COOPERATIVE BACTERIAL INTERACTIONS MEDIATE RESISTANCE TO COLONIZATION BY INTESTINAL PATHOGENS

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The diverse microbial populations constituting the intestinal microbiota promote immune development, nutrient digestion and colonization resistance against invading pathogens. Due to their complex metabolic requirements and the consequent difficulty culturing them, they remained, until recently, largely uncharacterized. In the past decade, deep nucleic acid sequencing platforms, new computational and bioinformatics tools and full genome characterization of several hundred commensal bacterial species have facilitated studies of the microbiota and revealed that differences in microbiota composition can be associated with a myriad of conditions and that the microbiota can be manipulated to prevent, reduce and even cure some of them. One of the most critical functions of the microbiota is to prevent the expansion of disease-causing organisms. Different bacterial species exert different roles within the intestine and therefore, the composition of the microbiota determines, in part, the level of resistance to infection. Perturbation of microbial communities with antibiotics renders the host susceptible to colonization by opportunistic antibiotic-resistant bacteria. The studies discussed in this dissertation aim to determine the impact that vancomycin-resistant Enterococcus (VRE) and carbapenem-resistant *Klebsiella pneumoniae*, two common hospital-acquired infections, have on each other and on the host and to identify intestinal bacteria responsible for colonization resistance against VRE. We demonstrate

that VRE and *K. pneumoniae* coexist within the intestine despite residing in very close proximity to each other and that colonization resistance against VRE can be mediated by two bacterial species despite antibiotic treatment. This work highlights the complex interplay between gut bacteria and has important implications for the development of new therapeutics to treat intestinal infections.

BIOGRAPHICAL SKETCH

Silvia G. Caballero completed a Bachelors of Arts in the Biological Sciences at Hunter College of the City University of New York in 2008 and matriculated into the Weill Cornell Immunology and Microbial Pathogenesis Program in 2009. She joined Dr. Eric Pamer's laboratory in 2010.

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PUBLICATIONS

Benakis C., Brea D., **Caballero S.,** Moore J., Murphy M., Sita G., Racchumi G., Ling L., Pamer E.G., Iadecola C. and Anrather J. (2016) Commensal microbiota affect ischemic stroke outcome by regulating the activity of intestinal γδ T cells. **Nature Med.**

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Ubeda C., Bucci V., **Caballero S.**, Djukovic A., Toussaint N.C., Equinda M., Lipuma L., Ling L., Gobourne A., No D., Taur Y., Jenq R.R., van den Brink M.R., Xavier J.B. and Pamer E.G. (2013) Intestinal microbiota containing Barnesiella species cures vancomycin-resistant *Enterococcus faecium* colonization. **Infect Immun.**

For my family most especially

my mother Lourdes,

my grandmother Irma and

my aunt Sonia, my biggest cheerleaders,

who instilled in me

the value of education from a

very young age. This is for you.

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LIST OF ABBREVIATIONS

MetaPhlAn	Metagenomic Phylogenetic Analysis
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
LefSe	Linear Discriminant Analysis with Effect Size
Th	T helper
Muc	Mucin
TLR	Toll-like receptor
MyD88	Myeloid differentiation factor 88
NLRP	Nod-like receptor Pyrin domain
IL	Interleukin
DC	Dendritic cell
CX3CR1	CX3C-chemokine receptor 1
CD	Cluster of differentiation
CCR2	C-C chemokine receptor type 2
Ly6C	lymphocyte antigen 6C
Gadd45a	Growth arrest and DNA damage 45a
RegIII	Regenerating islet-derived III
Ig	Immunoglobulin
ILC	Innate lymphoid cell
ID	Inhibitor of DNA binding 2

IFN	Interferon
ROR	RAR-related orphan receptor
MHC	Major histocompatibility complex
GM-CSF	Granulocyte-macrophage colony-stimulating factor
PD-1	Programmed cell death protein 1
TNF	Tumor necrosis factor
iNOS	Inducible nitric oxide synthase
NFIL	Nuclear factor regulated by IL-3
TGF	Tumor growth factor
C	Celsius

LIST OF SYMBOLS

γ	gamma
α	alpha
β	beta
μ	micro
°	degrees
%	percent

CHAPTER 1

INTRODUCTION

1.1. OVERVIEW

1.1.1. The gastrointestinal microbiota

The development in metazoans of a tubular tract for food digestion, nutrient absorption and waste expulsion is believed to have occurred over 500 million years ago (1). The evolution of the intestinal tract created a niche for microbial symbionts, with resulting complex microbial populations that are uniquely adapted to life in this environment and that, to greater and lesser extents, depend upon each other for survival. Although these microbial populations have, for hundreds of years, provoked curiosity among microscopists, microbiologists and physiologists, they remained largely uncultured and therefore uncharacterized, in part because they generally do not cause disease. The last decade, however, has seen remarkable progress in our understanding of the mammalian intestinal microbiota and its impact on immune system development and function (2,3). While much of the focus of microbiota and microbiome studies has been on microbiota composition of normal, healthy individuals (4-6), parallel studies have correlated microbiota composition with a wide range of diseases. The major messages emerging from this growing body of work are that healthy humans are colonized with diverse microbial populations that differ between individuals, that different microbes can have distinct interactions with the innate and adaptive immune systems of their mammalian host and that exogenous factors, in particular

medical interventions such as antibiotic administration, can lead to marked changes in the microbiota with downstream implications for health.

1.1.2. Bioinformatic and computational platforms for microbiota / microbiome analysis

Multi-parallel nucleic acid sequencing has greatly enhanced our understanding of commensal bacterial populations. Microbiota composition is generally determined by sequencing PCR-amplified bacterial 16S ribosomal RNA genes followed by analysis of sequencing data with bioinformatic programs such as Mothur (7) that assign taxonomic labels to each sequence. Other methods such as UniFrac enable investigators to compare complex samples and to correlate microbiota composition with specific experimental or clinical scenarios (8). Another method that has enabled investigators to identify bacterial taxa that differ between samples is LEfSe (linear discriminant analysis effect size), which supports high-dimensional class comparison between microbiomes obtained from different groups (for example colitis versus normal control samples (9). Programs such as MetaPhlAn (9) facilitate the determination of bacterial taxon prevalence in samples that have been shotgun sequenced, while PICRUSt enables investigators to estimate the representation of microbial metabolic pathways on the basis of 16S rRNA taxonomy (10). These platforms are well established and are commonly used for microbiota and microbiome analyses.

More recently, mathematical models have been used to predict shifts in microbiota composition following different perturbations and to identify interactions between distinct bacterial taxa. Using modified Lotka-Voltera equations, which were originally derived to mathematically model predator-

prey dynamics, a mathematical approach has been described that incorporates the growth rate of different bacterial taxa, their susceptibility to specific perturbations (such as antibiotic administration) and their impact on each other. If provided with quantitative data on the density of specific bacterial populations, and knowledge of their growth rate and susceptibility to a specific perturbation, one can calculate the strength of interactions between different bacterial populations (11). Using this approach, a sub-network of bacterial groups was identified that appears to confer protection against *Clostridium difficile* infection. Another mathematical approach studying reconstitution of germ-free mice with complex flora suggested that interactions between bacterial species range from competitive to parasitic, with the surprising result that mutualistic interactions were not detectable (12).

1.1.3. Microbiota composition and the intestinal environment

Using these computational tools, we have come to learn that the composition and density of the microbiota varies along the gastrointestinal tract. The stomach, due to its highly acidic pH, harbors around 10^1-10^3 bacteria per gram of content and consists, for the most part, of organisms belonging to the *Lactobacillus* genus and in some instance, disease-causing organisms such as *Helicobacter pylori* (13). The small intestine is most densely colonized in the distal region, where nearly 10^8 bacteria per gram of content reside. Most bacteria found there belong to the Firmicutes and Bacteroidetes phyla, the two most dominant phyla in the gastrointestinal tract (GI), and include members of the Lactobacillaceae, Enterococcaceae, Erysipelotrichiaceae, Clostridiaceae, and Bacteroidaceae families (14,15). The large intestine is the most diverse and colonized compartment of the GI tract harboring bacterial levels in the

10^{10}-10^{12} range. Similar to the small intestine, the large intestine is dominated by Firmicutes and Bacteroidetes. Other phyla, such as Proteobacteria, Verrucomicrobia and Actinobacteria are represented as well but in much lower frequencies (16). The majority of bacteria that make up the microbiota are strict anaerobes with complex nutritional requirements. Although rich in nutrients, the environment of the intestine is not uniform and just like the bacterial composition differs by compartment, so does the intestinal environment. For instance, pH and oxygen gradients vary along the GI tract, with the stomach being the most acidic and aerobic compartment while the colon is relatively neutral and anaerobic (16). Therefore, the low-acidity, low-oxygen, high-nutrient environment of the large intestine explains the large bacterial density found at this site. Variation in microbiota composition within healthy human populations has led to the enterotype hypothesis, which posits that humans can be divided into three groups on the basis of their fecal microbiota composition (6). The fecal microbiota of healthy individuals could be grouped into three enterotypes, one predominated by *Prevotella*, a second by *Bacteroides* and the third by *Ruminococcus*.

1.1.4. Bacterial interactions mediate the balance between health and disease

Our understanding of how microbiota complexity is maintained and how the individual bacterial taxa compete with and support each other is rudimentary but important new insights have emerged. Metabolism of host-derived polysaccharides by *Bacteroides fragilis*, an obligately anaerobic bacterial species that inhabits the human colon, is required for intestinal colonization and persistence and genetic deletion of the polysaccharide utilization locus

markedly reduces competitiveness in the gut (17). Commensal microbiota-mediated metabolism of host polysaccharides, for example the cleavage of sialic acid or fucose from host mucin, can promote infection with enterohaemorrhagic *Escherichia coli*, *Salmonella typhimurium* and *Clostridium difficile* by promoting virulence gene expression (18) or by enhancing growth (19).

The interactions between different bacterial taxa in the gut can be direct, i.e. resulting in the inhibition or support of bacterial species A by bacterial species B, or indirect, by modification of immunologic or physiologic host factors by bacterial species B which then either inhibit or support colonization by species A. Studies of these interactions are greatly facilitated by isolation, growth and characterization of the wide array of commensal bacterial species, a critical step that is both technically challenging and, given the marked genomic differences between bacterial strains belonging to the same species, daunting in terms of the massive number of potential strains to be studied. The importance of characterizing multiple strains was demonstrated in a study of four *Bifidobacterium longum* strains, of which only two provided resistance against an intestinal pathogen (20). Recent studies demonstrate that many colon-derived bacterial species can be cultured *in vitro* (21), including bacterial species that drive *in vivo* T cell differentiation (22,23). From an immunologic perspective, the impact of microbiota composition is increasingly recognized as important, with some bacterial taxa driving intestinal T-regulatory cells while others induce Th17 T cell development (22-25).

1.2. INTESTINAL EPITHELIAL BARRIER AND INNATE IMMUNE DEFENSES

1.2.1. Mucin and epithelial cell-mediated barriers of infection

While blood draining the gut flows to the liver, which protects the systemic circulation by filtering microbes that traverse the intestinal epithelium (26), the most important barrier to bacterial entry of deeper tissues is the intestinal epithelium. The mucin layer that coats luminal epithelial cell surfaces physically excludes microbes inhabiting the intestinal lumen. Direct bacterial contact with luminal epithelial cell surfaces can occur in the absence of intestinal mucin layers or when specific microbes can penetrate mucin. The predominant mucin secreted by goblet cells is Muc2, which is a large, highly glycosylated multi-domain protein that forms di-sulfide bonded trimers that are partially resistant to trypsin. Muc2 forms a dense, approximately 50mm thick mucin layer in the colon that is attached to underlying epithelial cells and a more dispersed outer layer (27). Some pathogens and commensal bacteria can penetrate the dense mucin layer by proteolytic degradation (28,29) and a subset of commensal bacteria can penetrate the outer mucin layer and break down and metabolize the O-linked glycans attached to Muc2 (30-32).

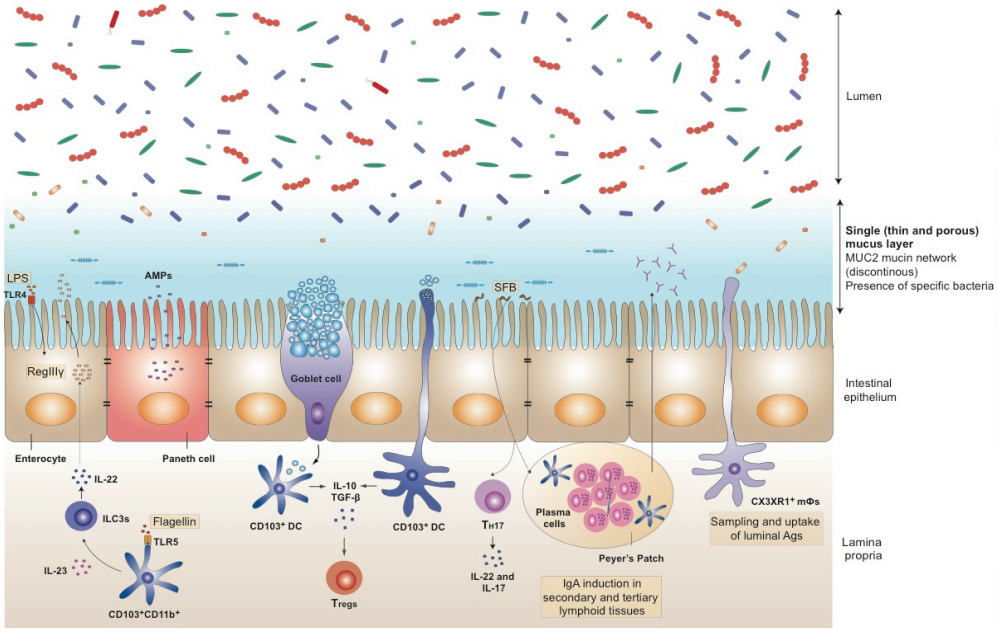
Commensal microbes enhance mucin production by goblet cells in a MyD88-dependent fashion, and germ-free or antibiotic-treated mice have markedly reduced levels of mucin. The thickness, density and composition of mucin differ in the stomach, small intestine and colon (33,34). The ability of bacteria to penetrate the mucus layers along the gut differs, with greater mucus penetration in small intestine than either stomach or colon (33). MUC1 expression in the stomach and small intestine has been implicated in

resistance to *Campylobacter jejuni* and *Helicobacter pylori* infection (35,36) while MUC2 provides resistance to enteric *Salmonella typhimurium* infection (37). In addition to functioning as a barrier against penetration by luminal bacteria, MUC2 also conditions lamina propria dendritic cells to become tolerizing, thereby reducing inflammatory responses to absorbed substances (38). Goblet cells have recently been shown to deliver antigens to lamina propria dendritic cells, providing a potential delivery pathway to facilitate mucin-mediated tolerization of antigen presenting cells (39).

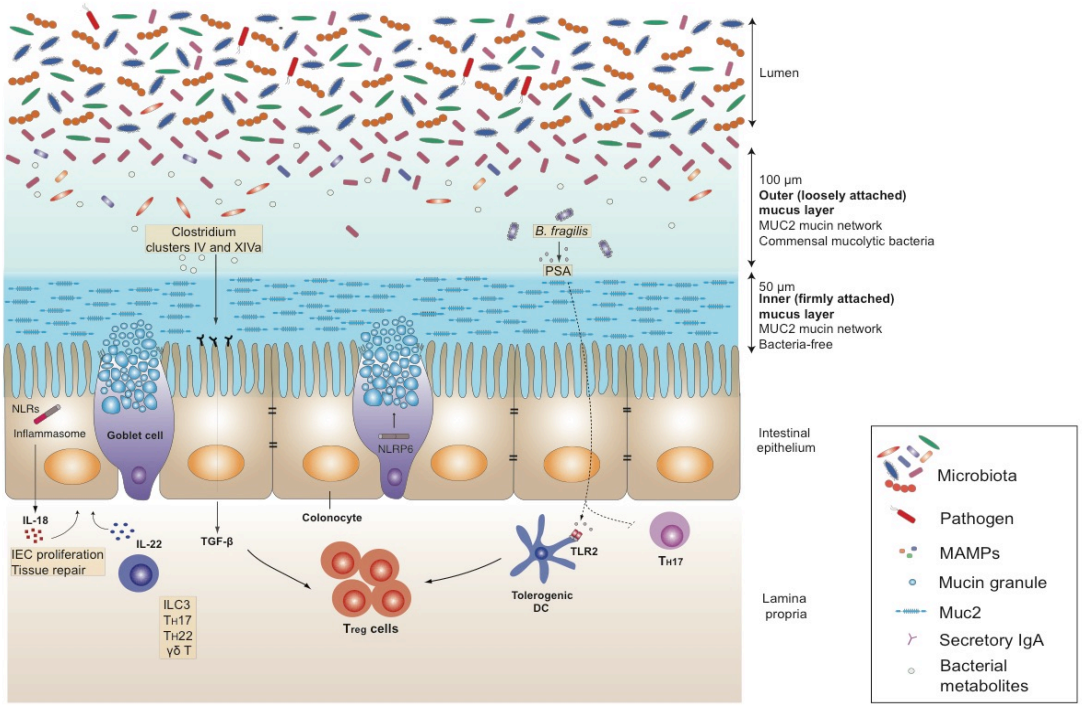
Below the mucin layer, intestinal epithelial cells form a continuous barrier that is one cell layer thick. Intestinal epithelial cells absorb a wide range of nutrients, including proteins, carbohydrates and fats, but exclude bacteria. Achieving the right balance between nutrient absorption and microbial exclusion is critical for optimal health, and deviation from this balance is deleterious to the host. Intestinal epithelial cells express innate immune receptors, but receptor positioning, i.e. apical versus basal, minimizes unnecessary activation by harmless commensals in the intestinal lumen while enabling rapid defensive responses when the barrier is breached. For example, TLR5, the innate immune receptor specific for bacterial flagellin, is not expressed on the apical surface of epithelial cells, where flagellin is presumably present at high concentrations (40). Deletion of MyD88 in intestinal epithelial cells reduces production of mucin and antibacterial peptides and increases susceptibility to infection (41). Certain components of the inflammasome, such as NLRP6, are selectively expressed by intestinal epithelial cells and can influence the composition of the intestinal microbiota by inducing IL18 expression (42) (**Figure 1.1**).

Figure 1.1. Maintenance of intestinal homeostasis in the GI tract. The intestinal epithelial surface is coated with a layer of mucus that has a pivotal role in intestinal barrier function. This mucus layer is organized by MUC2 mucin glycoproteins that polymerize into a gel-like structure preventing luminal bacteria from coming into contact with epithelial cells. In the large intestine, two mucus layers protect the colonic epithelia: a bacteria-free, dense, inner layer followed by an outer layer that harbors mucus-degrading bacteria. In contrast, a single loosely attached layer of mucus lines the small intestine. Mucins are produced by goblet cells and stored in secretory granules until appropriate stimulation, such as signaling through the NLRP6 inflammasome, prompts their release. Consistent with high mucus production in the colon, the number of goblet cells is much greater in the large intestine compared to the small bowel. The density and composition of luminal bacteria also differ between these two compartments. The small intestine harbors ~10^8 bacteria per gram of content, mostly *Lactobacillus* and *Erysipelotrichi* and to a lesser extent *Enterococcus* and Enterobacteriaceae. In mice, segmented-filamentous bacteria (SFB) adheres to the intestinal surface and enhances the development of Th17 cells and IgA production by B cells. Microbe-associated molecular patterns (MAMPS) stimulate the production of antimicrobial peptides (AMPs) from epithelial and Paneth cells through activation of pattern-recognition receptors. For example, induction of RegIIIγ, an antimicrobial protein that targets Gram-positive bacteria and maintains host-bacterial segregation, is mediated through lipopolysaccharide (LPS) and flagellin stimulation of $TLR4^+$ epithelial cells and $TLR5^+$ $CD103^+CD11b^+$ DCs, respectively. DCs and macrophages sample luminal antigens and stimulate cytokine production and T cell differentiation. Although $CX3CR1^+$ macrophages are the major antigen-sampling mononuclear phagocytes in the small intestine, CD103+ DCs have been shown to play a role as well. Nearly 10^{11}-10^{12} bacteria reside in the large intestine. The composition is diverse, comprised mainly of Lachnospiraceae, *Bacteroides* and *Clostridium* groups IV and XIV. Tissue repair and tolerance to commensal and food antigens are key factors for maintaining homeostasis in the gut. IL-22 and IL-18 promote epithelial cell proliferation and repair; however, excessive and aberrant signaling can lead to inflammation and colitis. Tolerance is accomplished by the induction of regulatory T cells (Tregs) through several mechanisms. In the small intestine, $CD103^+$ DCs take up mucus and stimulate Tregs through IL-10 and TGFβ. Although the mechanism has not been elucidated, it is possible that DCs take up mucus directly by extending dendrites into the lumen or that goblet cells transfer mucus to DCs, as it has been shown with antigen. In the colon, specific Treg-inducing bacteria have been identified. For instance, Clostridia spp. generate metabolites that upon receptor binding stimulate the production of TGFβ from epithelial cells. PSA from *Bacteroides fragilis* enhances Treg function (either through direct stimulation of $TLR2^+$ Tregs, or through a $TLR2^+$ DC intermediate) while inhibiting pro-inflammatory Th17 responses.

Small Intestine



Large Intestine



1.2.2. Lamina propria macrophages and dendritic cells

Immediately below the intestinal epithelial cell layer, complex cell populations make up the lamina propria. The lamina propria is a well-vascularized and lymph-drained tissue whose functions include facilitating systemic nutrient distribution, establishing immune tolerance towards innocuous antigens and microbes and providing immune defense against potentially pathogenic microbes that emerge from bacterial populations inhabiting the intestinal lumen. Mesenchymal stromal cells of the intestinal lamina propria help orchestrate immune responses by responding to microbe-derived molecules that trigger MyD88-mediated signals and potentially other innate immune signaling pathways (43). While the role of stromal cells in the lamina propria is likely underappreciated and under-investigated, great progress has been made in the last decade in our understanding of the myeloid-derived macrophage and dendritic cell populations of the gut (44,45).

Given the density and complexity of microbial populations in the intestinal lumen and the obvious benefits of mucus and epithelial cell-mediated segregation of these organisms from underlying tissues, the initial revelation that myeloid cells disrupt inter-epithelial cell tight junctions and extend processes into the intestinal lumen was both astounding and disquieting (46,47). Subsequent studies implicated CX3CR1-expressing myeloid derived cells as the key population accessing the intestinal lumen and taking up pathogenic organisms such as *Salmonella typhimurium* (47). $CD103^{+}$ dendritic cells of the lamina propria, which have been implicated in the transport of bacteria and antigens to draining mesenteric lymph nodes and in the induction of tolerance by producing retinoic acid (48) have also been found to patrol between intestinal epithelial cells and extend dendrites into the intestinal

lumen, taking up pathogenic bacteria such as *Salmonella typhimurium* and non-pathogenic commensal bacteria and transporting them to draining lymph nodes (49) (**Figure 1.1**). The contribution of the commensal flora to microbial transport by lamina propria residing myeloid derived cells was recently investigated, with the finding that antibiotic treatment or MyD88-deficiency increased the delivery of bacteria to mesenteric lymph nodes and enhanced specific T cell and antibody responses (50).

The identity of the cell populations capable of delivering bacteria to draining lymph nodes remains somewhat controversial, with some studies suggesting that CX3CR1^{+} mononuclear phagocytes transport bacteria (50) while most other studies implicate CD103^{+} dendritic cells (51). It is likely that this controversy in part stems from varied dendritic cell (DC) definitions and the plasticity of inflammatory monocytes infiltrating the intestinal lamina propria, particularly during intestinal inflammation. For example, CCR2^{+}Ly6C^{hi} monocytes give rise to CX3CR1^{+} mononuclear phagocytes, and, once differentiated, they appear to become non-migratory. However, under inflammatory conditions, CCR2^{+}Ly6C^{hi} monocytes can differentiate into migratory DCs and prime T lymphocytes (52,53).

1.2.3. Innate immune signaling in the intestinal wall

Activation of the innate immune system by microbial stimulation of innate immune receptors is an essential early step in defense against infection by pathogenic organisms. However, innocuous and potentially beneficial colonizing bacteria are composed of molecules that can bind innate immune receptors and potentially trigger inflammatory responses. Triggering of some innate immune receptors, however, can also induce tolerance by reducing

inflammatory responses. Indeed, development of the mucosal immune system is in part dependent on innate immune receptor triggering by commensal microbes in the intestinal lumen (54). Colonization of the murine gut with the anaerobic bacterium *Bacteroides fragilis* and its production of polysaccharide A (PSA) enhances T cell development and differentiation (55). PSA is believed to promote T regulatory cell development and inhibit Th17 responses by stimulating TLR2 (56) (**Figure 1.1**). In this setting, TLR2 signaling occurs in part via Gadd45a in intestinal dendritic cells upon exposure to *B. fragilis* outer membrane vesicles containing PSA (57). Although the specific DC population that responds to PSA stimulation in the colon has not been identified yet, a recent study suggests that plasmacytoid dendritic cells might have a role since transfer of $TLR2^{-/-}$ bone marrow-derived pDCs rendered mice susceptible to colitis (58).

Intestinal epithelial cells express antimicrobial molecules, including the bactericidal C-type lectin RegIIIγ, in response to gut colonization by commensal bacteria (59). Microbe-derived molecules drive RegIIIγ expression by stimulating TLRs (60,61). RegIIIγ expression reduces bacterial colonization of the dense mucus layer associated with intestinal epithelial cells, thereby reducing microbial contact with the epithelium (62,63). RegIIIγ binds to peptidoglycan by its lectin domain, and more recent studies of closely related RegIIIα demonstrated that bactericidal activity results from association with bacterial phospholipids and the formation of a hexameric structure that produces a pore in the bacterial membrane (64). In contrast to RegIIIα, which is inhibited by Gram-negative lipopolysaccharide (LPS), RegIIIβ has bactericidal activity against Gram-negative bacteria by lectin-mediated

association with LPS and, at least in part, pore formation in the Gram-negative outer membrane (65,66).

Which TLRs contribute to immune defense versus tolerance induction in the gut remains incompletely resolved. TLR5 has been investigated most extensively. While early studies suggested that TLR5 is expressed on the basal surface of intestinal epithelial cells, subsequent studies demonstrated that TLR5 is predominantly expressed on lamina propria DCs, and that TLR5-expression on lamina propria DCs facilitates the development the Th17 T cells and IgA-producing cells (67,68). Systemic administration of bacterial flagellin, the microbial ligand for TLR5, induces the expression of RegIIIγ by intestinal epithelial cells along the entire length of the small intestine, and provides significant protection against intestinal colonization by Vancomycin-resistant Enterococcus (VRE) (69) and infection by *Clostridium difficile* (70). Systemic flagellin rapidly induces expression of IL23 by $CD103^{+}CD11b^{+}$ lamina propria DCs, as noted above, which in turn stimulates IL22 production by ILC3 cells, which then leads to RegIIIγ expression by intestinal epithelial cells (71) (**Figure 1.1**).

Although TLR5 stimulation can promote innate immune defenses against intestinal infection, TLR5 signaling, under homeostatic conditions, reduces gut inflammation and metabolic syndrome (72). In some mouse colonies, TLR5-deficiency is associated with development of spontaneous colitis and massive weight gain with parallel development of metabolic syndrome. This association has been attributed to alteration of the intestinal microbiota, including expansion of proteobacterial populations, resulting from deficient TLR5 signaling (73). In the absence of TLR5, flagellin specific IgA levels are reduced which appears to increase the expression of flagellin and

invasion of flagellated bacteria into sub-epithelial tissues (74). While some studies have demonstrated that TLR5-deficiency results in the development of obesity and metabolic syndrome (72), this finding is not seen in all TLR5-deficient mouse colonies (75).

Flagellin and TLR5, while contributing to intestinal defense against microbial pathogens, only represents a part of the entire story. Oral administration of DNA isolated from the gut flora, for example, corrects innate immune antimicrobial responses in antibiotic treated mice (76) and oral administration of LPS corrects antibiotic-induced innate immune deficiency in the gut and enhances resistance against VRE (77). The extent to which TLR-mediated signals influence the composition of the intestinal microbiota is controversial. Comparison of wild type C57BL/6 mice with littermate control TLR2, TLR4, TLR5, TLR9 and MyD88 deficient mice did not detect differences in their luminal or mucosa-associated ileal, cecal or fecal microbiota under homeostatic conditions and following recovery from antibiotic treatment (78). This study demonstrated that microbiota composition was largely determined by familial transmission of the microbiota and that the composition of bacterial populations inhabiting the gut can drift over time as distinct breeding colonies are kept in isolation from each other.

Defects in innate immune signaling have also been implicated in microbiota-mediated development of inflammatory bowel disease, obesity and obesity-associated steato-hepatitis (79). Deficiency of the NLRP6 inflammasome subunit in mice is associated with a shift in colonic microbiota composition that leads to inflammatory bowel disease and steato-hepatitis (42,80). Transfer of the aberrant microbiota from NLRP6-knockout mice to wild type mice resulted in the development of these inflammatory diseases,

suggesting that loss of NLRP6 alters the microbiota into one that is pro-inflammatory or, in other words, that NLRP6 signaling in intestinal epithelial cells controls the composition of the microbiota and steers it into an anti-inflammatory state. In part, this process is mediated by IL18-mediated signaling (42). Another important mechanism by which NLRP6 modulates microbiota composition is by regulating intestinal goblet cell secretion of mucin (**Figure 1.1**). NLRP6 facilitates goblet cell autophagy and also exocytosis of mucin granules, and in its absence mucin production is markedly reduced (81). Reduced mucin production renders mice more susceptible to infection by intestinal pathogens, as noted above.

While inflammasome signaling in intestinal epithelial cells contributes to antimicrobial defense, inflammasome signaling following systemic infection can have adverse effects. For example, mortality following systemic spread of a pathogenic strain of *Escherichia coli* was mediated in part by the Naip5/Nlrc4 inflammasome (82). In contrast, systemic absorption of microbial molecules from the gut, such as peptidoglycan fragments, can trigger Nod2 signaling and activate circulating neutrophils, thereby enhancing resistance against infection by *Streptococcus pneumoniae* and *Staphylococcus aureus* (83). Nod2 signaling in the gut also acts locally, by promoting inflammatory monocyte recruitment into the gut during *Citrobacter rodentium* infection (84). Studies with germ-free mice demonstrated a systemic effect of microbial colonization on mononuclear phagocytes. Specifically, mononuclear phagocytes from germ-free mice were hypo-responsive to microbial stimulation, a result of aberrant chromatin modification of innate immune response genes (85). These studies are revealing the complexity of innate immune recognition and responses in mucosal tissues, and it is likely that many more examples of pro-

inflammatory antimicrobial responses and contrasting anti-inflammatory or tolerizing responses will be discovered in the coming years.

1.2.4. Innate lymphocytes and the intestinal microbiota

Innate lymphocytes derive from common lymphoid progenitors, depend on common γ-chain signaling, the ID2 transcriptional regulator and differentiate under the control of specific transcriptions factors that are shared with T lymphocytes into an array of phenotypes that are categorized as ILC1 (T-bet dependent and IFN-γ producing), ILC2 (GATA-3 dependent and IL4, IL13 producing) and ILC3 (RORγt dependent and IL17 and IL22 producing). ILCs represent an important arm of the innate immune defense system, at the level of orchestrating immune tissue development and, more directly, antimicrobial defenses (86).

A subset of ILC3 cells contributes to innate immune tissue development in the gut by producing lymphotoxin a and lymphotoxin b. The ILC3 cells involved in this process are referred to as Lymphoid Tissue inducer (LTi) cells and their existence depends on the RORγt transcription factor. Soluble lymphotoxin a facilitates the production of T cell dependent IgA in the gut, while lymphotoxin b on the surface of ILC3s promotes T cell independent IgA production (87). In utero development of LTi cells depends on maternal retinoid intake, and retinoic acid in the developing fetus is required for RORγt induction and LTi development (88).

ILCs play essential roles in defense against intestinal bacteria and also non-pathogenic bacteria that cross the intestinal epithelial barrier and reside in gut-associated lymphoid tissues. Upon deletion of IL22-producing ILC3s, lymphoid tissue-residing bacteria such as *Alcaligenes* can disseminate and

drive systemic flares of inflammation (89). Because ILCs and differentiated T lymphocytes can express overlapping phenotypes, and because depletion of ILCs often also results in depletion of T lymphocytes, it has been challenging to distinguish the roles of these cell populations in the induction of inflammation and during defense against infection. Using ILC-specific depletion strategies, it was found that the absence of ILC3s dysregulated T cell responses to the commensal flora. Furthermore, this process required MHC class II expression by ILC3s (90). While this finding suggests that responding T lymphocytes may interact directly with MHC class II-expressing ILC3s, the role of ILC3-mediated antigen-presentation remains unclear. IL22 expression by ILC3s is promoted by the aryl hydrocarbon receptor (Ahr), and mice lacking Ahr have markedly reduced numbers of IL22-producing ILC3s. Surprisingly, Ahr-deficiency results in increased numbers of Th17 cells, potentially the result of increased intestinal colonization by Segmented Filamentous Bacterium (SFB) (91) and also increases the risk of spontaneous colitis. ILC3s were recently also shown to be a major source of GM-CSF following stimulation by IL1β, which is produced by mononuclear phagocytes upon stimulation by microbial molecules. In turn, GM-CSF acts upon mononuclear phagocytes to enhance their effector functions (92). The interactions between myeloid cells, ILCs, T lymphocytes, B lymphocytes are complex and our knowledge of these relationships is far from complete. Indeed, novel cell populations, such as $CD71^{+}$ erythroid cells, which were recently demonstrated to attenuate inflammatory responses against newly colonizing intestinal bacteria in the neonate by producing arginase-2, are being discovered and assigned functional roles in the relationship between the host immune system and colonizing microbes (93).

1.3. ADAPTIVE IMMUNE DEFENSES AND THE INTESTINAL MICROBIOTA

Antibody and T lymphocyte mediated recognition of intestinal bacteria contributes to immune defense and to the pathogenesis of intestinal and systemic inflammatory diseases (94). In parallel with advances in our understanding of the complex microbial populations inhabiting the gut and the more detailed characterization of individual members of the microbiota, recent studies have begun to define the specificity of antibody and T cell responses to intestinal microbes and the mechanisms driving microbe-mediated differentiation of immune cells.

1.3.1. Secretory IgA

While the intestinal microbiota influences systemic antibody responses, as demonstrated by increased serum IgE levels in germ-free mice (95), induction of secreted IgA in response to intestinal bacteria is particularly important for antimicrobial defense and intestinal microbiota homeostasis. Secretory IgA provides defense against intestinal pathogens and its absence has been implicated in the expansion of some commensal bacterial populations such as SFB (96) and in the maturation of the intestinal microbiota during neonatal life (97). Colonization of the murine small intestine with SFB and its ability to induce IgA was established by studies over 15 years ago (98), and recent studies demonstrate that SFB induces Peyer's patch development and IgA secretion (99) (**Figure 1.1**).

The PD-1 inhibitory receptor regulates the development of T follicular helper cells in Peyer's patches and, in its absence, promotes precursor cells that produce IgA with lower affinity for intestinal bacteria, ultimately affecting

microbiota composition (100). Kinetic analyses of intestinal IgA responses against commensal bacteria suggest that specific antibodies can be secreted for prolonged periods but that they are also dynamic and that new IgA antibodies are generated following shifts in microbiota composition (101). Deep sequencing of the IgA antibody encoding genes of mice revealed a very broad repertoire, with many distinct specificities and with increasing diversity with aging (102). While secreted IgA, by virtue of its specificity, influences microbiota composition, some IgA-producing plasma cells also produce TNF and iNOS and depletion of these cells alter microbiota composition and decrease resistance against infection (103). Thus, IgA producing plasma cells may augment defensive strategies that have largely been attributed to myeloid-derived cell populations.

1.3.2. Th17 cells

SFB also contributes to the differentiation of Th17 cells in the murine small intestinal lamina propria (25,104). SFB appears to have a unique ability to penetrate the small intestinal mucus layer and directly contact the surface of intestinal epithelial cells, a characteristic that likely contributes to its ability to drive the differentiation of Th17 T cells (**Figure 1.1**). Whole genome sequencing of SFB revealed a reduced genome size compared to other Clostridiales and its dependence on exogenous amino acids, suggesting that this organism is highly adapted to and dependent upon its mammalian host (105). Two recent studies have investigated the antigen specificity of Th17 cells induced by SFB mono-colonization and discovered that the majority of T cells belonging to this subset are specific for SFB. Thus, without apparent invasion of the lamina propria, SFB induces a broad Th17 response directed

against several SFB encoded antigens (106) that depends on MHC class II presentation by lamina propria dendritic cells and is independent of secondary lymphoid tissues (107). While T cell responses to commensal bacteria can be induced by intestinal infections that destroy the epithelial cell layer and induce potent inflammatory responses (108), the response to SFB is distinct and occurs in the presence of an intact epithelial barrier and in the absence of apparent inflammatory cell infiltration of the lamina propria.

Although not specific to SFB, induction of IL1β in intestinal macrophages (a.k.a. mononuclear phagocytes) by gut microbiota has been implicated in the induction of Th17 T cells by binding to T cell expressed IL1 receptor (109). Recent studies have demonstrated that light-cycle disruption in mouse colonies can promote the differentiation of Th17 cells (110). This study demonstrated that the REV-ERBa transcription factor, which is implicated in regulation of the circadian clock, enhances NFIL3 activity, which in turn interferes with RORγt binding to its promoter. Thus, interference with REV-ERBa expression reduces NFIL3 activity and enhances Th17 development. This finding suggests that sleep abnormalities or frequent challenges to the baseline circadian rhythm of mammals may incrementally increase Th17 development and, potentially increase the risk of inflammatory diseases.

1.3.3. Regulatory T cells

The intestinal lumen contains a wide range of microbial inhabitants and ingested antigens that are potential inducers and targets of T cell responses. Regulatory T cells limit naïve T cell differentiation into effector cells that can target many of the innocuous antigens that are present in the intestinal lumen. In their absence, inflammatory diseases involving the gut occur rapidly and

can have lethal consequences. Recent studies have identified bacterial species that drive intestinal Treg development. Colonization of mice with altered Schaedler flora, which consists of 8 distinct bacterial species, induced colonic Treg cells in germ free mice (111) and prevented the development of colitis. Characterization of colonic Treg cells induced by commensal bacteria revealed that their TCR repertoire was distinct from Tregs in other sites, suggesting that colonic Tregs are derived from circulating naïve CD4 T cells (112). Remarkably, induction of colonic Treg cells can be driven by colonization with a single bacterial strain of *Bacteroides fragilis* that expresses polysaccharide A (113). The ability of bacteria belonging to the Bacteroidetes phylum to drive Treg cell differentiation was also demonstrated by colonizing germ-free mice with different mixtures of human fecal bacterial cultures that included *Bacteroides caccae, B. thetaiotaomicron, B. vulgatus, B. massiliensis* and *Parabacteroides distasonis* (114). Other studies have demonstrated that murine and human derived fecal microbes that belong to *Clostridium* clusters IV and XIVa drive Treg cell development in the murine colon (22,23) in part by inducing TGFβ production by intestinal epithelial cells (**Figure 1.1**). Optimal Treg development in mice can be induced by colonization with 17 human-derived strains belonging to these Clostridia groups (23,115). Trafficking of Treg cells to colonic lamina propria is mediated by the GPR15 heterotrimeric guanine nucleotide-binding protein coupled receptor, the expression of which is modulated by the gut microbiota (116). One mechanism by which Tregs are induced involves the intestinal microbiota up-regulation of Uhrf1 expression, which DNA methylates the cyclin-dependent kinase inhibitor p21 promoter, thereby inhibiting its transcription and promoting Treg proliferation (117).

1.4. DIET, VITAMINS, METABOLISM, IMMUNITY AND THE INTESTINAL METABOLOME

Food digestion, nutrient and vitamin absorption, immune defense and bacterial and host metabolism are interrelated and interdependent processes occurring in the gut. The implications of these interactions for the mammalian host are profound. The mechanisms by which commensal bacteria contribute to host metabolism are increasingly being defined (118). Given the complexity of intestinal microbial populations, one approach to determine the impact of specific bacterial species on host metabolism has been to colonize germ free mice with individual bacterial strains and measure the impact on host gene expression (119). This study demonstrated that two bacterial species, *Bifidobacterium longum* and *Bacteroides thetaiotaomicron* have distinct effects on gene expression by intestinal epithelial cells, but also influence each other's gene expression patterns. Ingestion of different dietary carbohydrates, such as fructose-based polysaccharides, can drive the selective expansion of bacterial species that encode the appropriate hydrolytic enzymes and can thereby alter microbiota composition (120). The impact of altered microbiota composition can be profound, in some cases leading to obesity that can be reversed by transfer and expansion of specific members of the Bacteroidetes phylum (121). Changes in diet can rapidly alter the composition of the human fecal microbiota (122), with animal-based diets reducing the density of bacteria belonging to the Firmicutes phylum and increasing organisms associated with bile tolerance. Pregnancy has also been associated with changes in the intestinal microbiota towards a composition that, upon transfer into germ-free mice, increases adiposity and reduces insulin sensitivity (123).

Vitamin A is an essential dietary component and is metabolized into retinoic acid, which plays an important role in T cell differentiation, including development of Treg cells (124-126). Ingested vitamins, such as vitamin B12, are also of benefit to the microbiota and recent studies revealed that the intestinal symbiont *B. thetaiotaomicron* expresses three non-redundant vitamin B12 receptors that can each confer competitive advantages to the bacterial strain, depending on the presence of distinct vitamin B12 analogs (127). Intestinal bacteria can also produce vitamins, such as vitamin B3 (a.k.a. niacin) and signal via G-protein-coupled receptor (GPR) 109A and reduce intestinal inflammation (128). Recent studies have demonstrated that a dietary switch from carbohydrates to tryptophan leads to production of tryptophan metabolites by intestinal microbes that enhance signaling via the aryl hydrocarbon receptor, in turn leading to greater IL22 production (129). The impact of microbiota-mediated metabolism of ingested nutrients on host health can also be negative, as demonstrated by studies of L-carnitine, a trimethylamine that is acquired by meat ingestion. Conversion of L-carnitine to trimethylamine-N-oxide by microbiota members that are expanded in meat-eating individuals led to increased progression of atherosclerosis in mice (130).

It is clear that intestinal microbes can dramatically alter the absorption and metabolism of orally administered medications. Recent studies have demonstrated, for example, that some strains of *Eggerthela lenta*, a member of the human microbiota, can inactivate the cardiac drug digoxin, and that changes in diet can alter in vivo metabolism of the drug, leading to different drug levels (131). For a drug like digoxin, which has a relatively narrow

therapeutic window of activity and potentially lethal toxicities, changes in its metabolism by intestinal microbes can have serious clinical implications.

While most studies of the microbiota have focused on microbiota composition, determined either by deep bacterial 16S rRNA gene sequencing or shotgun sequencing of fragmented bacterial DNA, recent studies have started to investigate the bacterial transcriptome and metabolome. Analysis of the bacterial transcriptome in the gut demonstrated that many genes and their respective transcripts are similarly abundant, while genes associated with sporulation and amino acid synthesis are often down-regulated and genes involved in ribosome biogenesis and methanogenesis are up-regulated (132). Administration of xenobiotics to mice can dramatically alter gene expression by intestinal microbes, with transcript fluctuations greatly exceeding the quantitative changes in the representation of different bacterial taxa (133).

Beyond determination of the microbiota transcriptome, mass spectrometric analysis of microbially produced or modified metabolic products promises to provide new insights into the role of intestinal microbes in health and disease (134). The impact of the microbiota on a wide range of metabolites was revealed by treatment of mice with antibiotics and demonstrated that over 87% of metabolites, including bile salts, eicosanoids and steroid hormones, were affected by antibiotic-induced shifts in the microbiota (135). The metabolomic changes induced by antibiotic administration have been implicated in the development of adiposity and have been suggested to contribute to the ongoing obesity epidemic (136). Administration of fecal microbiota from different humans to germ-free mice demonstrated conserved metabolite differences that could be detected by mass spectrometry-based metabolomic assays of urine samples (137).

1.4.1. Short-chain fatty acids and bile salts

Among the metabolites that are being characterized in the intestinal lumen, short-chain fatty acids (SCFAs) and bile salts stand out as having clearly demonstrable roles in immune development and defense against infection. SCFAa include acetate, butyrate and propionate and are produced by fermentation of fiber and other polysaccharides by a range of intestinal bacteria (138). Bacteria belonging to the *Bifidobacterium* genus can produce acetate, which reduces the susceptibility of the colonic epithelium to damage caused by the *Escherichia coli* O157:H7 Shiga toxin (20). In this setting, a *Bifidobacterium*-expressed transporter pumps acetate into the intestinal lumen (139). SCFAs produced by intestinal microbes stimulate the GPR43 expressed by host inflammatory cells and reduce inflammation, and mice with a deletion of GPR43 have enhanced colitis and arthritis upon induction of inflammation (140). SCFAs, and butyrate in particular, have recently been demonstrated to regulate extrathymic Treg cell development in a GPR43 dependent manner (141-143). Butyrate and propionate administration enhances histone acetylation in the promoter of the Foxp3 locus, potentially by inhibiting histone deacetylase function.

Bile salts released into the intestinal lumen can impact the composition of the microbiota, and commensal microbes can alter the composition of intestinal bile salts. Ingestion of milk fats, for example, increases production of taurocholate by the liver, which, in turn, leads to a bloom of the bacterial species *Bilophila wadsworthia* (144). *B. wadsworthia* can enhance the development of colitis in IL10 deficient mice, demonstrating the indirect mechanism by which dietary changes can alter the risk of inflammatory diseases. Antibiotic treatment, on the other hand, can alter the gut microbiota

and eliminate microbes that deconjugate primary bile salts and also dehydroxylate primary bile salts to produce secondary bile salts. The conversion of primary to secondary bile salts has been shown to be an important mechanism of colonization resistance against *C. difficile* (145) and may potentially be implicated in clearance of other intestinal pathogens.

1.5. MICROBIOTA-MEDIATED RESISTANCE TO INFECTION

The ability of the normal microbiota to confer resistance to infection became apparent in the decades following the introduction of broad-spectrum antibiotics with the discovery that antibiotic-treatment could render mice much more susceptible to a range of intestinal infections (146-149). In the last decade, significant progress has been made in our understanding of the mechanism by which the microbiota provides colonization resistance (150-152). These studies are revealing that colonization resistance can be mediated by direct interactions between different bacterial species or by indirect mechanisms that either involve stimulation of the host's innate immune system, which induces expression of antimicrobial factors, or modification of host metabolic products into molecules that inhibit pathogenic organisms.

Antibiotics can induce dramatic and long-lasting changes to the microbiota, with expansion of some species and loss of others (14,153,154). Antibiotic treatment enhances susceptibility to infection by *S. typhimurium* and *C. rodentium*, and resistance to these infections has been associated with the presence of obligate anaerobes belonging to the Bacteroidetes phylum (155-157). The emergence of highly antibiotic-resistant bacterial pathogens represents a growing clinical problem. Many of the most antibiotic-resistant pathogens densely colonize the intestinal tract of patients following treatment

with antibiotics (14,158). Vancomycin-resistant Enterococcus (VRE) is one such intestinal colonizer and can achieve very high densities in the small intestine and colon following antibiotic-mediated depletion of the microbiota.

Although innate immune activation via TLR4 or TLR5 stimulation can enhance resistance to VRE colonization by stimulating the production of RegIIIγ expression in the small intestine (69,77), it is not sufficient to provide complete protection against colonization. Administration of a diverse and antibiotic-naïve microbiota to VRE dominated mice eliminates VRE from the gut, demonstrating that colonization resistance can be re-established and bacteria can be cleared with normal flora administration. Obligate anaerobes are essential for clearance, as demonstrated by fractionation of the microbiota by anaerobic or aerobic culture and treatment with chloroform to eliminate non-spore forming organisms. In addition, transplantation of a microbiota containing bacteria from the *Barnesiella* genus correlated strongly with resistance to VRE colonization and with VRE clearance from the gut (159).

Fecal transplantation is one method to re-establish a complex microbiota and has been used in patients with recurrent *C. difficile* infection (160). This procedure, which involves obtaining fecal samples from healthy donors and administering them to individuals with recurrent *C. difficile* infection, is remarkably effective and, in the recipient, re-establishes a normal microbiota that confers resistance to infection. An important and obvious concern with fecal transplantation is the potential risk of transmitting unknown or uncharacterized infections that the donor may be resistant against but the recipient may be susceptible to (161). This concern is difficult to eliminate since the complete composition of a fecal sample is difficult to determine. To avoid this, defined mixtures of specific bacterial populations must be

developed for administration to individuals with specific intestinal infections. In the case of *C. difficile*, *Clostridium scindens*, a bacterium belonging to the Clostridia cluster XIVa, was recently found to increase resistance to infection when adoptively transferred into antibiotic-treated mice (145).

1.6. SUMMARY AND CHAPTER OUTLINE

Some of the many functions the intestinal microbiota exerts on the host include the acquisition of nutrients from diet, the development of the immune system and colonization resistance. Antibioitc treatment destroys the intestinal microbial ecology and increases the risk for pathogen colonization. The focus of this dissertation is to 1) determine the impact the opportunistic pathogens vancomycin-resistant Enterococcus (VRE) and carbapenem-resistant *Klebsiella pneumoniae* have on each other and on the host and 2) identify intestinal bacteria responsible for colonization resistance against VRE. In Chapter 2 we examine whether colonization resistance exists between VRE and *K. pneumoniae*, whether they affect the host and where they localize within the intestine. In Chapter 3 we characterize a healthy murine microbiota that is ampicillin-resistant and can prevent the deleterious effects of ampicillin upon transplant. In Chapter 4 we used this ampicillin-resistant microbiota as a tool to identify bacterial groups that are associated with resistance to VRE and demonstrated that colonization resistance can be restored with only two bacterial species, a discovery that has important implications for the development of novel probiotic treatments against VRE.

Notes

Adapted from Microbiota-mediated inflammation and antimicrobial defense in the intestine. Caballero S and Pamer EG. Annu Rev Immunol. 2015.

CHAPTER 2

DISTINCT BUT SPATIALLY OVERLAPPING INTESTINAL NICHES FOR VANCOMYCIN-RESISTANT ENTEROCOCCUS FAECIUM AND CARBAPENEM-RESISTANT KLEBSIELLA PNEUMONIAE *

2.1. INTRODUCTION

Antibiotic-resistant bacteria such as vancomycin-resistant *Enterococcus faecium* (VRE) and multi-drug resistant *Klebsiella pneumoniae* represent a growing concern in hospitals worldwide. In the United States, *Enterococcus* spp. and *K. pneumoniae* account for nearly 10% of all hospital-acquired infections and are common causes of bacteremia (162). Vancomycin resistance among enterococci has markedly increased since it was first described in the mid-1980s (163). Even more alarming, however, is the increasing prevalence of carbapenem-resistant Enterobacteriaceae, primarily *K. pneumoniae*, rendering treatment of these infections very challenging (164). Broad-spectrum antibiotic exposure, immune suppression and intravascular devices increase the risk for colonization and infection with one or more antibiotic-resistant bacteria (165). In hospitalized patients, the intestine can become densely colonized with drug-resistant organisms. While colonization by itself does not directly cause disease, in the event of injury to the mucosal barrier colonizing bacteria may translocate beyond the intestinal tract, leading to deep tissue and bloodstream infections (166).

* S. Caballero, R. Carter, X. Ke, B. Sušac, I. M. Leiner, G. J. Kim, L. Miller, L. Ling, K. Manova and E. G. Pamer. PLoS Pathog. 2015.

Consistent with this, studies have shown that intestinal colonization precedes bloodstream infection with VRE or *K. pneumoniae*, suggesting that loss of colonization resistance represents an early step in the progression of these infections (14,158,167).

Colonization resistance refers to the ability of the microbiota to prevent expansion and persistence of exogenously acquired bacterial species, a pivotal defense mechanism that can be impaired by antibiotic treatment (152,168). Changes in microbiota composition, mainly the elimination of specific groups of anaerobic bacteria, lead to VRE domination of the gastrointestinal tract in antibiotic-treated mice (14,159). Similarly, colonization of mice with *K. pneumoniae* is facilitated by antibiotic administration (169,170). Antibiotic-mediated depletion of commensals also decreases production of mucus and antimicrobial effector molecules, potentially increasing the risk for bacterial invasion of the intestinal epithelium (62,69,77,157,171).

The interactions between different bacterial species in the gut are complex. Studies of mice singly colonized with *Bacteroides thetaiotaomicron*, *Bifidobacterium longum* or co-colonized with both demonstrated that these distinct bacterial species impact each other's transcriptional profile (119). In some circumstances, bacterial species work together in assembly-line fashion to dismantle complex carbohydrates, where the product of one species becomes the substrate for another (172). In other circumstances, however, microbial species within the intestine compete for limited nutrients and space in order to persist. For example, similarities in carbon utilization by *Escherichia coli* and *Citrobacter rodentium* lead to competition between these species. Niche specialization also results in competition between the same, but not different, strains of *Bacteroides* spp. (17,173). Metabolic overlap and niche

restriction, however, can be shared by distinct bacterial species as is the case with *Salmonella typhimurium* and *Clostridium difficile* which benefit from the transient abundance of the same sugars following antibiotic treatment (19). Yet, the extent to which metabolic competition between antibiotic-resistant microbes contributes to their prevalence and persistence is unknown.

We sought to investigate the interactions between VRE, *K. pneumoniae* and the host in a murine model of intestinal colonization. Our goal was to determine whether intestinal domination by either VRE or *K. pneumoniae* would provide colonization resistance against the other. We found that VRE and *K. pneumoniae* were able to co-exist despite occupying the same intestinal sites, while restoration of a normal microbiota by means of a fecal transplant displaced both organisms effectively but at different rates. Antibiotic treatment of mice reduced the thickness of the colonic mucin layer, a defect that was corrected by *K. pneumoniae* but not VRE colonization. However, *K. pneumoniae* was more effective than VRE at invading the mucus layer and translocating to mesenteric lymph nodes. Our findings demonstrate that highly antibiotic-resistant bacteria such as VRE and *K. pneumoniae* non-competitively co-occupy the colon but establish distinct relationships with the mucosal epithelium.

2.2. RESULTS

2.2.1. VRE and *K. pneumoniae* coexist in the gastrointestinal tract

We previously showed that treatment with ampicillin, a broad-spectrum antibiotic, renders C57BL/6 mice from Jackson Laboratories highly susceptible to VRE colonization (14). Inoculation of ampicillin-treated mice with *K. pneumoniae* also resulted in dense colonization of the colon, with approximately 10^{10} colony-forming units (CFU) per gram of feces (**Supplementary Figure 2.1B**). To assess the impact of VRE and *K. pneumoniae* on each other's ability to colonize the gut, we performed a series of experiments in which ampicillin-treated mice were pre-colonized with VRE or *K. pneumoniae* by gastric lavage and challenged with the other species 3 days later, at which point the initial colonizer has become established in the intestine and reached maximum density (**Supplementary Figure 2.1B**). Fecal levels of *K. pneumoniae* and VRE were determined by culture beginning one day and continuing through 21 days post infection (p.i.) with the challenge species (**Figure 2.1A**). We found that dense colonization with VRE did not significantly impact *K. pneumoniae* colonization levels at any time point over the course of the experiment. However, fewer *K. pneumoniae* were recovered from the feces of co-colonized mice on day 1 p.i., suggesting a lag in establishment of *K. pneumoniae* in the presence of VRE shortly after infection (**Figure 2.1B**). On day 21, mono- and co-colonized animals had comparable *K. pneumoniae* burden in the proximal and distal small intestine (duodenum and ileum, respectively) as well as in the large intestine (cecum) (**Figure 2.1D**). Under this experimental condition, introduction of *K. pneumoniae* did not reduce VRE density in the intestine. Rather, VRE CFU levels were, for the

most part, similar in mono- and co-colonized mice although a trend toward increased VRE burden was observed on days 1, 14 and 21 post challenge (**Figures 2.1C** and **2.1E**).

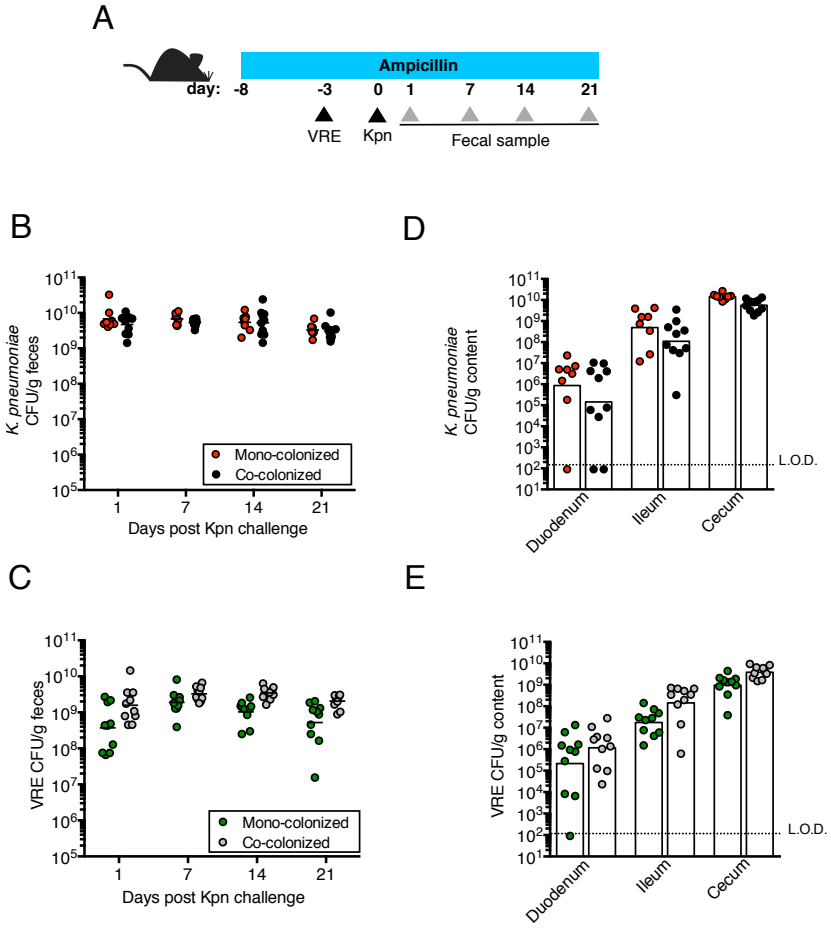


Figure 2.1. Pre-colonization with VRE does not prevent colonization by *K. pneumoniae*. (A) Experimental design. Mice were treated with ampicillin for 29 days. On day 5 of ampicillin treatment, mice were inoculated with $5x10^4$ colony-forming units (CFU) of VRE by oral gavage or left uninfected. Three days later, half of the VRE-infected mice and the uninfected group were challenged with $5x10^4$ CFU of *K. pneumoniae* (Kpn). (B, C) CFU of *K. pneumoniae* (B) and VRE (C) were quantified in fecal pellets collected at different time points post *K. pneumoniae* inoculation. (D, E) Mice were sacrificed 21 days post *K. pneumoniae* challenge. *K. pneumoniae* (D) and VRE (E) burden was quantified in the luminal contents from the duodenum, ileum and cecum. L.O.D., limit of detection. Data were pooled from two independent experiments (n=10 per group). (B-E) Data were analyzed by the Mann-Whitney test.

In the converse experiment, challenge of *K. pneumoniae*-dominated mice with VRE resulted in a transient but significant increase in VRE density 1 day p.i. but then equivalent density in all intestinal compartments when compared to antibiotic-treated mice lacking *K. pneumoniae* (**Figures 2.2A-B** and **2.2D**). In addition, we observed no difference in *K. pneumoniae* colonization between mice challenged with VRE and mice mono-colonized with *K. pneumoniae* (**Figures 2.2C** and **2.2E**). Overall, our results demonstrate that VRE and *K. pneumoniae* neither compete nor synergize with each other upon dense colonization of the murine gut.

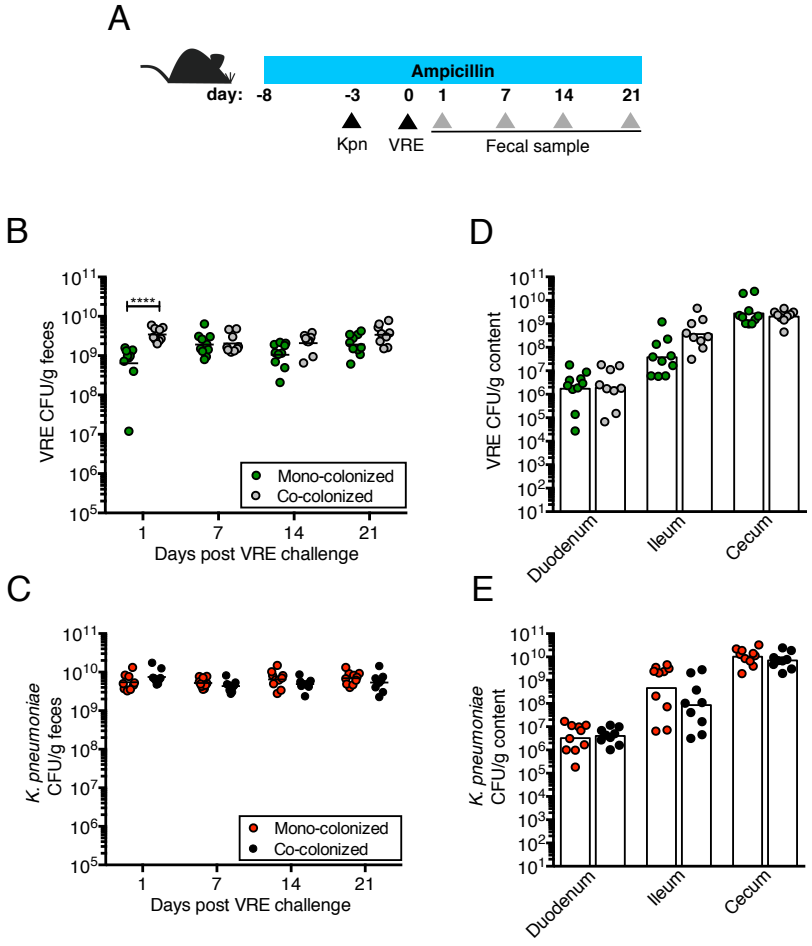


Figure 2.2. Pre-colonization with *K. pneumoniae* does not prevent colonization by VRE. (A) Experimental design. Mice were treated with ampicillin for 29 days. On day 5 of ampicillin treatment, mice were inoculated with $5x10^4$ colony-forming units (CFU) of *K. pneumoniae* (Kpn) by oral gavage o left uninfected. Three days later, half of the *K. pneumoniae*-infected mice and the uninfected group were challenged with $5x10^4$ CFU of VRE. (B, C) CFU of VRE (B) and *K. pneumoniae* (C) were quantified in fecal pellets collected at different time points post VRE inoculation. (D, E) Mice were sacrificed 21 days post VRE challenge. VRE (D) and *K. pneumoniae* (E) burden was quantified in the luminal contents from the duodenum, ileum and cecum. L.O.D., limit of detection. Data were pooled from two independent experiments (n=10 per group). (B-E) ****P<0.0001, by the the Mann-Whitney test.

2.2.2. VRE and *K. pneumoniae* achieve similar densities in the large intestine of co-colonized mice

Because VRE becomes the dominant member of the intestinal microbiota within days after administration to antibiotic-treated mice (14), we examined how colonization by *K. pneumoniae* and subsequent VRE challenge would impact their relative proportions at different time points p.i. On day 1 post challenge, VRE represented 30% of the total fecal bacteria in mono-colonized mice, with the remaining 70% constituting bacteria that remained and expanded following antibiotic treatment. In mice previously colonized with *K. pneumoniae*, VRE represented 10% of the microbiota and *K. pneumoniae* dominated. However, VRE rapidly expanded in the colon of co-colonized animals and within 4 days of inoculation, VRE and *K. pneumoniae* achieved roughly equal densities and remained stable for up to 21 days (**Figure 2.3A**). This ratio was specific to the large intestine since both bacterial species were equally abundant in the cecum and colon but VRE dominated over *K. pneumoniae* in the ileum (**Figure 2.3B**).

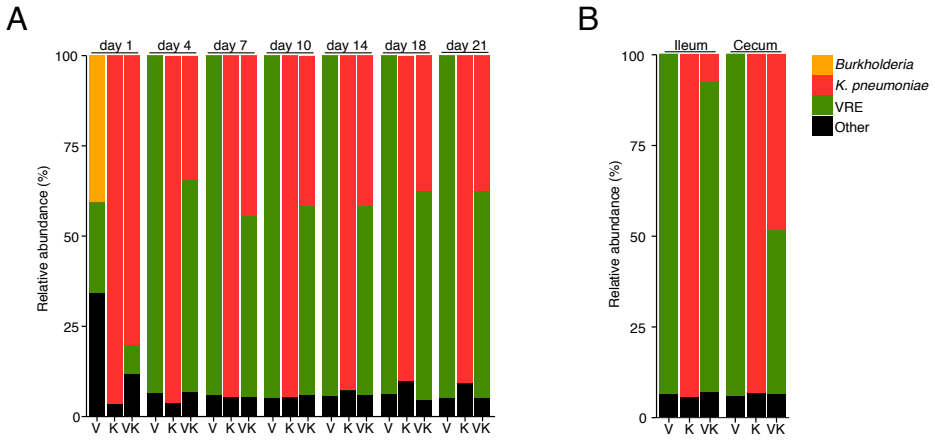


Figure 2.3. *K. pneumoniae* and VRE achieve similar densities in the large intestine of co-colonized mice.
Ampicillin-treated mice were inoculated with *K. pneumoniae* by oral gavage or left uninfected. Three days later, half of the *K. pneumoniae*-infected mice and the uninfected group were challenged with VRE. Microbiota composition of mice colonized with VRE alone (V), *K. pneumoniae* alone (K) or both (VK) was determined by sequencing of the V4-V5 region of the 16S rRNA genes. (A) Fecal microbiota composition at different time points post VRE challenge. (B) Ileal and cecal microbiota composition at day 21 of colonization. (A,B) Each stacked bar represents the average of five individually-housed mice per time point.

2.2.3. Fecal bacteriotherapy eliminates established VRE and *K. pneumoniae* intestinal domination

Transplantation of feces from donor mice that have not been treated with antibiotics can eliminate VRE from the intestine of densely colonized mice and, in humans, fecal transplantation from healthy donors cures patients with recurrent *Clostridium difficile* infection (159,174). To determine whether the kinetics of VRE and *K. pneumoniae* clearance from the murine intestine following fecal transplantation are similar or distinct, we colonized ampicillin-treated mice with VRE and *K. pneumoniae* concurrently, terminated ampicillin treatment and treated mice with fecal microbiota transplants (FMT) or PBS on three consecutive days (**Figure 2.4A**). VRE and *K. pneumoniae* colonization levels were similar in the feces before FMT administration and remained

elevated in mice that received PBS instead of FMT (**Figure 2.4B**). However, following FMT treatment, *K. pneumoniae* density in fecal pellets decreased within one day and became undetectable within 7 days in all mice (**Figure 2.4C**). VRE, on the other hand, was cleared in 60% of the mice but reduced by 3 logs in the remaining 40%. Increased colonization resistance against *K. pneumoniae* as opposed to VRE was also observed in mice that had not been treated with antibiotics (**Supplementary Figure S2.1A**). *K. pneumoniae* was also cleared more effectively than VRE from the duodenum, ileum and cecum of FMT-treated animals while the density of these bacterial species remained high in PBS-treated mice (**Figures 2.4D** and **2.4E**). These findings suggest that the mechanisms of microbiota-mediated colonization resistance against VRE and *K. pneumoniae* are distinct or that *K. pneumoniae* is more susceptible to colonization resistance.

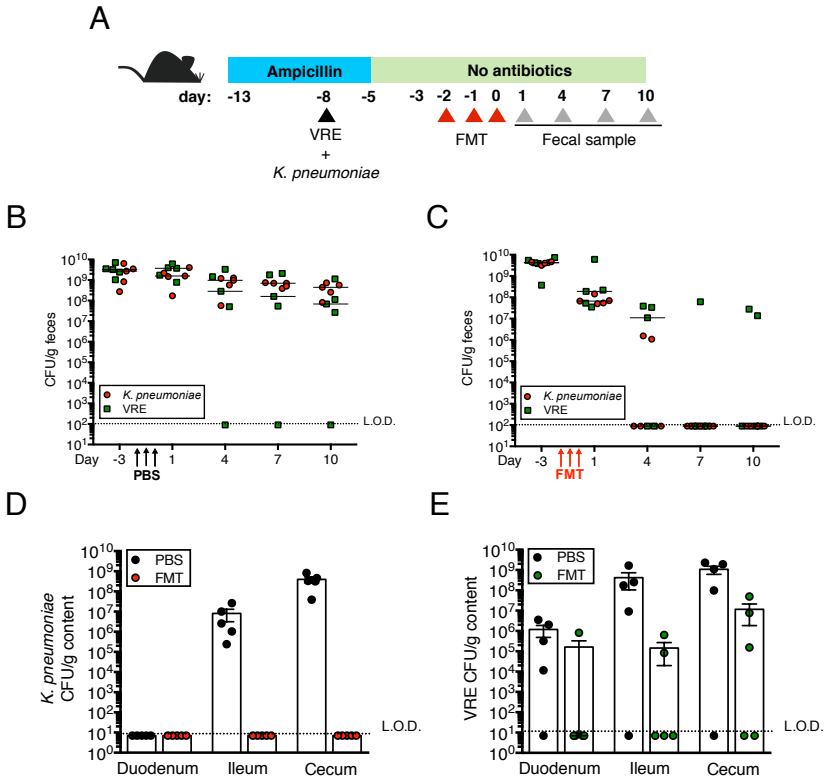


Figure 2.4. Fecal bacteriotherapy eliminates established *K. pneumoniae* and VRE intestinal domination.

(A) Experimental design. Mice were treated with ampicillin for 8 days. On day 5 of ampicillin treatment, mice were simultaneously infected with $5x10^4$ CFU of VRE and *K. pneumoniae* (Kpn). Three days post infection, ampicillin treatment was stopped. Mice were administered PBS or a fecal microbiota transplant (FMT) from an untreated mouse on three consecutive days starting on the third day after ampicillin cessation. (B, C) VRE and *K. pneumoniae* burden was quantified in fecal pellets at the indicated time points after the last PBS (B) or FMT (C) dose. (D, E) PBS- and FMT-treated mice were sacrificed on day 10 following the last treatment dose and numbers of *K. pneumoniae* (D) and VRE (E) CFU were quantified in the duodenum, ileum and cecum. L.O.D., limit of detection. n ≥ 5 per group. (B-E) $^{**}P<0.005$ by the Mann-Whitney test.

2.2.4. *K. pneumoniae* and VRE reside within the same intestinal regions but occupy distinct metabolic niches

The findings that *K. pneumoniae* and VRE do not interfere with each other's ability to colonize the gut lumen and that their elimination from the intestine following FMT differs suggest that these bacterial species occupy distinct intestinal niches. To localize bacteria within the colons of mice we performed fluorescence in situ hybridization (FISH) with a universal probe targeting bacterial 16S rRNA genes. In mice that had not been treated with antibiotics, we detected a dense and morphologically diverse bacterial microbiota that is almost completely depleted by ampicillin-treatment (**Figures 2.5A-B** and **Supplementary Figures 2.2A-B**). FISH analysis of antibiotic-treated mice colonized with *K. pneumoniae* (Kpn), VRE or both with species-specific oligonucleotide probes revealed that *K. pneumoniae* and VRE were most abundant in luminal areas adjacent to the colonic epithelial layer and that both organisms localized to the same intestinal sites (**Figures 2.5C-D** and **2.6A-D**).

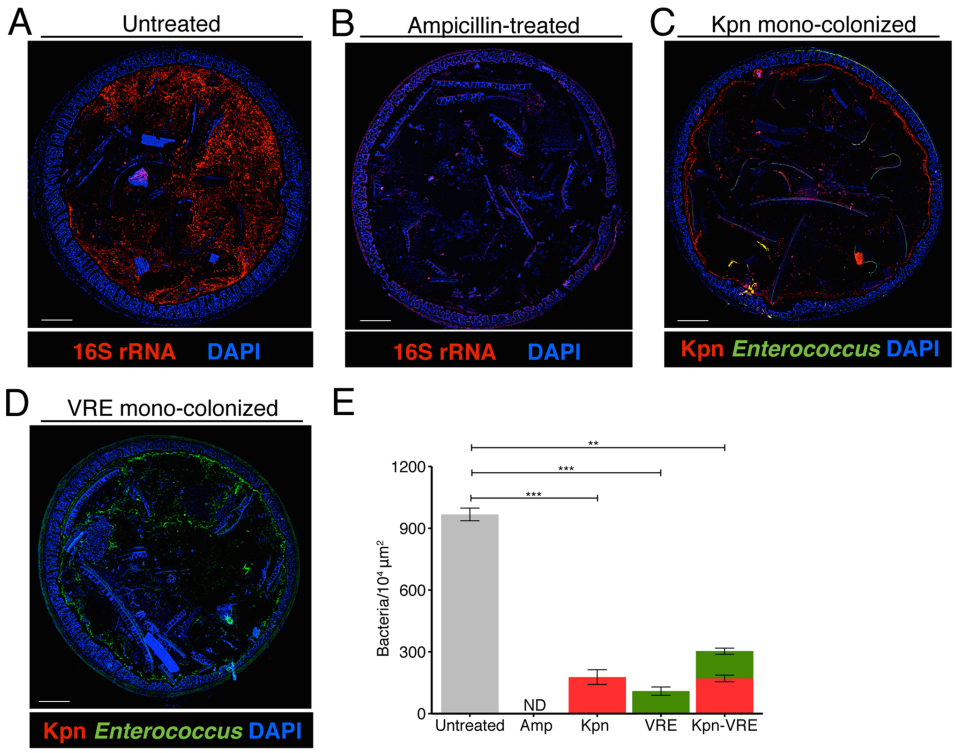
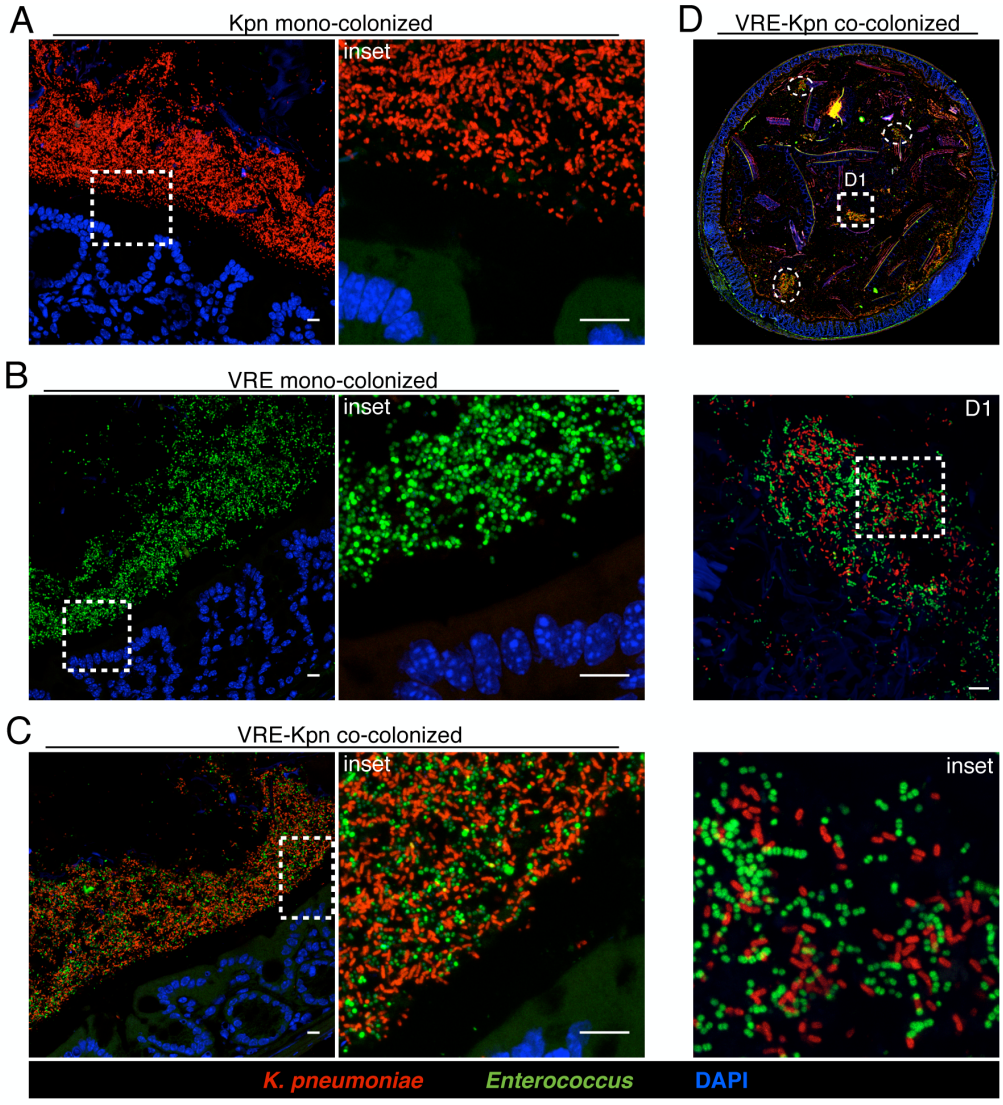


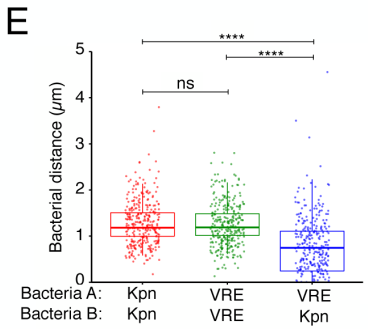
Figure 2.5. *K. pneumoniae* and VRE occupy a fraction of the total available space in the colon.
(A-E) Visualization of bacterial localization by FISH. Entire colon cross-sections from untreated mice (A) and mice treated with ampicillin for 3 weeks (B) were stained with a universal probe that targets the 16S rRNA gene of all bacteria. Cross-sections from ampicillin-treated mice colonized with *K. pneumoniae* (C) or VRE (D) for 21 days were hybridized with probes specific for *K. pneumoniae* (Kpn) and Enterococcus, respectively. Sections were counterstained with Hoechst dye to visualize nuclei. Images are representative of 5 mice per group. Scale bar, 500 µm. (E) Number of bacteria per unit area of whole colon cross-sections. n = 3 per group. ND = non-detectable. Error bars (mean ± SEM). $^{**}P<0.005$, $^{***}P<0.0005$ by the Mann-Whitney test.

Confocal microscopy-based quantification of VRE and *K. pneumoniae* in mono-colonized mice demonstrated that *K. pneumoniae* and VRE each only achieved 10% of the bacterial density detected in antibiotic-naive mice with a diverse microbiota (**Figure 2.5E**). In co-colonized mice, the densities of VRE and *K. pneumoniae* in the colonic lumen were additive, supporting the idea that VRE and *K. pneumoniae* do not interfere with one another and that their metabolic needs may differ (**Figure 2.5E**). Furthermore, *K. pneumoniae* and VRE were generally occupying overlapping regions within luminal areas

closest to the colonic epithelium, although islands of increased bacterial density were also detected more centrally in the colonic lumen (**Figures 2.6C-D**). To further assess whether VRE and *K. pneumoniae* compete for space within the regions they occupy in the colon, we measured distances between neighboring VRE bacteria, between neighboring *K. pneumoniae* bacteria and between neighboring VRE and *K. pneumoniae* bacteria. Supporting the notion that VRE and *K. pneumoniae* occupy different metabolic niches, intraspecies distances were significantly greater than interspecies distances between VRE and *K. pneumoniae*, suggesting that localized nutrient depletion may promote spatial avoidance among competing bacteria while non-competing bacteria ignore each other (**Figure 2.6E**).

Figure 2.6. *K. pneumoniae* and VRE reside within the same intestinal regions but occupy distinct metabolic niches.
(A-D) Spatial localization of *K. pneumoniae* and VRE in the colon. Colon sections from ampicillin-treated mice colonized for 21 days with *K. pneumoniae* alone (A), VRE alone (B) and *K. pneumoniae* together with VRE (C, D) were hybridized with probes specific for *K. pneumoniae* and Enterococcus. (D) VRE and *K. pneumoniae* islands (dashed circles and square) in the colonic lumen of co-colonized mice. (A-D) All sections were counterstained with Hoechst dye to visualize nuclei. Scale bars, 10 μm. Insets, 63X oil objective plus 4X digital zoom. Images are representative of at least 5 mice per group. (E) Minimum distance between neighboring bacteria determined by confocal microscopy. ns=non-significant; ****$P<0.0001$, by the Mann-Whitney test.


A
Kpn mono-colonized
inset
D
VRE-Kpn co-colonized
D1
B
VRE mono-colonized
inset
D1
C
VRE-Kpn co-colonized
inset
inset
K. pneumoniae
Enterococcus
DAPI


E
****
****
ns
Bacterial distance (μm)
0
1
2
3
4
5
Bacteria A:
Bacteria B:
Kpn
Kpn
VRE
VRE
VRE
Kpn

2.2.5. Antibiotic treatment and colonization with *K. pneumoniae* or VRE influences the thickness and integrity of the inner mucus layer

The murine colonic mucus layer consists almost exclusively of the mucin Muc2 (175). Upon release from goblet cells, Muc2 forms a gel-like layer that coats the intestinal epithelium and that is largely impenetrable to bacteria. Beyond the thick, epithelium-associated mucin layer is a loosely attached and less dense mucin layer that is readily penetrated by intestinal bacteria and that serves as a microbial habitat (175,176). Under normal homeostatic conditions, bacterial molecules released by the microbiota and host factors regulate the production and secretion of mucus (177) and thinning of the colonic mucus layer occurs following antibiotic treatment (157). The infectivity of some intestinal pathogens, such as *C. rodentium* and *Salmonella enterica sv Typhimurium*, is enhanced by disruption of the mucus layers in the colon (37,69).

To explore the impact of *K. pneumoniae* and VRE colonization on the colonic mucus layer, we stained colonic sections for Muc2 and measured the thickness of the inner mucus layer. While the dense mucus layer was significantly reduced in mice treated with ampicillin (**Figures 2.7A-B** and **2.7E**), mono-colonization of ampicillin-treated mice with *K. pneumoniae* but not VRE resulted in recovery of normal mucus layer thickness (**Figures 2.7C-E**). This indicates that bacterial species differ in their ability to promote mucin production and mucus layer generation in the murine colon.

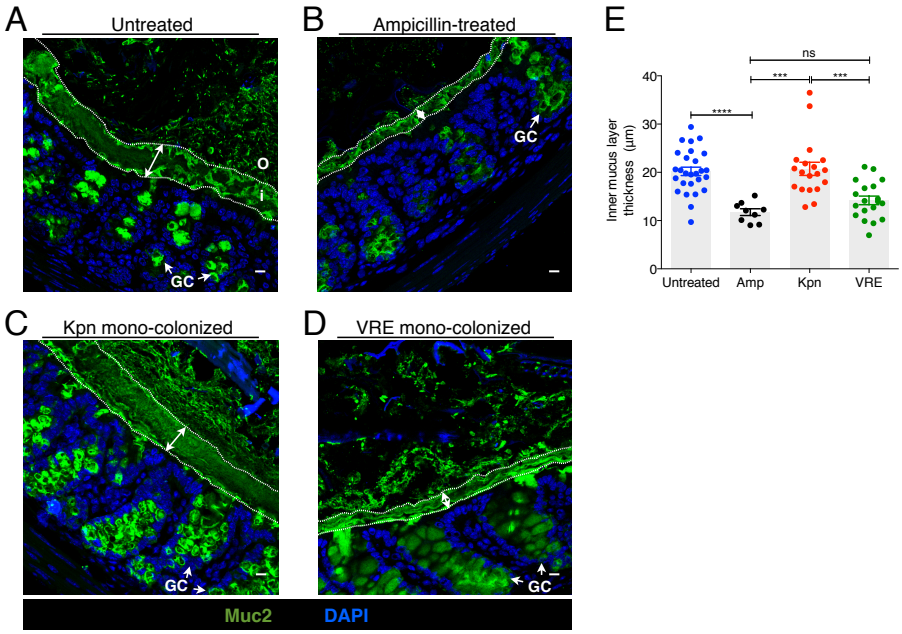


Figure 2.7. *K. pneumoniae* and VRE colonization influences the thickness of the inner mucus layer.

(A-D) Colon sections from untreated mice (A), ampicillin-treated mice (B) and ampicillin-treated mice mono-colonized with either *K. pneumoniae* (C) or VRE (D) for 21 days were stained with an anti-Muc2 antibody to visualize the inner and outer mucus layers along with goblet cells (arrows). Double arrows denote the inner mucus layer. i, inner mucus layer; o, outer mucus layer; GC, goblet cell. Scale bars, 10 µm. Images are representative of 5 mice per group. (E) Quantification of inner mucus layer thickness. Error bars (mean ± SEM). n=3-6 mice per group. ns=non-significant; ***P<0.0005, ****P<0.0001, by the unpaired Student t test.

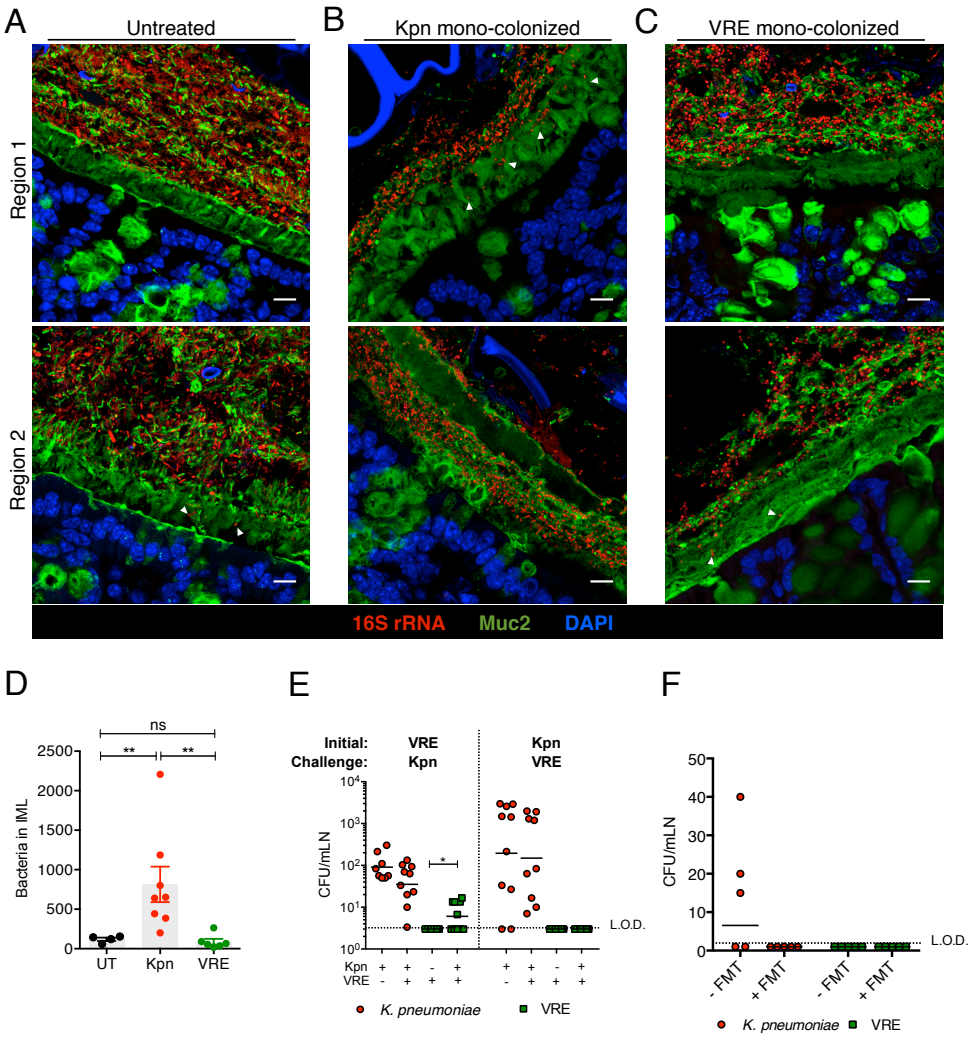
2.2.6. *K. pneumoniae* and VRE differ in their ability to invade the colonic mucus barrier and translocate to extra-intestinal sites

To investigate the interactions between *K. pneumoniae* and VRE with the colonic mucin layer, we mono-colonized ampicillin-treated mice and performed FISH in conjunction with Muc2 immunostaining to localize bacteria in relation to the inner mucus layer (IML). Very few bacteria were detected within the IML in antibiotic-naïve mice harboring a diverse microbiota (**Figure 2.8A**). On the other hand, *K. pneumoniae* was detected within the IML in mono-colonized mice, with the extent of mucin penetration varying in different regions of a colonic cross-section (**Figure 2.8B**). VRE also penetrated the IML at different regions but to a lesser extent than *K. pneumoniae* (**Figure 2.8C**). Image analysis of entire colon cross-sections revealed substantially higher bacterial numbers in the IML of *K. pneumoniae* mono-colonized mice compared to antibiotic-naïve or VRE mono-colonized animals (**Figure 2.8D**). Within the colonic lumen, however, *K. pneumoniae* and VRE were similarly associated with detached islands of mucus (**Figures 2.8B-C**).

To determine whether increased mucus layer penetration by *K. pneumoniae* is associated with increased invasion of deeper tissues, we cultured mesenteric lymph nodes (mLNs). In mono-colonized mice, we detected up to 10^4 live *K. pneumoniae* in mLNs three weeks p.i. (**Figure 2.8E**) Notably, infection of mLNs by *K. pneumoniae* was not affected by co-colonization with VRE, regardless of the order of microbial administration. In contrast, we did not recover any live bacteria from mLNs of VRE mono-colonized animals, perhaps reflecting their reduced penetration of the IML. However, challenge of VRE mono-colonized mice with *K. pneumoniae* resulted in VRE translocation to mLNs in 60% of the mice, suggesting that introduction

of *K. pneumoniae* may enhance the ability of VRE to traverse the mucin layer and gain access to the intestinal epithelium (**Figure 2.8E**). Administration of fecal microbiota transplants to colonized mice reduced *K. pneumoniae* infection of mLNs (**Figure 2.8F**), suggesting that isolation of live bacteria from mLNs of *K. pneumoniae*-colonized mice results from continuous seeding of the node rather than bacterial growth and persistence in mLNs. Our findings demonstrate that *K. pneumoniae*, upon achieving high density in the intestine, can penetrate the dense mucin layer of the colon. In contrast, VRE access to the epithelial layer appears to be more restricted by the mucin layer. Although the ability to penetrate mucus has been associated with pathogenic bacteria, our results suggest that among commensal bacterial species mucus penetration may correlate with their ability to disseminate beyond the gut lumen.

Figure 2.8. Differential mucus layer infiltration and translocation by VRE and K. *pneumoniae*. (A-C) Dual immunostaining of colon sections from untreated mice (A) and ampicillin-treated mice mono-colonized with either *K. pneumoniae* (B) or VRE (C) for 21 days using anti-Muc2 and a pan-bacterial 16S rRNA gene FISH probe. Sections were counterstained with Hoechst dye to visualize nuclei. Arrowheads indicate bacteria within the inner mucus layer. Scale bar, 10 μm. Images are representative of 5 mice per group. Boundaries of the inner mucus layer (IML) zone were determined by the density of Muc2 staining and the stratified organization characteristic of the inner, but not the outer, mucus layer. (D) Number of bacteria within the IML. UT, untreated. (E) Numbers of VRE and *K. pneumoniae* in mesenteric lymph nodes of mono-colonized mice and mice pre-colonized with either VRE or *K. pneumoniae* (initial strain) and challenged with the opposite strain at day 21 post challenge. Data were pooled from two independent experiments. (F) Numbers of VRE and *K. pneumoniae* in mesenteric lymph nodes of mice co-colonized with VRE and *K. pneumoniae* with or without a fecal transplant (FMT) 10 days after receiving the last of three FMT/PBS doses. Data were pooled from two independent experiments. L.O.D., limit of detection. (D-F) ns=non-significant; **P*<0.05, ***P*<0.005, by the Mann-Whitney test.


A
Untreated
B
Kpn mono-colonized
C
VRE mono-colonized
Region 1
Region 2
16S rRNA
Muc2
DAPI
D
Bacteria in IML
ns
**
**
2500
2000
1500
1000
500
0
UT
Kpn
VRE
E
Initial:
Challenge:
VRE
Kpn
Kpn
VRE
CFU/mLN
L.O.D.
Kpn
VRE
K. pneumoniae
VRE
F
CFU/mLN
50
40
30
20
10
0
L.O.D.
- FMT
+ FMT
- FMT
+ FMT
K. pneumoniae
VRE

2.3. DISCUSSION

VRE and *K. pneumoniae* are two of the most common highly antibiotic-resistant bacterial species that cause infections in hospitalized patients. Both organisms are oxygen-tolerant facultative anaerobes and principally reside in the lower gastrointestinal tract where, under normal circumstances, they are minor contributors to a colonic microbiota composed predominantly of oxygen-intolerant obligate anaerobes. Microbiota analyses have revealed that some patients undergoing allogeneic hematopoietic stem cell transplantation have marked expansion of VRE or *K. pneumoniae* in their colonic microbiota (14,158), to the point where in many cases these species constitute the overwhelming majority of the intestinal bacterial taxa. Our experiments with murine models demonstrate that both organisms can undergo massive expansion in the gastrointestinal tract of antibiotic-treated mice, occupy the same intestinal regions and coexist without exerting any colonization resistance against each other.

The mechanisms that determine bacterial density in the colon remain incompletely defined. During broad-spectrum antibiotic treatment the density of bacteria in the colon, as determined by quantitative 16S rRNA gene PCR, is reduced over 1,000 fold. Nevertheless, upon termination of antibiotic treatment, the compositionally-restricted residual flora re-expands to a density that is similar to pre-treatment levels (14,154). Along similar lines, we find that VRE and *K. pneumoniae* achieve densities of 10^{10} CFU per gram of colonic content in antibiotic-treated mice, at which point their growth stops, potentially resulting from nutrient depletion or quorum sensing mechanisms that remain undefined (178). Because VRE and *K. pneumoniae* grow independently in the colons of antibiotic-treated co-colonized mice, our findings suggest that distinct

mechanisms determine maximal bacterial density of these two bacterial species.

Competition for resources has been demonstrated in several in vivo colonization studies involving bacteria of the same or closely related species (17,173,179,180). In the intestine, heavily glycosylated mucus is a major source of energy for bacteria. Commensals that express mucus-degrading enzymes, such as *Bacteroides thetaiotaomicron*, can cleave sugars from host glycans and support the growth of other members of the colonic microbiota (18,19). Enterococci and γ-Proteobacteria do not express glycosidases that degrade mucosal polysaccharides, and thus their carbohydrate utilization is limited to less complex sugars (181,182). Some studies have demonstrated that mucus-derived carbohydrates, including sialic acid and fucose, transiently accumulate following antibiotic-treatment, presumably as a result of hydrolases released during bacterial cell lysis, and promote the growth of *Salmonella* and *Clostridium difficile* (19,183). However, given the transient release of these carbohydrates following initiation of antibiotic treatment, it is unlikely that this process is sustaining dense and prolonged colonization by either VRE or *K. pneumoniae*. Although *E. faecium* and *K. pneumoniae* can metabolize monosaccharides, and the range of potential carbon sources for K. pneumoniae is broad (184), the *in vivo* carbohydrate dependencies of these two bacterial species remain largely uncharacterized. The lack of competition between VRE and *K. pneumoniae* could potentially be explained by a difference in metabolic requirements (i.e. different sugar utilization) and the observation that bacteria can switch to alternative nutrient sources in the presence of competing strains (185). Further studies that examine the metabolic profiles of VRE and *K. pneumoniae* during mono- and co-

colonization will be necessary to identify the mechanisms determining their density in the intestinal tract.

In addition to serving as a nutrient source for intestinal bacteria, mucus also provides a barrier that keeps bacteria away from the colonic epithelium. The intestinal microbiota induces mucin production and antibiotic administration results in thinning of the mucus barrier, thereby increasing susceptibility to bacterial invasion (157). Pathogens such as *Salmonella* and *C. rodentium* can penetrate the mucus layer and in the process induce increased mucin production, a host defense mechanism that limits bacterial invasion (37,171). Consistent with these studies, we find that *K. pneumoniae* infiltrated the mucus layer while also restoring its thickness to pre-antibiotic treatment levels. VRE colonization of antibiotic-treated mice, on the other hand, did not induce mucus layer thickening. In addition, VRE did not invade the mucus layer to the same degree as *K. pneumoniae*. It remains to be determined whether enhanced mucus production is a consequence of bacterial invasion or rather, molecular differences between Gram-negative and Gram-positive bacteria.

In mice with a normal flora and intact TLR signaling, the dense mucus layer is largely devoid of bacteria (62,175). Antibiotic treatment, however, reduces the expression of antimicrobial molecules such as RegIIIγ (77), potentially enabling bacteria to gain access to and survive within the mucus layer. Since expression of RegIIIγ in the small intestine is markedly reduced as a result of antibiotic treatment (69,77), it is possible that a paucity of host-derived antimicrobial proteins in the mucin layer renders it more penetrable. A recent study showed that mice harboring high levels of bacteria belonging to the Proteobacteria and TM7 phyla have an inner mucus layer that is normal in

thickness but penetrable to bacteria (186). Thus, it is also possible that *K. pneumoniae* may induce mucins with abnormal glycosylation or that are structurally disorganized.

Although intestinal colonization with VRE and *K. pneumoniae* is asymptomatic, it increases the risk of extra-intestinal infection, including bacteremia (158,187). Consistent with the observed differences in mucus infiltration, we found increased *K. pneumoniae* translocation to mesenteric lymph nodes (mLNs) relative to VRE. Co-colonization with *K. pneumoniae* resulted in increased VRE translocation to mLNs, suggesting that *K. pneumoniae* may have opened barriers for the less invasive VRE. The high levels of *K. pneumoniae* observed in mLNs of intestinally dominated mice and the complete reduction of bacteria following FMT-mediated intestinal clearance of *K. pneumoniae* suggests that bacteria are not replicating in the mLNs but rather are delivered on a continuous basis. Although it is unclear how live bacteria are delivered to draining mLNs, recent studies with other bacterial pathogens suggest that cells within the colonic lamina propria, including $CD103^+$ and $CX3CR1^+$ dendritic cells, may capture *K. pneumoniae* from the luminal environment and carry them to mLNs (49,50).

Enterococci and γ-Proteobacteria constitute a minor population within the human and murine microbiota and are, for the most part, harmless to the host unless intestinal homeostasis is perturbed (176). In our murine model, intestinal colonization with VRE and *K. pneumoniae* did not result in detectable inflammation of the intestinal wall. In clinical scenarios, however, dense intestinal colonization with antibiotic-resistant bacteria is an important risk factor for systemic infection and for patient-to-patient spread. We found that introduction of a normal microbiota into VRE and *K. pneumoniae* co-colonized

mice resulted in clearance of both bacterial species, albeit at different rates. Current studies are focusing on the identification of commensal bacterial species that mediate clearance of antibiotic-resistant bacteria. Overall, the findings presented here uncovered previously unrecognized features of VRE and *K. pneumoniae* colonization and provide insight into the nature of pathogen coexistence, dissemination and ways to eradicate colonization.

2.4. MATERIALS AND METHODS

2.4.1. Mice, bacterial strains and infection. All experiments were carried out using 6-8 week-old C57BL/6 female mice purchased from Jackson Laboratories and housed in sterile cages with irradiated food and acidified water. For experiments involving antibiotic treatment, 0.5g/L ampicillin (Fisher) was administered to animals in the drinking water and changed every 4 days. Vancomycin-resistant Enterococcus faecium was purchased from ATCC (700221). Carbapenem-resistant *Klebsiella pneumoniae* was obtained from the Clinical Microbiology Laboratory at Memorial Sloan-Kettering Cancer Center and isolated from blood cultures of a patient. Both bacterial strains were grown at 37^{o}C in Brain Heart Infusion (VRE) or Luria-Bertani (*K. pneumoniae*) broth to early stationary phase and diluted in phosphate buffered solution (PBS) to 10^5 colony-forming units (CFU). For infection experiments, $5x10^4$ CFU of VRE and/or *K. pneumoniae* were administered by oral gavage in a 200µl volume on the fifth day of ampicillin treatment. For simultaneous infection of VRE and *K. pneumoniae* (**Fig 2.4**), inocula were mixed in a 1:1 ratio prior to administration. Mice were single-housed at the time of infection and kept on ampicillin until the end of the experiment unless otherwise specified. Animals were maintained in a specific pathogen-free facility at Memorial Sloan-Kettering Cancer Center. All mouse handling, cage changes and tissue collection were performed in a biosafety level 2 facility wearing sterile gowns, masks and gloves.

2.4.2. Quantification of VRE and *K. pneumoniae* burden. Fecal samples and intestinal contents from the duodenum, ileum and cecum were weighed

and resuspended in 1 ml of PBS. Tenfold dilutions were plated on Difco Enterococcosel (supplemented with 8 µg/ml vancomycin; Novaplus and 100 µg/ml streptomycin; Fisher) and Luria-Bertani (supplemented with 50 µg/ml neomycin; Sigma-Aldrich and 100 µg/ml carbenicillin; LabScientific) agar plates for the specific detection of VRE and *K. pneumoniae*, respectively. Mesenteric lymph nodes were mashed through a 40 µm filter, resuspended in PBS and plated directly onto selective plates.

2.4.3. Fecal microbiota transplantation (FMT). A fecal pellet from an untreated C57BL/6 mouse was resuspended in 1ml of PBS under anaerobic conditions and 200µl was administered per mouse via oral gavage starting on the third day of ampicillin cessation. This process was repeated on the fourth and fifth days for a total of three consecutive FMT doses.

2.4.4. DNA extraction, V4-V5 16S rRNA gene amplification, multiparallel sequencing and sequence analysis. Fecal samples and intestinal contents were frozen immediately after collection in a dry ice/ethanol slurry and stored at -80^{o}C. DNA was extracted using the phenol-chloroform and bead beating method described previously (14). The V4-V5 region of the 16S rRNA gene was amplified and sequenced with the Illumina Miseq platform as described previously (145). Sequences were analyzed using version 1.33.3 of the MOTHUR pipeline (7) as described in Buffie et al., 2015. Sequences with distance-based similarity of at least 97% were assigned the same OTU (operational taxonomic unit) and representative OTUs were classified using a modifiedfied Greengenes reference database (188).

2.4.5. Tissue preparation for histology analysis. Intestinal tissues with luminal contents were carefully excised and fixed in freshly made nonaqueous Methacarn solution (60% methanol, 30% chloroform and 10% glacial acetic acid) as previously described (62,189) for 6 hours at 4^{o}C. Tissues were washed in 70% ethanol, processed with Leica ASP6025 processor (Leica Microsystems) and paraffin-embedded by standard techniques. 5-µm sections were baked at 56^{o}C for 1 hour prior to staining.

2.4.6. Fluorescence in-situ hybridization (FISH). The hybridization method was adapted from Swidsinski et al., 2005 and Vaishnava at el., 2011. Briefly, tissue sections were deparaffinized with xylene (twice, 10 min each) and rehydrated through an ethanol gradient (95%, 10 min; 90%, 10 min) to water. Sections were incubated with a universal bacterial probe directed against the 16S rRNA gene or with probes specific to K. pneumoniae and Enterococcus at 50^{o}C for 3 hours. Probes were diluted to 5ng/µl in 0.9M NaCl, 20mM Tris-HCl at pH7.2 and 0.1% SDS prior to use. Sections were later washed twice in 0.9M NaCl, 20mM Tris-HCl at pH7.2 (wash buffer) for 10 min and counterstained with Hoechst (1:3000 in wash buffer) for nuclear staining. The following FISH probes were used: universal bacterial probe EUB338: [Cy3]-GCTGCCTCCCGTAGGAGT-[AmC7~Q+Cy3es] (190). *K. pneumoniae*-specific probe Kpn: [Cy3]-CCTACACACCAGCGTGCC-[AmC7~Q+Cy3es] (probe base accession number 00352, (191)); *Enterococcus*-specific probe Enfm93:[AminoC6+Alexa488]-GCCACTCCTCTTTTTCCGG [AmC7~Q+Alexa488] (192).

2.4.7. Muc2 immunofluorescence. Muc2 immunostaining was performed as previously described (63,175) with some modifications. Briefly, deparaffinized sections were incubated in 0.9M NaCl, 20mM Tris-HCl at pH7.2 and 0.1% SDS at 50^{o}C for 3 hours, rinsed in PBS and blocked with 5% goat serum in PBS for 30 min at room temperature to minimize non-specific binding. Sections were then washed in PBS for 10 min prior to overnight incubation at 4^{o}C with an anti-Muc2 rabbit polyclonal antibody (H300, Santa Cruz; 1:200 in PBS) (193). Following incubation with primary antibody, tissues were washed 3 times in PBS for 10 min and incubated with goat-anti-rabbit Alexa 488 secondary antibody (Life Technologies, 1:1000 in PBS) for 1 hour at room temperature. Sections were washed twice in PBS for 10 min and counterstained with Hoechst (1:3000 in PBS). For FISH-Muc2 dual staining, sections were briefly rinsed in wash buffer after FISH hybridization and incubated directly with the anti-Muc2 primary antibody diluted in wash buffer. Incubation with secondary antibody was carried out at 4^{o}C for 2 hours. A single 10 min PBS wash was performed after incubation with the primary and secondary antibodies before Hoechst nuclear staining and mounting with Mowiol solution.

2.4.8. Microscopy. Images were acquired with a Leica TCS SP5-II upright confocal microscope using a 63x oil immersion lens (NA 1.4, HCX PL APO) with or without digital zoom as a series of short Z-stacks. Maximum intensity projection processing of Z-stacks was done in Fiji (ImageJ) software. Mucus layer thickness was measured using the Leica distance measurement tool (LASAF). The width of the inner mucus layer was determined by the average of 4 measurements per field with 4 fields measured per section. Whole tissue

images were digitally scanned using the Zeiss Mirax Desk Scanner with 20x/0.8NA objective. Bacterial distance analysis was performed on colon images taken at 63x magnification with a 4x digital zoom by determining the XY coordinates of each bacterial cell in MetaMorph (Molecular Devices) software and measuring the distance from their center. For quantification of bacterial density and invasion into the mucus layer, whole tissue cross-sections were tile scanned in short Z-stacks using an inverted laser scanning confocal Zeiss LSM 5-Live microscope at 63x magnification. For bacterial quantification, a threshold based on the RGB color combination and intensity of each bacterial species was generated with the color thresholding option in MetaMorph. Thresholded objects of 1µm in size were counted as a single bacterial cell with MetaMorph's integrated morphometric analysis tool.

2.4.9. Statistics. The Mann-Whitney test was used for statistical analysis of intestinal CFU as well as image-based quantification of bacterial distance, density and infiltration into the mucus layer. Differences in mucus layer thickness were analyzed with the unpaired Student t test. All statistical tests were performed using the Graph-Pad Prism software package (version 6.0). P values less than 0.05 were considered to be significant.

CHAPTER 3

SPECIFIC BACTERIAL SPECIES CONFER COMMUNITY-WIDE ANTIBIOTIC RESISTANCE IN THE INTESTINE FOLLOWING LONG-TERM ANTIBIOTIC EXPOSURE

3.1. INTRODUCTION

Antibiotic administration can result in drastic changes to the intestinal microbiota that may compromise various aspects of gut function including protection against pathogen growth, a process known as colonization resistance (168). The extent by which antimicrobial agents disrupt the intestinal microbial ecosystem depends largely on the type of antibiotic used and the course of therapy. This was evidenced in recent infection studies where mice on a 3-day course of either ampicillin or enrofloxacin regained colonization resistance against *Clostridium difficile* as early as one day after antibiotic treatment, whereas mice treated with metronidazole and vancomycin or a single dose of clindamycin remained susceptible to infection for up to two to three weeks (145,154,194). Although re-establishment of colonization resistance suggests the return of key bacterial species, for the most part, the recovered microbiota does not resemble the former, pre-antibiotic, state. Despite regaining resistance to infection, clindamycin-treated mice exhibited decreased bacterial diversity that lasted for at least four weeks post treatment (154). In humans, a 7-day clindamycin course led to a dramatic reduction in the diversity of *Bacteroides* spp. that persisted even two years after cessation of antibiotic therapy (195,196). Similarly, treatment with ciprofloxacin decreased the overall bacterial diversity and despite the return of a substantial

portion of the microbiota four weeks following antibiotic administration, some Clostridiales members did not reappear (197). In addition, some antibiotic perturbations may persist and shift the microbiota to an alternative stable state in some individuals as a result of repeated clindamycin use (153).

Although these studies have shed considerable light on the long-term effects of short-course antibiotic therapy, the impact of prolonged antibiotic use on the composition of the microbiota and the stabilization of resistant strains is not well defined. Even after a short course on clindamycin, clindamycin-resistant *Bacteroides* spp. harboring erythromycin-resistant methylase (erm) genes were detected two years after treatment (195), while in a separate study, levels of erm genes remained significantly high even four years post treatment (198). These findings are consistent with the idea that the gastrointestinal tract serves as a reservoir for antibiotic-resistant genes (199,200) and may seem alarming given the increase in multidrug-resistant nosocomial pathogens (201). Nevertheless, the selection of antibiotic-resistant strains may confer a benefit to the entire microbial community when faced with continued antibiotic pressure. This benefit may come in the form of antibiotic-degrading enzymes released into the environment or factors that can increase overall bacterial fitness (202,203). Therefore, although persistent antibiotic treatment can be potentially detrimental, it can also push the microbiota to evolve mechanisms of resistance without compromising function.

In this study, we describe and characterize a murine microbiota that developed resistance as a result of ongoing treatment with amoxicillin (ampicillin) and clavulanic acid for longer than a decade. We found that this microbiota, despite differing markedly from antibiotic-naive intestinal populations, exhibits a high degree of microbial diversity and functionality as

demonstrated by the preservation of distinct aspects of gut homeostasis, including colonization resistance against the opportunistic pathogen vancomycin-resistant *Enterococcus*. Furthermore, we identified a small fraction composed of specific members of the *Parabacteroides* and *Bacteroides* genera responsible for conferring population-wide antibiotic resistance through the production of β-lactamases. Our findings constitute an example of the remarkable adaptation capacity of the microbiota and provide evidence that long-term antibiotic exposure can result in collective antibiotic tolerance in the gastrointestinal tract, an event that proved crucial for the maintenance of normal intestinal function.

3.2. RESULTS

3.2.1. Long-term antibiotic therapy leads to the emergence of a unique and functional antibiotic-resistant gut microbiota

The myeloid differentiation factor 88 (MyD88) is a critical signaling component of the innate immune system (204). As such, mice lacking MyD88 (MyD88$^{-/-}$) are highly susceptible to infection and exhibit high rates of infection-related mortality (205). To prevent this, generations of MyD88$^{-/-}$ mice were continuously treated with a combination of amoxicillin/ampicillin (β-lactam antibiotic) and clavulanic acid (β-lactamase inhibitor) in their drinking water for over 15 years, which resulted in the development of an ampicillin/clavulanic acid-resistant microbiota.

Taxonomic comparison of the fecal microbiota of MyD88$^{-/-}$ mice and C57BL/6 mice from Jackson laboratories, which carry an antibiotic-sensitive microbiota, showed similar proportions of Firmicutes and Bacteroidetes, the two dominant bacterial phyla, in MyD88$^{-/-}$ mice whereas 75% of the total bacterial population in C57BL/6 mice belonged to the Firmicutes (**Figure 3.1A-B**). Although we found bacterial families (namely, Clostridiaceae, Ruminococcaceae, S24-7 and Lachnospiraceae) previously associated with a healthy microbiota (206) and that are often lost following antibiotic treatment (14,194) represented in both mouse groups, S24-7 was the dominant family in MyD88$^{-/-}$ mice at 40% relative abundance compared to 5-10% in C57BL/6 animals. Conversely, over 50% of the C57BL/6 microbiota corresponded to the Clostridiaceae family, which was present at 15% abundance in MyD88$^{-/-}$ mice (**Figure 3.1A**).Finally, other bacterial families such as the Erysipelotrichiaceae, Bacteroidaceae, Prevotellaceae, Alcaligenaceae and Verrucomicrobiaceae

were found associated with MyD88$^{-/-}$, but not C57BL/6, animals. (**Figure 3.1A**). Despite these differences, MyD88$^{-/-}$ and C57BL/6 mice exhibited comparable bacterial diversity as measured by the inverse Simpson index (**Figure 3.1C**). However, the ileal compartment was significantly more diverse in MyD88$^{-/-}$ animals where, similar to the large intestine, S24-7 was the dominant family followed by Alcaligenaceae and Lachnospiraceae whereas the Lactobacillaceae and Clostridiaceae families were, for the most part, the major components of the C57BL/6 ileal microbiota (**Supplementary Figure 3.1A-B**).

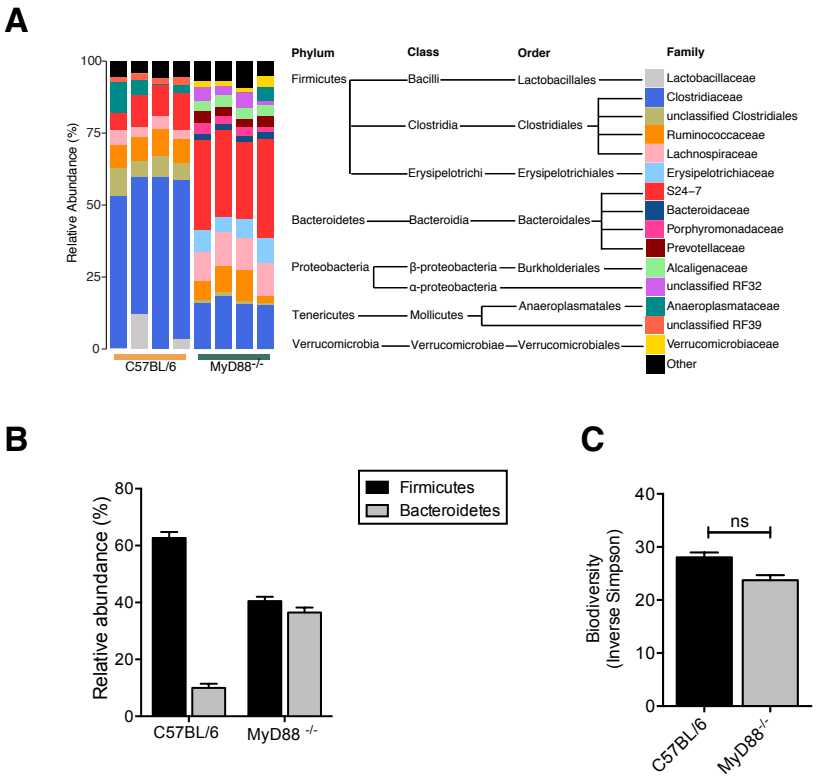
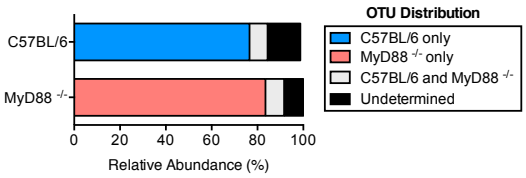


Figure 3.1. Taxonomic comparison between an antibiotic-tolerant and an antibiotic-sensitive microbiota. (**A**) Fecal microbiota composition of MyD88$^{-/-}$ and C57BL/6 mice. Each stacked bar corresponds to an individual mouse. OTUs at >0.01% relative abundance are shown. (**B**) Relative abundance of Firmicutes and Bacteroidetes OTUs. (**C**) Fecal microbiota alpha diversity. ns, non-significant by the Student's *t* test. n = 4 per group.

Analysis at the operational taxonomical unit (OTU) level in all intestinal compartments (ileum, cecum and feces) revealed that only 5% of all OTUs present at 0.1% abundance or higher were shared between MyD88$^{-/-}$ and C57BL/6 mice while 75-85% were unique to either mouse group (**Figure 3.2A**). Specifically, MyD88$^{-/-}$ mice harbored *Bacteroides sartorii*, *Parabacteroides distasonis* and multiple S24-7 OTUs within the Bacteroidia class while the Clostridia class consisted mainly of cluster XIVa members *Clostridium bolteae* and *Blautia producta* in addition to several uncharacterized *Clostridium* spp. (**Figure 3.2B**) Of note, these bacterial groups have been reported to be present in the microbiota of healthy individuals but were not detected in C57BL/6 animals (23,207). Our analyses demonstrate that at the species level, the composition of the MyD88$^{-/-}$ microbiota differs almost completely from that of C57BL/6 Jackson mice.

A



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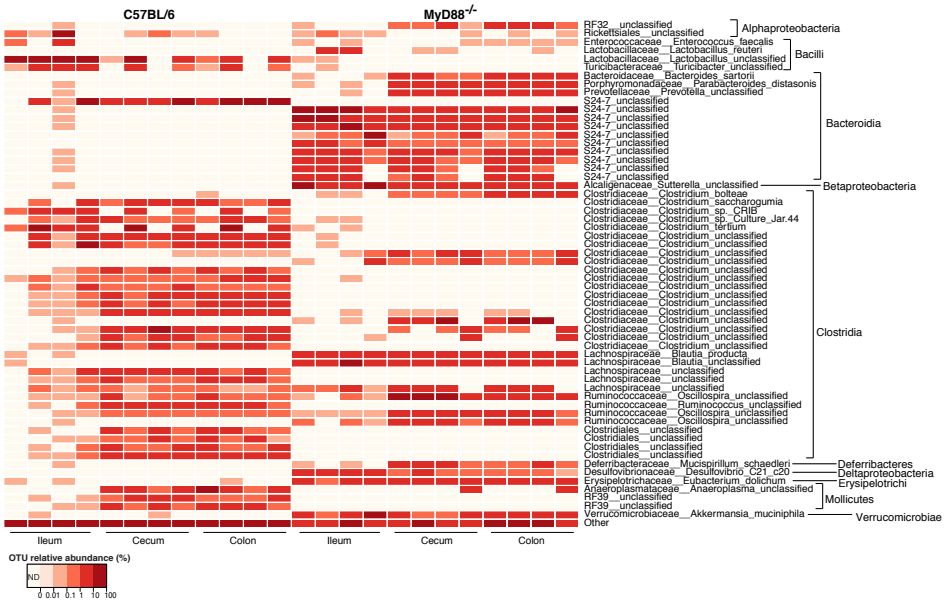


Figure 3.2. Distribution and composition of OTUs from MyD88$^{-/-}$ and C57BL/6 mice. (**A**) Distribution of fecal OTUs (≥97% similarity, >0.01% relative abundance). Data shown is the average of 4 mice per group. (**B**) The relative abundance of the top 60 OTUs comprising the small (ileum) and large (cecum and colon) intestines.

3.2.2. Transplantation of an antibiotic-resistant microbiota restores intestinal homeostasis in the presence of antibiotics

We investigated whether the ampicillin-resistant flora, which we will refer to as ARF from here on, harbored by MyD88$^{-/-}$ mice could prevent the deleterious effects of antibiotic treatment and restore normal intestinal function. To this end, we subjected C57BL/6 mice to a 2-week course of ampicillin in the drinking water and administered three daily doses of fecal ARF beginning 24 hours after the initiation of antibiotic treatment (**Figure 3.3A**). In line with previous evidence (14), we observed significantly reduced bacterial density as early as one day after ampicillin administration prior to any transplant (**Figures 3.3B**, Untreated vs. Amp). Nevertheless, we detected a 3-log increase in 16S copy numbers immediately after the last ARF dose (day 4 post ampicillin) all the way through day 14, demonstrating that transplant with ARF was able to bring total bacterial DNA load back to initial, pre-antibiotic, levels even after 2 weeks of ampicillin treatment (**Figure 3.3B**). Supporting this, we found that on day 4 post ampicillin treatment the bacterial composition of transplanted mice was nearly identical to the ARF input and remained stable throughout the duration of the experiment whereas control mice were depleted of most bacterial groups following antibiotic treatment (**Figure 3.3C**).

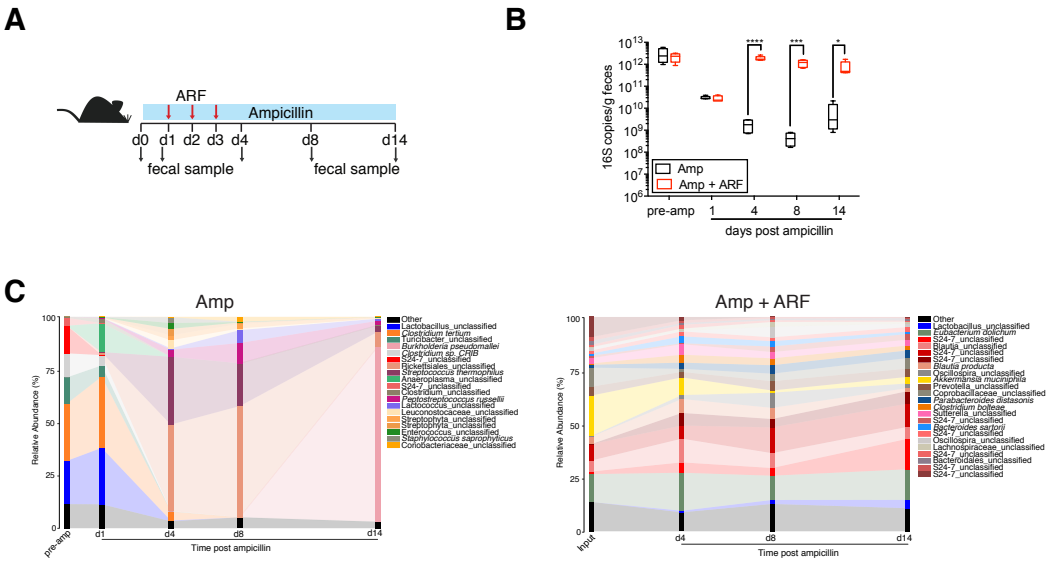


Figure 3.3. ARF administration fully reconstitutes the gut microbiota of antibiotic-treated mice. (**A**) Experimental layout for ARF transfer into antibiotic-treated mice. Three daily doses of fecal material from MyD88$^{-/-}$ mice were administered by oral gavage to ampicillin-treated C57BL/6 mice beginning one day after antibiotic initiation. Fecal samples were collected before antibiotics (d0) and on days (d) 1, 4, 8 and 15 after antibiotic administration for bacterial density and composition analyses (**B**) Density of 16S rRNA gene copies at different time-points during treatment (n = 5 mice per group per time point). Box plots show the median, 25th percentile and 75th percentile; whiskers represent minimum and maximum values. *p=0.0137, ***p=0.0009 and ****p>0.0001 by the Student' *t* test. (**C**) Microbiota composition of ampicillin-treated mice that received ARF or that were not manipulated. Each stacked bar represents the average of 5 separately-house mice. (**D**) Differences in cecum size in antibiotic-naive, ampicillin-treated and ARF-reconstituted ampicillin-treated animals.

Consistent with the reconstitution of a complex and diverse microbiota, cecum enlargement, a phenotype characteristic of antibiotic treatment due water accumulation in the lumen (208,209), was reversed in antibiotic-treated mice that received ARF transplants making the intestinal anatomy of these mice indistinguishable from that of antibiotic-naïve animals (**Figure 3.4A**). Furthermore, antibiotic-mediated depletion results in thinning of the colonic mucus layer and down-regulation of the antimicrobial protein RegIIIγ (69,157,176) which we also observed (**Figures 3.4**, Untreated vs. Amp). However, ampicillin-treated mice colonized with ARF exhibited an increase in mucus-producing goblet cells and consequently, restoration of the protective

inner mucus layer as well as dense bacterial staining restricted to the luminal compartment (**Figure 3.4B**). Finally, we also observed a rescue in RegIIIγ protein levels in ARF-treated animals, suggesting that ARF reconstitution can reset the normal immune tone of the intestine (**Figure 3.4C**). Taken together, our findings provide an example of how continued antibiotic pressure can drive intestinal microbial communities to develop antibiotic resistance and retain their complexity in order to maintain gastrointestinal health.

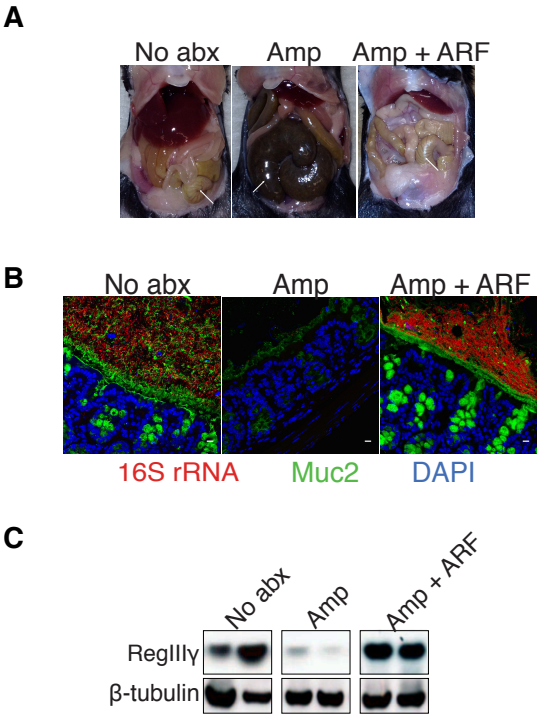


Figure 3.4. ARF reconstitution prevents antibiotic-induced changes in intestinal homeostasis. (**A**) Differences in cecum size in antibiotic-naive, ampicillin-treated and ARF-reconstituted ampicillin-treated animals. (**B**) Visualization of the inner mucin layer (Muc2) and bacteria (16S rRNA gene) in colon cross-sections. All sections were counterstained with Hoechst dye to visualize nuclei. Original magnification, 63X. Scale bars, 10 μm. (**C**) Western blot analysis of protein extracts from the distal region of the small intestine with RegIIIγ-specific antiserum and anti - β-tubulin as a loading control. (**A-C**) Shown are representative images and samples from 5 mice per group.

3.2.3. Efficacy of ARF bacteriotherapy against vancomycin-resistant Enterococcus

To expand our functional characterization studies, we assessed the ability of ARF to prevent and eliminate colonization by the nosocomial pathogen vancomycin-resistant Enterococcus (VRE). To examine whether ARF could preserve colonization resistance against VRE in antibiotic-treated animals, we infected mice the day following the last of three ARF transplants, the earliest time-point at which we observed complete engraftment of ARF (**Figures 3.3C**). While VRE had reached a density of 10^9 colony forming units (CFU) in the feces of PBS-treated mice starting at day 1 post infection (p.i.), VRE levels in ARF-treated mice were significantly lower on day 1 p.i (10^4 CFU) and became undetectable by day 3 p.i., demonstrating that ARF has the ability to re-establish colonization resistance despite ongoing antibiotic treatment (**Figure 3.5A**). Remarkably, fecal extracts from ARF-treated animals were also able to suppress VRE growth *ex vivo*, suggesting that colonization resistance against VRE is mediated exclusively by the intestinal microbiota and independent of the host (**Figure 3.5B**).

Murine models have shown that once VRE has colonized the intestine, it can persist for as long as two months even in the absence of antibiotics and clearance can only be achieved through transplantation with a healthy and diverse fecal microbiota (14,159,176). We tested the potential of ARF to eliminate established VRE by administering three consecutive ARF doses beginning 3 days following infection with VRE, at which point VRE becomes stable in the intestine (**Chapter 2, Supplementary Figure 1B**). Prior to treatment, VRE burden was similarly high in all mice and remained elevated in mice that went on to receive PBS instead of ARF whereas ARF-treated

animals showed a progressive reduction in VRE levels with clearance by day 15 post ARF administration (**Figure 3.5C**). The rate of VRE clearance with ARF mirrors the efficacy obtained with fecal transplants from C57BL/6 Jackson mice (159). Altogether, our findings showing that ARF can preserve intestinal homeostasis suggests that despite being compositionally different, ARF exerts the same protective effect as the C57BL/6 microbiota.

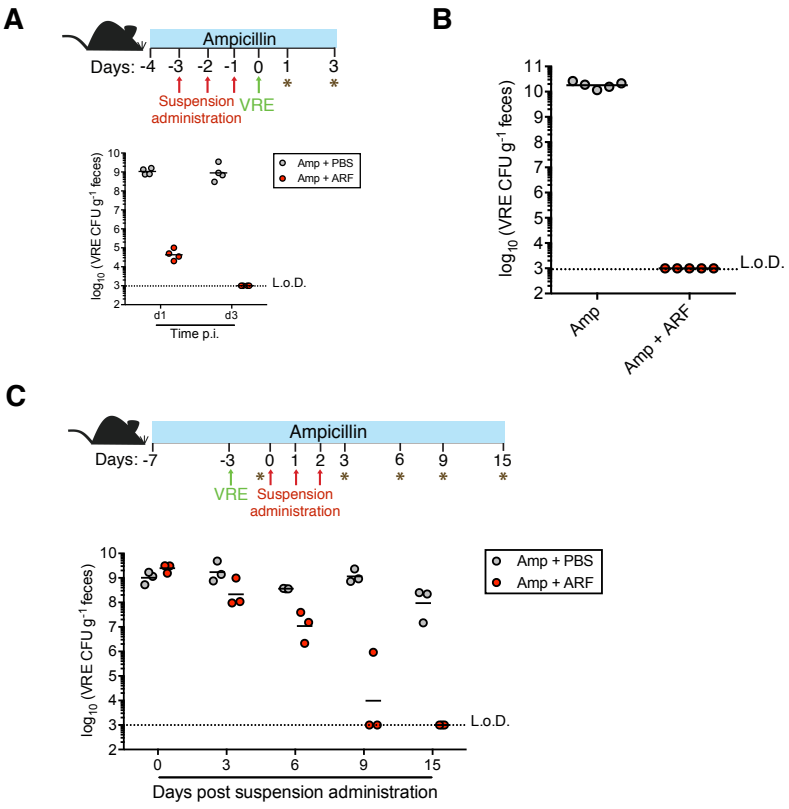


Figure 3.5. ARF promotes resistance against vancomycin-resistant Enterococcus (VRE) during antibiotic pressure. (**A**) Colonization resistance against VRE following PBS or ARF inoculation into antibiotic-treated mice. Fecal samples, denoted by *, were collected on days 1 and 3 post infection (p.i.) to quantify VRE intestinal expansion. (**B**) Fecal pellets from ampicillin-treated and ARF-reconstituted ampicillin-treated mice were inoculated with VRE and cultured for 16-24 hours to assess VRE growth. (**C**) VRE decolonization following PBS or ARF inoculation. Fecal samples (*) were collected before suspension administration (day 0) and on days 3, 6, 9 and 15 after the first dose to monitor VRE levels. L.o.D., limit of detection.

3.2.4. Collective antibiotic resistance is mediated by a few β-lactamase-producing bacterial strains within ARF

Evidence from *in vitro* studies has demonstrated that bacterial populations may owe their ability to survive antibiotic pressure to minor members with the capacity to resist killing by antibiotics. Although this effect can be direct, as in production of antibiotic-degrading enzymes (i.e. β-lactamases), resistant cells can also produce factors such as the cell-signaling molecule indole that allow their susceptible counterparts to withstand antibiotic stress (203,210,211). These observations prompted us to investigate whether the antibiotic resistance capability of ARF was a collective rather than individual endeavor. To address this, we plated a 10^5-fold diluted suspension of fecal ARF on plates containing increasing concentrations of ampicillin ranging from 0 to 0.5 mg/ml and examined the bacterial composition of each plate. We identified about 15 distinct bacterial on plates containing no ampicillin, which corresponded to about 20% of the biodiversity of ARF (**Figure 3.6A-B**). However, this diversity was lost at ampicillin concentrations as low as 0.01 mg/ml at which mostly *Parabacteroides distasonis*, *Clostridium bolteae* and some *Bacteroides* spp. (*B. sartorii, B. uniformis, B. acidifaciens*) were able to grow. The number of unique organisms was further reduced at higher ampicillin concentrations where an enrichment for *P. distasonis*, *B. sartorii* and *B. uniformis* was observed (**Figure 3.6A-B**).

We hypothesized that inoculation of bacteria grown under non-selective conditions into antibiotic-treated mice would lead to expansion of the ampicillin-resistant fraction and reduction of the ampicillin-sensitive one. Following our transplantation model, we administered mice three daily doses of the contents from each plate (Input) beginning on their second day of

ampicillin treatment and collected fecal samples 24 hours after the last dose (Output). Almost all Input bacteria underwent shifts in vivo but the ampicillin-sensitive fraction remained a significant portion of the population. In addition, a trend towards higher *C. bolteae* relative abundance in the output was observed for all the ampicillin concentrations that supported *C. bolteae* growth while *P. distasonis* and *B. sartorii*, which constituted over 90% of bacteria found on plates containing ampicillin concentrations of 0.1 mg/ml and higher remained the predominant populations in vivo (**Figure 3.6C**). Furthermore, we assessed whether the loss of ampicillin-sensitive bacteria would impact colonization resistance against VRE as a measure of normal intestinal function. We infected ampicillin-treated mice that had been inoculated with the different plate cultures and found that colonization resistance had been abrogated in all mice that had received bacteria grown under ampicillin selection, regardless of concentration (**Figure 3.6D**).

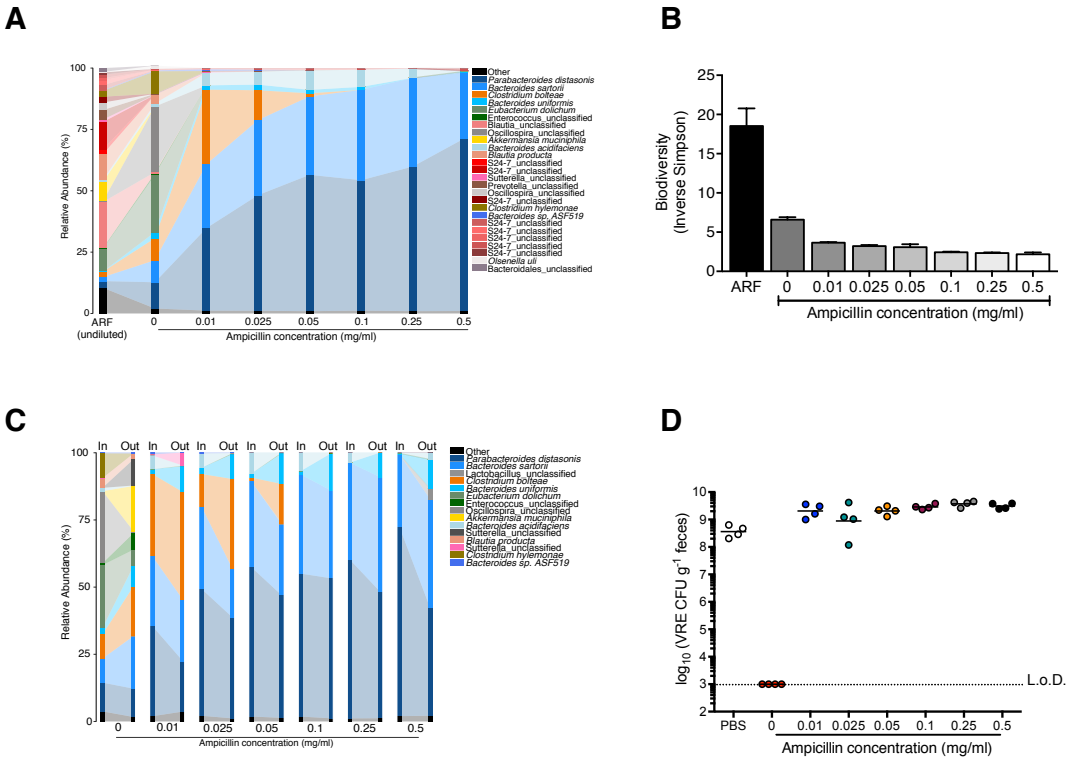


Figure 3.6. A minority of bacteria within ARF confers population-wide antibiotic resistance. Fecal suspensions from MyD88$^{-/-}$ mice were diluted 10^5-fold and grown on plates containing 0, 0.01, 0.025. 0.05, 0.1, 0.25 and 0.5 mg/ml of ampicillin. (**A**) Bacterial composition and (**B**) diversity of plate cultures. Data is representative of 3 samples. (**C**) Shifts in microbial community composition following transfer *in vivo*. Ampicillin-treated mice were gavaged with pooled bacterial contents from each plate (Input/In) daily for three days starting one day following antibiotic therapy. Fecal samples were collected from each mouse 24 hours after administration of the third bacterial dose (Output/Out). Each stacked bar (Out) represents the average of 4 separately-housed mice. (**D**) Assessment of colonization resistance against VRE with plate cultures grown under various antibiotic concentrations. Mice from (C) were infected with VRE 24 hours after administration of the third bacterial dose and VRE CFU were quantified three days p.i. L.o.D., limit of detection.

Resistance to β-lactams is primarily due to degradation of the β-lactam ring by β-lactamases (212). Using the chromogenic cephalosporin substrate, which changes from yellow to red when cleaved (213), we observed β-lactamase activity in fecal samples from MyD88$^{-/-}$ but not C57BL/6 mice (**Figure 3.7A**). Consistent with the results of our ampicillin susceptibility test, cultures from *P. distasonis*, *B. sartorii* and *B. acidifaciens* isolated from ARF exhibited strong β-lactamase activity whereas none was detected in cultures from a *Blautia producta* isolate (**Figure 3.7B**). Our observations demonstrate that culturable anaerobic bacteria comprising roughly 5% of the bacterial population in ARF are highly resistant to ampicillin and produce ampicillin-degrading enzymes that allow the survival of the ampicillin-sensitive fraction responsible for providing colonization resistance.

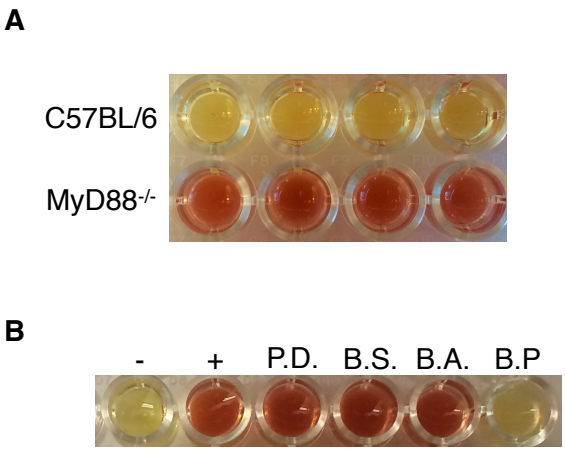


Figure 3.7. β-lactamase production by ARF and select isolates. β-lactamase activity of (**A**) fecal samples from MyD88$^{-/-}$ and C57BL/6 mice and (**B**) isolates cultured from MyD88$^{-/-}$ feces. P.D. *Parabacteroides distasonis*; B.S., *Bacteroides sartorii*; B.A., *Bacteroides acidifaciens*; B.P., *Blautia producta;* - , negative control (PBS); + , positive control (Amp^{R} E.coli DH5α).

3.3. DISCUSSION

Although antibiotics have proved critical in the fight against infection, they also exert widespread collateral damage to the body's microbiota. Even short courses of antibiotic therapy can induce long-lasting perturbations in the gut microbiota including loss of bacterial species and selection for antibiotic-resistant strains. This poses a serious health problem since loss of colonization resistance resulting from antimicrobial treatment can promote the intestinal expansion of disease-causing drug-resistant organisms (14,82,158). Because of this, it may seem counterintuitive to envision positive outcomes following long-term antibiotic therapy. Our study is, to the best of our knowledge, the first to demonstrate that continuous antibiotic exposure of laboratory mice for generations spanning 10+ years can result in a unique, complex and functional antibiotic-resistant microbiota. In our laboratory, treatment of MyD88$^{-/-}$ animals with amoxicillin and clavulanic acid was used as a measure to prevent spontaneous infection. Clavulanic acid enhances the efficacy of amoxicillin by inactivating β-lactamases produced by amoxicillin-resistant organisms and are often used in combination during antibiotic therapy (214). Since fecal samples were not collected prior and during antibiotic exposure, we cannot definitively know when and how resistance took place, how the MyD88$^{-/-}$ microbiota shifted during 15 years of antibiotic therapy and whether a reservoir for ampicillin-resistant strains existed prior to therapy. Despite these unknowns, our findings reveal that the MyD88$^{-/-}$ microbiota is composed of a diverse bacterial community that it's characterized by an overabundance of S24-7 (*Barnesiella*) species and low abundance of Clostridia. Although several studies have linked microbiota composition to host genotype, previous work from our laboratory revealed that compositional

differences among mice lacking distinct arms of innate immunity are a consequence of long-term breeding in isolation and vertical transmission rather than genetic mutation (78). Therefore, the unique microbiota of MyD88$^{-/-}$ animals is largely a result of husbandry conditions (i.e. exposure to antimicrobial agents) and not MyD88 deficiency.

The most striking aspect of our study was the discovery that after 15 years of antibiotic treatment, most of the MyD88$^{-/-}$ microbiota had remained susceptible to the antibiotic whereas a minor fraction was highly resistant. This phenomenon was termed 'bacterial cheating' by Lee et al. in a study showing that over time, *E.coli* cultures were able to withstand high norfloxacin concentrations even though individual clones were vulnerable to the antibiotic (203). In these scenarios, antibiotic-sensitive bacteria can be thought of as 'cheaters' since they benefit from the activity of antibiotic-resistant bacteria and may grow faster than the resistant population once the antibiotic has been inactivated (202,210). Although charity and cheating can be explored in great detail in homogenous populations, the complexity of the microbiota makes these processes difficult to study in vivo. We overcame this problem by culturing the MyD88$^{-/-}$ microbiota under rich, anaerobic conditions in the presence of increasing ampicillin concentrations. By doing this, we loss most of the diversity found on ampicillin-negative cultures suggesting that the majority of bacteria that make up the MyD88$^{-/-}$ microbiota cannot survive antibiotic stress in isolation.

The concept of charity and/or cheating implies that one population is responsible for the production of 'public goods' that other bacteria can benefit from without reciprocating. In this scenario, β-lactamases produced by *Bacteroides* and *Parabacteroides* spp. to protect themselves from antibiotic

toxicity became an asset to the entire bacterial community. These organisms, although normally present in small frequencies, can colonize the murine intestinal tract at densities comparable to that of in-vitro cultures indicating that the presence of the antibiotic-sensitive fraction is not required for their survival or maintenance. Based on these observations, it appears that in our model coexistence is a one-way street involving the degradation of ampicillin in the intestinal lumen by hydrolytic enzymes produced by a subset of the population, a process that, inadvertently but efficiently, ensures the survival of the remainder microbiota. One explanation for the limited number of ampicillin-resistant species despite the length of treatment is that antibiotic-resistant genes were present in these bacterial groups even before therapy. While resistance to ampicillin/amoxicillin is a common occurrence among *Bacteroides* and *Parabacteroides*, resistance to β-lactamase inhibitors such as clavulanic acid is rare (215-217). Nevertheless, administration of amoxicillin in combination with β-lactamase-inactivating agents has been significantly associated with the emergence of resistant strains in the clinic (218,219). Therefore, it is possible that *Parabacteroides* and *Bacteroides* spp. harbored ampicillin-resistance genes at the time of antibiotic treatment but addition of clavulanic acid selected for mutants that could grow in the presence of the β-lactamase inhibitor. In this way, continued antibiotic therapy may have pushed these bacterial groups to become increasingly resistant and produce large amounts of β-lactamases lowering the ampicillin concentration in the intestinal lumen, an event that may have taken place at the initial stages of treatment and prevented the development of resistance in other anaerobic organisms. The finding that within a community of hundreds of species *Bacteroides* and *Parabacteroides* are the only highly resistant strains after 15 years of antibiotic

treatment is contrary to the idea that the presence of antibiotic-resistant bacteria enhance horizontal transmission of antibiotic determinants the intestine (220). Although horizontal gene transfer is an important clinical and microbiological problem, most antibiotic-resistance genes in *Bacteroides* and *Parabacteroides* are chromosomally encoded (216) and their location may have prevented resistance from spreading to other organisms within the community.

Despite the difference in ampicillin susceptibility we show that by adoptively transferring suspensions from MyD88$^{-/-}$ stool samples into mice undergoing ampicillin therapy, many of the adverse effects caused by ampicillin including enlargement of the cecum, decreased production of mucins and antimicrobial peptides and loss of colonization resistance were reversed in all animals that received fecal transplants. Importantly, our findings demonstrate that the ampicillin-sensitive fraction is the one exerting such recovery, supporting the idea that the role of resistant *Bacteroides* and *Parabacteroides* spp. in this process is the production of ampicillin-degrading enzymes that keep the rest of the microbiota alive. This observation implies that administration of β-lactamase-producing strains during antibiotic treatment may help protect the intestinal microbiota and preserve gut function. In fact, studies have shown that oral administration of β-lactamase enzymes, a β-lactamase-containing microbiota, or a cephalosporinase-producing strain of Bacteroides thethaiotamicron can preserve colonization resistance in otherwise susceptible animals (221-223). These findings together with ours suggest that administration of β-lactamase-producing bacteria may have important clinical implications and. On the one hand, the presence of β-lactamase-producing *Bacteroides* may preserve the microbiota and many of its

functions in the face of antibiotic treatment. Such prophylactic treatments would provide a mechanism by which bloodstream infections could be treated with antibiotics while sparing the intestinal microbiota except in situations when non-β-lactam antibiotics are used. On the other hand, there is the potential for these organisms to become pathogenic in which case their treatment may be limited given their level of resistance. In addition, ampicillin-sensitive infectious agents may take advantage of the detoxification of the intestinal lumen and potentially cause disease.

In conclusion, our study shows that years of ongoing therapy with combination of a β-lactam agent with a β-lactamase inhibitor resulted in the development of a complex, functional and stable microbiota capable of withstanding antibiotic pressure collectively but not independently. Whether this can occur in patients undergoing long-term antibiotic therapy is unknown. The fact that our mice were subjected to antibiotic treatment for multiple generations makes it unlikely that the same level of resistance, diversity and functionality may arise in humans. Nonetheless, the generation of a bigger pool of antibiotic-resistant strains is a possibility. In our model, collective antibiotic resistance resulted from the activity of a small percentage of resistant bacteria comprising species of the *Bacteroides* and *Parabacteroides* genera that provided β-lactamase-mediated protection of the susceptible bacterial compartment and in this way, helped maintain normal gut function.

3.4. MATERIALS AND METHODS

3.4.1. Mouse strains, adoptive transfer and VRE infection. 16 week-old MyD88$^{-/-}$ male mice from our facility and age, gender-matched C57BL/6 mice purchased from Jackson Laboratories were used for taxonomic comparisons. For experiments involving microbiota transplantation and VRE infection, 6-8 week old female mice from Jackson Labs were treated with 0.5g/L ampicillin (Fisher) in their drinking water made fresh every 4 days. Fecal transplants were prepared by resuspending fresh stool pellets from a MyD88$^{-/-}$ donor in 1 ml pre-reduced PBS. 200µl of this suspension was administered into mice by oral gavage daily for 3 days. For VRE infection, 5x10^4 CFU of vancomycin-resistant *Enterococcus faecium* (ATCC 700221) were administered to mice by oral gavage in a 200µl volume. Mice were single-housed at the time of infection and kept on ampicillin until the end of the experiment. For VRE quantification, tenfold dilutions of pre-weighed fecal samples resuspended in PBS were plated on Difco Enterococcosel agar (supplemented with 8 µg/ml vancomycin; Novaplus and 100 µg/ml streptomycin; Fisher).

3.4.2. Bacterial DNA extraction. Intestinal samples were frozen in a dry ice/ethanol slurry immediately after collection and stored at –80 °C. Samples were resuspended in 500 µl of an extraction buffer (200 mM Tris pH 8.0/200 mM NaCl/20 mM EDTA), 200 µl of 20% SDS, 500 µl of phenol:chloroform:isoamyl alcohol (24:24:1) and 500 µl of 0.1-mm diameter zirconia/silica beads (BioSpec Products). Cells were lysed by mechanical disruption using a bead beater (2 min). DNA was extracted using a phenol/chloroform/isoamyl solution twice and precipitated with ethanol and

sodium acetate. DNA was resuspended in 200 µl of TE buffer containing 100 µg/ml RNase, purified with QIAmp Mini Spin Columns (Qiagen) and eluted in 100 µl water.

3.4.3. 16S rRNA amplification, multiparallel sequencing and sequence analysis. The V4–V5 region of the 16S rRNA gene was amplified and sequenced on an Illumina MiSeq platform using primers 563F (59-nnnnnnnn-NNNNNNNNNNN-AYTGGGYDTAAAGN G-39) and 926R (59-nnnnnnnn-NNNNNNNNNN-CCGTCAATTYHTTTR AGT-39). The PCR reaction consisted of 50 ng of purified DNA, 0.2 mM dNTPs, 1.5 µM $MgCl_2$, 1.25 U Platinum TaqDNA polymerase, 2.5 µl of 10X PCR buffer and 0.2 µM of each primer. A unique 12-base Golay barcode (Ns) preceded the primers for sample identification after pooling amplicons. One to eight additional nucleotides were added before the barcode to offset the sequencing of the primers. The cycling conditions were: 94 °C (3 min), 27 cycles of 94 °C (50 s), 51 °C (30 s) and 72 °C (1 min) followed by a final elongation step at 72 °C (5 min). Replicate PCRs were pooled and purified using the Qiaquick PCR Purification Kit (Qiagen) and Qiagen MinElute PCR Purification Kit. PCR products were quantified using the Illumina TruSeq Sample Preparation procedure and combined at equimolar amounts before Illumina barcodes and adaptors were added. The completed library was sequenced on an Ilumina Miseq platform as per Illumina specifications. Sequence analysis was performed using the mothur pipeline (version 1.33.3). Sequences were aligned using the Silva reference alignment as a template and chimeric sequences were eliminated using UCHIME (224). Sequences with a distance-based similarity of ≥97% were binned into OTUs using the furthest-neighbour algorithm. OTUs were classified using a modified

Greengenes 16S rRNA reference database [45]. OTU-based biodiversity was calculated by Inverse Simpson index. OTUs at relative abundance >0.01% were plotted.

3.4.4. Quantification of bacterial density by qPCR. Copies of the 16S rRNA gene were determined by performing quantitative PCR on total DNA extracted from fecal samples using primers specific to the V4 region of the 16S gene, 517F (5'-GCCAGCAG CCGCGGTAA-3') and 798R (5'-AGGGTATCTAATCCT-3'), at 0.2 μM concentration with the DyNAmo SYBR green RT-PCR kit (Finnzymes). Standard curves were prepared by serial dilution of the PCR blunt vector (Invitrogen) containing a single copy of the 16S rRNA gene. The cycling protocol was: 95 ^{o}C for 10 min, followed by 40 cycles of 95 ^{o}C for 30 s, 52 ^{o}C for 30 s, and 72 ^{o}C for 1 min.

3.4.5. FISH and Muc2 Immunofluorescence. Tissues were fixed in Methacarn solution (60% methanol, 30% chloroform and 10% acetic acid) to preserve intestinal architecture. Co-staining for bacteria and Muc2 was performed as previously described (175) using the universal EUB338 bacterial probe and anti-Muc2 rabbit polyclonal antibody (H300, Santa Cruz). Image acquisition was performed with a Leica TCS SP5-II upright confocal microscope using a 63x oil immersion lens as a series of short Z-stacks. Fiji (ImageJ) software was used for maximum intensity Z-stack projection.

3.4.6. Western blot analysis. A 2-cm segment from the distal portion of the small intestine was excised and stored in RNA stabilization solution (RNAlater) at 4^{o}C prior to homogenization with Trizol reagent (Invitrogen). Total protein

was extracted according to the manufacturer's specifications and resuspended in a solution of 8 mol/L urea, 1% sodium dodecyl sulfate (SDS) and 0.15 mol/L Tris-HCl at pH 7.5. Equal amounts of protein were denatured, loaded onto a 4-12% SDS-polyacrylamide electrophoresis gel (Nupage Bis-Tris gel, Invitrogen) and transferred to a nitrocellulose membrane. Protein blots were incubated with rabbit polyclonal RegIIIγ specific-antiserum and mouse anti-β-tubulin (Santa Cruz Biotechnology) antibodies, followed by horseradish peroxidase-conjugated anti-rabbit (GE Healthcare) and anti-mouse (Santa Cruz technology) antibodies.Protein bands were detected using chemiluminescence (GE Healthcare).

3.4.7. *Ex vivo* VRE suppression assay. Fecal samples from C57BL/6 ampicillin-treated mice that received 3 consecutive doses of ARF or PBS were collected, transferred to an anaerobic chamber and resuspended in reduced PBS at a concentration of 25mg/ml. VRE ($5x10^3$ CFU) or PBS was added to each sample and incubated at 37 ^oC anaerobically. VRE growth was assessed 16-24 hours later by plating on selective media.

3.4.8. β-lactamase detection assay. Fecal pellets from MyD88$^{-/-}$ and C57BL/6 animals were collected and resuspended in PBS at 25mg/ml. Samples were left undisturbed for 5 minutes to allow particulate matter to sediment. 100µl of the suspension was pipetted into a 96-well plate with 50µl of nitrocefin (0.2 mg/ml, Fisher) and incubated for 30 minutes at room temperature while protected from light. For detection of β-lactamase activity from individual isolates, 100µl from a 3-day anaerobic culture corresponding to 10^6 CFU was used.

3.4.9. ARF cultures and ampicillin susceptibility assay. Fecal samples from MyD88$^{-/-}$ mice were collected, transferred immediately to an anaerobic chamber, resuspended in 1 ml pre-reduced PBS. Fecal suspensions were titered by culturing 100µl from 10-fold serial dilutions on Columbia agar plates supplemented with 5% sheep blood and ampicillin concentrations in the 0-0.5mg/ml range. Plate cultures were incubated anaerobically at 37^{o}C for 3-5 days, bacterial colonies were scraped off the plates and resuspended in 5 ml pre-reduced PBS. 500µl of culture suspensions were removed for sequencing analysis and stored at -80^{o}C. For inocula preparation, glycerol was added to the remaining cultures at a final concentration of 15% and aliquoted before freezing at -80^{o}C.

3.4.10. Statistics. All statistical tests were performed with the unpaired Student's t test using the Graph-Pad Prism software package (version 6.0).

CHAPTER 4

DEFINED BACTERIOTHERAPY RESTORES RESISTANCE AGAINST VANCOMYCIN-RESISTANT ENTEROCOCCUS

4.1. INTRODUCTION

For many years, the prevention and treatment of nosocomial infections have relied heavily on the use of antimicrobial agents with varying spectra of activity. Despite the effectiveness of antibiotics in the treatment of infection (167), this practice has become increasingly less popular due to their impact on non-target, intestinal commensals and the potential for the expansion of multi-drug resistance pathogens (145). For instance, treatment of *Clostridium difficile*-infection (CDI) with metronidazole or vancomycin increases the risk for colonization by vancomycin-resistant Enterococcus and multi-drug resistant Enterobacteriaceae in humans and laboratory animals (194,225). Increased susceptibility to infection and colonization is a direct consequence of antibiotic-mediated depletion of beneficial microbes whose role is to mediate resistance against pathogens (14,154,168).

The detrimental effects caused by antibiotics have accelerated the search for safer and more efficient ways to treat intestinal infections. A therapy that has had tremendous clinical success in the treatment of CDI is fecal microbiota transplantation (FMT), a method that involves the adoptive transfer of feces from a healthy donor into afflicted individuals by stool enemas, colonoscopy or duodenal administration (226). Compared to conventional antibiotic therapies where the rate of efficacy was only 23-31%, the rate of CDI resolution with FMT was 81% after a single naso-duodenal dose and 94%

after a second one (174) while in patients receiving FMT through colonoscopy, a 91% cure rate was achieved within 90 days post transplant (227). Although FMT is most commonly used for the treatment of CDI, a case report showed that it can also eliminate colonization by other nosocomial pathogens, including VRE (228). In fact, murine studies have shown that VRE can be effectively eliminated with fecal transplants from a healthy, antibiotic-naïve mouse (159,176) or as discussed in Chapter 3 of this dissertation, a diverse antibiotic-resistant flora.

Despite its efficacy, a major caveat of FMT is the transmission of pathogens (bacteria, fungi or viruses) that may be undetectable at the time of transplant or have yet to be identified (161,226). Such risks can be prevented by a more targeted approach involving the identification of specific bacterial strains that mediate resistance. Several groups have made progress in this area in the context of murine CDI. One group found that a member of the Lachnospiraceae family could reduce *C. difficile* burden 100-fold (229) while another showed that a mixture of 6 distinct bacterial strains reduced *C. difficile* to levels below detection 15 days post treatment (230). More recently, it was demonstrated that *Clostridium scindens*, a Clostridia species from cluster XIVa, can significantly reduce *C. difficile* expansion, an inhibitory effect that was enhanced when administered in combination with three other strains (145). Despite these promising findings, commensal bacteria responsible for resistance against other intestinal pathogens such as VRE remain to be identified.

To address this, we expanded our preliminary findings showing that a culturable fraction of an ampicillin-resistant flora (ARF) could prevent colonization by VRE (Chapter 3). Using the dilution method as a strategy to

induce variability in protection, we identified a community of bacteria that were significantly correlated with resistance. Resistance-associated strains were isolated, assembled into different consortia and adoptively transferred into antibiotic-treated mice. The results presented here revealed that a two-bacteria consortium consisting of members of Clostridia cluster XIVa, *Clostridium bolteae* and *Blautia producta*, exert complete colonization resistance against VRE and eliminate established VRE colonization.

4.2. RESULTS

4.2.1. Select bacterial communities are associated with resistance to VRE

Our previous findings that plate cultures from a 10^5-fold diluted ARF suspension provided full colonization resistance against VRE suggested that bacterial species responsible for VRE protection could be cultured in vitro under non-selective, anaerobic conditions. To identify resistance-associated organisms, we performed an additional 1/10 dilution of ARF to reduce community complexity further and generate variability in VRE protection. Compared to the 10^{-5} dilution, increased abundance levels of *Bacteroides* spp. and an unclassified *Blautia* OTU were recovered from the 10^{-6} dilution cultures. In addition, we detected slightly higher levels of OTUs belonging to the S24-7 family in the 10^{-6} dilution while other OTUs, including one corresponding to *Clostridium hylemonae*, were only present in the 10^{-5} dilution or undiluted ARF suspensions (**Figure 4.1A**). We harvested bacterial cultures from single 10^{-5} or 10^{-6} dilution plates, gavaged them into antibiotic-treated mice for three consecutive days beginning on the second day of ampicillin treatment and infected mice with VRE the day after the last bacterial or PBS dose. As shown previously, mice that received the 10^{-5}-dilution cultures had undetectable VRE levels at day 3 p.i. whereas PBS-treated animals were densely colonized. Consistent with our predictions, variability in colonization was observed in mice gavaged with cultures from the 10^{-6} dilution, suggesting the loss of key bacterial species (**Figure 4.1B**).

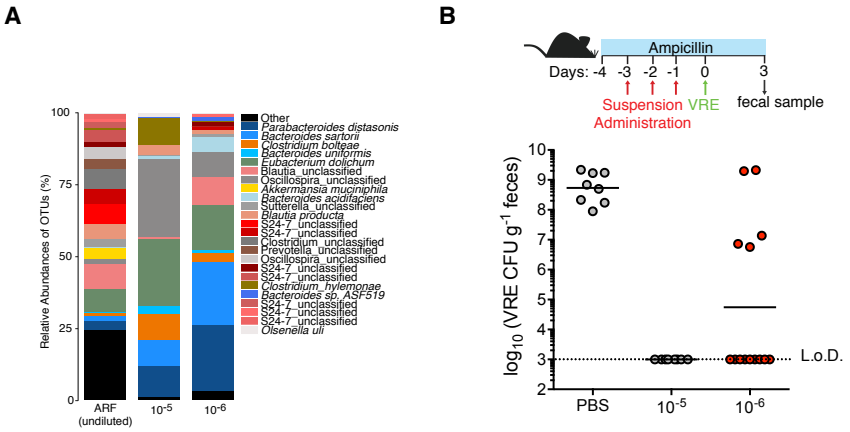


Figure 4.1. Adoptive transfer of cultured ARF exerts varying degrees of colonization resistance against VRE (**A**) Bacterial composition of MyD88$^{-/-}$ fecal microbiota (ARF) and plate cultures from 10^{5}- and 10^{6}- fold ARF dilutions. Each stacked bar corresponds to an individual sample (n=3 per group). OTU, operational taxonomical unit (≥ 97% similarity, relative abundance > 0.01%) (**B**) Ampicillin-treated mice were inoculated with three daily doses of PBS, 10^{-5} or 10^{-6} dilution cultures beginning one day after antibiotic initiation and infected with VRE 24 hrs after the last dose. Colonization resistance was assessed 3 days p.i. (n=8-14 per group). L.o.D., limit of detection.

As expected, antibiotic-treated mice that received PBS instead of a bacterial suspension exhibited markedly lower biodiversity than the pre-antibiotic state. However, this reduction in diversity was significantly increased following administration of bacterial cultures from both the 10^{-5} and 10^{-6} dilutions (**Figure 4.2A**). Low bacterial diversity increases the likelihood of intestinal infection and colonization (158,159). Although this was indeed the case for all PBS-treated animals, within the 10^{-6} dilution group colonization resistance was observed in mice harboring a microbiota less diverse than that of some susceptible mice (**Figure 4.2B**). The finding that low biodiversity does not always correlate with infection susceptibility was previously reported (145) and suggests that the presence of specific bacterial species, rather than the degree of diversity, is the determinant factor in preventing colonization. To identify candidate bacteria, we stratified mice from the 10^{-6} dilution group by their VRE density and plotted the abundance of the top 20 OTUs on a heat map (**Figure 4.2C**). While most OTUs were present in all resistant mice at

various frequencies, several of them including members of the Clostridia (*Blautia* spp., *Oscillospira* spp. and *Clostridium bolteae*), Mollicutes (*Eubacterium dolichum*), Bacteroidia (*Bacteroides* spp., S24-7 and Rikenellaceae families) and β-proteobacteria (*Sutterella* spp.) classes were undetectable in susceptible animals (**Figure 4.2C**). Of these, we identified 8 bacterial OTUs that correlated significantly with resistance to VRE colonization according to the Spearman's rank correlation test (**Figure 4.2D**). Importantly, the second OTU most strongly correlated with resistance was an unclassified strain from the S24-7 family also known as *Barnesiella*, confirming our previous findings that a *Barnesiella*-containing microbiota was significantly associated with VRE clearance (159).

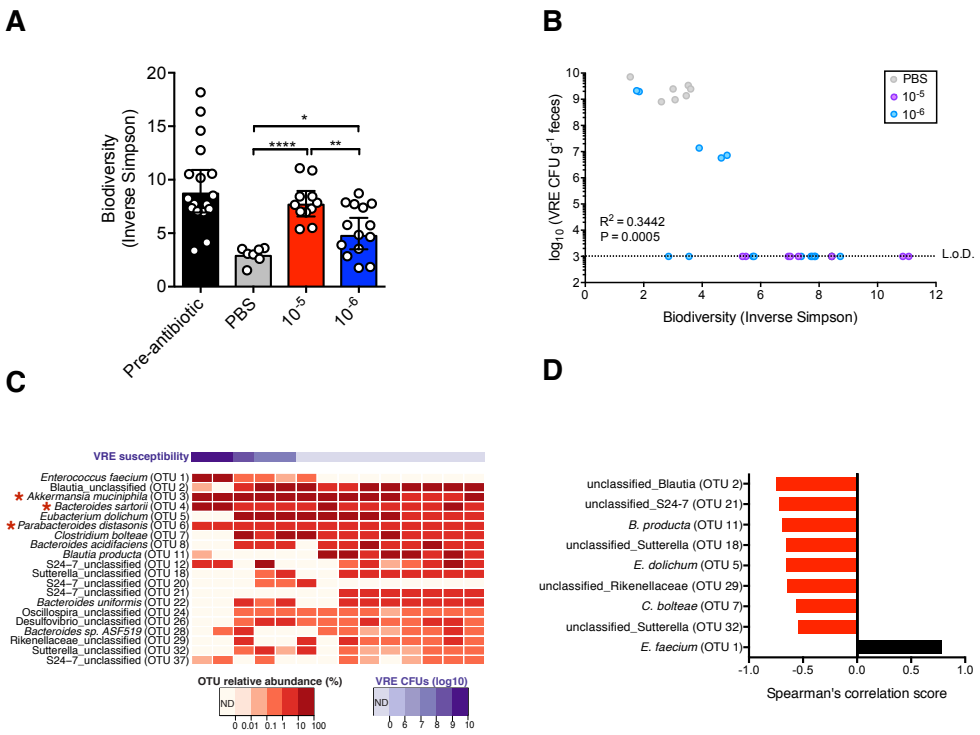


Figure 4.2. Specific bacterial groups correlate with protection against VRE. (**A**) Alpha-diversity of the fecal microbiota of antibiotic-naive mice and ampicillin-treated mice inoculated with three doses of PBS, 10^{-5} or 10^{-6} dilution cultures. Error bars (mean ± SEM). *P=0.0193, **P=0.0071, ****P<0.0001, by the Student's *t* test. (**B**) Linear correlation between VRE levels and biodiversity. L.o.D., limit of detection. (**C**) Relative abundance of the top 20 OTUs (>0.01% relative abundance) and corresponding VRE susceptibility 3 days p.i. * denotes high abundance OTUs not significantly associated with protection but present in all mice. (**D**) Correlation of OTUs with resistance to VRE colonization. OTUs with P values < 0.05 are plotted.

4.2.2. Defined resistance-associated bacterial groups restore colonization resistance against VRE

In order to isolate bacterial strains responsible for VRE protection, we picked 250 colonies from 10^{-5} dilution plates, the highest dilution that was able to re-establish resistance in all mice. Sanger sequencing and BLAST classification of the 16S rRNA gene of isolated colonies revealed the presence of at least 19 distinct bacterial species belonging to the Bacteroidaceae, Clostridiaceae, Porphyromonadaceae, Coriobacteriaceae, Rikenellaceae, Lachnospiraceae, unclassified_Clostridiales and Verrucomicrobiaceae families with considerable redundancy among isolates (**Table 4.1**). To determine whether any of the 8 resistance-associated OTUs were present in our library, we aligned Miseq and Sanger sequencing reads and found 100% sequence similarity between 4 OTUs and some of our isolates, namely unclassified *Blautia, Blautia producta, Clostridium bolteae* and *Eubacterium dolichum*, whose 16S sequence was identical to that of isolates classified as *Clostridium innocuum*. Of note, we detected OTUs corresponding to *Akkermansia muciniphila, Bacteroides sartorii* and *Parabacteroides distasonis* at very high densities in susceptible and resistant animals alike (**Figure 4.2C**). These organisms are well known for their ability to digest complex polysaccharides, including mucin (172,231). In addition, we previously showed that *B. sartorii* and *P. distasonis* are highly resistant to ampicillin. Therefore, even though these OTUs did not reach statistical significance in our correlation analysis, we hypothesized that they could potentially play an important role in the establishment and maintenance of resistance-associated strains by providing a direct source of nutrients and inactivating ampicillin.

TABLE 4.1. 16S rRNA gene analysis of isolated strains. The full length of the 16S ribosomal RNA gene was amplified by colony-PCR . Amplified 16S genes were Sanger-sequenced and classified using the 16S ribosomal RNA Sequence Database in BLAST. Close relative species, % similarity and the similarity to resistance-associated OTUs are shown.

Strain	Sequenced length (bp)	Close relatives	Similarity (%)	Match length (bp)	Isolated clones	OTU similarity (100 %)
Strain_01	912	Bacteroides sartorii strain JCM 17136 Bacteroides chinchillae strain JCM 16487	99 99	909 907	9	**OTU 4**
Strain_02	886	Blautia producta strain JCM 1471 Blautia coccoides strain JCM 1395 Blautia producta strain ATCC 27340	98 98 98	867 867 865	15	**OTU 11**
Strain_03	833	Clostridium innoccuum strain B-3 Eubacterium dolichum strain JCM 10413	97 92	805 769	17	**OTU 5**
Strain_04	916	Bacteroides acidifaciens strain JCM 10556 Bacteroides acidifaciens strain A-40	98 98	896 895	5	
Strain_05	914	Parabacteroides distasonis strain ATCC 8503 Parabacteroides distasonis strain JCM 5825	98 98	892 892	56	**OTU 6**
Strain_06	911	Parabacteroides goldsteinii strain JCM 13446 Parabacteroides goldsteinii strain WAL 12034	99 98	902 877	3	
Strain_07	892	Akkermansia muciniphila strain ATCC BAA-835 Akkermansia muciniphila strain Muc2	99 99	882 881	2	**OTU 3**
Strain_08	881	Clostridium hylemonae strain TN-272 Clostridium hylemonae strain TN-271	95 96	838 789	12	
Strain_09	838	Clostridium bolteae strain JCM 12243 Clostridium bolteae strain 16351	99 98	826 825	3	**OTU 7**
Strain_10	890	Bacteroides uniformis strain JCM 5828 Bacteroides rodentium strain JCM 16496	99 99	887 884	10	
Strain_11	858	Flavonifractor plautii strain 265 Flavonifractor plautii strain Prevot S1	99 99	853 851	32	
Strain_12	873	Enterococcus gallinarum strain LMG 13129	99	865	1	
Strain_13	462	Anaerostipes caccae strain L1-92	97	447	7	
Strain_14	457	Erysipelatoclostridium ramosum strain JCM 1298	99	456	4	
Strain_15	1069	Blautia schinkii strain B Blautia glucerasea strain HFTH-1	94 94	1006 954	3	**OTU 2**
Strain_16	958	Barnesiella viscericola	82	784	10	
Strain_17	921	Barnesiella intestinihominis strain JCM 15079	88	811	4	
Strain_18	919	Alistipes senegalensis strain JC50	95	874	1	
Strain_19	1015	Olsenella umbonata strain lac31 Olsenella uli strain DSM 7084	94 94	950 935	5	

Using this rationale, we assembled a suspension of 7 ARF isolates: unclassified *Blautia, B. producta, C. bolteae, E. dolichum, A. muciniphila*, *B. sartorii* and *P. distasonis*. To examine the potential for this bacterial cocktail to restore colonization resistance during antibiotic treatment, we used our well-described transplantation model and orally administered a mixture of the 7 strains (7-mix) or PBS into mice for three consecutive days beginning on their second day of ampicillin, infected them with VRE the day following the last gavage dose and collected fecal samples 1, 3 and 6/8 days p.i to monitor colonization. Out of 10 mice that received the 7-mix, 8 had undetectable VRE levels as early as 1 day p.i. and 2 had a VRE burden of 10^6-10^8 that had decreased by 2-logs on day 6/8 p.i. whereas VRE levels in PBS-treated animals ranged between 10^8-10^{10} CFU at all time points p.i. (**Figure 4.3A**)

To assess the extent by which each strain in the 7-mix contributed to colonization resistance, we treated mice with mixtures containing all but one bacterium. We excluded *A. muciniphila, E. dolichum*, *unclassified Blautia, C. bolteae* or *B. producta* but kept *P. distasonis* and *B. sartorii* in every group for their ampicillin-degrading capacity. We found that exclusion of unclassified *Blautia* or *E. dolichum* did not impact the protection ability exerted by the remaining strains whereas an intermediate effect was observed in the absence of *A. muciniphila* (**Figure 4.3B**). Remarkably, complete loss of colonization resistance was observed in mice gavaged with bacterial mixtures deficient in *C. bolteae* or *B. producta* (**Figure 4.3B**). These findings indicated that within our 7 bacterial consortium, unclassified *Blautia* and *E. dolichum* were not directly involved in colonization resistance and were therefore excluded from future experiments, while on the other hand *C. bolteae* and *B. producta* appeared to be critical for the 7-mix protective effects against VRE. To further examine this, we treated mice with different combinations of the remaining 5 strains and found that a combination of *C. bolteae, B. producta, P. distasonis* and *B. sartorii* was the smallest consortium that fully restored colonization resistance in antibiotic-treated animals (**Figure 4.3C**). Subsets from this 4-mix showed that mice colonized with *P. distasonis* and *B. sartorii* or *C. bolteae* and *B. producta* exhibited VRE levels comparable to that of control animals suggesting that the β-lactamase-producing strains do not, on their own, inhibit VRE colonization but are required for the survival of ampicillin-sensitive *C. bolteae* and *B. producta* (**Figure 4.3C**).

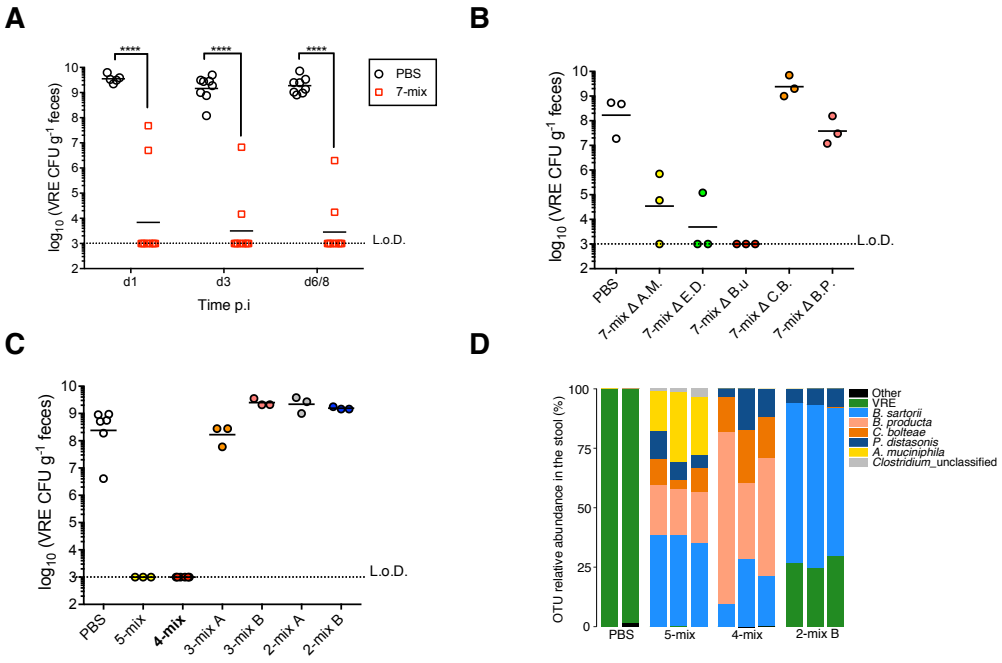


Figure 4.3. Adoptive transfer of defined bacterial consortia restores colonization resistance against VRE in antibiotic-treated mice. (A-C) Ampicillin-treated mice were inoculated with PBS or combinations of 7 bacteria isolated from ARF cultures on three consecutive days beginning the day following antibiotic initiation. Mice were infected with VRE the day after the last PBS/bacterial dose and VRE expansion was assessed at different time points p.i. Ampicillin treatment was continued throughout. L.o.D., limit of detection. (**A**) Suspension administered: PBS or 7-mix (*A. muciniphila*, *E. dolichum*, unclassified *Blautia*, *C. bolteae*, *B. producta*, *P. distasonis* and *B. sartorii*).****p<0.0001 by the Mann-Whitney test. (n=10 mice per group).(**B**) Suspension administered: PBS or 7-mix excluding *A. municiphila* (A.M.), *E. dolichum* (E.D.), unclassified *Blautia* (B.u), *C. bolteae* (C.B.) or *B. producta* (B.P.) Data corresponds to d5 p.i. (**C**) Suspension administered: PBS, 5-mix (*A. muciniphila*, *C. bolteae*, *B. producta*, *P. distasonis* and *B. sartorii*), 4-mix (*C. bolteae*, *B. producta*, *P. distasonis* and *B. sartorii*), 3-mix A (*C. bolteae*, *P. distasonis* and *B. sartorii*), 3-mix B (*B. producta*, *P. distasonis* and *B. sartorii*), 2-mix A (*C. bolteae*, *B. producta*) and 2-mix B (*P. distasonis* and *B. sartorii*). Data corresponds to d5 p.i. (n=3-6 mice per group). (**D**) Fecal microbiota composition on d5 p.i. OTUs at >0.01% relative abundance are shown.

Importantly, the same protection pattern was observed in the distal portion of the small intestine (**Supplementary Figure 4.1A**). Finally, we show that neither *C. bolteae* nor *B. producta* is able to restore colonization resistance when administered individually even in the presence of *P. distasonis* and *B sartorii*, supporting the idea that both strains work together to suppress VRE growth (**Figure 4.3C**). Because ampicillin was administered throughout the duration of the experiment, it is unlikely that native bacteria

would reappear. However, a possibility exists that administration of the 4-mix may enhance the recovery of indigenous bacteria directly responsible for colonization resistance. To determine if this was indeed the case, we assessed the microbiota composition of mice colonized with the various bacterial consortia. Strikingly, we found that while PBS-treated mice were fully dominated with VRE, mice colonized with the 4-mix harbored only the 4 administered bacteria with *B. producta* representing 30-70% of the total bacterial composition. While all 4 bacteria were detected in the feces, the small intestine consisted mostly of *B. producta* (65-98%) and *C. bolteae* (1-35%) (**Figure 4.3D** and **Supplementary Figure 4.1B**). Similarly, the microbiota of mice colonized with the 5-mix consisted of the 5 input bacteria in addition to an unclassified *Clostridium* OTU which expanded considerably in the ileum. On the other hand and in agreement with our CFU data, mice colonized with *P. distasonis* and *B. uniformis* exhibited high VRE relative abundance levels in the stool while the ileum was completely dominated VRE (**Figure 4.3D** and **Supplementary Figure 4.1B**). These findings demonstrate that colonization with *P. distasonis*, *B. sartorii, B. producta* and *C. bolteae* directly provides full colonization resistance against VRE.

4.2.3. Bacterial consortia prevent VRE growth *ex vivo* and *in vitro*

We previously showed that ARF could inhibit VRE growth ex vivo as efficiently as in vivo (**Chapter 3, Figure 3.4**). Treatment with metronidazole, an antibiotic that specifically targets anaerobic bacteria, completely abrogated the inhibitory effect or ARF, an effect that was not observed with aminoglycosides (gentamicin, streptomycin) that have limited anaerobic coverage (**Supplementary Figure 4.2A-B**). Loss of ARF-mediated suppression was also observed with heat treatment of fecal samples prior to VRE inoculation (**Supplementary Figure 4.2C**). Together these data suggest that the anaerobic compartment is the one responsible for mediating colonization resistance against VRE and that anaerobes need to be alive to exert such effect, observations that are consistent with the results obtained with our 4-mix experiments and with previous findings (159). Because all of this evidence demonstrates that resistance to VRE is exclusively mediated by the microbiota, we sought to determine whether the in vivo effects observed with our bacterial consortia could be recapitulated ex vivo and in vitro. To this end, we collected fecal pellets from ampicillin-treated mice colonized with the different bacterial mixtures, resuspended them in PBS under anaerobic conditions, inoculated them with VRE and assessed VRE expansion the following day. Similar to our in vivo observations, we found that VRE was significantly inhibited in fecal samples from mice colonized with the 7- and 4-mix while it expanded to a density of 10^{10} CFU/g feces in stool from mice colonized with *P. distasonis, B. sartorii* and *B. producta* (3-mix B); *P. distasonis* and *B. sartorii* (2-mix A); *C. bolteae* and *B. producta* (2-mix B) as well as PBS-treated animals (**Figure 4.3A**). In contrast to our in vivo findings,

fecal pellets from mice colonized with *P. distasonis, B. sartorii* and *C. bolteae* (3-mix A) were inhibitory to VRE.

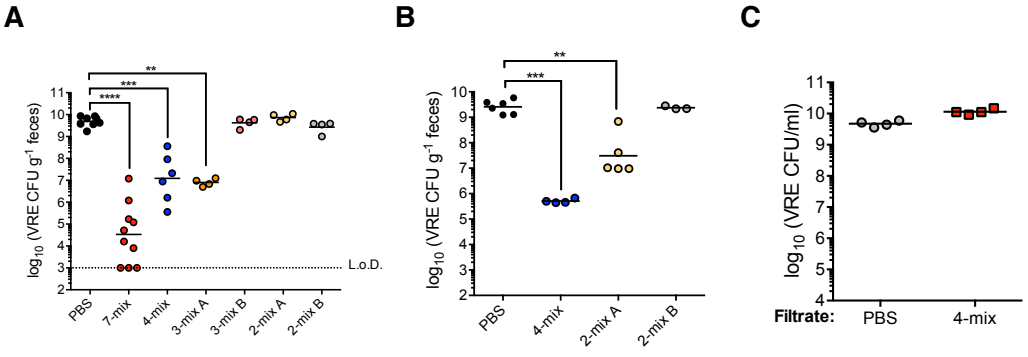


Figure 4.4. Bacterial isolates suppress VRE growth *ex vivo* and *in vitro*. (**A**) Fecal pellets from ampicillin-treated mice colonized with different bacterial mixtures were inoculated with VRE and incubated overnight. **P=0.0040, ***P=0.0007, ****P<0.0001, by the Mann-Whitney test. L.o.D. Limit of detection. (**B**) Fecal pellets from ampicillin-treated mice were inoculated with bacterial isolates from each mix in addition to VRE. VRE growth was assessed after 16-24 hours. **P=0.0043, ***P=0.0009, by the Mann-Whitney test. (**C**) Filtrates from fecal cultures in (B) were inoculated with VRE an incubated overnight. (**A-C**) 7-mix (*A. muciniphila*, *E. dolichum*, unclassified *Blautia*, *C. bolteae*, *B. producta*, *P. distasonis* and *B. sartorii*), 4-mix (*C. bolteae*, *B. producta*, *P. distasonis* and *B. sartorii*), 3-mix A (*C. bolteae*, *P. distasonis* and *B. sartorii*), 3-mix B (*B. producta*, *P. distasonis* and *B. sartorii*), 2-mix A (*C. bolteae*, *B. producta*) and 2-mix B (*P. distasonis* and *B. sartorii*).

Similarly, incubation of fecal pellets from ampicillin-treated mice with the 4 strains resulted in suppression of VRE growth by 4 logs, while incubation with *P. distasonis* and *B. sartorii* did not have any effect on VRE expansion (**Figure 4.4B**). Interestingly, introduction of *C. bolteae* and *B. producta* inhibited VRE albeit not as efficiently as the 4-mix, suggesting that residual ampicillin in the feces may suppress their activity and that both strains may be sufficient to prevent VRE growth (**Figure 4.4B**). Since a change in pH and competition for nutrients could explain the inhibitory effect of the 4 strains, we filtrated the 4-mix/fecal cultures and re-inoculated them with VRE. We found that VRE expanded to 10^{10} CFU in filtrates from fecal suspensions that had been incubated with either the 4 strains or PBS (**Figure 4.4C**). Not only do these observations demonstrate that nutrient limitation is not the mechanism

by which these 4 strains exert colonization resistance but also that the factors critical for protection are either insoluble or short-lived.

4.2.4. *C. bolteae* and *B. producta* eliminate established VRE from the gastrointestinal tract

Our in vitro findings that *C. bolteae* and *B. producta* had a significant inhibitory effect on VRE suggested the possibility that *P. distasonis* and *B. sartorii*, the other two components of the 4-mix may not be required. Members of the Bacteroidetes phylum, mainly *Bacteroides* spp., possess glycoside hydrolases that break down complex carbohydrates into simple sugars that serve as a nutrient source for other members of the community (172). Therefore, it is possible that in addition to their ampicillin-degrading properties, *P. distasonis* and *B. sartorii* may increase the nutrient availability in the gut and as a consequence, enhance the engraftment and growth of ampicillin-sensitive *C. bolteae* and *B. producta*. To address whether *P. distasonis* and *B. sartorii* were required solely for the degradation of ampicillin or had a more essential role in the protection exerted by the 4-mix, we pre-treated mice with ampicillin for 1 week, infected them with VRE and stopped antibiotic treatment the day of infection. We waited 3 days to allow VRE to colonize and ampicillin to clear from the gut prior to administering the first of 3 daily doses of PBS, a mixture of *C. bolteae* and *B. producta* or a mixture of all 4 bacteria (*C. bolteae, B. producta, P. distasonis* and *B. sartorii*). To determine how these two bacterial combinations would affect VRE colonization, we collected fecal samples before administration of the first dose (day 0) and on days 3, 6, 9 and 12 post treatment (**Figure 4.5A**). The purpose of this experiment was two-fold: 1) to determine whether the 4-mix, in addition to providing colonization resistance,

could clear established VRE and 2) to determine whether *P. distasonis* and *B. sartorii* were dispensable in the absence of antibiotic pressure. We found that 3 days following infection (day 0), all mice were densely colonized with VRE. In contrast to PBS-treated animals, which remained colonized throughout the duration of the experiment, mice inoculated with *C. bolteae* and *B. producta* or the 4-mix exhibited a progressive reduction in VRE levels and were able to clear VRE by day 12 post treatment from the small and large intestines (**Figure 4.5B-C**), demonstrating that the same level of decolonization efficiency obtained with FMT (159,176) can be achieved with only two bacterial species.

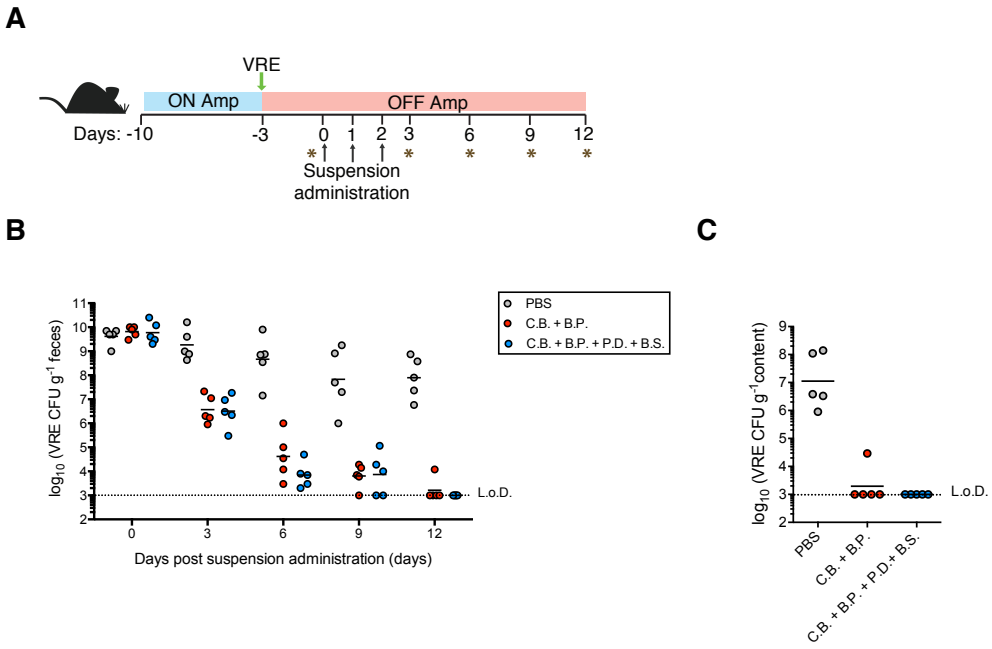


Figure 4.5. Administration of *C. bolteae* and *B. producta* clears VRE colonization. (**A**) Model for adoptive transfer. * denotes fecal pellets collected at indicated time points. (**B-C**) VRE burden in (**B**) the large intestine at various time points post transfer and (**C**) small intestinal content on day 12 post suspension administration. L.o.D. Limit of detection. C.B., *C. bolteae*; B.P., *B. producta*; P.D., *P. distasonis*; B.S., *B. sartorii*.

4.3. DISCUSSION

Enterococci are extremely hardy organisms. They can survive for extended periods of time on surfaces such as doorknobs, faucets and medical equipment and have been shown to be tolerant to heat and alcohol-based solutions (232,233). The resilience and multi-drug level resistance of these organisms make the prevention and treatment of Enterococcus infections incredibly difficult. VRE bacteremia is a frequent complication in the clinic, particularly in patients with a deficient immune system and severe microbiota perturbations such as those undergoing allogeneic bone marrow transplantation (14,158). Once in the bloodstream, VRE can reach the heart and cause infective endocarditis, a condition that is potentially lethal if not treated in a timely manner (163). A risk factor for VRE bacteremia is colonization of the intestine (158). Patients that either harbor VRE in their guts or acquire it from the environment can become colonized if colonization resistance has been lost due to antibiotic treatment. Complications that weaken the intestinal barrier can result in translocation of VRE from the intestine into the blood. Therefore, efficient colonization resistance as well as decolonization strategies must be implemented in order to prevent VRE dissemination.

Fecal microbiota transplantation (FMT) provides full recovery from various intestinal infections (228). However, the concern exists that undetectable pathogens may be transmitted from donor to recipient. This issue can be overcome through the use of keystone bacteria within FMT that mediate resistance to infection. Using the ampicillin-resistant flora (ARF) as a model system, we identified bacteria that correlated strongly with resistance to VRE. This was accomplished by diluting the colonization resistance capacity

of ARF to generate variation in VRE protection. Adoptive transfer of isolated resistance-associated strains into antibiotic-treated mice revealed that a bacterial cocktail comprising two species, namely *Clostridium bolteae* and *Blautia producta*, was sufficient to prevent VRE from colonizing the intestine and to eradicate it once it became established. The ampicillin susceptibility of *C. bolteae* and *B. producta* required the addition of the β-lactamase-producing strains *P. distasonis* and *B. sartorii* during antibiotic treatment. Importantly, colonization with these strains did not have any significant impact on VRE although in the ileum, the density of VRE was higher than that of control animals. This observation is in line with previous findings showing that metabolic by-products of *Bacteroides* spp. enhance the growth of S. *typhimurium* and *C. difficile* (19,183).

Previously, we showed that the presence of bacteria belonging to the genus *Barnesiella* (S24-7) positively correlated with resistance to VRE in humans and in mice (14). Consistent with these findings, we identified *Barnesiella* OTUs in our model that were significantly associated with VRE protection. However, none of our isolated strains matched the sequence of those OTUs. Although we were able to restore colonization resistance with bacterial mixtures that did not contain *Barnesiella*, it is possible that specific *Barnesiella* strains may be inhibitory as part of other bacterial combinations. Alternatively, the presence of *Barnesiella* may indicate an association with a healthy microbiota capable of providing colonization resistance rather than a direct association with VRE protection.

Supporting the notion that bacteria work in collaboration and not in isolation, we showed that neither *C. bolteae* nor *B. producta* provided protection against VRE when administered separately. This observation

suggests that metabolic by-products of *C. bolteae* or *B. producta* may serve as substrates for the opposite bacterium which is then equipped to produce VRE-inhibitory factors. On the other hand, both organisms may have the capacity to inhibit VRE but their interaction is required for this effect. Another possibility is that the localization of these bacteria within the intestine may impact their behavior. For instance, whereas mice colonized with *C. bolteae, P. distasonis* and *B. sartorii* were susceptible to colonization, their fecal pellets inhibited VRE growth *ex vivo*, suggesting that pathways involved in colonization resistance are functional in these animals. One explanation for this discrepancy is compartmentalization of protective strains and consequently, different mechanisms of resistance. While *C. bolteae* seems to be the major player in the large intestine, it is possible that *B. producta* alone or together with *C. bolteae* mediates resistance to VRE in the small intestine. Supporting this hypothesis, we found that *B. producta* represented 65-98% of the total bacterial population in the small intestinal microbiota of mice colonized with all 4 strains (*B. producta, C. bolteae, P. distasonis* and *B. sartorii*). Therefore, in the presence of *C. bolteae* but absence of *B. producta*, VRE may colonize the small intestine and lead to continued VRE seeding in the colon, an event that may override the inhibitory effects of *C. bolteae*. On the other hand, the lack of *in vivo* and *ex vivo* protection following treatment with *B. producta* in the absence of *C. bolteae* suggests that *C. bolteae* may *aid B. producta* to engraft and expand within the intestine in order to be effective against VRE.

Importantly, we showed that the mechanism of colonization resistance exerted by *C. bolteae* and *B. producta* do not involve nutrient depletion and changes in environmental pH. These observations point in the direction of bacterial metabolites generated through fermentation, such as short chain fatty

acids (SCFAs), and conversion of host-derived bile salts. SCFAs are the end products of bacterial fermentation by intestinal microbiota. Acetate, a major constituent of intestinal SCFA, was shown to be protective against enterohaemmorhagic *E.coli* through reinforcement of the intestinal barrier (20). Importantly, *C. bolteae* and *B. producta* strains isolated from human feces were shown to produce large amounts of acetate *in vitro* (115). Aside from SCFAs, the conversion of primary into secondary bile salts by another member of the Clostridia cluster XIVa, *Clostridium scindens*, has recently been implicated in protection against *C. difficile* (145). In addition to SCFAs and bile salts, bacteriocin-producing bacteria have been shown to outcompete *Enterococcus faecalis in vivo.* We are currently investigating these possibilities in our laboratory.

Lastly, we demonstrated that the rate of VRE clearance by *C. bolteae* and *B. producta* mimics that obtained with FMT, indicating that a feat as great as colonization resistance, normally afforded by a complex and diverse microbiota, can be mediated by at least two bacterial species. Altogether, our findings provide empirical support for the development of novel probiotic therapies using well-characterized commensal bacteria to target intestinal infection, a clinical application that provides a promising and safer alternative to FMT without sacrificing efficiency.

4.4. MATERIALS AND METHODS

4.4.1. Mice and infections. 6-8 week old female mice from Jackson Laboratories were treated with 0.5g/L ampicillin (Fisher) in their drinking water made fresh every 4 days. For VRE infection, $5x10^4$ CFU of VRE (ATCC 700221) were administered to mice by oral gavage. Mice were single-housed at the time of infection and kept on ampicillin until the end of the experiment unless otherwise noted. Tenfold dilutions of pre-weighed intestinal samples resuspendend in PBS were plated on Difco Enterococcosel agar (supplemented with 8 µg/ml vancomycin; Novaplus and 100 µg/ml streptomycin; Fisher).

4.4.2. DNA extraction, multiparallel sequencing and sequence analysis. DNA from intestinal samples was extracted using the phenol-chloroform extraction method (14). The V4-V5 region of the 16S rRNA gene was amplified and sequenced with the Illumina Miseq platform as described previously (145). Sequences were analyzed using version 1.33.3 of the MOTHUR pipeline (7) as described in Buffie et al., 2015. Sequences with distance-based similarity of at least 97% were grouped into OTUs. A modified Greengenes reference database (188) was used for OTU classification. The Inverse Simpson index was used to compute sample biodiversity at the OTU-level.

4.4.3. Bacterial culture and isolation. To isolate bacteria from ARF, fecal samples from $MyD88^{-/-}$ mice were collected, transferred immediately to an anaerobic chamber, resuspended in 1 ml of sterile pre-reduced PBS. Samples were serially diluted and 100 μl from the 10^{-5} and 10^{-6} dilutions were plated on

non-selective Columbia agar supplemented with 5% sheep blood (BD Biosciences) and at 37^{o}C anaerobically for 3-5 days. For inocula preparation of dilution cultures, bacteria were harvested and resuspended in PBS and glycerol at a final concentration of 15% glycerol. To isolate bacteria, colonies were picked from 10^{-5} plates and streaked onto fresh agar to ensure purity. Isolated strains were resuspended in PBS and glycerol (15%) and stored at -80^{o}C. The 16S rRNA gene was amplified by colony PCR using primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5' –GGTTACCTTGTTACGAC TT-3'). The resulting PCR product was Sanger sequenced with primers spanning the full 16S gene (8F and 1492R) as well as primers specific to the V4-V5 region (517F, 5'-GCCAGCAGCCGCGGTAA-3') and classified using BLAST. For colonization experiments, resistance-associated bacteria were cultured on Columbia blood agar plates for 3 days under anaerobic conditions, scraped off and mixed in a 1:1 ratio (10^{7}-10^{8} CFU per isolate) in PBS and glycerol (15%). All bacterial cultures were orally gavaged into mice in a 200 μl volume.

4.4.4. *Ex vivo* and *in vitro* VRE suppression assay. For *ex vivo* assays, fecal samples from C57BL/6 ampicillin-treated colonized with different bacterial mixtures were transferred to an anaerobic chamber and resuspended in pre-reduced PBS at a concentration of 25 mg/ml. For in vitro assays, 10^{7}-10^{8} CFU of each resistance-associated strain were added to fecal pellets from antibiotic-treated mice and incubated overnight prior to VRE inoculation. VRE ($5x10^{3}$ CFU) or PBS was added to each sample and incubated at 37 ^{o}C anaerobically. VRE growth was assessed 16-24 hours later by plating on selective media. Fecal filtrates were obtained by filtering overnight fecal

cultures of VRE grown in the presence or absence of resistance-associated strains over a 0.22 μm filter prior to VRE re-inoculation. For antibiotic treatment, fecal pellets from ampicillin mice colonized with ARF or not were resuspended in reduced PBS and metronidazole (Sigma-Aldrich), gentamicin (Fisher) or streptomycin (Fisher) at 0.5 mg/ml was added at the time of VRE inoculation.

4.4.5. Statistics. Statistical analyses were performed using R Statistical Language (version 3.1.1) and Graph-Pad Prism (version 6.0). Two-tailed unpaired Student's t test was used to determine if VRE levels were statistically significant among different groups. The Mann-Whitney nonparametric test was to determine statistical significance in biodiversity scores. Spearman's rank correlation test (two-tailed) was used to determine statistical correlations between two variables. The Benjamini-Hochberg method was applied to control for false discovery rate. For the Student's *t* test, Mann-Whitney test and Spearman's correlation test, P values < 0.05 were considered significant.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

One of the most critical roles of the microbiota is to prevent pathogen expansion. Treatment with antimicrobial agents kills a good fraction of the intestinal bacterial community and renders the host susceptible to colonization by multidrug-resistant organisms including VRE and *K. pneumoniae*. The work presented here outlines different aspects of host-bacterial and bacterial-bacterial interactions with a focus on colonization resistance.

In Chapter 2 we showed that VRE and *K. pneumoniae* expand to similar densities in the intestine during co-colonization and induce different levels of mucus production. Given the extent to which these organisms colonize together with the limited amount of resources in the intestine as a result of antibiotic-mediated depletion of the microbiota, we had originally hypothesized that they might compete with each other in the gut. However, their close proximity to each other suggests that they most likely interact despite not interfering with each other's growth. How VRE and *K. pneumoniae* adapt to the intestinal environment when alone or in the presence of the other is currently under investigation.

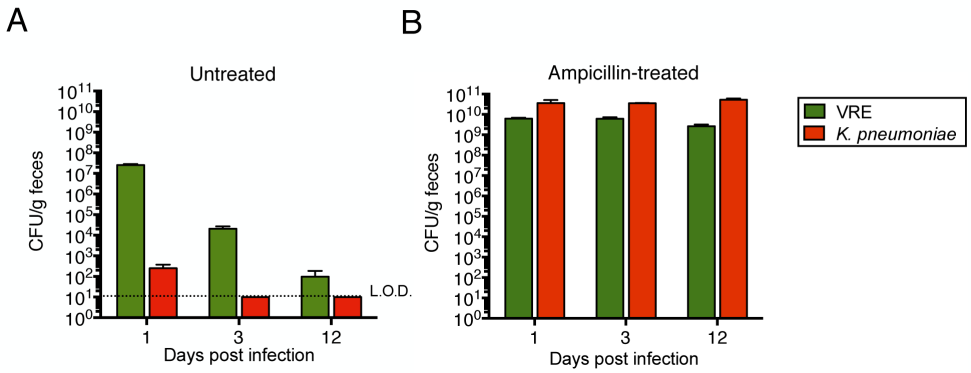
Colonization by these highly antibiotic-resistant pathogens is known to precede bacteremia in patients undergoing harsh treatments such as chemotherapy. Therefore, novel therapies to prevent and/or eliminate colonization are of great need in the clinic. One approach is the identification of bacteria that directly mediate colonization resistance and the underlying

mechanisms by which they do so. The work presented in Chapters 3 and 4 of this dissertation provides evidence of a causal relationship between specific bacterial strains and colonization resistance against VRE. In Chapter 3 we describe the development of population-wide antibiotic resistance in the mammalian gut as a result of long-term antibiotic treatment. Within this complex bacterial community, we identified a combination of two strains, *C. bolteae* and *B. producta*, that together were able to completely restore protection against VRE (Chapter 4). Importantly, both of these organisms are components of a healthy microbiota in humans (23). Whether human-derived *C. bolteae* and *B. producta* exert the same protective roles as their murine counterparts remains to be explored.

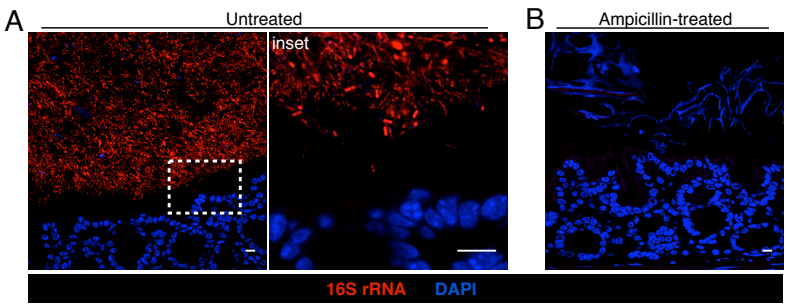
An important aspect of using a defined set of strains as therapy is pathogen specificity. Despite the remarkable ability to target VRE, *C. bolteae* and *B. producta* do not provide any protection against multidrug-resistant *K. pneumoniae* and *E.coli* (data not shown). Therefore, the players and rules involved in colonization resistance against different bacterial groups are not the same. Since Enterococci and Enterobactericeae can co-colonize the intestine, an ideal treatment would involve the administration of several commensal bacteria that can target multiple pathogens at one. The purpose of these studies is to enhance our understanding of how intestinal bacterial populations behave and how they impact resistance or susceptibility to VRE, making defined bacteriotherapy against VRE and other intestinal pathogens a real possibility.

APPENDIX 1

SUPPLEMENTARY FIGURES FOR CHAPTER 2



Supplementary Figure 2.1. Ampicillin treatment leads to VRE and *K. pneumoniae* expansion in the intestine.
Untreated mice (A) and mice treated with ampicillin in the drinking water for 1 week (B) were colonized with 10^8 CFU of VRE or *K. pneumoniae* and bacterial burden was quantified in the feces on days 1, 3 and 7 post infection. Ampicillin-treated animals were kept on ampicillin for the duration of the experiment. L.O.D., limit of detection.

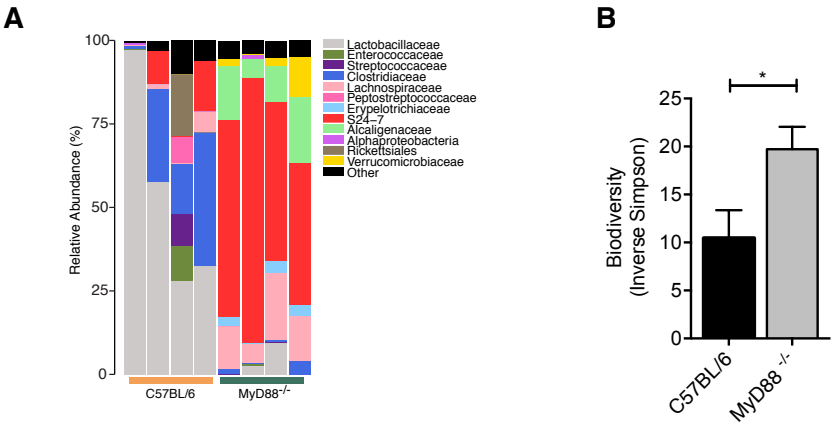


Supplementary Figure 2.2. Visualization of morphologically distinct intestinal bacteria by FISH

Colon sections from untreated mice (A) and mice treated with ampicillin in the drinking water for 3 weeks (B) were hybridized with a universal bacterial probe directed against the 16S rRNA gene and counterstained with Hoechst dye to visualize nuclei. Scale bars, 10 μm. Inset, 63X oil objective plus 4X digital zoom. Images are representative of 5 mice per group.

APPENDIX 2

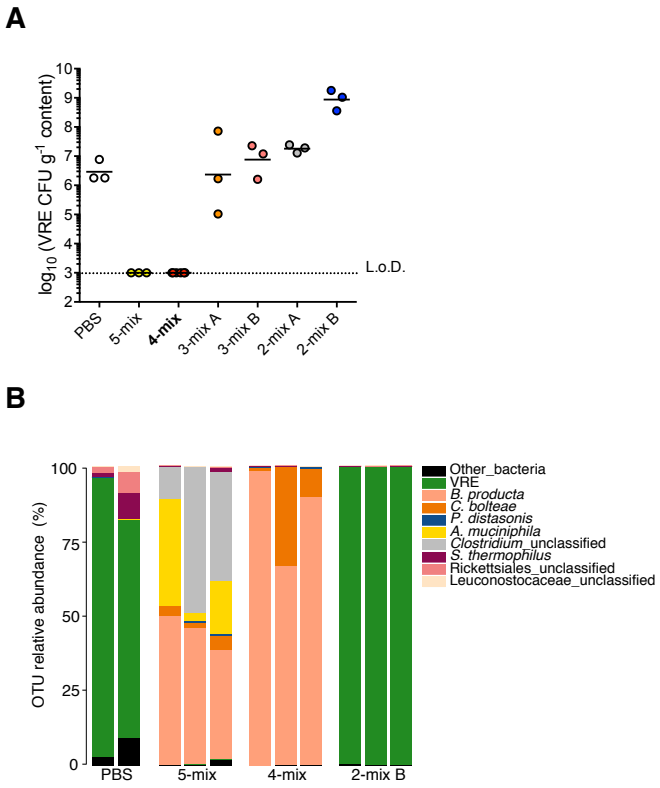
SUPPLEMENTARY FIGURES FOR CHAPTER 3



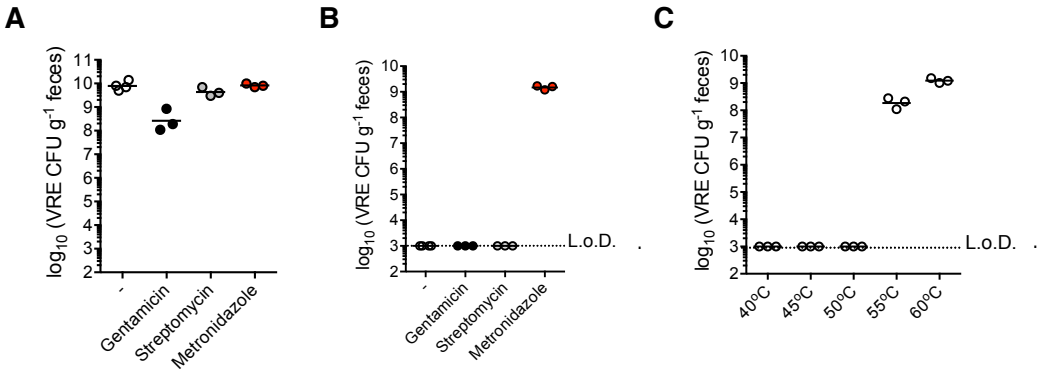
Supplementary Figure 3.1. Bacterial composition of the ileal microbiota of MyD88$^{-/-}$ and C57BL/6 mice. (**A**) Family-level classification of OTUs present at >0.01% relative abundance. Each stacked bar corresponds to an individual mouse. (**B**) Alpha biodiversity of the ileal microbiota (n=4 per group). Error bars (mean ± SEM). *p=0.0137 by the Student's *t* test.

APPENDIX 3

SUPPLEMENTARY FIGURES FOR CHAPTER 4



Supplementary Figure 4.1. Defined bacterial consortia restore colonization resistance against VRE in the small intestine. (A-C) Ampicillin-treated mice were inoculated with PBS or combinations of 5 bacteria isolated from ARF cultures on three consecutive days beginning the day following antibiotic initiation. Mice were infected with VRE the day after the last PBS/bacterial dose. Data corresponds to d5 p.i. Ampicillin treatment was continued throughout. (n=3-6 mice per group). L.o.D., limit of detection. (**B**) Ileal microbiota composition. OTUs with a mean relative abundance >0.01% were plotted. (**A-B**) Suspension administered: PBS, 5-mix (*A. muciniphila*, *C. bolteae*, *B. producta*, *P. distasonis* and *B. sartorii*), 4-mix (*C. bolteae*, *B. producta*, *P. distasonis* and *B. sartorii*), 3-mix A (*C. bolteae*, *P. distasonis* and *B. sartorii*), 3-mix B (*B. producta*, *P. distasonis* and *B. sartorii*), 2-mix A (*C. bolteae*, *B. producta*) and 2-mix B (*P. distasonis* and *B. sartorii*).



Supplementary Figure 4.2. Live anaerobic bacteria suppress VRE expansion *ex vivo*. (**A-B**) Fecal pellets from (A) ampicillin-treated mice and (B) ampicillin-treated mice colonized with ARF were treated with gentamicin, streptomycin, metronidazole or no antibiotics (-) and inoculated with VRE. (**C**) Fecal samples from ARF-colonized ampicillin-treated mice were incubated at the indicated temperatures for 15 min prior to VRE inoculation. (**A-C**) VRE expansion was measured 16-24 hours later. L.o.D., limit of detection.

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