APPLICATION OF ECOLOGICAL THEORIES TO THE GUT MICROBIOME AND BIFIDOBACTERIAL COMMUNITIES

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ABSTRACT

This doctoral thesis presents two studies that utilize infant-gut associated bifidobacteria as a study system to test theories in community ecology. Bifidobacteria are the first colonizers of the human gut microbiome and a critical taxon that is said to significantly affect host health and physiology. The first study (Chapter II) examines the role of priority effects in the initial structuring of bifidobacterial communities. The second study (Chapter III) evaluates the role of bifidobacteria after the gut microbial community is established. This study tests the efficacy of bifidobacteria as a probiotic species and its role in the ecological recovery after antibiotic-induced disturbance.

The first study described in Chapter II uses four infant-gut associated bifidobacterial species (*Bifidobacterium bifidum* JCM 1254, *Bifidobacterium breve* UCC2003, *Bifidobacterium longum* subspecies *longum* MCC 10007, and *Bifidobacterium longum* subspecies *infantis* ATCC 15697^{T}) to examine the role of priority effects, or the effect of species arrival order on community composition. Human milk oligosaccharides (HMOs) were used as a carbon source. Consistent with the genomic analysis performed in this study, the four bifidobacterial species displayed a variety of species-specific HMO assimilation phenotypes and competitive abilities in HMO-supplemented environments. Results of culturing experiments showed that assembly history and priority effects significantly affected community structure, as the identity of the first colonizer and the second colonizer had a significant effect on the outcome of the community. Priority effects allowed *B. breve*, a species predicted to be a weak competitor in terms of HMO assimilation, to dominate. Further analysis with previously published *in vivo* gut microbial community composition data from a cohort of 41 infant-mother pairs also suggested that *B. breve* benefitted from priority effects. The results of this study found that infant gut-associated microbes are subject to assembly history and priority effects, and that colonization during the early stages of assembly is critical for the development of the community.

The second study described in Chapter III examines the efficacy of *B. bifidum* JCM 1254 as a probiotic strain. Using mouse models, repeated disturbance to the gut microbiome was introduced through three different antibiotics, and *B. bifidum* was administered after each disturbance event. The effect of antibiotic administration diminished with repetitive use, showing that the gut microbiome retains memory of past disturbances. Furthermore, the response of the gut microbiome varied by each antibiotic type, which consequently affected the efficacy of *B. bifidum* administration. Results show that although *B. bifidum* was not effective in recovering species diversity, it reduced gut inflammation when antibiotic administration caused an increase in proinflammatory species. The findings show that different types of disturbances determine the identity of the taxa that survive in the community, which ultimately affects the response of the gut microbiome to subsequent recovery interventions.

In this doctoral thesis, the author presents interdisciplinary studies that combine concepts and experimental methods from molecular biology, community ecology, and bioinformatics. Using bifidobacteria as a study system, the two studies described above explore how theories in community ecology can be used to understand the dynamics within host-associated microbial communities and their role in host health and disease. Furthermore, the author hopes that the application of ecological theories could be utilized to improve currently available microbiome-based therapies that are aimed at restoring or maintaining the ecosystem services provided by healthy gut microbial communities.

LIST OF ABBREVIATIONS AND TERMS

Abbreviation	Term
2'-FL	2'-Fucosyllactose
3-FL	3-Fucosyllactose
AA	Anthranilic Acid
AAD	Antibiotic-Associated Diarrhea
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
CAGR	Compound Annual Growth Rate
DFAST	DDBJ Fast Annotation and Submission Tool
DNA	Deoxyribonucleic Acid
EFA	Exploratory Factor Analysis
ESBL	Extended-Spectrum β-Lactamase
FDH	Fucose Dehydrogenase
FMT	Fecal Microbiome Transplant
Fuc	Fucose
Gal	Galactose
GAM	Gifu Anaerobic Medium
GH	Glycoside Hydrolase
Glc	Glucose
GlcNAc	*N*-Acetylglucosamine
HMOs	Human Milk Oligosaccharides
HPLC	High-Performance Liquid Chromatography
IBD	Inflammatory Bowel Disease
IgA	Immunoglobulin A
ITS	Internal Transcribed Spacer
JCM	Japan Collection of Microorganisms
Lac	Lactose
LDFT	Lactodifucotetraose
LNB	Lacto-*N*-biose I
LNDFH	Lacto-*N*-difucohexaose
LNFP	Lacto-*N*-fucopentaose
LN*n*T	Lacto-*N*-*neo*tetraose

LNT	Lacto-*N*-tetraose
MCC	Morinaga Culture Collection
MRS	De Man, Rogosa, Sharpe (Medium)
NCBI	National Center for Biotechnology Information
NCIMB	National Collection of Industrial Food and Marine Bacteria
NEC	Necrotizing Enterocolitis
NGS	Next-Generation Sequencing
NMDS	Non-Metric Multidimensional Scaling
OD	Optical Density
ORF	Open Reading Frame
OTU	Operational Taxonomic Unit
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative Polymerase Chain Reaction
rm-ANOVA	Repeated Measures Analysis of Variance
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RPKM	Reads Per Kilobase of exon per Million mapped reads
SCFA	Short-Chain Fatty Acids
SE	Standard Error
SRA	Sequence Read Archive
subsp.	Subspecies
UCC	University College Cork Culture Collection
YCFA	Yeast, Casitone, Fatty Acids (Medium)

Chapter I

GENERAL INTRODUCTION

The human gastrointestinal tract is host to an abundant and diverse community of microbes, collectively referred to as the gut microbiome. A study by Sender et al. (2016) estimates that the gut microbiome consists of around 4×10^{13} microbial cells, which is approximately as many as the number of human cells in the body. Given the abundance of microbes within the gut microbiome, it is no surprise that they have a significant potential to influence host physiology. The gut microbiome is known to aid in food digestion and nutrient absorption by breaking down carbohydrates that are otherwise inaccessible to the host, such as dietary fibers (Daïen et al., 2017) and host-produced complex glycans like human milk oligosaccharides (HMOs) and mucin (Sela et al., 2008). Other studies have shown associations between the composition of the gut microbiome and the development of the immune system (Round and Mazmanian, 2009; Kau et al., 2011). Any imbalances in a healthy microbiome (dysbiosis) on the other hand, have been linked to diseases such as obesity (Ley et al., 2005), inflammatory bowel disease (IBD) (Nishida et al., 2018), and type 2 diabetes (Qin et al., 2012).

With the advancement in next-generation sequencing (NGS) and metagenomic technologies, studies elucidating the relationship between the gut microbiome and host health have increased significantly over the last two decades. Furthermore, the use of gnotobiotic animal models has allowed studies to experimentally determine correlations between specific taxa and disease states. For example, a study by Jiang et al. (2002) found that the development of IBD was accelerated in mice mono-associated with *Helicobacter muridarum.* Another study by Fei and Zhao (2013) found that the inoculation of *Enterobacter cloacae* B29 isolated from a morbidly obese human individual induced obesity in gnotobiotic mice. Although gnotobiotic mice are a useful tool for examining the effect of specific taxa on host physiology, studies often present casual relationships and lack mechanistic explanations. Moreover, some studies indicate that certain disease states do not develop in the absence of other gut microbes (Bäckhed et al., 2007). This suggests that the mechanisms of pathology within the gut microbiome are rarely simple, but rather highly context-dependent and influenced by community interactions within microbiomes (Walter et al., 2020). However, studies have only recently begun to examine the gut microbiome within the context of community ecology.

The goal of community ecology is to explain and predict the mechanisms that determine temporal and spatial patterns of species diversity. However, most current ecological theories are developed based on experimentation with higher eukaryotic communities and have yet to be tested in host-associated microbial communities. Ecological communities both shape and are shaped by their environments, but unlike eukaryotic communities and free-living microbial communities, host-associated microbial communities are distinct in that they form mutualistic relationships with their host environments, thereby becoming a holobiont (Margulis, 1991). The human holobiont is an ecological system under selective pressure that minimizes conflict between host and microbe, creating a symbiotic relationship. In this

symbiosis, the human host provides a niche for the gut microbial community to occupy. The selective pressure exerted by the host can also affect microbe to microbe interactions, as selection can favor competitive outcomes that are beneficial to the host. In turn, the gut microbiome as a community provides ecosystem services that increase the fitness of their host. This mutualistic relationship accelerates host-microbe coevolution, making the gut microbiome a unique system in which theories in community ecology are currently underexplored.

To address the research gaps in both gut microbiome studies and community ecology, this doctoral thesis examines two concepts: assembly theory and disturbance ecology. In this thesis, I used host-associated gut microbial communities, with a focus on infant gut-associated bifidobacteria. Bifidobacteria are Gram-positive and anaerobic bacteria with high guanine and cytosine (G + C) content (> 50 %) (Shah, 2011), belonging to the Actinobacteria phylum. Bifidobacteria were first isolated by Henri Tissier in 1899 from breastfed infant feces (Tissier, 1900) and are one of the first colonizers and symbionts of the human gut. The guts of infants, especially breastfed infants, are rich in bifidobacteria due to selection by HMOs found in breastmilk (Roger et al., 2010). HMOs are a group of oligosaccharides with a degree of polymerization greater than 3 and are the third most abundant solid component in breastmilk after lactose and lipids (Urashima et al., 2012). Despite being an abundant component in breastmilk, it provides no nutritional value to the host as it is resistant to pancreatic digestion (Kunz et al., 2000; Urashima et al., 2012). HMOs pose a strong selective pressure in the gut microbiome as bifidobacteria and a limited subset of gut microbes possess the enzymatic genes to utilize them (Macrobal et al., 2010; Ruiz-Moyano et al., 2013; Katayama, 2016; Matsuki et al., 2016; Sakanaka et al., 2020). Furthermore, the composition of the initial gut microbiome that forms during infancy is said to have consequences for host health that last throughout adulthood. Although the available literature is mostly correlative data, studies suggest that the lack of a bifidobacterial community is linked to the development of allergy and atopic dermatitis, as well as an impairment in the immune system (Kalliomäki et al., 2001; Di Gioia et al., 2014). In addition to being a critical species in the initial formation of the human gut microbiome, bifidobacteria have gained attention as probiotics. Bifidobacteria are said to confer several health benefits to the host, such as the prevention and treatment of colorectal cancer (Sekine et al., 1985; Le Leu et al., 2010), anti-obesity effects (Kondo et al., 2010; Stenman et al., 2014; Moya-Pérez et al., 2015), and the suppression of inflammation (Riedel et al., 2006; Medina et al., 2008). Due to their unique role as both a pioneer species of the human gut and a commonly used probiotic taxon, bifidobacteria proved to be an ideal model system to test both assembly theory and disturbance ecology, as described below.

Assembly Theory

Chapter II of this dissertation examines the process by which infant gut-associated bifidobacterial communities are formed. Bifidobacteria are one of the first colonizers of the human gut microbiome and a focal species of initial community assembly. However, due to limited opportunities for experimentation with human subjects, elucidating the factors and mechanisms of gut microbiome assembly remains difficult. Chapter II addresses this issue by utilizing a reductionist approach and examining the mechanisms of community assembly with infant gut-associated bifidobacteria, with a focus on priority effects. According to a synthesized framework proposed by Vellend (2010), community assembly is driven by four basic processes: dispersal, diversification, drift, and selection (Table I-1). Dispersal allows for taxa to colonize local habitats from a regional species pool. Within the local habitat, diversification adds new genetic variation and taxa. Then, deterministic selection based on species traits and stochastic drift shapes the relative abundances of local taxa. The following sections will briefly review the available literature regarding the gut microbiome and mechanisms of community assembly.

Dispersal

Dispersal refers to the movement of individuals across space and it is a fundamental process by which diversity accumulates in local microbial communities. Through dispersal, the niches available in the infant gut microbiome is colonized. The infant gut is generally considered sterile at birth and microbial colonization is thought to begin postpartum. Several factors contribute to the initial community assembly, such as mode of delivery (Bokulich et al., 2016; Akagawa et al., 2019), use of antibiotics by mother and infant (Yassour et al., 2016), and feeding method (breastfeeding or formula) (Ho et al., 2018). After birth, microbes from a variety of species pools can colonize the gut, but maternal microbes are one of the most significant sources. A recent study has shown that the neonatal oral fluid that infiltrates the infant nasal cavity during vaginal delivery is a possible transmission route for bifidobacteria (Toda et al., 2019). This is further corroborated by studies that show that the gut microbiome of infants born vaginally is dominated by taxa found in the mother's vagina, while the gut microbiome of infants born through cesarian section is dominated by taxa found on the human skin (Palmer et al., 2007; Dominguez-Bello et al., 2010). These studies strongly suggest that the maternal microbial reservoir is an important source of microbe acquisition for the early infant microbiome.

Diversification

Diversification refers to the generation of new genetic variation within a local community. Compared to communities of higher eukaryotes, local diversification occurs rapidly in the gut microbiome due to large population sizes and high growth rates. A recent study by Zhao et al., (2019) examined the within-microbiome evolution of *Bacteroides fragilis* in a cohort of 12 healthy individuals and found that *B. fragilis* adapted through *de novo* mutations. Thus, diversification was observed within individuals. Bacteria can also inherit mutational changes through horizontal gene transfer, and data from the Human Microbiome Project suggests that horizontal gene transfer occurs frequently within the adult human gut microbiome. A study by Liu et al. (2012) identified 13,514 horizontal gene transfer genes, most encoding for catalytic functions and metabolic processes, in 308 prokaryotic species. However, diversification occurs as a result of persistent selective pressure, and the infant gut microbiome experiences frequently changing selective pressures (i.e., host immune system development, breastfeeding, and the introduction of solid foods). Consequently, the extent to which horizontal gene transfer and diversification affects community assembly in the infant gut remains poorly understood (Lerner et al., 2017; Sprockett et al., 2018).

Selection

Selection creates differences in community structure based on fitness and niche differences among taxa. As a host-associated microbial community, the infant gut microbiome faces frequently shifting sources of selective pressures, primarily from diet and the immune system. At birth, the infant's main source of food is breastmilk. HMOs found in breastmilk are a group of structurally diverse oligosaccharides with more than 200 distinct structures (Ninonuevo and Lebrilla, 2009) that are resistant to enzymatic digestion and low gastric pH (Engfer et al., 2000). As a result, HMOs reach the intestine intact and act as a substrate for gut microbes. However, only a subset of microbes (*Bifidobacterium* species, some *Bacteroides* species, and a limited group of *Clostridium* species) possess the genes to utilize HMOs (Macrobal et al., 2010). Consequently, HMOs provide a competitive advantage to HMO-utilizing taxa over other gut microbes, and the guts of breastfed infants are often dominated by *Bifidobacterium* species (Tannock et al., 2013; Matsuki et al., 2016). Furthermore, the most drastic change in community composition occurs at the cessation of breastfeeding, suggesting a sudden shift in selective pressure (Yatsunenko et al., 2012). In addition to diet, the oxygen concentration within the infant gut exerts selective pressure. The gut microbiome becomes increasingly anaerobic postpartum, which selects for facultative or strict anaerobes. A study by Ferretti et al. (2018) found that microbes that

originate from the maternal gut, the majority of which are anaerobic strains, are more persistent and ecologically better adapted to the infant gut than microbes from other maternal body sites. The immune system could also exert selective pressure on the microbiome, causing microbial communities to become more body-site specific. Furthermore, as the gut microbiome can affect host health, selection is expected to favor microbial traits that are beneficial to the host.

Drift

While selection is a deterministic force that alters community structure, ecological drift refers to the random changes in the relative abundance of species. However, distinguishing the effect of drift from other processes is challenging, and very few studies examine its effect within the gut microbiome. One study using synthetic bacterial communities found that the importance of ecological drift increases when selection is high and dispersal is low (Fodelianakis et al., 2020). Drift also becomes important when species abundance is low, as low-abundance species are more likely to be randomly pushed to extinction (Gilbert and Levine, 2017). A study examining the rate of evolution in a commensal *Escherichia coli* strain observed limited genomic diversity and found little evidence of selection, possibly due to small population sizes (Ghalayini et al., 2018). Although there are other possible explanations, the absence of diversification and selection for this *E. coli* population suggests that drift plays a larger role in bacterial adaptation for taxa with small population sizes. However, this study did not explicitly test for ecological drift. Consequently, ecological drift remains difficult to characterize in the gut microbiome, as multiple confounding factors could contribute to the low abundance of microbial populations, such as dispersal limitation, high susceptibility to disturbance, or low competitive ability.

Table I-1 | Summary of the ecological processes involved in community assembly.

Process	Definition	Examples in the Gut Microbiome
Dispersal	Movement of individuals across space	Possible vertical transmission of vaginal microbes to the infant's gut during vaginal delivery (Toda et al., 2019)
Diversification	Locally generated, new genetic variation	Adaptive evolution of *Bacteroides fragilis* in healthy individuals (Zhao et al., 2019)
Selection	Deterministic force that alters community structure based on fitness differences among taxa	Enrichment of taxa that can utilize HMOs (bifidobacteria) in breastfed infant guts (Fallani et al., 2010; Macrobal et al., 2010)
Drift	Stochastic change in relative abundance of taxa	Documented examples in the gut microbiome limited
Priority Effects	Effect of assembly history on final community composition	The relative abundance of *Bacteroides* at birth affects the three-year maturational trajectory of the gut microbiome (Yassour et al., 2016)
Niche Pre-emption	Early arriving species inhibit colonization by later colonizers by reducing resources available	Prior colonization by *Bacteroides thetaiotaomicron* in the colonic crypt physically prevents later-arriving isogenic strains from colonizing (Lee et al., 2013; Whitaker et al., 2017)
Niche Modification	Early arriving species change the nature of available niches, consequently changing the identities of later arriving species	Depletion of oxygen by Enterobacteriaceae in the infant's gut may be facilitating colonization by obligate anaerobes (Bokulich et al., 2016)

The processes described above often exert interactive effects, and the relative importance of each process is difficult to isolate in the gut microbiome. For example, feeding mode (formula or breastmilk) affects both dispersal and selection. Breastmilk contributes to dispersal as it harbors its own microbiome, with the majority of the taxa belonging to the Proteobacteria, Firmicutes, and Actinobacteria phyla (Moossavi et al., 2019). In addition to the selective pressures posed by HMOs, the antimicrobial factors in breastmilk, such as lactoferrin and immunoglobulin A (IgA), can further select the members of the gut microbiome. As a result, the gut microbiome of formula-fed infants experiences altered dispersal and selection compared to that of breastfed infants.

Another example of such interactive effects is priority effects, which will be discussed in detail in Chapter II. Priority effects, or the effect of dispersal history and species arrival order on community structure, operate through two mechanisms. The first mechanism, niche pre-emption, occurs when the early arriving species sequester and reduce the amount of resources available for later-arriving species. The second mechanism, niche modification, occurs when the early arriving species alters the types of available niches and consequently determines which later-arriving species can colonize the niche (Fukami, 2015). Priority effects can determine the nature of species interactions within the community, and consequently alter how selection, drift, and diversification operate. Several recent papers have highlighted the need to examine the role of priority effects in the initial assembly of the human gut microbiome (Martínez et al., 2018; Sprockett et al., 2018). However, because experimental opportunity with infant subjects is limited, the study described in Chapter II will examine the role of priority effects in a controlled laboratory setting using four infant gut-associated *Bifidobacterium* strains. With medium supplemented with HMOs as the carbon source, the colonization history was manipulated to examine the effect of assembly history on community composition.

Disturbance Ecology

Chapter III addresses the role of bifidobacteria in established gut microbial communities. Specifically, the chapter explores its role in the recovery after ecological disturbance. In addition to assembly history, the history of disturbances is another factor that shapes the gut microbiome. An ecological disturbance is defined as an abiotic or biotic event that causes sudden structural changes to the community composition. Disturbance events remove some portion of resident individuals and create an opportunity for the remaining community members to increase in abundance, or for new colonists to establish within the community (Sousa, 1984). The type of disturbance determines which individuals and traits are selected for over time, and communities are predicted to adapt over evolutionary time. Furthermore, the community's response to disturbance reveals features that are otherwise difficult to detect, such as stability and resilience, as well as interspecies interactions and dependencies. The stability of an ecological community can be described through engineering resilience and ecological resilience. The former refers to the rate at which a community returns to a single steady-state after disturbance, while the latter refers to the amount of disturbance a community can tolerate before its trajectory is altered to a different stable state (Peterson et al., 1998). While resilience is often described in terms of species diversity and community composition, assessing resilience in terms of ecosystem functioning and services may be especially important for gut microbiome studies (Costello et al., 2012), given the importance of the gut microbiome for human health.

Within the gut microbiome, antibiotics are a common source of disturbance. Approximately 30 % of patients in the United Kingdom are prescribed antibiotics at least once a year (Shallcross et al., 2017), and up to 3 % of the population in the European Union is exposed to pharmacologic doses of antibiotics daily (Goossens et al., 2005). In Japan, approximately 1.6 % of the population is prescribed antibiotics on a given day (Japan Ministry of Health Labour and Welfare, 2016). More than 20 classes of antibiotics have been produced since 1930 (Coates et al., 2002), and the spectrum and mode of activity vary by each antibiotic. Consequently, the effect of antibiotics on the gut microbial community varies significantly by the type, dosage, and frequency of administration. While many studies focus on the emergence of antibiotic-resistant strains, relatively few studies have addressed the long-term adverse effects of antibiotics on the structure and composition of the gut microbial community. A study by Jernberg et al. (2007) found that antibiotic administration can have lasting consequences on the gut microbiome. For example, the administration of clindamycin, a broad-spectrum antibiotic that targets anaerobes, caused a decline in *Bacteroides* that lasted for two years. A different study by Dethlefsen and Relman (2011) examined the effect of repeated antibiotic exposure using ciprofloxacin, a fluoroquinolone antibiotic with a broad spectrum of activity, in human subjects and found that while the gut microbiome recovered relatively quickly to its initial state, repeated antibiotic use caused the recovery to be incomplete. These examples show that antibiotic disturbance can have significant and often lasting effects on the community composition of the gut microbiome.

Recent studies suggest that the administration of probiotics, or microbial strains such as *Bifidobacterium* and *Lactobacillus* that are consumed for therapeutic purposes, can alleviate the negative effects of antibiotic-induced dysbiosis (Grazul et al., 2016; Ekmekciu et al., 2017). Probiotics are said to aid in correcting dysbiosis and prevent antibiotic-associated diarrhea (AAD) by filling in the niche cleared by antibiotics and preventing pathogen colonization (Guo et al., 2019). A study by Gueimonde et al., (2007) suggested that probiotics can physically inhibit pathogen colonization by attaching to the host's epithelial cells. Other studies have shown probiotic species to act through the production of short-chain fatty acids and other acidity-related mechanisms (Fukuda et al., 2011), production of bacteriocins (Corr et al., 2007), and disruption of quorum sensing (Medellin-Peña et al., 2007). However, the research regarding the efficacy of probiotics after antibiotic-induced dysbiosis remains inconclusive. For example, a study by Suez et al. (2018) also found that some probiotic strains produce anti-microbial peptides, which inhibited, rather than promoted, recovery after antibiotic disturbance. One of the issues with current probiotic research is that probiotics are often considered as a homogenous entity, even though the purported effects can vary significantly not only at the species level but also at the strain level. Many studies utilize a pre-mixed blend of probiotic species, and the specific role of bifidobacterial species in the recovery after antibiotic-induced disturbance warrants further research. Therefore, Chapter III examines

the efficacy of a probiotic strain, *Bifidobacterium bifidum* JCM 1254, against three antibiotics with differing spectrums and modes of action.

Despite recent advances in sequencing technologies, questions remain regarding how to best sample and test for factors that affect structural, functional, and temporal variations in the gut microbiome. There is a growing interest in examining the gut microbiome through the lens of community ecology, but the development of theory-based, microbiome-specific hypotheses are limited (Costello et al., 2012; Koskella et al., 2017). To address these research needs, the studies presented in this dissertation utilizes approaches from both microbiology and community ecology, as well as bioinformatics, to empirically test for ecological theories in host-associated microbial communities.

Chapter II

THE ROLE OF PRIORITY EFFECTS IN BIFIDOBACTERIAL COMMUNITIES

Summary

Bifidobacteria are one of the first colonizers of the human gut, and human milk oligosaccharides (HMOs) found in breastmilk are crucial for the formation of a bifidobacteria-rich gut microbial community in infants. While the presence of bifidobacteria during infancy has been linked to a variety of health-promoting effects, little is known about how bifidobacterial communities are initially formed in the infant gut. This study focused on four infant-gut associated bifidobacteria *(Bifidobacterium bifidum* JCM 1254*, Bifidobacterium breve* UCC2003*, Bifidobacterium longum* subspecies *longum* MCC 10007*, Bifidobacterium longum* subspecies *infantis* ATCC 15697^{T}) that employ a variety of species-specific strategies for HMO consumption. Through genomic analysis and monoculture experiments, phenotypes of each strain were first characterized. Co-culturing experiments in medium supplemented with HMOs were performed to test for priority effects, by manipulating colonization histories. Pairwise culturing experiments revealed differences in competitive outcomes between each species pair, which were significantly affected by inoculation order as well as species traits. Four-species assemblages also showed that the structure of bifidobacterial communities, as well as the behavior of growth curves, was significantly influenced by assembly history and subject to priority effects. Bifidobacterial communities extracted from publicly available metagenome data from a cohort of infant-mother pairs were examined for further analysis. Results show that the identity of colonizers during the early stages of community assembly affects community outcomes. Furthermore, the findings of this study suggest that *B. breve* can significantly benefit from priority effects, as it dominated in both *in vitro* and *in vivo* systems. In conclusion, the results of this study show that priority effects are prevalent in infant gut-associated microbes, highlighting the importance of initial community assembly and its implications for the maturation trajectory of the gut microbiome.

Chapter III

THE ROLE OF BIFIDOBACTERIA IN THE RECOVERY AFTER REPEATED DISTURBANCES

Summary

Antibiotic administration can disturb the ecological balance of the gut microbiome and have long-term consequences. Probiotics have been proposed as a remedy for antibiotic-induced disturbance, but their efficacy remains uncertain. Thus, the effect of specific antibiotic-probiotic combinations on the gut microbiome and host health warrants further research. To test theories in disturbance ecology within the context of the gut microbiome, I used murine models and examined the effect of three antibiotics: vancomycin, a glycopeptide antibiotic that targets Gram-positive bacteria; amoxicillin, a moderate spectrum antibiotic; and ciprofloxacin, a broad-spectrum antibiotic that targets Gram-negative bacteria. Antibiotic administration was followed by one of the three following recovery treatments: *Bifidobacterium bifidum* JCM 1254 as a probiotic (PR); fecal transplant from healthy donor mice (FT); or natural recovery (NR). Antibiotic administration and recovery treatments were each repeated three times. The efficacy of each treatment was evaluated by measuring gut microbiome diversity, and recovery was assessed using the Bray-Curtis Index of Dissimilarity, which quantified the magnitude of microbial shift. Community composition was determined by sequencing the V3–V4 regions of the 16S ribosomal RNA gene. To assess host health, I measured body weight and cecum weight, as well as mRNA expression of inflammation-related genes by reverse-transcription quantitative PCR. Results show that community response varied by the type of antibiotic used, with vancomycin having the most detrimental consequences. As a result, the effect of probiotics and fecal transplants also varied by antibiotic type. For vancomycin, the first antibiotic disturbance substantially increased the relative abundance of taxa associated with gut inflammation, such as *Proteus* and other species in the phylum Proteobacteria. However, the effect of subsequent disturbances was less pronounced, suggesting that the response of the gut microbiome is affected by past disturbance events. Furthermore, although gut microbiome diversity did not recover, probiotic supplementation effectively limited cecum size enlargement and colonic inflammation caused by vancomycin. However, for amoxicillin and ciprofloxacin, the relative abundances of proinflammatory species were not greatly affected. As a result, the effect of probiotic supplementation on community structure, cecum weight, and expression of inflammation-related genes was comparatively negligible. The results of this study show that probiotic supplementation is effective, but only when antibiotics cause an increase in proinflammatory taxa, suggesting that the necessity of probiotic supplementation is strongly influenced by the type of disturbance introduced to the community.

Introduction

The ecological balance maintained by the gut microbial community has been shown to play an important role in host metabolism (reviewed by Rowland et al. 2018), nutrition (Yatsunenko et al., 2012), and immune function (Round and Mazmanian, 2009; Kau et al., 2011). However, dysbiosis, or a disturbance in the healthy microbiome, is suggested to cause a variety of health issues such as obesity (Ley et al., 2005), diabetes (Qin et al., 2012; Kostic et al., 2015), asthma (Stokholm et al., 2018), and inflammatory bowel disease (IBD) (Petersen and Round, 2014). While the gut microbiome is relatively stable over time and resilient to isolated disturbance events (Faith et al., 2013), the long-term effects of repeated disturbance remain poorly understood.

The gut microbiome can be disturbed through various events, such as the consumption of a high-fat diet (He et al., 2018), jet lag (Thaiss et al., 2014), and the use of medications, especially antibiotics (Theriot et al., 2014). While antibiotics are important in combating diseases caused by pathogenic bacteria, an unintended consequence is that they can also affect other beneficial and commensal species in the gut (Jernberg et al., 2007). Overuse of antibiotics can also lead to clinical issues such as the emergence of antibiotic-resistant strains (Levy and Marshall, 2004), weight gain (Cho et al., 2012; Gerber et al., 2016), and antibiotic-associated diarrhea (Hogenauer et al., 1998; Wiström et al., 2001; Elseviers et al., 2015). Furthermore, repeated antibiotic use can alter the composition of the gut microbiome long term (Dethlefsen and Relman, 2011).

Recently, probiotics, or live microbes exogenously administered for therapeutic purposes, have been suggested as a promising remedy for antibiotic-induced dysbiosis (Korpela et al., 2016; Ekmekciu et al., 2017). Probiotics have become increasingly popular — with a compound annual growth rate (CAGR) of 7.0 %, the global probiotics market is expected to reach 63 billion USD (approximately 6.6 trillion JPY) by 2023 (Global Market Insights, 2016). However, the efficacy of probiotic therapies is debated, as many probiotic strains do not remain in the gut long term (reviewed by Suez et al., 2019). Furthermore, a recent study showed that probiotics possibly inhibit, rather than promote, recovery, while autologous fecal microbiome transplants effectively restored gut microbiome diversity (Suez et al., 2018).

Fecal microbiome transplants (FMT) have been used as a treatment for severe antibiotic-induced dysbiosis (Shahinas et al., 2012) and provide relatively rapid recovery from dysbiosis (Suez et al., 2018). However, despite increasing reports of successful treatments, the methodology is unstandardized (Goldenberg et al., 2018), and challenges for clinical implementation remain. Furthermore, several side effects, such as weight gain and diarrhea, have been reported (Alang and Kelly, 2015). In 2019, a death from an infection caused by *Escherichia coli* strains that produce extended-spectrum β-lactamase (ESBL) after FMT was reported (U. S. Food and Drug Administration, 2019). While both probiotics and FMT are

promising therapeutic microbiome-based treatments, studies often report conflicting results, indicating a need for further research.

One of the difficulties with probiotics research is the variety of probiotic strains available, leading to variability in reported results. Species in the *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Escherichia*, *Lactobacillus*, *Saccharomyces*, and *Streptococcus* genera are most often used in probiotic products. However, purported effects can vary not only at the species level but also at the strain level. When formula-fed infants were given either *Bifidobacterium longum* subspecies *infantis* (*B. infantis*) or *Bifidobacterium animalis* subspecies *lactis*, *B. infantis* was more effective in increasing fecal bifidobacteria and decreasing γ-Proteobacteria due to its superior ability to colonize the infant gut (Underwood et al., 2013). In a study by Gotoh et al., the addition of different *Bifidobacterium bifidum* strains to fecal cultures increased fecal bifidobacteria, but the ability of *B. bifidum* to increase the abundance of other bifidobacterial species varied by strain (Gotoh et al., 2018; Katoh et al., 2020).

Despite the diversity in both antibiotics and probiotic species, many studies utilize a single combination of broad-spectrum antibiotics and pre-made probiotic blends. Consequently, the effect of specific antibiotic-probiotic combinations remains relatively understudied. The type, intensity, and frequency of disturbance are important factors that shape ecological communities and their response to subsequent recovery treatments. Therefore, I introduced a repeated disturbance to the gut microbiome with three types of antibiotics that have different bacterial targets and modes of action: vancomycin, amoxicillin, and ciprofloxacin. As a probiotic, I used *Bifidobacterium bifidum* JCM 1254, an infant-gut associated species that extracellularly degrades complex sugars, such as human milk oligosaccharides (HMOs) and mucin *O*-glycans (Gotoh et al., 2018). Presented here is a comparative analysis of repeated antibiotic disturbance on the gut microbiome and the subsequent effect of probiotics on recovery in a lab-controlled experiment using mouse models.

Materials and Methods

Animals and Housing

A total of 40 female C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) at 8–10 weeks of age. Mice were housed individually in polycarbonate cages with bedding and given free access to drinking water and a basal diet, Oriental MF (Oriental Yeast Co., Ltd., Tokyo, Japan). The mice were kept under controlled conditions of humidity (70 %), lighting (12-h light/dark cycle), and temperature (22℃). The experiment began after a 2-week acclimation period. The protocols of the

experiment were approved by the Kyoto University Animal Experimentation Committee (Lif-K18009 and Lif-K19022). Animal experiments were performed from August 21, 2018, to June 17, 2019.

Antibiotics

Three different types of antibiotics were selected: vancomycin hydrochloride (Nacalai Tesque Inc., Kyoto, Japan), amoxicillin (LKT Laboratories, Inc., Minnesota, USA), and ciprofloxacin (LKT Laboratories, Inc., Minnesota, USA). The antibiotics were selected for their varied spectrum of activity and reported effects on the gut microbial community (Table III-1). Antibiotics were administered in drinking water for mice to ingest *ad libitum*. Concentrations of each antibiotic were calculated and adjusted for mice based on human dosages suggested by the US Food and Drug Administration (GlaxoSmithKline, 2006; Baxter Healthcare, 2007; Bayer HealthCare, 2017).

Table III-1 | The characteristics of the antibiotics used in this experiment.

Antibiotic	**Class**	**Bacterial Target**	**Dosage**
Amoxicillin	Penicillin	Moderate spectrum	0.22 mg / mL
Ciprofloxacin	Fluoroquinolone	Broad-spectrum, Gram-negatives	0.19 mg / mL
Vancomycin	Glycopeptide	Gram-positives	0.25 mg / mL

Experimental Design

Mice were divided into 10 groups (Table III-2), with one control group and 9 different antibiotic-recovery combinations, with four biological replicates per group. The sample size was calculated based on power analyses and the resource equation approach (Arifin and Zahiruddin, 2017). Each group received antibiotics (vancomycin, amoxicillin, or ciprofloxacin) in drinking water for one week (antibiotics week). After antibiotics were administered, mice were switched to normal water without antibiotics and were given one of the following recovery treatments for one week (treatment week): natural recovery (NR); *Bifidobacterium bifidum* JCM 1254 as a probiotic (PR); or fecal transplant from control mice (FT). The treatment week was followed by one week with no treatments to allow the mice to recover (recovery week). With the treatment week and recovery week combined, mice were given two weeks to recover from antibiotic administration, as past studies have reported that the gut microbiome

recovers within 1-2 weeks after disturbance (David et al., 2014; MacPherson et al., 2018). This was repeated three times for a total of three 3-week phases (Figure III-1). Mice in the control were provided with water without antibiotics throughout the 9-week experiment.

Table III-2 | List of treatment groups (antibiotic and recovery treatment combinations).

Treatment Group	Antibiotics	Recovery
Control		
A	Amoxicillin	Natural Recovery (NR)
AB	Amoxicillin	*B. bifidum* JCM1254 (PR)
AF	Amoxicillin	Fecal Transplant (FT; from Control)
P	Ciprofloxacin	Natural Recovery (NR)
PB	Ciprofloxacin	*B. bifidum* JCM1254 (PR)
PF	Ciprofloxacin	Fecal Transplant (FT; from Control)
V	Vancomycin	Natural Recovery (NR)
VB	Vancomycin	*B. bifidum* JCM1254 (PR)
VF	Vancomycin	Fecal Transplant (FT; from Control)

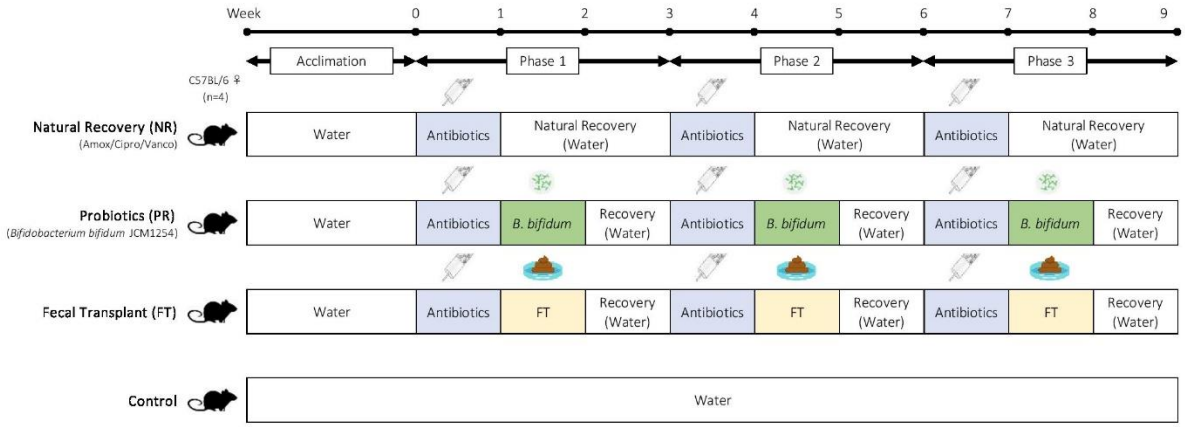


Figure III-1 | **Experimental design.** A total of 40 adult female C57BL/6 mice were used in this experiment. After a 2-week acclimation period, mice were given one of the following antibiotics for one week: amoxicillin, ciprofloxacin, or vancomycin. Antibiotic treatment was followed by one of the following recovery treatments: natural recovery (NR); *Bifidobacterium bifidum* JCM1254 as a probiotic (PR); and fecal transplant (FT). The recovery treatment was followed by 7 days of no treatments to allow the mice to recover. Each 3-week cycle was repeated three times during this 9-week experiment. Mice in the control were provided with water alone throughout the experiment. This figure is used with permission from the authors (Ojima et al., 2020).

To prepare for probiotic administration, Gifu Anaerobic Medium (GAM, Nissui Pharmaceutical, Tokyo, Japan) was inoculated with *B. bifidum* each day and incubated at 37 ℃ overnight. From the overnight cultures, bacterial suspensions were diluted in phosphate-buffered saline (PBS) at a concentration of 5 x 10^9 CFU per mL. 200 μL of the bacterial suspensions were administered to each mouse via oral gavage daily during the treatment weeks. For fecal transplants, a mixture of fresh feces collected from age-matched control mice was suspended in PBS at a concentration of 40 mg/mL and vortexed for 3 minutes. The mixture was then allowed to settle, and 200 μL of the supernatant was given to the mice via oral gavage daily during the treatment weeks. As a control, mice in the control and NR groups were given 200 μL of anaerobic PBS via oral gavage daily during the treatment weeks.

Body weight was measured as an indicator of feed intake and health. Fecal samples were collected from each mouse at the end of each week and stored at −30 ℃ and freeze-dried within a few days of collection. Freeze-dried fecal samples were stored at −30 ℃ until use for DNA extraction. At the end of the experiment, animals were humanely euthanized by cervical dislocation. Immediately after death, a midline incision was made to exteriorize the intestine and cecum. Cecum weight was measured, and intestinal tissue samples were stored in RNA*later* (Invitrogen, Taastrup, Denmark) at 4 ℃ until use for RNA extraction.

DNA Extraction

Freeze-dried fecal samples were placed in 2 mL plastic tubes with one 5.0 mm stainless steel bead and approximately 200 mg of 0.1 mm zirconia beads. The tubes were then vigorously shaken for 10 minutes at 1,500 rpm using the Shake Master NEO (Bio Medical Science, Tokyo, Japan) before extraction, as described previously (Sakanaka et al., 2019). Genomic DNA was extracted using a Qiagen QIAamp® DNA Fast Stool Mini Kit (Hilden, Germany) according to the manufacturer's instructions with a few modifications. Extracted DNA samples were stored at −30 ˚C until use.

Quantification of Total Bacterial Load Using Quantitative PCR

After genomic DNA extraction, the total bacterial load was quantified by measuring the number of copies of the 16S ribosomal RNA (16S rRNA) gene by quantitative PCR (qPCR). qPCR was performed with a Thermal Cycler Dice Real-Time System (TaKaRa Bio., Kyoto, Japan). Each reaction mixture (total volume: 15 μL) contained the following: 7.5 μL of TB Green® Premix Ex Taq™ II (TaKaRa Bio, Kyoto, Japan), 0.6 μL (10 pmol) of each forward (5'- ACTCCTACGGGAGGCAGCAGT -3') and reverse (5'- ATTACCGCGGCTGCTGGC -3') primers, 1 μL of extracted DNA (diluted to 5

ng/μL), and 5.3 μL of water. The cycling conditions included an initial denaturation of 10 min at 95 °C followed by 40 cycles of 95 °C for 30 s and 68 °C for 1 min. Known concentrations of genomic DNA extracted from *Bacteroides thetaiotaomicron* were used as reference curves for DNA quantification.

Microbiome Analysis

Microbiome analysis was performed with the support of Morinaga Milk Industry, Co. Ltd. Sequencing of the V3-V4 region of the 16S rRNA gene was performed with an Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) as described previously (Odamaki et al., 2019). After removing sequences consistent with data from phiX reads from the raw Illumina paired-end reads, the sequences were analyzed using the QIIME2 software package version 2017.10 (https://qiime2.org/). After trimming of the forward and the reverse reads (30 bases from the 5' region and 90 bases from the 3' region, respectively), the paired-end reads were joined, and potential chimeric sequences were removed using DADA2 (Callahan et al., 2016). The taxonomical classification was performed using a Naive Bayes classifier trained on the Greengenes 13.8 16S rRNA reference set with a 99 % threshold of OTU full-length sequences. When possible, species were determined by Blastn analysis of the representative OTU sequences, for which the NCBI rRNA database was used.

Quantification of Inflammation-Related Gene Expression Using Reverse-Transcription qPCR

The expression of inflammation-related genes was quantified with the support of H. Takada from Kyoto University. Intestinal tissue samples were placed in 2 mL plastic tubes with one stainless steel bead (5.0 mm) and approximately 200 mg of 0.1 mm zirconia beads. Samples were homogenized by vigorous shaking for 20 minutes at 1500 rpm using the Shake Master NEO (Bio Medical Science, Tokyo, Japan). Following RNA extraction using NucleoSpin® RNA (TaKaRa Bio., Kyoto, Japan) according to the manufacturer's instructions, cDNA was synthesized from 500 ng of total RNA by reverse transcription (RT) using PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa Bio., Kyoto, Japan). To measure the expression of inflammation-related genes in the intestinal tissue, qPCR was carried out with a Thermal Cycler Dice Real-Time System (TaKaRa Bio Each RT-qPCR reaction contained the following: 7.5 μL of TB Green® Premix Ex Taq™ II (TaKaRa Bio., Kyoto, Japan), 0.6 μL (10 pmol) of each forward and reverse primers, 1 μL of the appropriately diluted cDNA solution, and 5.9 μL of water. The specificity of all primers was confirmed by analyzing the melting curves after the PCR was run. The cycling conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and a dissociation phase with 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Standard curves were created for respective

genes using the PCR-amplified fragments as templates with oligo-dT primers. The primers were designed using Primer3 Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), and the primer sets are listed in Table III-3.

Table III-3. Primer sets used for the quantification of inflammation-related gene expression with qPCR

Gene	Forward Primer (5'- 3')	Reverse Primer (5'- 3')
Actb	GCTCTTTTCCAGCCTTCCTT	AGGGAGACCAAAGCCTTCAT
Infg	CCTTTGGACCCTCTGACTTG	CGCAATCACAGTCTTGGCTA
Il1b	TGAAATGCCACCTTTTGACA	CTGCCTAATGTCCCCTTGAA
Il6	CACGGCCTTCCCTACTTCAC	TTCCAAGAAACCATCTGGCTA
Il10	CCAAGCCTTATCGGAAATGA	CATTCCCAGAGGAATTGCAT
Tnf	CCACATCTCCCTCCAGAAAA	CTCCCTTTGCAGAACTCAGG

Diversity/Similarity Metrics and Statistical Analysis

Statistical analyses were performed using R ver. 3.6.0 (www.r-project.org). Species richness (α diversity) of the samples was estimated by the number of OTUs in each microbial profile using the Shannon Index (Shannon and Weaver, 1949) and the Chao1 Index. Diversity indexes used in this study are summarized in Table III-4. Two-Way Repeated Measures ANOVA (rm-ANOVA) with Tukey's HSD post hoc test was used to determine the effect of each treatment over time. To determine the recovery of microbial communities, the magnitude of the microbial shift was quantified by comparing the microbiome profiles at baseline (Week 0) with profiles from other time points using the Bray-Curtis Dissimilarity Index. Community structure was further analyzed using principal components analysis (PCA) and exploratory factor analysis (EFA). One-Way ANOVA with post-hoc Dunnett's test was used to determine the statistical differences in cecum weight and expression of inflammation-related genes. Pearson's correlation analysis was used to identify specific taxa that were positively or negatively associated with cecum weight and expression of inflammation-related genes.

Table III-4. Summary of diversity indexes used in this study.

Index	Description
Shannon	An index that accounts for both the number of species and their respective relative abundances. Higher values indicate higher diversity and evenness within the community (Shannon and Weaver, 1949).
Chao1	An estimate for species richness based on the abundance of OTUs. Higher values indicate a higher diversity within a given community (Chao, 1984).
Bray-Curtis Dissimilarity	A measure of β-diversity that quantifies the dissimilarity between two different sites based on community composition. Values are bound between 0 and 1. If 0, the two sites similar and share all the same species. If 1, the sites are dissimilar and do not share any species (Bray and Curtis, 1957).

Results and Discussion

The goal of this study was to assess the efficacy of the probiotic strain, *Bifidobacterium bifidum* JCM 1254, in the recovery period after the repeated antibiotic disturbance. Using mouse models, the effect of three different antibiotics with varying bacterial targets and spectrum of activity were tested. A subsequent recovery treatment consisted of natural recovery, *B. bifidum* administration, or fecal transplants from healthy, age-matched donor mice from the control group. The key findings of this study are as follows: (1) the response of the gut microbiome varies significantly with the type of disturbance; (2) *B. bifidum* is most effective when antibiotic disturbance increases proinflammatory species; (3) probiotic supplementation does not restore the diversity of the gut microbiome to baseline levels but can contribute to the recovery of host health. These results provide insight into how disturbance ecology affects the gut microbial community and its response to recovery treatments.

Vancomycin significantly alters the gut microbiome and increases proinflammatory species

First, the effect of repeated exposure to vancomycin, ciprofloxacin, or amoxicillin on the structure of the gut microbiome was compared (see Table III-1 for spectrum and mode of action). To do so, each antibiotic was administered in drinking water for a week, and the mice were given two weeks of natural

recovery. This process was repeated three times (Natural Recovery; Figure III-1). Although statistically insignificant, the percent body weight increase tended to be greater for all antibiotics compared to control (Supplementary Figures III-1 A–C). The fecal microbiome was analyzed by meta-16S rRNA sequencing. For all antibiotic types, significant variation in bacterial load during the experiment was not observed (Supplementary Figures III-1 D–F). This may be because fecal samples were collected after 7 days of antibiotic administration, which could have allowed the taxa unaffected by the antibiotics to proliferate during that time. Similar trends with vancomycin (Cheng et al., 2017) and amoxicillin (Cabral et al., 2019) have also been previously reported.

However, the differences between antibiotics were clear when comparing α-diversity using the Shannon Index (evenness; Figure III-2 A) and the Chao1 Index (species richness; Figure III-2 B). The results of Two-Way rm-ANOVA show that the type of antibiotic had differential effects on α-diversity (Supplementary Table III-1). For ciprofloxacin, antibiotic administration had no significant effect on α-diversity over time. Ciprofloxacin has been shown to significantly alter the gut microbiome in human subjects (Dethlefsen and Relman, 2011), but has a limited effect on community structure in murine models (Schubert et al., 2015). Furthermore, ciprofloxacin is considered to have limited activity against anaerobic microbes (Goldstein and Citron, 1988). For amoxicillin, α-diversity was significantly reduced in terms of both evenness (> 34 % reduction; Figure III-2 A) and species richness (> 60 % reduction Figure III-2 B) after the first antibiotic disturbance event but recovered to control levels within two weeks. While this pattern continued after the second and third antibiotic disturbance events for species richness, evenness was not significantly affected after the first disturbance event, as amoxicillin is a β-lactam antibiotic that affects both Gram-positive and -negative bacteria. Of the three antibiotics, vancomycin had the strongest effect on α-diversity. The first antibiotic disturbance significantly reduced evenness (> 52 % reduction; Figure III-2 A) and richness (> 81 % reduction; Figure III-2 B), both of which did not recover throughout the experiment.

In addition to α-diversity, recovery was measured by quantifying the magnitude of the microbial shift from baseline (Week 0) using the Bray-Curtis Index of Dissimilarity (Figure III-2 C), and the results of Two-Way rm-ANOVA show that the differences in the antibiotic type significantly affected the microbial communities during recovery (Supplementary Table III-2). For this study, communities that returned to baseline community structures based on this index were considered as “recovered.” For both ciprofloxacin and amoxicillin, dissimilarity increased with each antibiotic disturbance event and gradually decreased over the following two weeks.

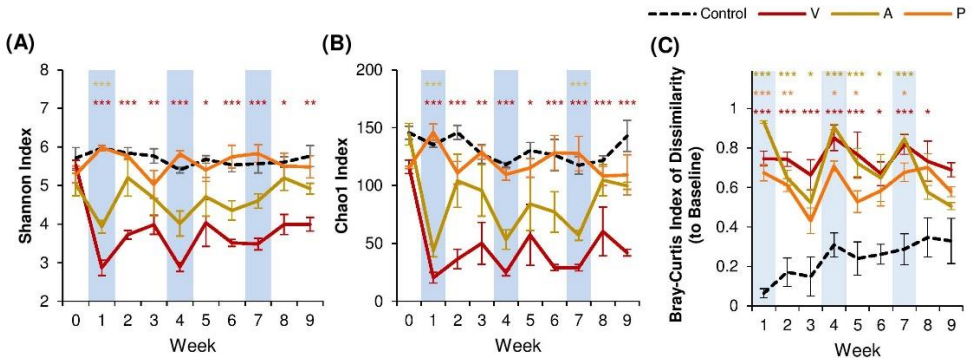


Figure III-2 | Comparison of the effects of vancomycin, ciprofloxacin, and amoxicillin on diversity metrices. Each antibiotic was administered for 7 days every 3 weeks, and changes to the fecal microbiome over time were observed by meta-16S rRNA sequencing. Alpha diversity measured by (A) Shannon Index and (B) Chao1 Index for each treatment over time ± standard error. (C) Bray-Curtis Index of Dissimilarity vs baseline for each treatment over time ± standard error. The Bray-Curtis Index was used to quantify the amount of microbial shift from the first day of the experiment (baseline) for each mouse. Colored asterisks indicate significance vs control for NR, PR, and FT groups based on Two-Way rm-ANOVA and Tukey's HSD post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Data for the control samples are indicated as the black dotted line. Natural recovery data for vancomycin is in red, amoxicillin in yellow, and ciprofloxacin in orange. Figure modified with permission from Ojima et al., (2020).

Examination at the phylum level showed that each antibiotic disturbance event increased the relative abundance of Proteobacteria (12 %, ciprofloxacin; 20 %, amoxicillin), which then decreased over time (Figure III-3 A). Although some level of recovery was observed, the microbial communities did not return to baseline levels throughout the experiment, which is consistent with previous studies that report that repeated antibiotic use leads to incomplete recovery (Dethlefsen and Relman, 2011). With vancomycin, the microbial communities displayed patterns consistent with α-diversity, and community dissimilarity remained high throughout the experiment after the first antibiotic disturbance. At the phylum level, the relative abundance of Proteobacteria increased significantly after the first antibiotic disturbance event (70 %). Although this increase was diminished after the second (19 %) and third (8 %) antibiotic disturbance events, the presence of Proteobacteria was persistent throughout the experiment (Figure III-3 A). For all antibiotics, the increase in Proteobacteria was less pronounced with repeated disturbances. Further examination with principal components analysis (PCA) based on the microbial community composition corroborated these observations. For vancomycin- and amoxicillin-treated groups, PCA revealed that communities after the first antibiotic administration formed a separate cluster (Supplementary Figures III-2 A, B). The subsequent second and third antibiotic treatments for vancomycin and amoxicillin clustered closer to the control communities, lending further evidence to the fact that the gut microbiome retains the memory of past disturbance events (Dethlefsen and Relman, 2011). For ciprofloxacin-treated groups, however, the different treatments did not create clear clusters (Supplementary Figure III-2 C).

Further examination using exploratory factor analysis showed that the increase in Proteobacteria can be attributed to *Escherichia coli* for all antibiotics (Figure III-3 B, Supplementary Figure III-3, Supplementary Table III-3). However, for vancomycin, there were also increases in proinflammatory species associated with dysbiosis. For example, after the first antibiotic disturbance event, there was a notable increase in *Proteus* (34.7 %, relative abundance; Figure III-3 B, Supplementary Figure III-3), a genus associated with the onset of colitis (Shin et al., 2015). There was also an increase in the abundance of Desulfovibrionaceae (Figure III-3 B, Supplementary Figure III-3), a family of sulfate-reducing bacteria often associated with high-fat diets (Clarke et al., 2012) during Week 2 (a 350-fold increase compared to baseline). In disturbance ecology, the type of disturbance is a critical factor that determines which specific members of the community are selected for over time (Relman, 2012), and these results indicate that gut microbiome responses vary significantly by antibiotic type, with vancomycin having the most detrimental effects. Previous studies have shown that vancomycin is a particularly potent antibiotic that significantly reduces gut microbiome diversity (Vrieze et al., 2014) and causes intestinal dysbiosis (Cheng et al., 2017). Therefore, the following sections focus on vancomycin and how different treatments (fecal transplants or probiotic administration) could contribute to the recovery of the gut microbial community.

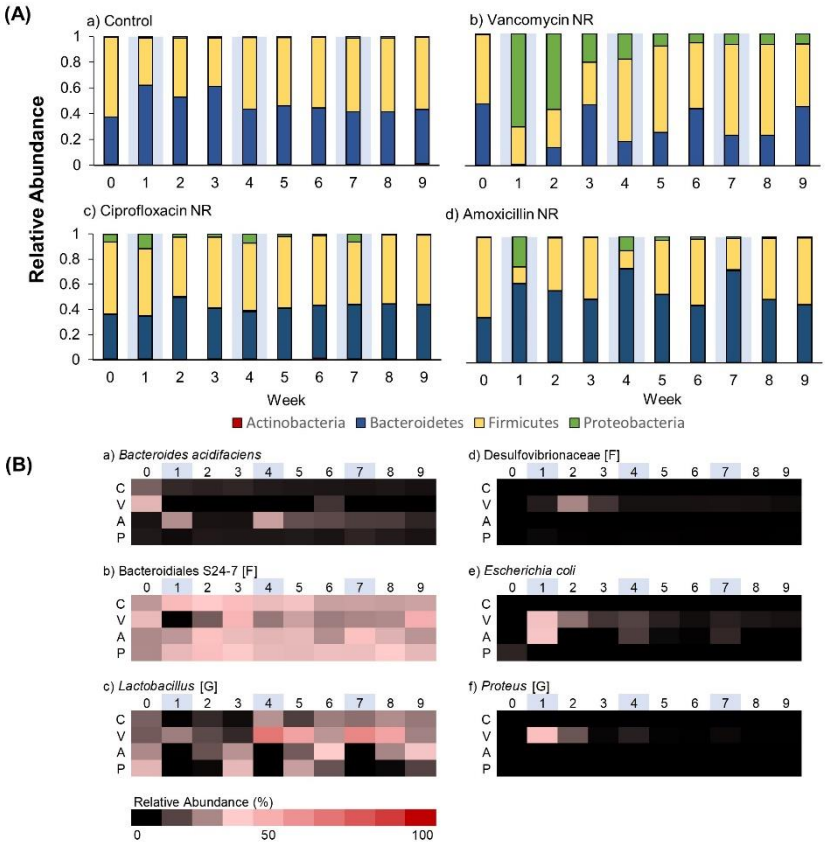


Figure III-3 | Comparison of the effects of vancomycin, ciprofloxacin, and amoxicillin on the gut microbiome. The community composition of the gut microbiome at each time point for each treatment was observed using meta-16S rRNA sequencing. (A) The microbial community at each time point at the phylum level for (a) control, (b) vancomycin, (c) ciprofloxacin, and (d) amoxicillin. (B) Heat map of taxa that significantly changed after antibiotic administration. Significant taxa were identified using factor analysis (factor loading > 0.2). The lowest taxonomic rank for which information was available is indicated in square brackets (F: family, G: genus). Weeks shaded in blue indicate weeks in which antibiotics were administered. Figure modified with permission from Ojima et al., (2020).

Fecal transplants restore gut microbiome diversity after vancomycin administration

Past studies have indicated that fecal transplants contribute to relatively rapid recovery after antibiotic-induced dysbiosis (Ekmekciu et al., 2017; Suez et al., 2018). After each vancomycin administration, I administered fecal transplants from healthy, age-matched control mice for a week. As expected, the fecal transplants produced a significant effect on both α-diversity metrics, as well as community dissimilarity (Supplementary Tables III-4, 5). α-Diversity was reduced after the first antibiotic disturbance event but completely recovered to control levels within two weeks of fecal transplants, and this pattern was observed for the subsequent disturbance events as well (Figures III-4 A, B). A similar pattern was observed for community dissimilarity, where each antibiotic administration increased dissimilarity, but fecal transplants restored community structures to baseline levels within two weeks

(Figure III-4 C). Examination of community membership revealed that, compared to the natural recovery groups (V), fecal transplants were effective in reducing the Proteobacteria populations that had increased with each vancomycin administration (Figure III-5 A). While Proteobacteria populations persisted in the natural recovery groups (relative abundance > 8 %), Proteobacteria were nearly undetectable within two weeks after fecal transplants (relative abundance < 1 %). The increase in inflammatory taxa such as *Proteus* was also suppressed (Figure III-5 B). These results are consistent with previous studies, which have shown that fecal transplants are effective in correcting dysbiosis and reducing inflammation. Furthermore, a recent study by Burrello et al. (2018) demonstrated that fecal transplants promote recovery by stimulating immune cells to produce IL-10 and that the beneficial effects of fecal transplants seem to be correlated with the persistence of protective taxa such as Lactobacillaceae, Bifidobacteriaceae, Erysipelotrichaceae, Ruminococcacceae, and Bacteroidales S24-7.

Bifidobacterium bifidum does not restore diversity but reduces intestinal inflammation

In addition to fecal transplants, probiotic supplementation has been linked to a variety of positive effects, such as reduced incidences of diarrhea in infants (Hotta et al., 1987), improvement in immune functions (Mohan et al., 2008), anti-obesity effects (Kondo et al., 2010; Stenman et al., 2014; Moya-Pérez et al., 2015), and recovery after antibiotic disturbance (Grazul et al., 2016; Ekmekciu et al., 2017). However, how effective probiotics are in restoring the disturbed gut microbial community after antibiotics remains a topic of debate (Suez et al., 2019). To assess the efficacy of probiotics in recovery after vancomycin administration, *Bifidobacterium bifidum* JCM 1254 was administered to mice via oral gavage for one week. Probiotic administration seemed to have little effect on recovery. Like the natural recovery groups, α-diversity did not return to baseline levels after the first antibiotic disturbance event (Figures III-4 A, B), and community dissimilarity remained high (Figure III-4 C). Similarly, Suez et al. (2018) also reported that a probiotic blend including *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, and *Streptococcus* genera did not promote recovery after antibiotic-induced dysbiosis. These results suggest that species commonly called “probiotics” may be insufficient for community recovery.

The results of exploratory factor analysis showed that the relative abundance of *Lactobacillus* species, *Proteus* species, *E. coli*, and Bacteroidales S24–7 contributed significantly to community structure (Supplementary Table III-6). Although α-diversity did not recover, the first *B. bifidum* supplementation caused a two-fold increase in the relative abundance of Bacteroidales S24–7 (Figure III-5 B), a family of fermenters often associated with a healthy microbiome in mice that produce short-chain fatty acids (SCFA) and vitamin B (Evans et al., 2014; Rooks et al., 2014; Ormerod et al., 2016). While not as effective as fecal transplants, probiotics were also able to suppress the increase of Proteobacteria,

such as *E. coli* and *Proteus* populations (Figure III-5 B). Previous studies have also reported the reduction of Proteobacteria after *Bifidobacterium* supplementation. For instance, the administration of *Bifidobacterium longum* decreased the relative abundance of Proteobacteria and reduced the expression of the gene encoding TNF-α in mice (Lee et al., 2019), and *B. infantis* supplementation decreased γ-Proteobacteria in infants (Underwood et al., 2013). Furthermore, I observed that recovery treatments had a significant effect on cecum size (One-Way ANOVA: $F(3,12) = 5.513$, $p < 0.05$; Figure III-6 A). While mice in the natural recovery group (V) had a significantly larger cecum compared to the control (post hoc Dunnett's test: $p < 0.05$), the difference was insignificant for groups given *B. bifidum* (VB) and fecal transplants (VF), suggesting that cecal enlargement was corrected by *B. bifidum* administration and fecal transplants. A previous study has also reported an enlargement in the cecum of antibiotic-treated mice, possibly because of a decrease in intestinal motility (Puhl et al., 2012). Cecal enlargement may also have been caused by the increase of pro-inflammatory species. Further analysis with Pearson's correlation analysis revealed that there were strong positive correlations between cecum weight and the abundance of inflammatory taxa such as *Proteus* ($r = 0.91$, $p < 0.001$), and *E. coli* ($r = 0.84$, $p < 0.001$). Cecum weight and the expression of genes encoding IL-1β also showed a significant positive correlation ($r = 0.70$, $p < 0.05$).

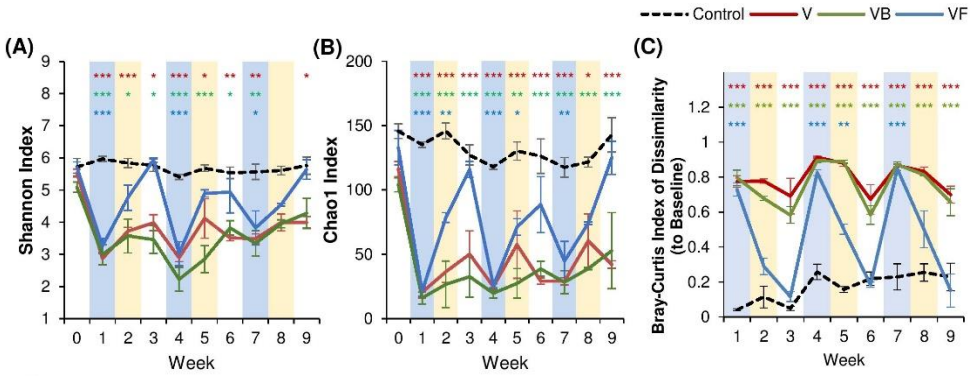


Figure III-4 | The effect of recovery treatments on the gut microbiome after vancomycin. Vancomycin administration was followed by either natural recovery (NR), *Bifidobacterium bifidum* (PR), or fecal transplants (FT), and changes to the gut microbiome were observed over time. Alpha diversity measured by (A) Shannon Index and (B) Chao1 Index for each treatment over time ± standard error. (C) Bray-Curtis Index of Dissimilarity vs baseline for each treatment over time ± standard error. The Bray-Curtis Index was used to quantify the amount of microbial shift from the first day of the experiment (baseline) for each individual. Colored asterisks indicate significance vs control for NR, PR, and FT groups based on Two-Way rm-ANOVA and Tukey's HSD post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Data for the control samples are indicated as the black dotted line, with NR (V) groups in red, PR (VB) groups in green, and FT (VF) groups in blue. Figure modified with permission from Ojima et al., (2020).

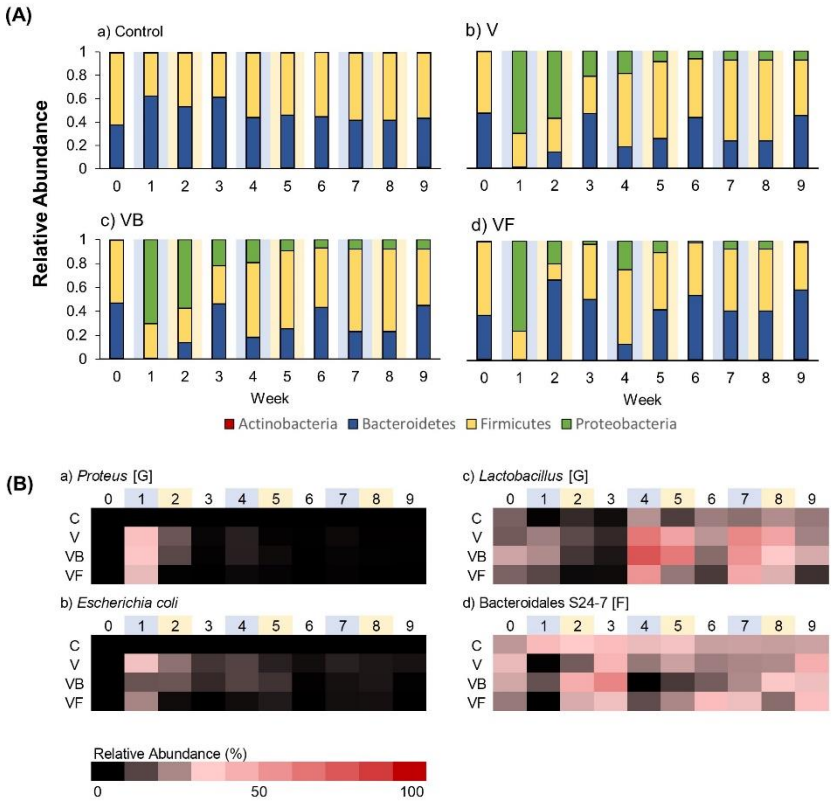


Figure III-5 | The effect of recovery treatments on the gut microbiome after vancomycin. The community composition of the gut microbiome at each time point for each treatment was observed using meta-16S rRNA sequencing. (A) The microbial community at each time point at the phylum level for (a) control, (b) V, (c) VB, and (d) VF. (B) Heat map of taxa that significantly changed after antibiotic administration. Significant taxa were identified using factor analysis (factor loading > 0.4). The lowest taxonomic rank for which information was available is indicated in square brackets (F: family, G: genus). Weeks shaded in blue indicate weeks in which antibiotics were administered, and weeks shaded in yellow indicate weeks in which a recovery treatment (natural recovery, probiotics, or fecal transplants) were administered. Figure modified with permission from Ojima et al., (2020).

Additionally, results of RT-qPCR indicate that the expression of genes encoding proinflammatory cytokines (IL-1β and INF- γ) in the colon was significantly increased in the natural recovery groups (Figures III-6 B, C), while expression was suppressed by probiotic administration and fecal transplants. Verma et al. (2019) recently identified *B. bifidum* cell surface polysaccharides as a factor that suppresses inflammation in the gut. Another possible anti-inflammatory mechanism may be modulated by indole-3-lactic acid (ILA) produced by *Bifidobacterium* species. ILA is an aromatic lactic acid and a metabolite of aromatic amino acids such as tryptophan and serves as a ligand for the aryl hydrocarbon receptor (AhR) that regulates intestinal homeostasis. Meng et al. (2019) found that ILA produced by *B. infantis* had anti-inflammatory effects on infant enterocytes *in vitro*. This metabolite is also reported to be produced by *B. bifidum* (Sakurai et al., 2019). A recent study by Laursen et al. (2020) has shown that *Bifidobacterium* species possess a specific enzyme that converts aromatic pyruvates, precursors of aromatic amino acids,

into aromatic lactic acids. Given these results, it is possible that *B. bifidum* supplementation suppresses the increase of proinflammatory species and ultimately reduces gut inflammation.

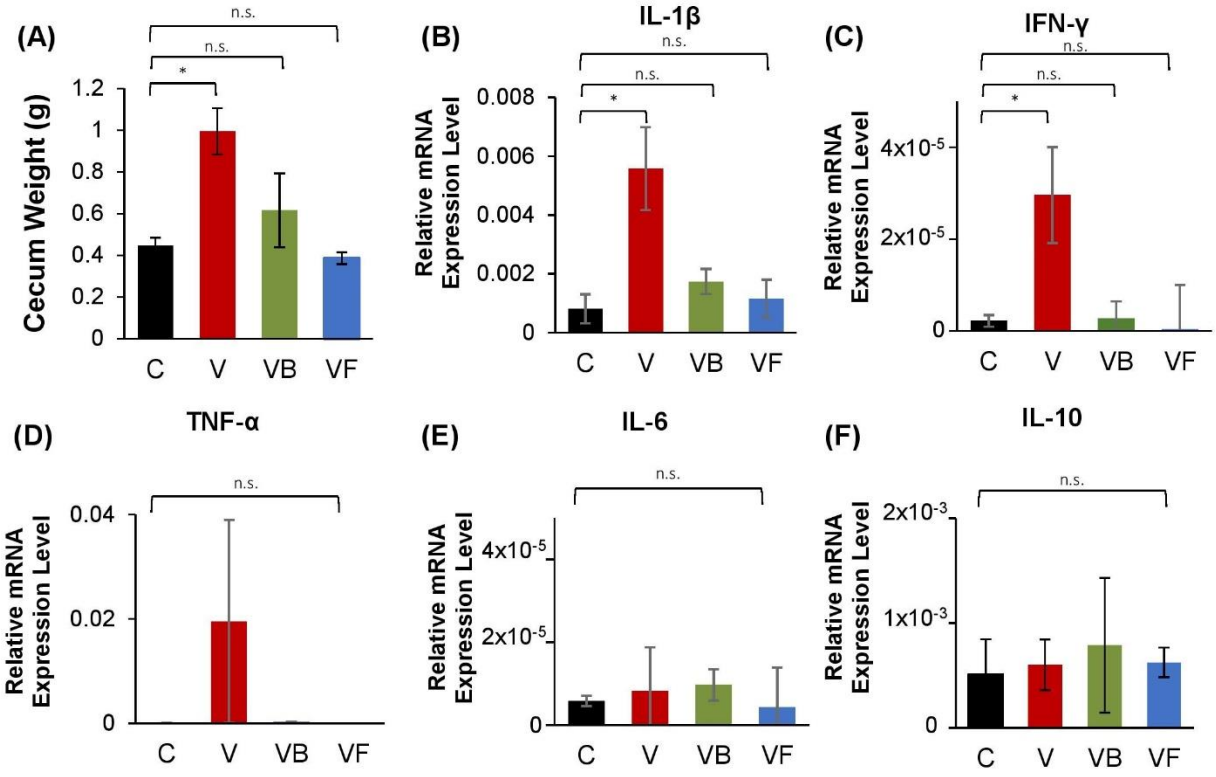


Figure III-6 | Changes in cecum size and expression of inflammation-related genes in vancomycin-treated mice. At the end of the experiment, cecum weight and the mRNA expression levels of inflammation-related genes in the large intestine for vancomycin-treated mice (C: control, V: vancomycin + natural recovery, VB: vancomycin + *B. bifidum*, VF: vancomycin + fecal transplant from control mice) were measured. (A) Cecum weight, relative mRNA expression of genes encoding (B) IL-β, (C) TNF-α, (D) INF-γ, (E) IL-6, and (F) IL-10 for vancomycin-treated mice, using Actb as a reference gene. Error bars indicate standard error, and significance was determined by One-Way ANOVA and Dunnett's test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). This figure is used with permission from Ojima et al., (2020).

The expression of genes encoding TNF-α, IL-6, and IL-10 were also examined, but there were no significant differences between treatments (Figures III-6 D–F), suggesting that antibiotic-induced dysbiosis leads to the induction of specific inflammatory cytokines. This experiment was repeated for amoxicillin- and ciprofloxacin-treated mice. However, the effects of neither fecal transplants nor probiotics differed significantly from NR groups for α-diversity and community structure (Supplementary Figures III-6, 5), and no cecum enlargement was observed, even for antibiotic-treated groups (Supplementary Figures III-6 A, 7 A). As neither amoxicillin nor ciprofloxacin caused a bloom in pro-inflammatory species (Supplementary Figures III-4 B–F, 5 B–F), probiotics did not affect the expression

of inflammation-related genes after antibiotic administration. In the absence of inflammatory species, the effect of fecal transplants and probiotic supplementation was negligible.

Increase in Lactobacillus abundance potentially delays gut microbiome recovery

One common event observed for all antibiotics was the expansion of *Lactobacillus*, particularly for vancomycin- treated groups (Figure III-3 B). The increase in *Lactobacillus* abundance was especially noticeable after the second antibiotic administration, possibly because of its superior capability to tolerate disturbances. Past studies have shown *Lactobacillus* species to have a high level of vancomycin resistance (Gueimonde et al., 2013), as well as a relatively high tolerance to low pH (Corcoran et al., 2005; O'May et al., 2005). Furthermore, Suez et al. (2018) found that *Lactobacillus* was a microbiome-inhibitory species. Although unconfirmed in this study, the increased relative abundance of *Lactobacillus* may have contributed to the inhibited recovery from antibiotic administration.

Limitations

One limitation of this study is that it utilized a human-derived *Bifidobacterium* strain in murine models. Even in the human gut microbiome, the inability of probiotics to colonize the gut is a longstanding issue (Suez et al., 2019), but the lack of colonization was particularly evident in this study. Although samples were collected within 24 h of *B. bifidum* administration, its detection was limited in the 16S metagenomic analysis. Furthermore, prebiotics were not administered, possibly making colonization by *B. bifidum* in the gut even more difficult to achieve. A recent study by Cabral et al. (2019) has shown that the addition of fiber protected gut microbes from antibiotics, suggesting that the carbohydrates consumed in the diet alter the gut microbiome's response to disturbances. Therefore, to develop more efficient probiotic therapies, future studies should also consider the type of diet and prebiotics that are co-administered with antibiotics and probiotics.

Conclusions

Despite these limitations, this study provides insight into how the gut microbiome responds to repeated disturbances and subsequent recovery treatments. In clinical settings, antibiotics are prescribed both frequently and repeatedly. A study based in the United Kingdom found that approximately 30 % of patients are prescribed antibiotics at least once a year (Shallcross et al., 2017), although a different class of antibiotics is often re-prescribed with repeated use. Nonetheless, the results of our study elucidated

how the repeated use of different types of antibiotics affects the response of the gut microbiome to recovery treatments. The type of disturbance (i.e., affected species, frequency, magnitude, and duration) is a key factor in community structuring, and its effect should be considered when examining the gut microbial community. The disturbance type determines which specific taxa and functions within the gut microbiome are selected for (Relman, 2012); moreover, it also affects how the gut microbiome responds to the addition of probiotics. The results of this study showed that probiotics were effective in reducing gut inflammation without recovering gut microbiome diversity. Additionally, this study showed that probiotics were most effective when antibiotic disturbance caused an increase in proinflammatory species. The results of the study could be applied to clinical settings, where predicting the response of the gut microbiome to different recovery treatments after dysbiosis would offer potential benefit.

Supplementary Figures and Tables

Supplementary Table III-1 | Two-Way rm-ANOVA results for α-diversity metrics for natural recovery groups.

Shannon	Chi-Sq	Df	p value	Significance
Antibiotic-Type	142.7184	3	< 2e-16	***
Week	0.1738	1	0.6768	
Antibiotics × Week	3.1144	3	0.3743	

Chao 1	Chi-Sq	Df	p value	Significance
Antibiotic-Type	94.9894	3	< 2e-16	***
Week	6.0514	1	0.0139	*
Antibiotics × Week	0.3801	3	0.9443	

Significance codes: *p < 0.05, **p < 0.01, ***p < 0.001

Supplementary Table III-2 | Two-Way rm-ANOVA results for based on Bray-Curtis Index of Dissimilarity for natural recovery groups.

	Chi-Sq	Df	p value	Significance
Antibiotic-Type	65.9684	3	3.11E-14	***
Week	0.3231	1	0.5698	
Antibiotics × Week	25.8446	3	1.03E-05	***

Significance codes: *p < 0.05, **p < 0.01, ***p < 0.001

Supplementary Table III-3 | Factor loadings based on exploratory factor analysis for natural recovery groups. Note that the absolute values for blank entries are less than 0.2, but not zero.

	Factor 1	Factor 2	Factor 3
Lactobacillus [G]	0.926644	-0.27507	
Proteus [G]		0.392107	-0.32282
Escherichia coli		0.499274	-0.45402
Desulfovibrionaceae [F]			-0.21764
Bacteroides acidifaciens			0.457251
Bacteroidales S24-7 [F]	-0.24224	-0.64117	-0.50964

Supplementary Table III-4 | Two-Way rm-ANOVA results for α-diversity metrics for vancomycin-treated groups.

Shannon	Chi-Sq	Df	p value	Significance
Treatment	138.5642	3	< 2e-16	***
Week	0.3842	1	0.5354	
Treatment × Week	1.0472	3	0.7898	

Chao1	Chi-Sq	Df	p value	Significance
Treatment	177.8706	3	< 2e-16	***
Week	3.4694	1	0.06251	
Treatment × Week	3.2894	3	0.3743	

Significance codes: *p < 0.05, **p < 0.01, ***p < 0.001

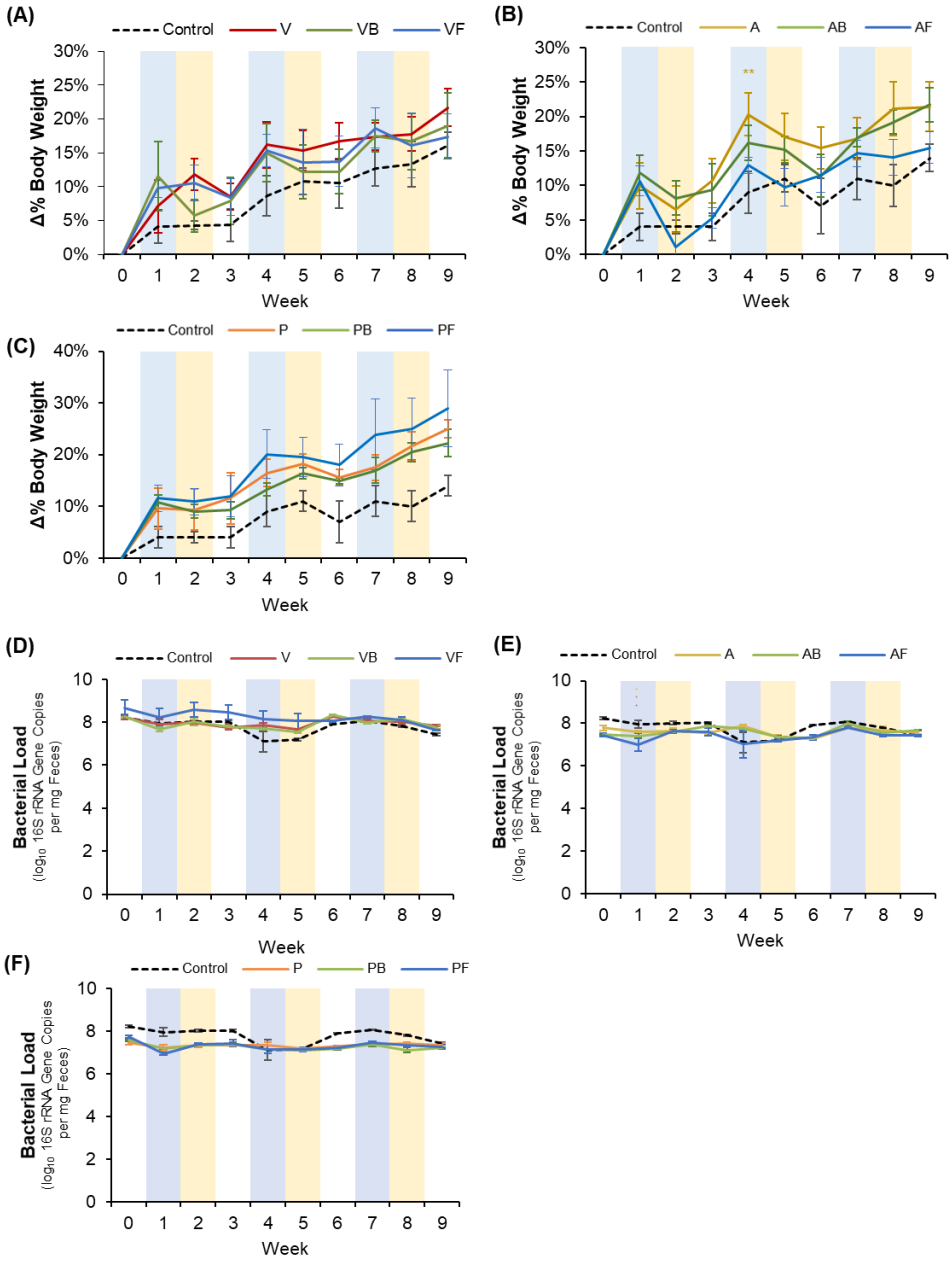
Supplementary Table III-5 | Two-Way rm-ANOVA results for based on Bray-Curtis Index of Dissimilarity for vancomycin-treated groups.

	Chi-Sq	Df	p value	Significance
Treatment	65.9684	3	3.11E-14	***
Week	0.3231	1	0.5698	
Treatment × Week	25.8446	3	1.03E-05	***

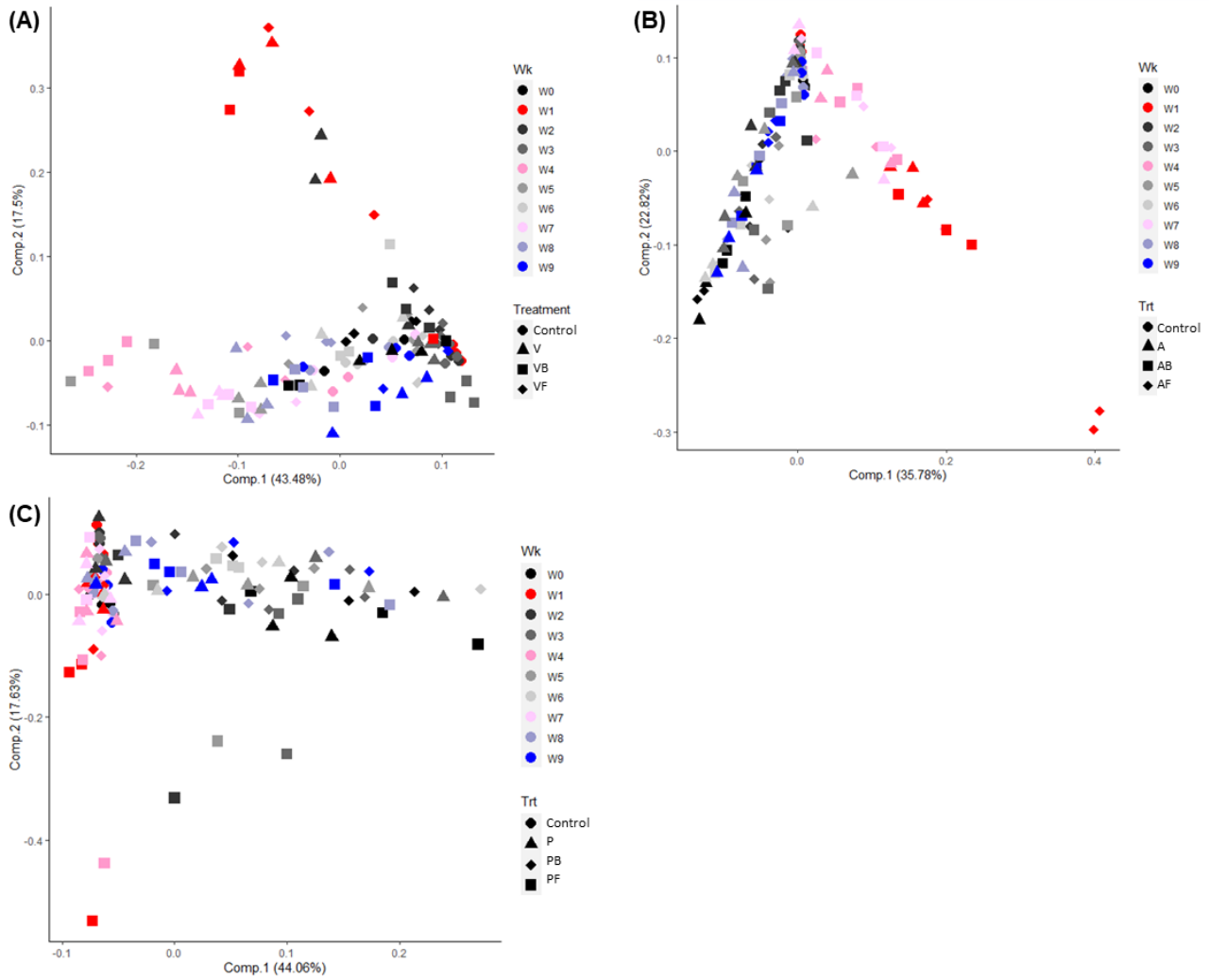
Significance codes: *p < 0.05, **p < 0.01, ***p < 0.001

Supplementary Table III-6 | Factor loadings based on exploratory factor analysis for vancomycin treated groups. Note that the absolute values for blank entries are less than 0.2, but not zero.

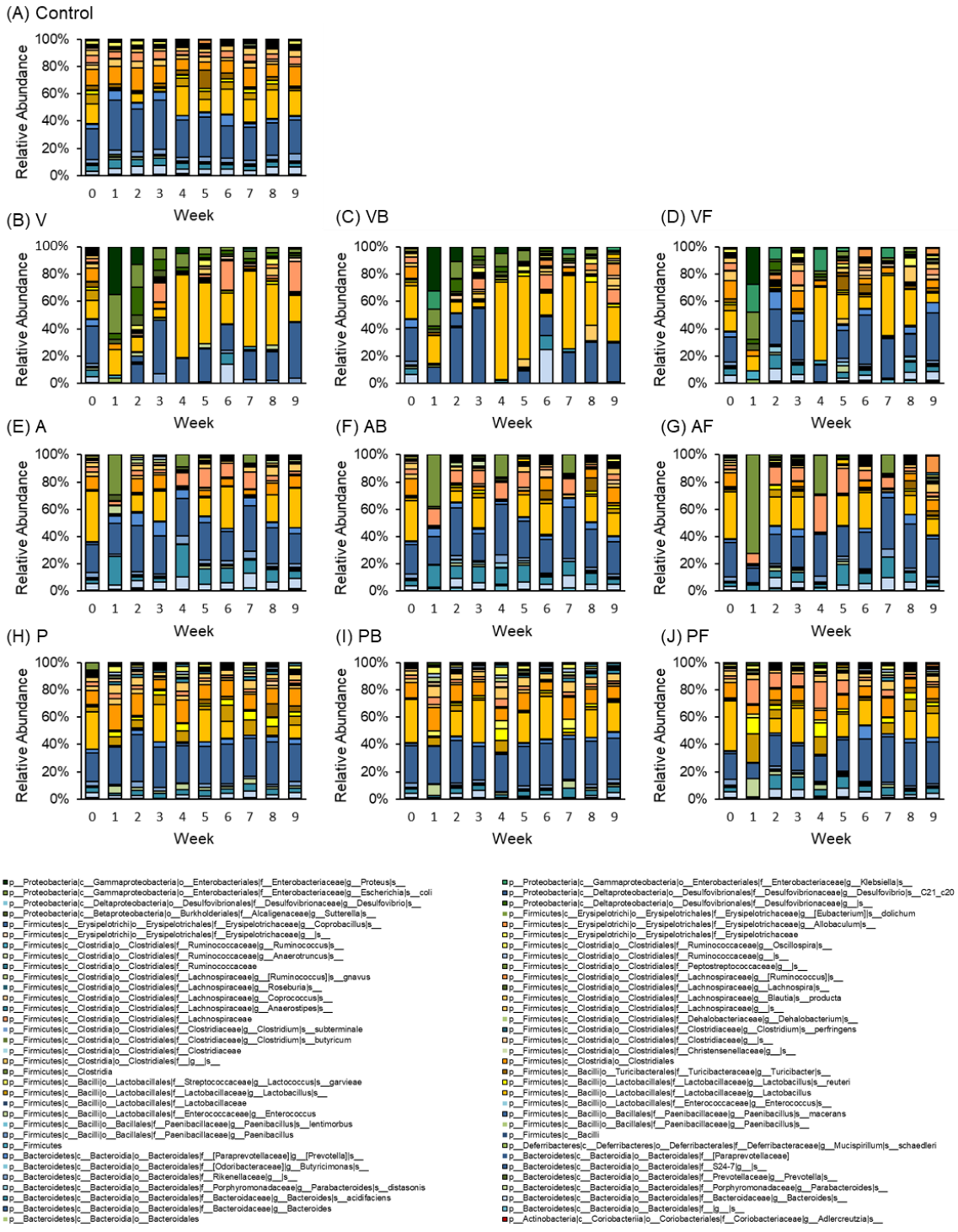
	Factor 1	Factor 2	Factor 3
Lactobacillus [G]	0.867991	-0.39684	
Proteus [G]		0.655285	0.403186
Escherichia coli		0.356525	0.300518
Bacteroidales S24-7 [F]	-0.40856	-0.51367	0.650558



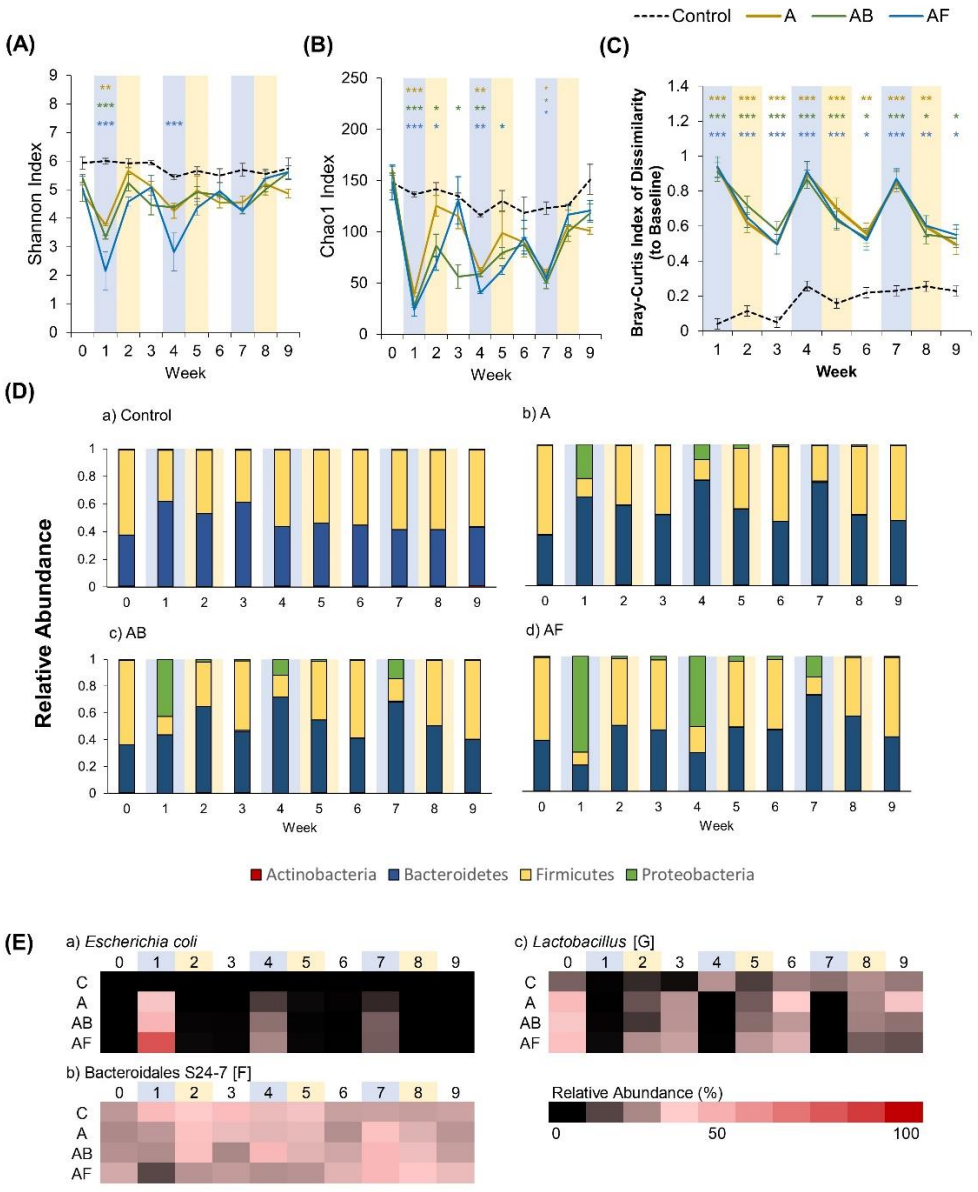
Supplementary Figure III-1 | Change in body weight and bacterial load over time. Percent change in body weight over time for A) vancomycin, B) amoxicillin, and C) ciprofloxacin. Total bacterial load measured by the number of 16S rRNA gene copies per mg of feces using qPCR D) vancomycin, E) amoxicillin, and F) ciprofloxacin. Weeks shaded in blue indicate weeks in which antibiotics were administered, and weeks shaded in yellow indicate weeks in which recovery treatments (natural recovery, probiotics, or fecal transplants) were administered. This figure is used with permission from Ojima et al., (2020).



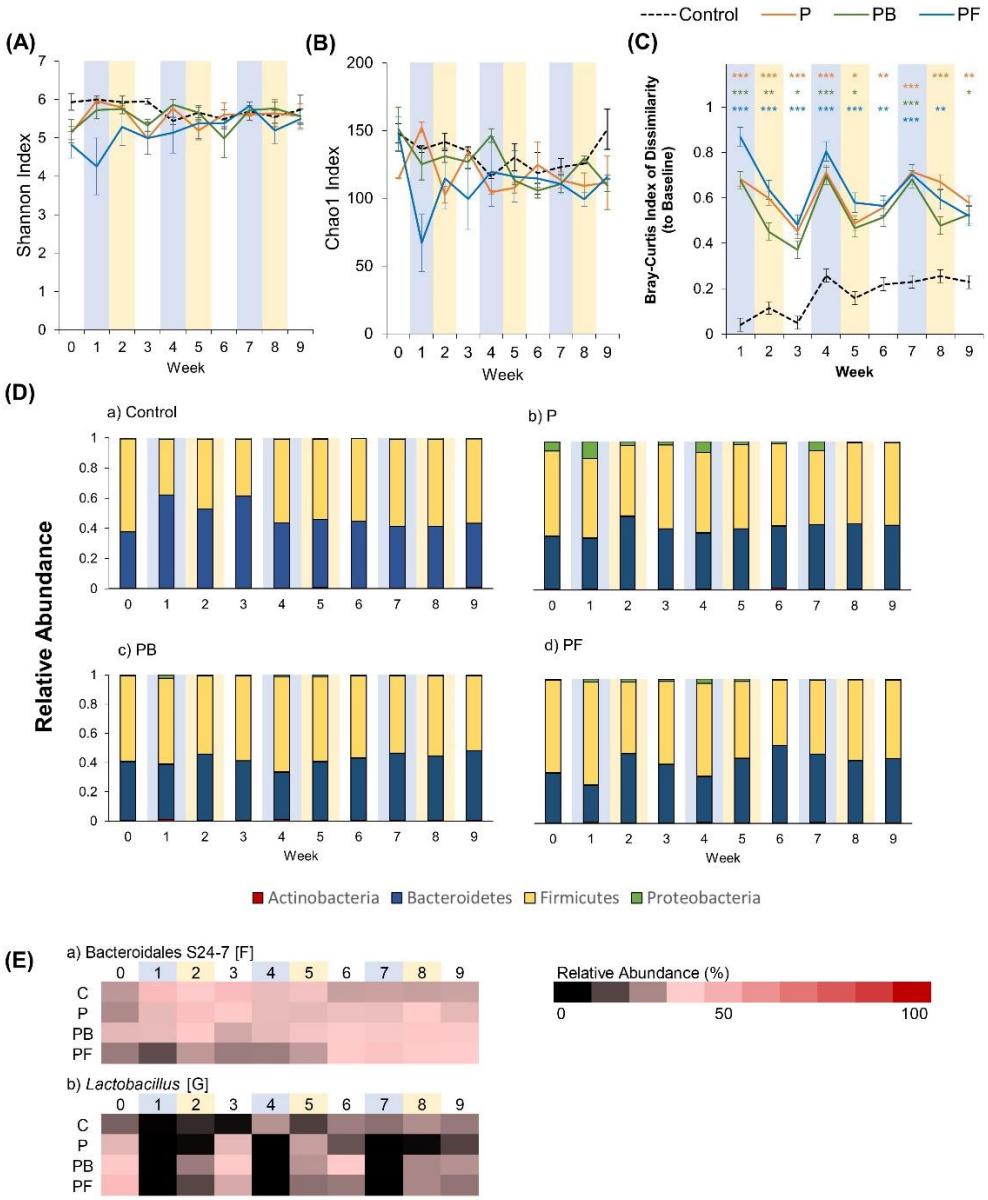
Supplementary Figure III-2 | Principal components analysis plot based on the microbial community composition. Principal components analysis was performed based on microbial community composition for A) vancomycin-treated groups, B) amoxicillin treated groups, and C) ciprofloxacin treated groups. Treatment is denoted by different shapes (circle: control, triangle: natural recovery, diamond: *B. bifidum*, square: fecal transplant) for each antibiotic group. Baseline communities (Week 0) are indicated in black, communities after the first antibiotic treatment are indicated in red, and the final communities are indicated in blue. The subsequent second and third antibiotic treatments are indicated in progressively lighter shades of red, and interim periods between antibiotic courses are indicated in progressively lighter shades of grey. This figure is used with permission from Ojima et al., (2020).



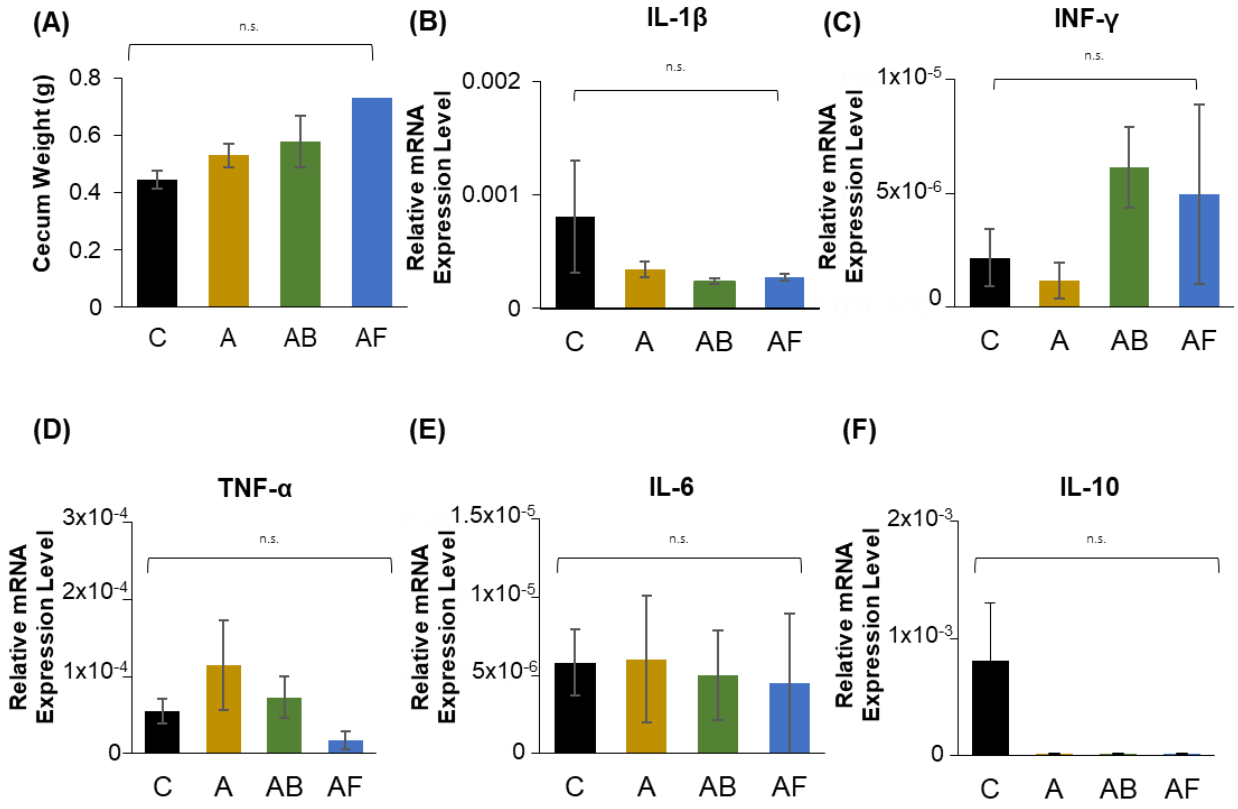
Supplementary Figure III-3 | Microbial community composition based on sequencing data of the V3-V4 regions of the 16S rRNA gene. The composition of the gut microbial community over time was determined based on sequencing data of the V3-V4 region of the 16S rRNA gene. For each treatment, relative abundance (%) of each taxon is shown (the lowest taxonomic rank for which information was available). A) C: control, B) V: vancomycin + natural recovery, C) VB: vancomycin + *B. bifidum*, D) VF: vancomycin + fecal transplant, E) A: amoxicillin + natural recovery, F) AB: amoxicillin + *B. bifidum*, G) AF: amoxicillin + fecal transplant, H) P: ciprofloxacin + natural recovery, I) PB: ciprofloxacin + *B. bifidum*, J) PF: ciprofloxacin + fecal transplant. This figure is used with permission from Ojima et al., (2020).



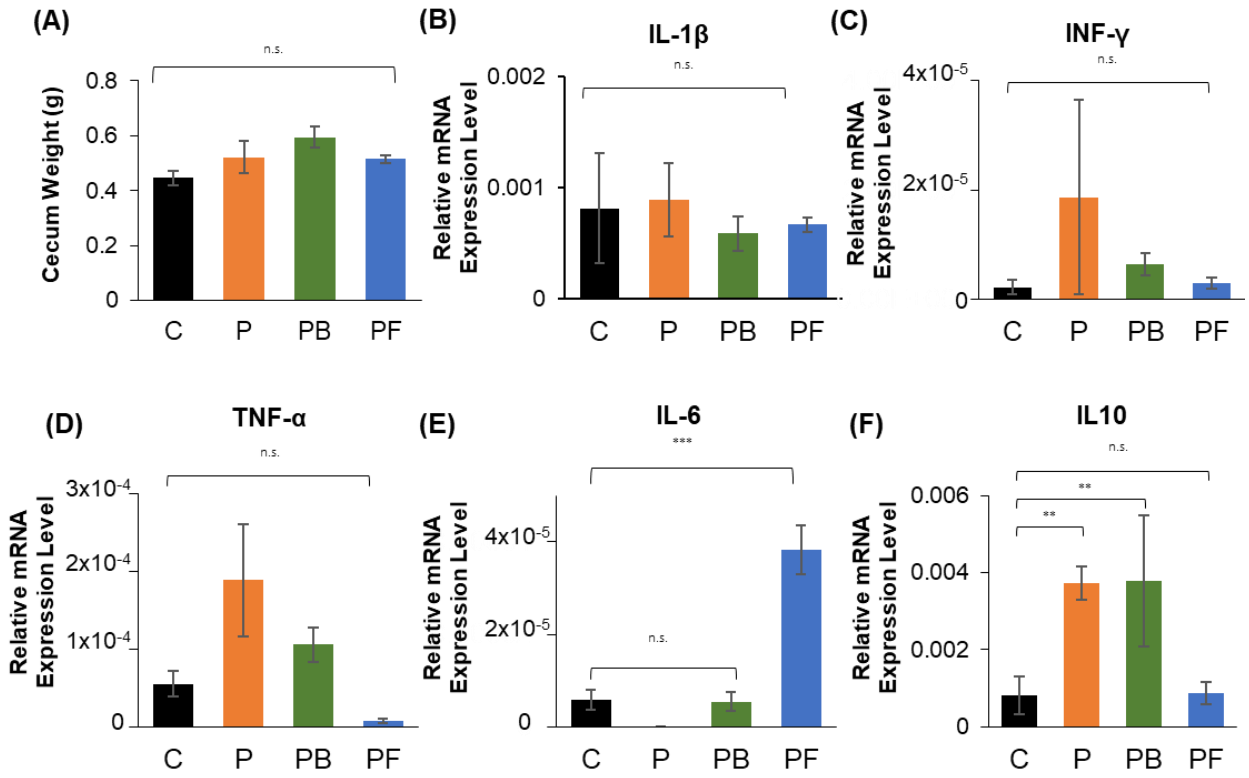
Supplementary Figure III-4 | The effect of recovery treatments on the gut microbiome after amoxicillin. Amoxicillin administration was followed by either natural recovery (NR), *Bifidobacterium bifidum* (PR), or fecal transplants (FT), and changes to the gut microbiome were observed over time.
Alpha diversity measured by A) Shannon Index and B) Chao1 Index for each treatment over time ± standard error. C) Bray-Curtis Index of Dissimilarity vs baseline for each treatment over time ± standard error. The Bray-Curtis Index was used to quantify the amount of microbial shift from the first day of the experiment (baseline) for each individual. Colored asterisks indicate significance vs control for NR, PR, and FT groups based on Two-Way rm-ANOVA and Tukey's HSD post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data for the control samples are indicated as the black dotted line, with NR groups in red, PR groups in green, and FT groups in blue.
D) The microbial community at each time point at the phylum level for a) control, b) NR (A), c) PR (AB) and d) FT (AF). E) Heat map of taxa that significantly changed after antibiotic administration. Significant taxa were identified using factor analysis (factor loading > 0.2). The lowest taxonomic rank for which information was available is indicated in square brackets (F: family, G: genus). Weeks shaded in blue indicate weeks in which antibiotics were administered, and weeks shaded in yellow indicate weeks in which recovery treatments (natural recovery, probiotics, or fecal transplants) were administered. This figure is used with permission from Ojima et al., (2020).



Supplementary Figure III-5 | The effect of recovery treatments on the gut microbiome after ciprofloxacin. Ciprofloxacin administration was followed by either natural recovery (NR), *Bifidobacterium bifidum* (PR), or fecal transplants (FT), and changes to the gut microbiome were observed over time.
Alpha diversity measured by A) Shannon Index and B) Chao1 Index for each treatment over time ± standard error. C) Bray-Curtis Index of Dissimilarity vs baseline for each treatment over time ± standard error. The Bray-Curtis Index was used to quantify the amount of microbial shift from the first day of the experiment (baseline) for each individual. Colored asterisks indicate significance vs control for NR, PR, and FT groups based on Two-Way rm-ANOVA and Tukey's HSD post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data for the control samples are indicated as the black dotted line, with NR groups in red, PR groups in green, and FT groups in blue.
D) The microbial community at each time point at the phylum level for a) control, b) NR (P), c) PR (PB) and d) FT (PF). E) Heat map of taxa that significantly changed after antibiotic administration. Significant taxa were identified using factor analysis (factor loading > 0.2). The lowest taxonomic rank for which information was available is indicated in square brackets (F: family, G: genus). Weeks shaded in blue indicate weeks in which antibiotics were administered, and weeks shaded in yellow indicate weeks in which recovery treatments (natural recovery, probiotics, or fecal transplants) were administered. This figure is used with permission from Ojima et al., (2020).



Supplementary Figure III-6 | Changes in cecum size and expression of inflammation-related genes in amoxicillin-treated mice. At the end of the experiment, cecum weight and the mRNA expression levels of inflammation-related genes in the large intestine for amoxicillin-treated mice (C: control, A: amoxicillin + natural recovery, AB: amoxicillin + *B. bifidum*, AF: amoxicillin + fecal transplant from control mice) were measured. A) Cecum weight, relative mRNA expression of genes encoding B) IL-β, C) TNF-α, D) INF-γ, E) IL-6, and F) IL-10 for amoxicillin-treated mice, using *Actb* as a reference gene. Error bars indicate standard error, and significance was determined by One-Way ANOVA and Dunnett's test. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). This figure is used with permission from Ojima et al., (2020).



Supplementary Figure III-7 | Changes in cecum size and expression of inflammation-related genes in ciprofloxacin-treated mice. At the end of the experiment, cecum weight and the mRNA expression levels of inflammation-related genes in the large intestine for ciprofloxacin-treated mice (C: control, P: ciprofloxacin + natural recovery, PB: ciprofloxacin + *B. bifidum*, PF: ciprofloxacin + fecal transplant from control mice) were measured. A) Cecum weight, relative mRNA expression of genes encoding B) IL-β, C) TNF-α, D) INF-γ, E) IL-6, and F) IL-10 for ciprofloxacin-treated mice, using *Actb* as a reference gene. Error bars indicate standard error, and significance was determined by One-Way ANOVA and Dunnett's test. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). This figure is used with permission from Ojima et al., (2020).

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