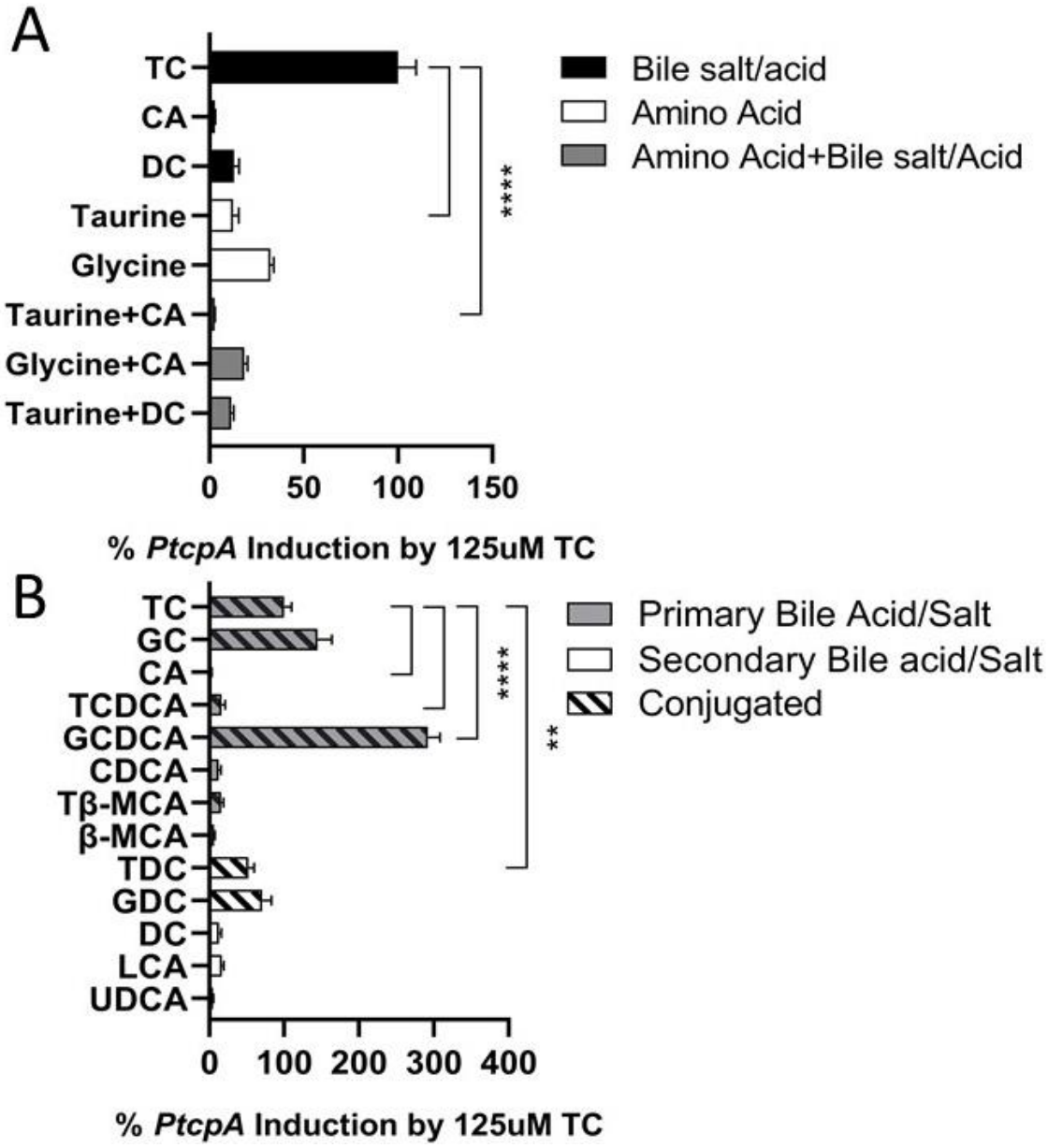

Figure 13: Ability of indicated bile acid species(B) and amino acids (B) to activate tcp gene expression in *V. cholerae*. P*tcpA-sh ble* induction results were normalized to PBS with 125μM TC control. Error bars calculated using ±SEM based on unpaired t-test.

DISCUSSION

The mammalian small intestine is a complex cellular and molecular milieu that enteropathogens must navigate to protect themselves from bactericidal and bacteriostatic effects of host components and the activity of commensal microbes. However, some of the molecules in this environment also act as signals that provide important timing cues for virulence gene regulation. The ability of pathogens to sense these signals ensures that metabolically expensive virulence regulons are only expressed in suitable environments. For example, in *V. cholerae*, utilization of bile acids and microaerophilic/anaerobic conditions to induce CT and TCP activation (*28, 31*, *34*). Important signals like bile acids however are chemically diverse and variable in composition between individuals in part due to the ability of native gut microbial communities to process bile acids.

Through *in vitro* and *ex vivo* screening, mass spectrometry, and genomic analysis, we were able to identify gut community members that deconjugate TC through *bsh* activity. This screen also revealed gut strains that contain putative *bsh* genes but do not deconjugate TC, supporting studies have shown *bsh* variation in both sequence and function, with *bsh* enzymes being broadly grouped into 8 phylotypes by amino acid sequence identity and biochemical activity against specific bile species (*51*, *53*). Therefore, different communities may exhibit varying levels of cumulative *bsh* activity. It is still unclear how differences in community *bsh* function, specificity, and activity translates into the final bile pool composition of the gut. However, it is reasonable to hypothesize that a host's microbiome diversity and abundance of these enzyme phylotypes decreases or disappear completely, as seen in microbial communities with

patients experiencing diarrhea or antibiotic treatment, would lead to changes in the deconjugated bile acid profile. Humans with dysbiotic gut microbiota have been shown to have a decrease in bacterial diversity, and therefore I propose there would be correlated decreases in *bsh* homologs (*84, 85, 97*).

One study that indirectly supports this proposition examined patients infected with *Clostridium difficile* and profiled the microbiome and bile acid pools before and after fecal transplant therapy. Prior to treatment, individuals had decreased diversity in their gut microbiome, higher conjugated bile acids, and lower levels of secondary bile acids. After the patient gut microbiomes were restored via fecal transplants, microbial and bile acid pool diversity increased (*97*). In germ-free mice that lack all of their gut microbes exhibit a similar pattern and have been shown to have bile acid pools that are predominated by conjugated bile acids and secondary bile acids are virtually absent with the exception of taurine conjugated UDCA (*98*).

Given that bile acids are important virulence signals for *V. cholerae* and that resident microbes can alter the composition of bile acids present, we examined how microbial communities of different composition might affect *V. cholerae* colonization. Of the bile acids tested on *V. cholerae,* our results saw primary conjugated bile acids having the greatest effect on the expression of *tcp*. These results suggest that communities with a higher abundance of *bsh* genes or 7-dehydroxylation pathways would create an environment unfavorable for *V. cholerae* colonization by a repression of virulence activation. Conversely, we expect that communities with lower abundance of the genes involved in these pathways to great a more favorable environment for *V. cholerae*

colonization. We propose that dysbiotic gut communities, like those found in malnourished or having recently experienced diarrhea would be favorable for colonization because these patients generally have significantly lower bacterial diversity and are likely to in turn have lower diversity of *bsh* genes or 7-dehydroxylation pathways. This could result in conjugated primary bile acids encompassing a larger percentage of the total bile acid pool and secondary forms decreasing in abundance.

Through studies performed by Salmasadat Alavi and studies in this thesis, *B. obeum* and, specifically, the *bsh* gene *RUMOBE_00028* are shown to have a detrimental effect on *V. cholerae* virulence activation and colonization. This enzyme efficiently deconjugates TC and converts TC into CA. *S. salivarius* lacked significant *bsh* activity under our conditions and had a no or a slightly positive effect on *V. cholerae* virulence activation (Figure 4, 6, 7). I also identified additional bacterial strains that had significant effects on *tcp* activation (*B. uniformis, B, vulgatus, B. longum, D. longicatena* and *E. rectale*) and the regulation and activity of these enzymes will be the subject of further research in the lab. Curiously, *S. infantarius* from our DS model community encodes an intact *bsh* gene and demonstrated deconjugation of TC *in vitro*. However, the presence of this microbe did not act to inhibit *V. cholerae* infection *in vivo* as we might expect. One possible reason is that the expression or activity level *in vivo* of this enzyme is absent or down regulated. Another possible explanation is that different species establish differently in the small intestines causing bsh to be more abundant in different sections.Very little work has addressed the possibility of *bsh* regulation, and it is possible

that bile concentration is not the only determinant in *bsh* expression, nor may every bile acid be equal in terms of regulating *bsh*.

It is important to note that, while examining the genomes of our defined model communities, a functional search revealed that *V. cholerae* contains a *bsh* gene (Locus: VCA0877). To our knowledge, no studies are available that describe the ability of this predicted *V. cholerae* enzyme to process bile acids. Homologs of *bsh* have also been found in other pathogenic bacteria, specifically *Listeria monocytogenes* and *Brucella abortus*, and a search in UniProt found homologs in *Salmonella enterica* and *Yersinia enterocolitica* (*101*). Given the necessity of gut-resident organisms to deal with the anti-microbial effects of bile, the ability of pathogens to process bile into less toxic forms is not surprising. What is not known is how these enzymes, and specifically the enzyme in *V. cholerae* is able to work on particular components of the bile acid pool; *V. cholerae* BSH was closest to the BSH type 6 (BSH-T6) phylotype as defined by Song et al. (Table 4). BSH-T6 enzymes have demonstrated no deconjugation capabilities to GCA or TCA, bile the acids we have shown to be strong activators of *tcp*, while deconjugating secondary bile acids that have bacteriostatic function instead (*89*). It is reasonable to hypothesize that this is a beneficial adaptation by deconjugating potential inhibitors of growth while sparing those bile acids that are used for *tcp* activation. *B. obeum's* BSH was closest to the BSH-T1 phylotype. This is in line with Song et al. with BSH-T1 phylotype demonstrating almost 100% deconjugation of TCA and the fact *B. obeum* demonstrated one of the highest BSH activity against TC in our community (*89*).

BSH phylogenetic Group	*V. cholerae* Percent Identity	*B. obeum* Percent Identity
BSH-T0	25.42	26.78%
BSH-T1	22.63%	76.31%
BSH-T2	21.94%	59.69%
BSH-T3	22.58%	47.38%
BSH-T4	22.74%	39.18%
BSH-T5	24.72%	28.08%
BSH-T6	45.53%	23.05%
BSH-T7	22.58%	24.18%

Table 4: Comparison of *V. cholerae* and *B. obeum* BSH amino acid sequence against a reference BSH enzyme from each phylotype as described by Song et al. Highlighted percent identity indicate closest similarity to phylotype. Percent identity determined by NCBI BLASTp.

Future work will focus on the other microbial species in the communities. The establishment of phylotypes of the *bsh* has led to a broad classification of deconjugation ability in these genes. More species found within these *bsh* types should be tested to determine if the enzymes effect on conjugated bile acids are the same; additionally, these phylotypes should be tested against all conjugated bile acid types found in humans to get a better understanding as to why *bsh* has specificity, and how the expression of these enzymes are regulated. Studies to look into *bsh* gene regulation would include incubation with different concentrations and types of bile acids, coupled with qRT-PCR to determine if bile acids are acting directly on *bsh* expression. It is also important to note that deconjugation can be an intracellular or extracellular process (*51*). This can be tested by comparing cytoplasmic, membrane, and extracellular fractions of bacteria with a functional *bsh*. Other studies to be performed on complex communities' effect on *V. cholerae* colonization through *bsh* intervention focusing on having bacteria with *bsh* genes from all eight phylotypes and their effect on the bile acid pool as well as profiling the genes expressed in these communities. Additional studies will focus on microbial 7α/β-dehydroxylation should be studied. This process creates the secondary bile acids seen in the human gut and our results demonstrate that secondary bile acids demonstrated lower *tcp* expression than primary, possibly leading to an additive effect on *V. cholerae* colonization over and above the degradation of virulence inducing bile acid species (*36*).

In addition, we will examine the role of *bsh* in providing protection from bile acids (*46*). By creating Δ*bsh* mutants in *V. cholerae*, the effects bile acids have on these bacteria can be tested in *in vitro* and *in vivo* assays. Also, a more comprehensive

examination on conjugated bile acids should be performed on *tcp* expression. Our bile acid screen demonstrated the effect different bile acids conjugates and amino acids had on *V. cholerae tcp* expression. However, this screen gave an incomplete picture of the bile acids seen in the intestinal tract and should be expanded to include conjugated forms of LCA, UDCA and other murine forms. For example, we expect that LCA conjugates would have lower induction as it is considered more toxic and am interested about the effect UDCA has as it is a non-toxic bile acid (*32*, *36*).

Taken together, my results suggest that *B. obeum* ATCC 29174 are antagonistic to *V. cholerae* infections, both by production of quorum sensing molecules but also through activity of its *bsh* enzyme, providing new insight on how colonization of *V. cholerae* is affected by the gut microbiome (*84*). It is important to note that this antagonistic activity may not be not refer to the species as a whole as differences are seen in BSH amino acid sequence and function within species (see Figure 8) (*51*). Many individuals in cholera endemic areas exhibit a disrupted microbiome due to malnutrition and non-cholera infectious diarrhea. My results suggest that this may in turn feed back into infection susceptibility by laying the framework for a *V. cholerae* infection due to an increase in conjugated primary bile acids (*97*). Based on reports in the literature, and our results, establishing and maintaining a resistant microbiome with bacteria containing *bsh* genes from the different phylotypes could decrease the risk of infection; one way to accomplish this is through the development of probiotics made up of cultures that have *bsh* genes and species that participate in dehydroxylation. By establishing these microbiomes, the bile acid pool can be manipulated in that secondary bile acids are created by the microbiome

and increasing the diversity of conjugated bile acid pool, bile acids that do not positively affect virulence expression in *V. cholerae.* There are other ways of manipulating bile acid composition, such a high fat diet and cholestyramine; these avenues would not be feasible as the acquisition of foods high in fat is more difficult in these developing areas and cholestyramine maybe more detrimental by affecting malnourished individuals ability to absorb and retain fat needed for survival. These results not only affect the microbial field, but also highlights the need for dietary and medical intervention in order to reduce the incidences of infection and maintaining a resistant microbiome in individuals.

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