Exploring Early Life Microbial Interventions and Subsequent Disease Resistance in a Swine-derived Bacterial Gnotobiotic Mouse Model

by

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Abstract:

Many associations between the gut microbiota, host health, and disease have been revealed, however, mechanistic research to determine causality is necessary. Gnotobiotic animal models, where all organisms are known, are the gold standard to examine mechanisms of causality and gnotobiotic mice are the most widely implemented animal model. The overall objective of this thesis was to create a novel gnotobiotic mouse model colonized with a swine defined community (DC) to study the effects of early life *Escherichia coli* and amoxicillin administration on subsequent *Salmonella* resistance previously investigated in conventional pigs. To create a DC, a swine-derived bacterial culture collection with 35 species spanning 15 genera, 12 families, and 6 phyla was generated. The curation of a representative pig DC with whole genomes consisting of 16 species was established based on the proposed swine core microbiota and prominent bacteria in pre-weaning piglets. Germ-free C57BL/6J male mice (N = 8) were colonized with the pig DC to determine the colonization ability and pattern in a mouse model. The majority (14/16 species) of the DC colonized the mouse gut except for *Prevotella copri* and *Streptococcus hyointestinalis*. The β diversity in the ileum significantly differed from the cecum, colon, and feces, which was driven by the enrichment of *Streptococcus* and *Lactobacillus* in the ileum versus *Bacteroides* in the lower tract; and the total bacteria load in the ileum was significantly lower than the other regions ($P < 0.05$). Similar biogeographical colonization and bacterial loads are observed in pigs.

Preliminary results suggesting that *E. coli* and amoxicillin administration in early life can enhance piglets' immune responsiveness to intraperitoneal heat-killed *Salmonella enterica* serovar Typhimurium (S. Tm) were tested in our swine DC mouse model. Female C57BL/6J mice colonized with the swine DC with or without *E. coli* were bred and during the first two

weeks of their pups' lives, dams were either administered sterile amoxicillin drinking water or sterile drinking water. The four pup treatment groups (*E. coli* + Amoxicillin, n = 5; *E. coli*, n = 8; Amoxicillin, n = 8; and Control, n = 8) were then challenged with live S. Tm SL1344 at six weeks of age, after normalization of the gut microbiota on post-natal day 21. The S. Tm load in both the ileum and liver significantly differed by treatment, with the lowest loads observed in the EA group, suggesting that the combined neonatal exposure to *E. coli* and amoxicillin does facilitate local and systemic pathogen burden later in life ($P < 0.05$). At 48 hrs post-infection, there were no differences in inflammatory cytokine levels between treatments. Furthermore, sex differences in S. Tm load and cytokine production were observed without accompanying microbiota differences. Males suffered from significantly greater S. Tm loads in the ileum, cecum, and liver with significantly increased IL-6 and IL-10 ($P < 0.05$). While largely underexplored in S. Tm infection, sexual dimorphism was consistent with other enteric infections and may be due to a greater innate and cell-mediated immune response without excessive proinflammatory cytokine production. While these results show that early life microbial and antibiotic interventions have long lasting impacts on S. Tm resistance, further research is needed to unravel the mechanisms behind early life *E. coli* and amoxicillin exposure on immune system development and subsequent disease resistance.

Preface

This thesis is an original work by Hannah Lantz. This research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board under AUP00000671.

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1.1 Fundamentals of the Intestinal Microbiota:

1.1.1 Microbial Membership, Colonization History, and Succession Patterns

Over time, both humans and animals have coevolved with commensal microbes (Moeller et al., 2016). The population of organisms in the gut (microbiota) are largely comprised of bacteria, and as such, bacteria have been widely studied for their role in host health and disease. Colonizing bacteria augment their host through protective and metabolic functions via metabolism of substrates inaccessible to host enzymes, preventing infection by pathogen exclusion, and modulating the host's immune system development and subsequent response (Cabreiro & Gems, 2013; Erkosar et al., 2013). These bacteria reside throughout the host, both externally and internally, with the densest populations found within the gastrointestinal (GI) tract (GIT) (Sender et al., 2016). The GIT is home to an abundant reservoir of various microbes, which consists of bacteria, archaea, fungi, microbial eukaryotes, protozoa and viruses that interact in a spatial and temporal manner (Koenig et al., 2011; Yatsunenko et al., 2012). Collectively, the microbiota and their genetic repertoire make up the microbiome and for the purpose of this thesis, the terms "microbe(s)" and "microbiota" will refer strictly to bacterial taxa.

The total number of bacterial cells is estimated to be 3.8×10^{13} in the human body with the majority residing in the GIT (Sender et al., 2016). Consisting of just over 1000 bacterial species, the human gut microbiota is highly diverse (Qin et al., 2010; Rajilić-Stojanović & de Vos, 2014). To grasp the scope of the microbiome's genetic content Tierney et al. (2019) identified over 22 million non-redundant genes in the human gut through a meta-analysis comprising 3,655 samples from 13 studies. To put these numbers in perspective, when the

human genome was sequenced, 26,600 protein encoding transcripts were identified (Venter et al., 2001). In comparison to humans, deep metagenome sequencing of fecal DNA from 287 pigs identified 7, 685, 872 non-redundant genes (Xiao et al., 2016). Clearly, the gut microbiome is vast and there is much research needed to determine their roles in the body.

The human gut microbiota is comprised of five core phyla, with Bacteroidetes and Firmicutes largely dominating (Huttenhower et al., 2012; Ottman et al., 2012; Qin et al., 2010). Bacteroidetes are Gram-negative bacteria that have an extra outer membrane layer containing lipopolysaccharides (LPS), which elicit a variety of immunological responses in the host. This phylum includes the genera *Prevotella* and *Bacteroides*, whose abundances are usually inversely correlated to each other due to substrate preferences (Vogt et al., 2017). Firmicutes is the largest phylum comprised of genera such as *Ruminococcus*, *Clostridium*, *Lactobacillus*, *Roseburia*, *Eubacterium*, *Faecalibacterium*, and *Mycoplasma*. These bacteria have a Gram-positive cell wall with a thick peptidoglycan layer. The remaining phyla, Actinobacteria (which includes *Bifidobacterium*), Proteobacteria (which includes *Escherichia*), and Verrumicrobia (which includes *Akkermansia*) make up much smaller fractions of the human microbiota at approximately ~4% abundance total (Huttenhower et al., 2012; Qin et al., 2010). The porcine microbiota is commonly composed of three main phyla, Firmicutes, Bacteroides, and Proteobacteria which account for over 90% of the microbiota, the rest of the microbiota is comprised mainly of Spirochetes, Fusobacteria, Actinobacteria, and Tenericutes (Holman et al., 2017; Kim et al., 2011; Kim & Isaacson, 2015; Mach et al., 2015; Schokker et al., 2014; Song et al., 2017). Despite the abundance of these phyla in the GIT, these microbes represent a small portion of all taxa on earth and many of these taxa are unable to replicate outside of the GIT highlighting their adaptation to this particular niche; as does the fact that the relative abundance

of bacteria and the dominant species found along the tract varies (Lawley & Walker, 2013; Ley et al., 2008; Liu et al., 2019; Suzuki & Nachman, 2016; Xiao et al., 2018).

The organisms found within each phylum are not represented equally in all individuals due to genetic, environmental, lifestyle and age differences (Qin et al., 2010). Notably, only about 10% of species have been estimated to be present in every individual, which makes defining a core microbiota difficult (Qin et al., 2010). Early in life, priority effects dictate that the timing and colonization order of species during community assembly cause specific variations in both the structure and function of communities (de Meester et al., 2016; Fukami, 2015). A study using a specific pathogen free (SPF) piglet model of microbial assembly showed that 2 batches of identically reared piglets developed different microbial communities (Merrifield et al., 2016). This variation came down to the colonization of Clostridia during the first day of life, however, it should be noted that the second batch was not litter matched, therefore piglets could have been exposed to different microbes at birth (Merrifield et al., 2016). Furthermore, the timing and frequency of exposure is important as multiple exposures during early life can result in a different microbial profile than a single exposure to the same microbial consortium (Schmidt et al., 2011).

Four concepts of community assembly: dispersal, selection, drift, and diversification, have been proposed to explain patterns of microbial succession, distribution, and abundance (Vellend, 2010). These four concepts are involved in community assembly and maintenance throughout an individual's life, and a multitude of factors play a role in these processes. Dispersal, the movement of organisms to an ecosystem, is a prominent force at birth as the infant is exposed to the maternal microbiota (Chu et al., 2017). In 2016, Nayfach and colleagues determined that 91% of strains found in the stool of mothers were also present in their newborns,

but only 55% of those strains were found a year later. After exposure to a microbe, its growth and abundance can be shaped by both selection and ecological drift. Fitness and niche differences among taxa select for the reproduction and survival of certain species, which is largely driven by abiotic conditions like environmental conditions within the GIT, biotic interactions between organisms, and host factors like the immune system and diet (Zhou & Ning, 2017). For example, human milk oligosaccharides in milk select for mucus-adapted species that possess glycoside hydrolases that can metabolise this nutrient (Marcobal et al., 2011). The concept of drift encompasses stochastic events, which Zhou and Ning (2017) defined as "random changes in the community structure with respect to species identities and/or functional traits due to stochastic processes of birth, death, immigration and emigration, spatiotemporal variation, and/or historical contingency". The effects of drift are more profound on low abundance taxa as they are infrequent colonizers and their small populations subject them to extinction and subsequent replacement by other species (Nemergut et al., 2013; Shade et al., 2012). A final important concept is diversification, where microbes rapidly adapt to various conditions within the body by generating new genetic variations that confer support survival. Rapid mutations and horizontal gene transfer are examples of diversification that microbes employ when facing inhospitable environments to promote their ecological stability and survival (Shapiro et al., 2012). Together, these concepts of community assembly facilitate the colonization of microbes and dictate the structure and composition of these microbes throughout the host's life.

1.1.2 The Human Intestinal Microbiota

The microbiota colonizes early in life through exposure to factors like the mother's microbiota, the environment, and diet until the succession of microbes reaches a more stable "climax" community (Bäckhed et al., 2015; Koenig et al., 2011; Palmer et al., 2007). Genetics

play less of a role than the aforementioned factors, as previous studies estimated that genetic factors account for less than 10% of the variation in gut microbiota (Falony et al., 2016; Rothschild et al., 2018). For human infants, the first 3 years of life are crucial for microbial colonization and represents a critical period to improve growth and development (Arrieta et al., 2014; de Filippo et al., 2010; Koenig et al., 2011; Lim et al., 2016; Rodríguez et al., 2015; Yatsunenko et al., 2012). Within the first 3 years of life, the gut microbiota is dynamic, and infants are dominated by fewer bacterial species than their older counterparts (Bäckhed et al., 2015; Yatsunenko et al., 2012). However, the gut microbiota is more highly variable between individuals during infancy than in adulthood (Avershina et al., 2014; Bäckhed et al., 2015; Palmer et al., 2007; Yatsunenko et al., 2012). Various factors such as genetics, gestational age, mode of delivery, diet, sanitation, and early life antibiotic treatment all influence which microbes are present and account for these shifts (Milani et al., 2017). At birth, the intestines are aerobic, which favors the growth of bacteria from the phyla Proteobacteria especially taxa from the *Enterobacteriaceae* family, like *Escherichia coli* (Bäckhed et al., 2015; del Chierico et al., 2015; Milani et al., 2017; Qin et al., 2010). *Lactobacillus* species are also common in babies delivered vaginally as the vaginal microbiota is heavily dominated by lactobacilli, and *Staphylococcus* species are early colonizers as they are widespread on mucosal and skin surfaces (Aagaard et al., 2012; Arrieta et al., 2014; Avershina et al., 2014). Shortly after birth, the intestinal lumen becomes anaerobic allowing strict anaerobic genera such as *Bacteroides*, *Bifidobacterium*, and *Clostridium* to colonize (Matamoros et al., 2013). Then, the dominant families in the first few weeks of an infant's life are *Bifidobacteriaceae, Clostridiaceae, Enterococcaceae, Lactobacillaceae*, and *Streptococcaceae* (Fallani et al., 2011; Koenig et al., 2011). The diet of young infants consists of milk, however, once weaned solid foods are the main

food source and shifts the microbial community towards taxa that metabolize complex carbohydrates, starch, and other novel substrates (Koenig et al., 2011; Laursen et al., 2016).

During weaning, alpha diversity increases while beta diversity decreases, and the dominant phyla shift towards Firmicutes and Bacteroidetes (Fallani et al., 2011; Koenig et al., 2011). With the increase in the variety of nutrients like microbially metabolized polysaccharides, the relative abundance of the taxa *Bifidobacterium*, *Clostridium*, *Ruminococcus*, *Lachnospiraceae*, and *Enterobacteriaceae* increases (Fallani et al., 2011; Koenig et al., 2011). During the toddler stage, increased protein intake corresponds to greater *Lachnospiraceae*, while a decrease in *Bifidobacteriaceae* parallels a decrease in milk oligosaccharides (Laursen et al., 2016). Furthermore, two important species, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* begin to increase in abundance around 12 to 24 months (Yassour et al., 2016). An adult-like gut microbial structure in humans starts to stabilize after the age of 3 (Bergström et al., 2014; Koenig et al., 2011; Yatsunenko et al., 2012).

1.1.3 The Swine Intestinal Microbiota

Much like human infants, piglets are colonized by microbes immediately following birth, and although the establishment of the swine GIT microbiota has not been studied for as long or in as much detail as in humans, their colonization patterns occur in dependence of many of the same factors (Fouhse et al., 2016). Variables such as genetics, exposure to the sow, the environment the animal is raised in, and importantly, the diet they are fed are central in establishing intestinal microbial communities (Bian et al., 2016; Pajarillo et al., 2014; Xiao et al., 2018). Many studies have examined the GIT microbial composition at various stages in pre and post weanling pigs. However, one must note that the dominant microbes at these various life stages are not always consistent between studies due to a multitude of factors, with the most notable being the “study”

itself and GIT location sampled (Holman et al., 2017). Like humans, initially, aerotolerant organisms including *E. coli* and *Streptococcus* species colonize the GIT and transform the intestines into an anaerobic environment to facilitate colonization by more anaerobic organisms (Bian et al., 2016). Although the human GIT microbiota fluctuates throughout the first 3 years, the microbiota is surprisingly stable in nursing piglets and fluctuates in subsequent weeks (Frese et al., 2015; Kim et al., 2011). To possibly explain this difference, conventional pigs are derived in similar conditions and nurse within their litter and therefore are likely exposed to the same microbes. Conversely, human infants are exposed to more variable conditions regarding mode of birth, environment, and diet. Within the first couple of days, the early piglet colonizers belong to the genera *Escherichia*, *Clostridium*, *Fusobacterium*, and *Streptococcus*, while in the subsequent days, colonizers include *Lactobacillus*, *Bacteroides*, *Prevotella*, and *Ruminococcus* species (Bian et al., 2016; Kubasova et al., 2017). Notably, *Lactobacillaceae* is increased in weaned pigs, however, but *Lactobacillus* species are still quite prominent in pre weaned piglets (Frese et al., 2015; Guevarra et al., 2019; Song et al., 2017; Yang et al., 2019). As in humans, the alpha diversity increases while the beta diversity decreases as pigs age (Guevarra et al., 2019). In pigs, weaning occurs around post-natal day (PND) 21 and is a major contributor to the shift in microbial species found in the GIT (de Rodas et al., 2018; Frese et al., 2015; Holman et al., 2017). Prior to weaning, piglets consume milk rich in monosaccharides and oligosaccharides, but at weaning, cereal-based diets high in complex carbohydrates and fibre are introduced (Navarro et al., 2019). As a result of these substrate differences, when nursing, *Bacteroides* species are abundant (Kim et al., 2011; Pajarillo et al., 2014). Conversely, after weaning, the relative abundance of the genus *Prevotella* increases compared to that of *Bacteroides* due to an increase fibre intake (de Rodas et al., 2018; Frese et al., 2015; Kim et al., 2011; Lamendella et al., 2011; Liu et al., 2019; Mach et al., 2015;

Pajarillo et al., 2014; Wang et al., 2019; Yang et al., 2019). In fact, *Prevotella* can account for relative abundances over 30% in weaned pigs, making it a genus of great interest (Kim et al., 2011; Mach et al., 2015). To determine if a "core" microbiota exists in pigs, Holman et al (2017) performed a metanalysis using 20 data sets from high throughput 16S ribosomal ribonucleic acid (rRNA) gene sequence studies of pigs aged ~3 weeks to ~24 weeks of age. This study defined a "core" microbiota to require a particular taxon to be present in more than 90% of all GI samples (Holman et al., 2017). The genera *Clostridium*, *Blautia*, *Lactobacillus*, *Prevotella*, *Ruminococcus*, and *Roseburia*, the RC9 group, and *Subdoligranulum* met those criteria and are suggested to make up the core microbiota of pigs (Holman et al., 2017). One limitation to this study is that no data from young nursery pigs was included as the data sets start at approximately 3 weeks of age, around the time of weaning. Nonetheless, these results provide a framework for researchers to manipulate the swine gut microbiota for potential benefits to host health.

1.2 Studying the Gastrointestinal Microbiota:

1.2.1 Culture and Sequence-based Methods

At the dawn of microbiological research, culture dependent techniques were pioneered by Robert Koch in the 1880's. Studying a microbe depended on the ability for it to be cultured in a pure isolate on a surface of agar media before being analyzed. New microbial species were typically identified by Gram staining and microscopy then put through a series of other physiological and biochemical tests. The growth and characterization of novel organisms at the time was largely limited to organisms that could grow in aerobic environments. However, with the advancement of culture techniques, the cultivation of microbes was improved with the creation of various methods to enrich the growth of specific organisms. Selective media with specific nutrients (amino acids, metals, vitamins, complex nutrient sources) and selective inhibitors (antibiotics,

metabolic inhibitors, and toxic compounds) were designed, and selective culture conditions (pH, temperature, salinity, and gas composition) were employed. Then, with the invention of anaerobic culture methods (Hungate tubes and anaerobic chambers), the cultivation of strict anaerobes residing in the gut took off as strict anaerobes were able to be grown in an anoxic environment (Aranki & Freter, 1972; Hungate et al., 1966). These culture advancements complement the more recent endeavour to study the impacts of gut microbiota on host health. To study such effects, obtaining pure isolates using various culture techniques is imperative. Therefore, creating culture collections consisting of such microbes allows researchers to study their ecological roles along with their characterizing biology.

A century later the advent of culture independent techniques, known as sequencing-based techniques, propelled microbiome research. Since then, microbial profiling through sequencing-based techniques have been quite popular due to the ability to collect great amounts of data relatively quickly. The basis of these methods is to analyze extracted DNA from a sample rather than cultivating a pure isolate *in vitro*, then information about taxonomic classification can be deduced from the DNA sequence and metagenomic analyses can be performed to infer function and possibly predict characteristics of the community as a whole. The earliest methods include fluorescent in situ hybridization (FISH) and polymerase chain reaction (PCR) (Amann et al., 1995; Mullis et al., 1992; Saiki et al., 1992). By creating amplicons of a highly conserved gene found in all bacteria, the 16S rRNA gene, taxonomic identification of gut microbes has been made possible (Olsen et al., 1986). Between species, the 16S rRNA gene varies in sequence, operon size, and secondary structures of 3 rRNA subunits, 16S, 23S, and 5S, which allows for differentiation between species (Maidak et al., 1997; Yarza et al., 2014). In 1977, Woese & Fox discovered that rRNA could be a marker for taxonomy and thus phylogenetic trees could be constructed by

comparing the 16S rRNA gene, as it consists of highly conserved and hypervariable regions that allow universal PCR primers to amplify distinct regions of this gene. By amplifying the 16S rRNA gene, researchers are then able to employ various techniques, like Sanger sequencing, which provides a sequence of the microbe that can then be compared to a reference database using platforms like the Basic Local Alignment Search Tool (BLAST) for further analysis (Altschul et al., 1990; Sanger et al., 1977; Sanger & Coulson, 1975).

As the molecular era of modern sequencing technologies emerged and made culture relatively obsolete for the time being, high throughput sequencing was introduced (Metzker, 2005). These next generation sequencing methods (NGS) were favored over more traditional methods as they were easier, less costly, and whole bacterial genomes could be sequenced in a timely manner (Metzker, 2005; Petersen et al., 2020). In addition to high throughput sequencing, multiple "omics" strategies such as proteomics, transcriptomics, and metabolomics are employed to complement metagenomic sequencing. Such advances came around the same time that the Metagenomics of the Human Intestinal Tract (MetaHIT) project and the Human Microbiome Project (HMP) were implemented (Qin et al., 2010; Turnbaugh et al., 2007). These multimillion-dollar projects compiled a catalog of the average composition of the gut microbiota from hundreds of humans to serve as a reference for microbiome researchers. Now, third generation sequencing, using instruments like the MinION nanopore device (Oxford Nanopore Technologies) and the PacBio RS II system (Pacific Biosciences) have been implemented to produce average read lengths over 500 base pairs, and depending on the device, at a fraction of the cost (Petersen et al., 2020). Without the need to amplify samples, amplification errors and biases are avoided, however, these technologies are not without their disadvantages (Petersen et al., 2020). The accuracy of these platforms ranges as higher rates of insertions and deletions due to the resulting high coverage

occur, and the PacBio RS II system has comparably high costs and turnaround times as NGS (Petersen et al., 2020).

For many years, culture-independent approaches dominated microbial research as various disease states have been correlated to 16S rRNA gene amplicon sequencing, and the ability to investigate microbial composition and the dynamics of these complex microbial communities has been made possible. Marker gene-based methods, like 16S rRNA sequencing, allow the analysis of community structure and taxonomic assignment down to the species level; and with bioinformatic approaches, predictions of functional metagenome composition compared to a reference database can be made. On the other hand, whole gnome sequencing allows taxonomic assignment down to strain level and functionality can be inferred through the analysis of genes present. Having said this, sequencing data alone does not necessarily reflect the actual biologically relevant phenotypes and functions of a microbial community, especially if DNA sequencing methods are employed as the results do not indicate which genes are actually being expressed like in RNA based sequencing methods (Kukurba & Montgomery, 2016). In 2018, Tramontano and colleagues revealed that metabolic models of gut microbial species based on NGS did not accurately predict growth requirements on these species. In fact, only 10 of the models predicted growth of the same bacterial species in different media (Tramontano et al., 2018). Researchers have identified other major drawbacks to NGS such as bias across studies due to depth bias, varying target regions and primers for 16S rRNA amplification, sequence heterogeneity within species, and the inability to detect some organisms. To study sequencing depth bias, Lagier and colleagues (2012) compared the detection of bacterial isolates using culturomic and metagenomic NGS approaches. Sixty five percent of the bacterial species identified with culture methods were below the level of detection thresholds of metagenomic technologies (Lagier et al., 2012). As

such, samples with low bacterial DNA may not be accurately sequenced. Furthermore, other studies have shown that NGS methods have failed to detect pathogenic organisms such as shigatoxigenic *E. coli* O104:H4, and lowly abundant bacteria due to insufficient sequencing depth (Lagier et al., 2012; Li et al., 2019; Loman et al., 2013). It has also been repeatedly confirmed that universal primers are biased against Actinobacteria, specifically, Bifidobacterial sequences are misrepresented depending on the choice of primers and the variable region of the 16S rRNA that is sequenced (Farris & Olson, 2007; Walker et al., 2015). Recently, Browne and colleagues (2020) analyzed GC-dependent coverage biases using the Illunima MiSeq, Next seq, HiSeq, Oxford Nanopore, and Pacific Biosciences sequencing platforms using high throughput sequencing based shotgun metagenomics. The only platform not affected by GC bias was the Nanopore, however, the other 4 platforms had considerable GC bias which resulted in underrepresentation of GC-poor microbes (Browne et al., 2020). With this knowledge, it is not unreasonable to suggest that these platforms may provide skewed abundance estimates in metagenomic studies, thus providing incorrect abundance data. Preparation of samples for sequencing should also be considered as DNA isolation kits are not all equal. The DNA extraction method is a critical factor influencing bacterial composition as it has been shown that depending on which kit is used, that the ratio of Gram-negative bacteria to Gram-positive bacteria may be skewed (Costea et al., 2017; Videnska et al., 2019). It should also be noted that proper controls and mock communities, depending on the type of NGS, should be used to standardize results (Knight et al., 2018). Furthermore, the completeness of reference databases should be considered when using whole metagenome shotgun sequencing or sequencing based on conserved marker sequences, as the diversity of microbiota extends past what is present in these databases. Additionally, reference genome databases are not as extensive as those of rRNA databases, thus improper assignment or unassigned sequences may

result. It has been reported that contaminant sequences are also present in published genomes. In fact, in 2019 the NCBI Refseq database revealed that 2250 bacterial genomes were contaminated by human sequences (Breitwieser et al., 2019). Likewise, discrepancies between databases can result in incorrectly linking sequences to microbial taxonomy (Park & Won, 2018; Yilmaz et al., 2014). Using public mock community data, Park and Won (2018) tested the ability of 16S rRNA databases (Greengenes, Silva, and EzBioCloud) to accurately assign taxonomy. EzBioCloud was the most successful database at the genus and species level, followed by Silva then Greengenes, as the number of true positives was higher than any other database (Park & Won, 2018). Additionally, the number of false-positives and false-negatives was lower for EzBioCloud than the other databases (Park & Won, 2018). It should be noted that typical microbial community analysis is from samples with many more species and their composition is not uniform, thus, this study may not necessarily reflect the best database for gut microbial samples. Given the aforementioned limitations of sequence-based methods, their use should complement other "omic" approaches and culture-based techniques.

1.2.2 Bringing Culture Back

The surge in genome-based studies proved to generate copious amounts of data providing new insights into bacterial life. Although, as a result of years of neglecting bacterial culture, a relatively low number of species had been isolated (Curtis et al., 2002; Locey & Lennon, 2016). Recent estimates have revealed that over 70% of all bacteria from the body with reconstructed genomes remain to be described due to not being isolated, while others estimate that 35-65% of species detected through sequencing have cultured representatives (Lagkouvardos et al., 2017; Pasolli et al., 2019). These uncultured bacteria have been suggested to have crucial functions in hosts, but without a pure isolate, mechanistic roles are difficult to elucidate (Chijiiwa et al., 2020;

Kenny et al., 2020; Lloyd et al., 2018). In 2013, Dubourg showed that pyrosequencing results underpredicted the number of bacterial species isolated from feces, while other studies reported that only 15% of phylotypes were common between culture and pyrosequencing, and that pyrosequencing neglects some Gram-negative prokaryotes (Hugon et al., 2013; Lagier et al., 2012). Even with the advance of sequencing techniques, there is still a mismatch between the representation of cultured species by NGS and vice versa (Gupta et al., 2019). As it was re-realized that pure bacterial isolates were required to test genome-based predictions about their biological characteristics as well as their roles in host health and disease, researchers have begun to ironically transition back to culture-based methods.

To complement metagenomic efforts, Lagier and colleagues (2012) created high throughput culturing methods coupled with Matrix-Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS), which they termed “culturomics”. The basic principle of MALDI-TOF MS is that bacteria have unique spectral signatures that are used to identify bacteria to the genus or even species level. Where MALDI-TOF MS is not able to discriminate between bacterial species within a genus, 16S rRNA approaches are successfully employed (Seng et al., 2009; Uchida-Fujii et al., 2020). In 2012, Lagier and colleagues employed culturomics to isolate 174 previously unreported human gut microbiota using over 200 culture conditions with various compositions. As such, culturomics has filled the gaps from sequence-based methods alone, as many “uncultured” microbes have since been assigned to unassigned operational taxonomic units (OTU) (Lagier et al., 2016). Culturomics can be a timely and laborious pursuit as many colonies must be selected to reveal new species. To highlight this, Lagier and colleagues (2016) screened almost a million colonies to ultimately culture just over 1000 species. Furthermore, there is no universal culture method to select for all microbes, thus so called “non-

cultivatable" bacteria may be missed (Greub, 2012; Lagier et al., 2012). With technological advances allowing isolation on a large scale, improved public repositories of isolates, and combining molecular techniques, some of the problems associated with culturomics can be mitigated (Lagkouvardos et al., 2017).

Prior to bacterial culture making a comeback, isolates spanning the families of *Enterobacteriaceae* (Proteobacteria) and *Veillonellaceae* (Firmicutes) were the most frequently cultivated bacteria from the gut, likely due to their partiality to the culture conditions used at the time (Rajilić-Stojanović et al., 2007). Now, 97% of isolated bacteria belong to the phyla, Bacteroidetes, Proteobacteria, Firmicutes, and Actinobacteria (Reimer et al., 2019). Today the percent of cultured fractions of mammalian gut microbiota is estimated to be around 50%, which is substantially higher than the predicted 20% of bacterial species in the gut that were once proposed to be cultivatable (Lagkouvardos et al., 2017; Ward et al., 1990). To determine the cultured fraction of bacterial communities the number of 16S rRNA gene sequences that can be assigned to cultured isolates is calculated (Lagkouvardos et al., 2017). With this approach Lagkouvardos and colleagues (2017) determined the median estimates of cultured fractions in humans and mice, which was 20-30% higher in humans than in mice. In terms of cumulative relative abundance of sequences, cultured fractions at the species level were over 40% and over 50% at the genus level (Lagkouvardos et al., 2017). With great efforts, especially from the labs of Didier Raoult (France), Trevor Lawley (England), and Thomas Clavel (Germany), the number of isolated gastrointestinal bacteria has increased drastically. A comprehensive review of isolated human bacteria was conducted by Bilen et al 2018, where they reported that 2,776 bacterial species belonging to 11 phyla have been cultured to date. Many of these bacteria were isolated using culturomics, highlighting the need for the continuation of these techniques (Bilen et al., 2018).

Such complete reviews are lacking in other host species and moreover, are required to update the cultured bacterial species in the GIT of hosts other than humans.

1.2.3 Factors Influencing Cultivability

The discordance between cultivatable colonies by culture dependent methods and the bacteria identified by culture independent means, known as the 'great plate count anomaly,' has long been documented (Staley & Konopka, 1985). This anomaly can be explained by the fact that many microbes present in the gut are adapted for growth in specific environments, which are difficult to replicate in laboratory settings. As the field progresses, new culture methods are developed, and bacteria once thought to be 'unculturable' have been cultivated from various hosts in the past decade. Notably, recent efforts combining various "omic" strategies coupled with culture, the first taxa from the family S24-7 were isolated (Lagkouvardos et al., 2016, 2019). With cultured representatives, researchers were able to explore the ecology and functional potential of these isolates, as well as propose taxonomic descriptions of the three genera in the proposed '*Muribaculaceae*' family (Lagkouvardos et al., 2019). By combining culture techniques with next generation sequencing, various studies have closed the gap between these two approaches as evidenced by the 50% of the OTUs detected through 16S rRNA gene amplicon sequencing being cultured (Goodman et al., 2011; Rettedal et al., 2014). Other studies have demonstrated that with great culture efforts, a representative proportion of the overall microbial community can be cultured (Bilen et al., 2018; Browne et al., 2016; Creevey et al., 2014; Crhanova et al., 2019; Fenske et al., 2020; Forster et al., 2019; Goodman et al., 2011; Ito et al., 2019; Lagier et al., 2012, 2016; Lagkouvardos et al., 2016; Lau et al., 2016; Moote et al., 2021; Rettedal et al., 2014; Wylensek et al., 2020). Furthermore, culturing efforts have revealed that bacteria not captured by culture independent techniques can be cultivated (Rettedal et al., 2014). One explanation why

culture independent methods miss these bacteria may be due to the cell wall composition, which may be more difficult to lyse during the DNA extraction process (*Staphylococcus* and *Enterococcus*) or the ability to form spores (*Clostridium*) (Esteban et al., 2020; Rettedal et al., 2014; Santiago et al., 2014). However, culture strategies do not always recapitulate the metagenomic community in the inoculum (Fenske et al., 2020). For example, in one study, the genus *Prevotella* was identified as the most highly abundant in Tamworth pigs, however, it was only isolated 7 times out of 500 colonies selected (Fenske et al., 2020). This highlights the fact that not all bacteria can be cultured on a common medium and that selective culture conditions need to be designed to more reliably cultivate these fastidious members of the microbiota. As such, this section will explore some of the factors influencing bacterial cultivability and various innovative culture techniques.

Sampling and Storage Methods

The viability of bacteria relies on the efficacy of the sampling and storage methods of gastrointestinal content. Such factors include duration of collection and transport, collection medium, and preservation method. Anaerobic bacteria vastly outnumber aerobes in the gut and varying levels of anaerobicity pose challenges for researchers to preserve the majority of abundant strict anaerobic bacteria. For example, the facultative anaerobe, *E. coli,* can survive in ambient oxygen for a minimum of 21 days while *Roseburia faecis*, a strict anaerobe, can only survive for two minutes (Browne et al., 2016; Duncan et al., 2006). It has been suggested that to culture the greatest proportion of the bacterial community in a sample, the ideal time between collection and sample processing should be less than two minutes and not exceed one hour without protection (Bellali et al., 2019; Brusa et al., 1989; Mata et al., 1969). Typically, GI content is collected into sterile containers or tubes where anaerobiosis can be achieved through use of anaerobic bags

(Guilhot et al., 2018). To further limit the loss of strict anaerobes, samples are often collected into a transport medium with negligible oxygen (Bellali et al., 2019). Cary-Blair, Stuart, and Amies' transport mediums prevent toxic superoxide formation and do not oxidize, allowing greater survival and possibility of cultivation (Amies, 1967; Cary & Blair', 1964; DeMarco et al., 2017; Stuart et al., 1954). Additionally, culture mediums can simply be gassed with a specific gas composition, or degassed to remove oxygen, however, with degassing, other gasses such as CO_2 are also removed, which may be undesirable during collection (Guilhot et al., 2018). Fresh GI content (4°C storage until plating) is preferred for cultivation; however, this is not always possible for various reasons (Musser & Gonzalez, 2011; Vandeputte et al., 2017). Thus, a few studies have looked at the effects of storage conditions on culturability (Curran et al., 2020; Fouhy et al., 2015; Hoefman et al., 2012). The current gold standard of collection, if immediate plating is not possible, is to immediately snap freeze samples in dry ice or liquid nitrogen then store them at -80°C, as ultra-low temperatures confer high stability and viability to bacterial cells (Hoefman et al., 2012; Vandeputte et at., 2017). Cryoprotectants are often used in longer term storage as they protect against cell lysis and increased salt concentrations due to freezing, allowing for more reliable revival upon plating (Prakash et al., 2020). Dimethyl sulphoxide and glycerol are popular cryoprotectants, but many other additives such as alcohols, saccharides and polysaccharides, proteins, and complex compounds have been used (Hubálek, 2003; Prakash et al., 2020). It should be noted that the efficacy of these protectants is not universal for every species, thus, optimizing the storage method to conserve the greatest diversity of bacteria is of the utmost importance when dealing with GI samples for culture (Bircher et al., 2018). While it has been shown that storage at -70°C in 25% glycerol preserves the viability of anaerobic organisms for up to three years, storage

time should also be considered when determining preservation of samples (Bryukhanov & Netrusov, 2006).

Non-Selective Vs. Selective media

Bacteria, especially anaerobic bacteria are demanding as they require rich media containing elements other than oxygen to produce their energy. As anaerobic bacteria account for up to 99.9% of total bacteria in the gut, designing media that facilitates their growth has been an important topic for their isolation (Bennett et al., 2014). Non-selective media contain compounds to facilitate the growth of many bacterial genera. These media can either be made in a liquid or an agar form. The isolation of bacteria is not plausible in liquid broth, as single colonies cannot be selected; however liquid media are used in initial incubations, as nutrients are more accessible (Lagier et al., 2016). Conversely, agar media produce single colonies but have less accessible nutrients, and agar in excessive quantities can inhibit the growth of certain bacteria. Non-selective media such as fastidious anaerobic agar (FAA), columbia agar, brain heart infusion (BHI) agar, schaedler agar, and CDC anaerobe agar have long been used to culture many different bacteria from the gut (Baron, 2015; Schaedler et al., 1965; Sokatch, 2014). More recently, culture media that simulates the natural environment of bacteria, like gut microbiota medium (GMM) and yeast extract, casitone and fatty acid (YCFA) medium, which are both rich in short chain fatty acids found in the gut, have been employed to culture and isolate gut bacteria (Browne et al., 2016; Forster et al., 2019; Goodman et al., 2011; Lau et al., 2016; Pereira & Cunha, 2020; Wylensek et al., 2020; Yousi et al., 2019). In 2011, Goodman and colleagues created GMM to generate an extensive human gut microbiota collection and Browne et al 2016 used YCFA to isolate 137 bacterial species from humans, of which 68 were novel. Having said this, most culturomic studies employ numerous media and culture conditions. In the pioneering study by Lagier and colleagues (2012), 212 culture

conditions were used to isolate 340 species of bacteria. Since, they have identified the conditions that bacteria were cultured at least once using 18 of the 212 culture conditions (Lagier et al., 2016). These culture conditions include both enriched non-selective mediums and selective mediums. Enriched mediums include growth factors that facilitate the growth of bacteria with specific needs. These growth factors include, but are not limited to, blood, antioxidants, vitamins, mucin, and rumen fluid. Blood and blood derivatives are commonly used as they provide nutritional supplements and protect against toxic oxygen radicals. Antioxidants also protect against oxidative stress and have been used to culture strict anaerobic bacteria under aerobic conditions (Dione et al., 2016; la Scola et al., 2014; Lagier et al., 2015). For example, two bacteria of great research interest, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*, have been noted to require specific growth factors. *F. prausnitzii* requires vitamins such as cobalamin, biotin, and folic acid, as well as various short chain fatty acids to grow *in vitro* (Duncan et al., 2002). While the isolation of *A. muciniphila* was facilitated by the supplementation of gastric mucin as the sole nitrogen source (Derrien et al., 2004). Media supplemented with rumen fluid is a common approach to culture rumen microbes and other anaerobes, as it simulates the environment in which these microbes are naturally found (Caldwell & Bryant', 1966; Kaeberlein et al., 2002; Moote et al., 2021; Zehavi et al., 2018). Furthermore, it has been shown that the combination of rumen fluid and sheep blood supplementation in media yields the largest number species total cultured and the greatest number of novel isolates (Diakite et al., 2020; Lagier et al., 2016). With the various strategies employed to culture microbes, selective culture conditions are used to isolate specific bacterial species or genera. Selective mediums contain inhibitors to increase the odds of selecting the particular bacteria of interest by eliminating unwanted organisms. The use of organic and inorganic compounds has been used to select against classes of bacteria, as has the use of

antibiotics and to a lesser extent, antiseptics (Lagier et al., 2015). Interestingly, squalamine from the dogfish shark, has been shown to present a wide variety of antimicrobial activities, and as such has been used as a Gram-positive bacterial inhibitor (Alhanout et al., 2010; Lagier et al., 2015). Two classic examples of media with inhibitory substances are MacConkey (MAC) agar and xylose lysine deoxycholate (XLD) agar. Crystal violet and bile salts present in MAC agar selects for coliforms, while sodium deoxycholate in XLD selects against Gram-positive bacteria. Having said this, antibiotics are the most used selective agents as their effects on different classes of bacteria are well known and their combination can be quite successful at selecting for target organisms (Bonnet et al., 2020). To illustrate, a study used 16 antibiotics in various combinations to select for previously uncultivated bacteria (Rettedal et al., 2014). In the swine GI tract, *E. coli* is relatively abundant and when plated will grow on a wide selection of media; therefore, to combat *E. coli* overgrowth antibiotics are often added to select against them. Lytic bacteriophages have also been used to limit the growth of *E. coli* from fecal samples and yielded the isolation of a new species of *Enterobacter* that was not detected in any other culture condition (Lagier et al., 2012, 2013). With this knowledge, it is accepted that a combination of enrichment factors and inhibitors are necessary to isolate novel bacteria.

Selective Culture Conditions

Selective culture conditions such as temperature, duration of incubation, preincubation, pre-treatment, and gas conditions further facilitate the cultivability of gut bacteria. The vast majority of studies cultivating bacteria from the gut incubate samples at 37°C, both aerobically and anaerobically, as the body temperature of most mammals ranges from 36°C to 39°C. Studies cultivating microbes from birds also typically incubate microbes at 37°C; however, the internal body temperature of most birds is 40°C, which may lend to the idea that poultry samples may be

better cultivated and represented at 40°C as some species are host adapted (Duar et al., 2017; Warriss et al., 1999). To date, no study has looked at the compositional differences in response to varying incubation temperatures. Furthermore, few microbes have been isolated below 37°C from the gut, but some bacteria isolated at 28°C include *Bacillus massiliosenegalensis, Pytheasella massiliensis, Stoquefichus massiliensis, Nocardioides massiliensis,* and *Nigerium massiliensis* (Dubourg et al., 2014; Lagier et al., 2016; Pfleiderer et al., 2013; Ramasamy et al., 2013; Traore et al., 2016). The duration of incubation is an important factor for slower growing bacteria as 24-72 hr incubation periods are sometimes too short to capture these bacterial isolates (Lagier et al., 2012, 2016; Wylensek et al., 2020). These species that take a longer time to cultivate are often anaerobes and can take up to 30-40 days to grow visible colonies (Diakite et al., 2020; Lagier et al., 2012, 2016; Lagkouvardos et al., 2016; Wylensek et al., 2020). Preincubation steps have also proved to be valuable for cultivating gut microbiota. In the pioneering culturomics study in 2012, Lagier and colleagues developed a preincubation technique where fresh human stool was pre-incubated in aerobic or anaerobic blood culture bottles for 1, 5, 10, 14, 21, 26, and 30 days before plating. Of the 29 bacterial species undetected by standard culture conditions, 24 of these species were anaerobic (Lagier et al., 2012). Since then, the preincubation of samples has been employed in various labs and is considered a valuable tool to capture slower growing bacteria (Guilhot et al., 2018). Another reason that bacteria may not be cultivatable is that they are in a state of dormancy. Dormancy is used by bacteria to survive and plays a role in transmission from one host to the next in some cases of bacteria within the phylum Firmicutes (Buerger et al., 2012). The most common form of dormancy is the bacterial endospore (spore), which has been suggested through metagenomic sequence analysis to be a mechanism of dormancy potentially employed by over half of the gut microbiota (Browne et al., 2016). To stimulate these viable spore formers to actively

grow, faecal samples are treated with ethanol to kill ethanol-sensitive vegetative cells or treated with high heat (pasteurization) to release spores in response to heat stress (Browne et al., 2016; Lagier et al., 2015; Pereira & Cunha, 2020).

It has been determined that the atmosphere in the gut is relatively hypoxic and the oxygen levels that are present in the gut drops along the radial axis from the intestinal submucosa to the lumen, supporting the notion that oxygen tolerant bacteria consume oxygen at the mucosal surface (Albenberg et al., 2014). A recent study validating the oxygen levels in the intestine determined that they may not even exceed 1 mmHg in the cecum, therefore further confirming the reason that anaerobes constitute the majority of the gut microbiota (Albenberg et al., 2014; Zheng et al., 2015). In 1969 Loesche determined the oxygen tolerance of two classes of anaerobes, which he termed "moderate" and "strict" anaerobes. Moderate anaerobes were capable of growing in 2-8% oxygen levels at room temperature for up to 90 minutes, while strict anaerobes could not withstand oxygen levels greater than 0.5% oxygen (Loesche, 1969). Currently, bacteria are classified into 5 classes of anaerobicity, obligate aerobe, microaerophilic, facultative anaerobe, aerotolerant, and obligate anaerobe, based on their oxygen tolerance. Since there is relatively low oxygen in the gut, anaerobes depend on other gases such as hydrogen, nitrogen, and carbon dioxide for energy but the ratios of these gasses and their effects on the growth of gut bacteria is largely understudied. Anaerobic incubation systems, such as anaerobic chambers and anaerobic jars provide an anoxic atmosphere. However, the gas conditions employed in high throughput culturomics are not consistent from lab to lab and various conditions have been implemented (Table 1.1). From these studies, it can be hypothesized that certain anaerobes may grow better and might even be selected for with different gas compositions; *Prevotella copri*, a strict anaerobe, highlights this notion. *P. copri* was first isolated from human feces in an atmosphere containing 100% CO_2 using the 'plate-

in-bottle' method (Hayashi et al., 2007). Given the importance of *P. copri* in the GIT of both pigs and humans, (Franke & Deppenmeier, 2018)(2018) analyzed the growth behavior and central metabolic pathways of *P. copri* to determine that it relies heavily on the addition of CO_2 for biomass formation. With this knowledge, our lab has investigated the impact of gas composition (20% CO_2 and 80% N_2 vs 5% CO_2, 90% N_2 and 5% H_2) using selective and non-selective media on the composition of bacteria cultivated from swine fecal samples.

1.3 Germ-Free Models:

1.3.1 Germ-free to Gnotobiotic Basics

Germ-free (GF) animals provide researchers an invaluable tool to study host-microbe interactions, specifically regarding causative roles of microbes. Truly GF animals, those that are free from all microbes, are referred to as axenic but the term is frequently interchanged with gnotobiotic. Once colonized with one or more bacterial species, these previously GF animals are termed "gnotobiotic". Gnotobiotic animals are ones where all the bacterial species are known. Various animals have been rendered GF such as zebrafish, dogs, chickens, and pigs; however, GF rodent models are the most frequently used (Furuse & Okumura, 1994; Melancon et al., 2017; Nance & Cain, 1968; Vlasova et al., 2018). GF rodents have been popular in microbiota research due to their ease of rearing, cost compared to other animals, and established protocols. The differences between GF and conventionally raised mice have been well studied. Briefly, GF mice exhibit organ level changes (larger cecum, narrower villi, abnormal accessory organ morphology), increased transit rate, altered immune system function (reduced antimicrobial peptides, decreased immune cells), and impaired tolerance to commensal bacteria (Kennedy et al., 2018). GF pigs also show differences in comparison to conventionalized pigs. The relative weights of organs like the heart, lungs, and liver are significantly lower than their conventionalized counter parts; as are blood

parameters such as white blood cells, neutrophils, lymphocytes eosinophils, and basophilic granulocytes (Zhou et al., 2021). Likewise, the intestinal morphology is altered as lower villus heights and shallower crypts in the ileum have been noted in GF pigs compared to conventionalized (Zhou et al., 2021). Furthermore, due to the absence of bacteria, enzyme activity, digestibility of nutrients, biochemical markers, and short chain fatty acid concentrations differ (Zhou et al., 2021). These GF models also show a reduction or absence of some diseases and associated symptoms, but these phenotypes can often be restored once GF animals are colonized (Hansen et al., 2014; Hörmannsperger et al., 2015). It is clear that the microbiota has important roles in the etiology of certain diseases and GF/gnotobiotic models allow for an in depth look at what part these microbes play.

Gastrointestinal morphology, metabolism, and immunity are also altered in gnotobiotic piglets (Chowdhury et al., 2007; Haverson et al., 2007; Shirkey et al., 2006; Zhou et al., 2021). However, these differences in piglets are not necessarily identical to that of gnotobiotic mice. For example, gnotobiotic piglets have been shown to have increased numbers of mucus secreting goblet cells, immunoglobulin A (IgA) producing cells, and helper T (Th) cells when colonized with human microbiota; while gnotobiotic mice transplanted with human microbiota were not observed to have the same immune activation (Che et al., 2009; Imaoka et al., 2004). Having said this, the composition of the inoculum in these studies were not identical, therefore it can be postulated that different members of the microbiota may exert varying effects on the immune system, or the results may be attributed to the underlying differences of the respective host.

1.3.2 Humanized Murine and Porcine Gnotobiotic Models

While rodents are simpler to rear and can be genetically modified to predispose them to various disease states, these animals have notable differences in microbial composition and

physiology compared to humans (Nguyen et al., 2015; Park & Im, 2020). Human microbiota associated (HMA) mice are those colonized with fecal microbes from the human GI tract. While it has been observed that the human and mouse gut microbiota share approximately 90% similarity at the genus level, the relative abundances differ as do the dominant species (Krych et al., 2013). Typically at the phylum level, humans have a greater Firmicutes to Bacteroidetes ratio compared to mice and the families that make up those phyla differ (Krych et al., 2013; Ley et al., 2005; Nagpal et al., 2018). Furthermore, the presence of exclusive taxa, such as murine-segmented filamentous bacteria in mice, poses another hurdle as these bacteria exhibit host-specific physiological influences (Chung et al., 2012). As such, selecting a GF piglet model may be more appropriate to study human diseases associated with the gut microbiota. Pigs are a suitable model for human research as they not only share similar GIT physiology, but also immunological, and microbiological parameters (Gonzalez et al., 2015; Pabst, 2020; Xiao et al., 2016). Notably, pigs have been estimated to share about 96% of the functional pathways found in humans and the porcine immune system resembles that of humans in more than 80% of analyzed parameters, which is 70% more compared to mice (Dawson et al., 2011; Xiao et al., 2016). While gut microbial communities do differ between pigs and humans, colonization of human gut microbiota to gnotobiotic piglets was successful, including certain species of Bifidobacteria that are not typical habitants of the piglet gut (Pang et al., 2007; Zhang et al., 2014; Zhang et al., 2013). A recent study investigated the ability of HMA piglet and mouse models to harbor and maintain humanized gut microbial community structure (Aluthge et al., 2020). The human fecal inoculum from adults established more successfully in the HMA piglet model, whereas half of the infant fecal donor microbiota was favored by the HMA mouse model (Aluthge et al., 2020). Differential colonization and persistence of members within the Firmicutes phylum drove the transplantation success in the

HMA piglets compared the HMA mice, which may be explained by the fact that mice typically have a lower Firmicutes to Bacteroidetes ratio (Aluthge et al., 2020; Krych et al., 2013; Ley et al., 2005; Nagpal et al., 2018). Furthermore, the HMA piglets were colonized by a higher percentage of core donor amplicon sequence variants (ASV) and harbored a higher percentage of persistent colonizers on average (Aluthge et al., 2020). With this knowledge, using HMA piglet models to study host-microbe interactions may increase the translational potential of these studies. However, rearing GF piglets is a laborious and costly endeavor, so the use of this model may not be feasible for most labs. Thus, using a gnotobiotic mouse model to test preliminary associations may be warranted.

1.4 *Salmonella* Infection:

1.4.1 *Salmonella* Overview

Salmonella enterica is a flagellated rod shaped Gram-negative facultative anaerobe that belongs to the *Enterobacteriaceae* family. The species, *S. enterica*, is subdivided into 6 subspecies with at least 2500 serotypes that are distinguished by different somatic (O) and flagellar (H) antigens. There are 3 major diseases caused by *Salmonella,* which are non-invasive non typhoidal Salmonellosis, invasive non-typhoidal Salmonellosis, and typhoid fever/paratyphoid fever. *Salmonella enterica* serovar typhimurium (S. Tm) is one of the most common non-typhoidal strains of *Salmonella* (NTS) and is most commonly contracted from consuming contaminated animal products and produce (Painter et al., 2013). It has been estimated that there are at least half a million cases of NTS each year and of those cases 15% are fatal (Stanaway et al., 2019). Furthermore, the greatest numbers of cases are seen in third world countries in areas of low sociodemographic development (Stanaway et al., 2019). In humans, NTS manifests as gastroenteritis, however, mice develop a systemic typhoid-like illness from S. Tm and piglets can

develop both enteritis and the systemic form. Due to the low cost, ease of rearing, ability for genetic manipulation, and capacity for GF derivation mice are the most widely implemented model to study salmonellosis (Gal-Mor et al., 2014). To more reliably ensure that mice become infected with *Salmonella* and to mimic human intestinal *Salmonellosis*, pre-treatment with streptomycin is often implemented (Barthel et al., 2003; Nilsson et al., 2019). However, streptomycin disturbs the host gut microbiota, which typically provides a level of colonization resistance and may not be suitable for certain studies (Rivera-Chávez et al., 2016). The susceptibility of mice to *Salmonella* also depends on the strain of mouse, with C57BL/6J mice being highly susceptible while 129/Sv are rather resistant (Brown et al., 2013). Although susceptibility to *Salmonella* is multifactorial, the natural resistance-associated macrophage protein 1 (Nramp1) encoded by the *Slc11a1* gene plays an important role in both mice and pigs (Govoni et al., 1996; Huang et al., 2018). Nramp1 transports divalent cations out of phagosomes, which limits iron for microbes, such as *Salmonella* that rely on iron to grow (Deriu et al., 2013; Nairz et al., 2009; Sassone-Corsi et al., 2016). C57BL/6J mice have a non-functional Nramp1 protein whereas transgenic B6 mice (C57BL/6 Nramp1^{G169D}) express a fully functional Nramp1 protein. Typically, B6 mice experience a systemic infection without adequate colonization of the GI tract, which causes these mice to succumb to the disease no more than 5 days post infection (Govoni et al., 1996; Nilsson et al., 2019). B6N mice, however, persist for a few weeks following oral infection and can be used to study both innate and adaptive immune responses to *Salmonella* (Brown et al., 2013; Nilsson et al., 2019).

1.4.2 *Salmonella* typhimurium Etiology and Host Immune Response

Salmonella utilizes Type-III secretion systems (T3SS) that are encoded on SPI-1 and SPI-2 pathogenicity islands to invade and circulate through the host's body. Once in the gut, effectors

secreted by T3SS/SPI-1 cause rearrangement of the cytoskeleton, cell membrane, and epithelial cell junctions facilitating entry into microfold (M) cells in the intestinal epithelial layer (Clark et al., 1994; Park et al., 2018). In *Salmonella* susceptible mice, the cecum is infected with levels exceeding 10^8 colony forming units (CFU)/g of luminal content within 8-12 hours (Barthel et al., 2003; Sellin et al., 2014). In mice, the cecum lacks a continuous mucus layer, leaving exposed cecal epithelial tips which are highly permissive and represents the primary sight of both motile and non-motile S. Tm invasion in the gut (Furter et al., 2019). Conversely, the colon is covered by a dense inner mucus layer that reduces the infection efficiency by 5-10-fold as only motile S. Tm penetrate the inner colon mucus layer at certain sites (Furter et al., 2019; Jakobsson et al., 2015). Furthermore, the colon typically shows enteropathy after 1 day post infection, whereas the small intestine may not show any signs of disease until 4 days post infection due to rapid flow of digesta that makes it difficult for bacteria to colonize the small intestine (Ren et al., 2009).

Dissemination from the intestinal tract to other tissues is facilitated by two main pathways. The first pathway is the lymphatic system and Peyer's patches (PP) where *Salmonella* spreads to the mesenteric lymph nodes (MLN) as early as 12 hours post infection (Monack et al., 2004; Sellin et al., 2014). MLN's are a major site of infection in mouse models and confinement to these lymph nodes delays *Salmonella* from reaching systemic sites early in the infection (Voedisch et al., 2009). The second relies on phagocytic cells such as dendritic cells (DCs), macrophages, monocytes, and neutrophils that carry intestinal bacteria directly into the bloodstream (Tam et al., 2008). These phagocytes are primarily found in the upper part of the PP, which are located under the M cells (Hopkins et al., 2000). Once phagocytosed, *Salmonella* utilizes T3SS/SPI-2 to inject effector proteins into the cytoplasm of these cells which direct the development of *Salmonella* containing vacuole (SCV) to allow for intracellular replication (Steele-Mortimer et al., 2002). Of these

phagocytes, macrophages are thought to be the cells most responsible for disseminating *Salmonella* into systemic tissues, as these cells are the primary sites of *Salmonella* replication (Gogoi et al., 2019; Mastroeni et al., 2009). During the systemic phase of infection, the *Salmonella* cells spread from PP to lymph nodes and then are subsequently phagocytosed by resident phagocytes in the spleen and the liver. Interleukin (IL) 10, an anti-inflammatory cytokine produced by T and B cells, is required by S. Tm to cause systemic infection in mice (Arai et al., 1995; Neves et al., 2010; Salazar et al., 2017). The administration of an IL-10 neutralizing antibody to mice, reduced the ability of S. Tm to turn into a systemic infection (Arai et al., 1995). It has been further validated that IL-10 is essential to S. Tm systemic infection as the transfer of B cells from mice unable to produce IL-10 increased resistance to infection (Neves et al., 2010). Thus, it has been suggested that S. Tm may promote tolerogenic mechanisms through IL-10 to allow for systemic invasion of these cells (Salazar et al., 2017). S. Tm also exerts virulence factors to induce intestinal inflammation. They produce reactive oxygen species (ROS) that react with thiosulphate to form tetrathionate which is a respiratory electron acceptor utilized by S. Tm, but not commensal bacteria as they lack the requisite metabolic pathway (Winter et al., 2010).

To clear infections, the innate immune system is the first responder to *Salmonella* invasion in the intestinal epithelium. *Salmonella*, being a Gram-negative bacterium, contains LPS in its cell wall, which is recognized by toll like receptor (TLR) 4 subsequently causing the release of cytokines and chemokines that recruit neutrophils and inflammatory monocytes. Neutrophils contribute to early killing of *Salmonella* after infection and are a source of IFNγ, especially in the cecal mucosa (Conlan, 1996; Spees et al., 2014). Inflammatory monocytes produce antimicrobial factors such as inducible nitric oxide synthase (iNOS), tumour necrosis factor alpha (TNF-α), and IL-1β, while resident macrophages in infected tissues produce the pro-inflammatory cytokines IL-

18 and IL-1β through the NLRC4 and NLRP3 inflammasomes upon the recognition of cytosolic flagellin (Broz et al., 2010; Rydström & Wick, 2007). Both inflammasomes activate Caspase-1, which cleaves proIL-18 and proIL-1β to their active forms, IL-18 and IL-1β, and causes pyroptosis (Franchi et al., 2009). IL-18 induces the release of IFNγ from T cells and the cytolytic activity of natural killer (NK) cells that are essential to *Salmonella* clearance (Kupz et al., 2013; Srinivasan et al., 2007). IFNγ is central to resolving the infection as it has been demonstrated that mice lacking the IFNγ receptor or treated with anti-IFNγ antibodies lack the ability to clear S. Tm and the mice succumb to the infection (Gulig et al., 1997; Hess et al., 1996). Furthermore, it is important to note that the type of cytokine response to *Salmonella* infection is vital to its successful clearance. As such, resolving the infection relies on the induction of naive helper T cell to type I helper T lymphocytes (Thl) through IL-12 and Th17 cells driven by IL-23 and Il-17 (Hsieh et al., 1993; Maclennan et al., 2004; Manetti et al., 1993; McGeachy & McSorley, 2012). Other proinflammatory cytokines that have been implicated in the clearance of *Salmonella* are IFNγ, TNF-α, IL-1, IL-2, IL-12, and IL-18 (Kurtz et al., 2017; Lalmanach & Lantier, 1999; Mittrucker & Kaufmann, 2000; Nauciel & Espinasse-Maes, 1992). Conversely, Il-10 and IL-4 can be detrimental to clearing this pathogen, as IL-10 is important for systemic dissemination of *Salmonella,* while IL-4 inhibits the differentiation and activity of Th1 cells that are vital to clear the infection (Arai et al., 1995; Everest et al., 2021; Neves et al., 2010; Salazar et al., 2017).

DCs are one link between the innate and adaptive immune responses to *Salmonella* infection as they recognize *Salmonella* LPS and flagellin, which increases antigen presentation and induces their migration to T cell dense populations in lymphoid tissues to initiate the adaptive phase of the immune response (Swart & Hensel, 2012). B and T cells help to clear *Salmonella* during the adaptive phase infection and subsequently provide protective immunity against

secondary infections. The role of B cells in not well understood and it is thought that resolution of *Salmonella* infection is not reliant on B cells as mice lacking B cells resolve primary infection with attenuated *Salmonella* through similar kinetics to wild type mice (Mastroeni et al., 2000; Mcsorley & Jenkins, 2000). However, it has been shown that mice deficient in B cells had higher bacterial burdens in primary and secondary infections compared to wild type mice, thus B cells may play a role in controlling bacterial replication (Mittrücker et al., 2000). B cell deficient mice have also been correlated with reduced IFNγ production from $CD4^+$ T cells in an antibody independent fashion as mice with B cells unable to produce antibodies were not deficient in IFNγ, nor did they show an increased susceptibility to *Salmonella* infection (Nanton et al., 2012). As such, B cells likely provide some cross-talk between humoral immunity and T-cell mediated immunity during Salmonellosis.

The role of T cells is more well defined, especially $CD4^+$ T cells. $CD4^+$ T cells produce IFNγ, which signals phagocytes and activates JAK/STAT signaling to stimulate iNOS expression, which reacts with L-arginine and oxygen to produce nitric oxide to damage *Salmonella* DNA (Blanchette et al., 2003). Depleting $CD4^+$ T cells in both *Salmonella* resistant and susceptible mice highlights the importance of these cells as mice lacking $CD4^+$ T cells have significant increases in *Salmonella* burdens in various organs (Johanns et al., 2010; Loomis et al., 2021). Furthermore, $CD4^+$ T cells express T-bet, which is required for the development of Th1 cells and the suppression of Th2 cells from naïve T lymphocytes (Ravindran et al., 2005). Given their role in Th1 cell development, $CD4^+$ T cells have also been implicated in antibody production. Th1 cells produce IgG2a, a *Salmonella* specific antibody, and mice deficient in Th1 cells as a result of being T-bet deficient were more susceptible to infection due to reduced IgG2a (Ravindran et al., 2005). Cytotoxic T cells ($CD8^+$) on the other hand have been proposed as non-essential to the clearance

of *Salmonella,* however, their role during *Salmonella* infection remains unclear. $CD8^+$ T cells rely on the major histocompatibility (MHC) class I antigen presentation pathway to mount a response against *Salmonella,* and it has been demonstrated that mice lacking MHC class I expression are still able to resolve the infection (Hess et al., 1996). Having said this, early studies lacked the ability to clearly isolate the role of specific MHC class I restricted T cells from other cell populations that express $CD8^+$ and assumed that reduced clearance was due to a lack of $CD8^+$ T cells. It has since been validated that $CD8^+$ T cells can play a role in primary infection at the late stages of bacterial clearance by restricting the growth of *Salmonella* (Lee et al., 2012). In this same study, the authors investigated previous claims of a protective role for $CD8^+$ T cells during secondary infection but found that mice lacking MHC class Ia molecules were not deficient in bacterial clearance upon secondary infection (Lee et al., 2012). It should be noted that many studies have employed mouse models that succumb to Salmonellosis within a week, and as such attenuated *Salmonella* strains have been used that lack key virulence factors to stimulate the adaptive immune system in a similar fashion as virulent strains (Hess et al., 1996; Lee et al., 2012; Lo et al., 1999). To address this issue, a study using virulent S. Tm in a resistant mouse model found that $CD8^+$ T cells were important in the clearance of chronic, virulent *Salmonella* infection (Patel & Sad, 2016). It is clear that *Salmonella* infections are complex and that the intricate functions of the innate and adaptive immune systems are still being elucidated.

1.4.3 *Salmonella* Infection, *E. coli*, and Antibiotics

The intestinal microbiota provides colonization resistance against pathogens limiting pathogen invasion in various sites of the host (Baümler & Sperandio, 2016; Sorbara & Pamer, 2019). Colonization resistance encompasses two mechanisms with the first being indirect mechanisms of colonization resistance which include alteration in the host epithelium, immune

cell function, and by activating antimicrobial immune pathways in the host (Baümler & Sperandio, 2016; Sassone-Corsi & Raffatellu, 2015). Direct methods of colonization resistance limit pathogen invasion by direct microbe-microbe competition for the same niche and nutrients as well as the production of bacteriocins, antimicrobial proteins and Type IV secretion systems (T6SS) (Sorbara & Pamer, 2019). Community species richness and community composition have been suggested to facilitate colonization resistance, as highly diverse microbial communities and certain microbes within those communities have been shown to provide protection against *Salmonella* (Brugiroux et al., 2016; Jakobsson et al., 2015; Lawley et al., 2008; Miki et al., 2017; Thiemann et al., 2017; Wotzka et al., 2019). One of these microbes that has been shown to provide colonization resistance against *Salmonella* is commensal *E. coli* (Deriu et al., 2013; Fouhse et al., 2019; Hudault et al., 2001; Thiemann et al., 2017; Wotzka et al., 2019). As such, multiple researchers have investigated the mechanisms by which *E. coli* reduces invasion by *Salmonella*. One of the mechanisms by which *E. coli* provides colonization resistance to *Salmonella* is through competition for essential micronutrients like iron. During infections, *Salmonella* scavenges for iron with salmochelin, a siderophore, in the inflamed gut to promote colonization. However, *E. coli* strains also employ iron uptake systems/siderophores that may scavenge for iron more effectively than those of *Salmonella* (Deriu et al, 2013). To demonstrate that the effects of *E. coli* Nissle on *Salmonella* reduction is due to iron acquisition, a mutation was introduced in the *tonB* gene, which provides the energy for active transport of iron-laden siderophores and heme of *E. coli* Nissle (Deriu et al., 2013). As expected, *Salmonella* infected mice did not experience a decrease in pathogen load when administered *E. coli* Nissile *tonB,* nor when *Salmonella* lacking the IroN receptor was used (Deriu et al., 2013). As such, it is clear that the competition for iron is an integral part of *E. coli* Nissle's probiotic activity against *Salmonella*. Furthermore, the production of small antimicrobial proteins

called microcins by certain *E. coli* strains have been shown to limit the expansion of *Salmonella* (Sassone-Corsi et al., 2016). Microcin expression is induced by iron scarcity and these microcins essentially target susceptible bacteria by mimicking an iron-siderophore complex, and once inside the cell the microcins either bind essential enzymes or interact with the inner membrane to elicit bactericidal effects (Rebuffat, 2012). Another mechanism by which *E. coli* may provide colonization resistance to *Salmonella* is through aerobic respiration (Brugiroux et al., 2016). Brugiroux and colleagues (2016) tested this theory by adding facultative anaerobes, such as *E. coli*, to their strictly anaerobic defined community and challenged mice with *Salmonella.* This defined community indeed restored colonization resistance to levels similar to conventional mice, which was not seen in the defined community lacking facultative anaerobes (Brugiroux et al., 2016). In the same study, hierarchical KEGG module clustering was performed and showed that the *E. coli* included in the community was highly similar to *Salmonella,* suggesting that *E. col*i Mt1B1 may also provide colonization resistance by merely occupying the preferred niche of *Salmonella*. FISH revealed that *E. coli* Mt1B1 was significantly enriched at the epithelium compared to the lumen (Brugiroux et al., 2016) Conversely, in the absence of *E. coli*, *Salmonella* occupied both niches with no preferentiality between the epithelium or lumen (Brugiroux et al., 2016). These results suggest that *E. coli* can provide colonization resistance by merely occupying this oxygen rich niche to restrict the colonization of *Salmonella* throughout the gut. Overall, given the ability of certain *E. coli* strains to prevent the colonization of *Salmonella*, there is potential to develop prophylactic microbial interventions to reduce the incidence of Salmonellosis.

Recently, the interplay between early life antibiotic administration, the gut microbiota, and subsequent S. Tm resistance has been explored (Costa et al., 2020; Fouhse et al., 2019). These studies have shown that pigs treated with amoxicillin during the first two weeks of life had better

outcomes than their counterparts when challenged with both live and heat killed S. Tm (Costa et al., 2020; Fouhse et al., 2019). In both studies, there was a more pronounced pro-inflammatory response in the amoxicillin treated groups. This pro-inflammatory response was highlighted by the activation of immune-related pathways associated with IL-2 production, nitric oxide production, and B cell receptor activation (Costa et al., 2020). Fouhse et al (2019) found that there was a more rapid nuclear factor kappa B (NF-kB) translocation in infiltrating leukocytes and the pro-inflammatory cytokines IL-2, IFNγ, TNF-α were significantly higher in amoxicillin treated piglets at PND 49 when intraperitoneally challenged with heat killed S. Tm. Interestingly, the results were accompanied by a transient 10 times expansion of *E. coli* during amoxicillin administration that normalized by the end of treatment (Fouhse et al., 2019). This bloom of *E. coli* has been previously linked to antibiotic administration in both pigs and mice (Antonopoulos et al., 2009; Fouhse et al., 2019; Looft et al., 2012). To possibly explain these results, it has been suggested that *E. coli* primes the immune system for subsequent enteric infection. One such proposed mechanism is through *E. coli* derived LPS that primes $CD4^+$ T cells and conditions the response of gut epithelial cells to subsequent TLR stimulation (Chassin et al., 2010; Lotz et al., 2006; Mcaleer & Vella, 2008; Vatanen et al., 2016). Nonetheless, research investigating the interplay between amoxicillin induced *E. coli* blooms and improved disease resistance later in life is it its infancy and warrants further exploration. Many questions remain surrounding how *E. coli* and amoxicillin influence the developing immune system, and while beyond the scope of this thesis, will be important to the development of targeted management strategies to shift microbial populations to recapitulate this more *Salmonella* resilient phenotype without actually administering unnecessary antibiotics.

1.5 Hypotheses and Objectives:

This thesis aimed to establish an effective model system to investigate the relationship between early life changes in the microbiota, amoxicillin administration, and subsequent S. Tm resistance with the following hypotheses and objectives.

Hypotheses:

Chapter 2. In the absence of competition from resident mouse microbiota, the swine-derived bacterial defined community will colonize the germ-free murine gut.

Chapter 3. *E. coli* is required for enhanced protection against subsequent *Salmonella* challenge provided by early life amoxicillin exposure.

Objectives:

Chapter 2.

1) Generate a swine culture collection of prominent bacterial taxa in the pig gut.

2) Select a representative community to create a defined pre-weaning piglet community with whole-genome sequences for gnotobiotic research.

3) Determine the colonization ability and pattern of the swine defined community in a germ-free mouse model.

Chapter 3.

1) Determine the colonization of the swine defined community when *E. coli* is present.

2) Investigate the effects of early life *E. coli* and amoxicillin administration on subsequent disease resistance when challenged with S. Tm later in life.

Table 1.1 Notable high-throughput culture studies from human and swine gastrointestinal samples in the past decade (2011-2021).

Study	Host	Culture atmosphere	Number of culture conditions	Gas composition	Temperature	Incubation period	Number of species cultured & colonies selected
Goodman et al., 2011	Human	Anaerobic	1	75% N_2, 20% CO_2, 5% H_2	37°C	7 days	316 Not stated
Lagier et al., 2012	Human	Aerobic, anaerobic, & microaerophilic	212	Not stated. 2.5-5% CO_2 for aerobic	4°C, 25°C, 28°C, 37°C, 45°C, and 55°C	Up to 40 days	340 32,500 colonies
Dubourg et al., 2013	Human	Aerobic, anaerobic, & microaerophilic	70	Not stated. 2.5-5% CO_2 for aerobic	37°C & 28°C	Up to 3 months	39 4,000 colonies
Rettedal et al., 2014	Human	Anaerobic	10 mediums & 16 antibiotics	95% N_2 and 5% H_2	37°C	7 days	26 192 colonies
Browne et al., 2016	Human	Anaerobic	2	10% CO_2, 10% H_2, 80% N_2	37°C	72 hours	137 2000 colonies
Lagier et al., 2016	Human	Aerobic, anaerobic, & microaerophilic	70	Not stated. 2.5-5% CO_2 for aerobic	28°C, 37°C, 45°C, 55°C, and 57°C	Up to 50 days	860 901,364 colonies
Lau et al., 2016	Human	Anaerobic & aerobic	66	5% CO_2, 5% H_2, 90% N_2. 5% CO_2 for aerobic	37°C	3-5 days	79 Not stated
Forster et al., 2019	Human	Anaerobic	Not stated	Not stated	37°C	Not stated	273 Not stated
Ito et al., 2019	Human	Aerobic, anaerobic, & microaerophilic	27	Not stated. 3-5% CO_2 for aerobic	25°C, 30°C, 35°C, 37°C, 42°C	Up to 7 days	Not stated Not stated
Diakite et al., 2020)	Human	Aerobic & anaerobic	58	Not stated	28 & 37°C	Up to 30 days	497 Not stated
Fenske et al., 2020	Pig	Anaerobic	10	Not stated	37°C	48-72 hours	46 2000 colonies

Wylensek et al., 2020	Pig	Aerobic & anaerobic	26 mediums	N_2 (89.3%), CO_2 (6%), and H_2 (4.7%) for anerobic. 6% CO_2 for aerobic	37°C	Up to 40 days	110 Not stated
Wang et al., 2021	Pig	Aerobic & anaerobic	53	85% N_2, 10% CO_2, and 5% H_2; not stated for other conditions	37°C	Up to 7 days	148 1,299 colonies
Moote et al., 2021	Pig	Anaerobic	Not stated	85% N_2, 10% CO_2, and 5% H_2; 90% CO_2 and 10% H_2	37°C	Up to 13 weeks	234 1,523 colonies

Chapter 2: Bacterial Isolation, Defined Community Creation, and Germ-free DC Colonization

2.1 Introduction:

The composition and structure of the gut microbiota are critical for host health and disease due to the vast effects bacteria have on pathogen colonization, host immune system, and metabolism (Pickard et al., 2017). Shifts in the gut microbiota have been linked to various infections and disease states, while other alterations have been observed to facilitate host health (Kho & Lal, 2018). However, many studies looking at the gut microbiota and its effects on the host are observational, which lack the ability to parse out which members are directly responsible for the compositional and functional changes and the mechanism by which they induce these effects. To overcome this drawback, follow up studies can utilize gnotobiotic models to determine if the previous results can be replicated and further investigate the underlying mechanisms.

Germ-free (GF) mice have been widely used to study the casual relationship between microbes of interest and host outcomes. Compared with mono-colonized animals, defined microbial communities made up of multiple species emulates a more "normal" interplay between endogenous bacteria and the host by mimicking the relationships between the microbiota and host under conventional conditions (Freter & Abrams, 1972; Schaedler et al., 1965; Syed et al., 1970). These defined communities are often based on the most prevalent and abundant taxa, however, other factors such as metabolic capacity, availability of whole-genome sequences, and the ability of selected bacteria to colonize the experimental host may be considered (Becker et al., 2011; Brugiroux et al., 2016). With advancements in culturomics, the rapid expansion of cultured species has improved the construction of defined communities as previously

unculturable strict anaerobes that are important members of the gut microbiota have been included (Hibberd et al., 2017; Kovatcheva-Datchary et al., 2019). By generating a defined community (DC) reflecting at large the typical functional abilities and interactions in the host gut allows for a well-controlled model to study host-microbe interactions.

One of the first defined communities, the Schaedler flora was used to colonize GF mice (Schaedler et al., 1965). The Schaedler flora has been manipulated multiple times; the altered Schaedler flora (ASF), composed of eight anaerobic bacteria, was designed to provide a baseline for gnotobiotic murine models and to allow for simple detection of contaminants (Orcutt et al., 1987). Since, the ASF has been further altered as it has come under criticism for being too different from that of wild mouse gut microbiota (Norin & Midtvedt, 2010). A newer murine defined community, Oligo-MM^{12} was designed to prevent the colonization of *Salmonella enterica* serovar Typhimurium (S. Tm) with 12-15 strains selected from the 5 most prevalent and abundant phyla in the laboratory mouse intestine, similar to another simplified mouse microbiota (GM15) consisting of 15 strains from the 7 of the most prevalent families represented in C57BL/6J mice (Brugiroux et al., 2016; Darnaud et al., 2019). Other defined communities in rodents have been created based on the prevalence of specific microbes in humans (humanized models) and in conjunction with important biochemical activities, such as the simplified human intestinal microbiota (SIHUMI) or the simplified intestinal microbiota (SIM) (Becker et al., 2011; Kovatcheva-Datchary et al., 2019).

Nonrodent defined communities have been created for work in GF pig models as the majority of ASF members have not been shown to readily colonize GF piglets (Laycock et al., 2012). As such, the Bristol microbiota was developed using 1 bacterial strain from each of the 4 most frequently identified taxa in the ileum, cecum, and colon of 12–18-week-old pigs of which

successfully colonized GF piglets (Laycock et al., 2012). It should be noted that this DC was constructed based on older sequencing data (Leser et al., 2002). Therefore, with better sequencing techniques and in-depth meta-analyses of gut microbial communities in pigs there is room to improve these defined communities. Additionally, with the improvements in culturomics, researchers are able to culture greater numbers of bacterial species allowing for more robust defined communities (Fenske et al., 2020; Wylensek et al., 2020). Currently, there are limited studies that have generated culture collections of pig-originated gut microbes; thus, the objectives of this work were to 1) generate a culture collection of the prominent taxa in the pig gut 2) select a representative community to create a defined piglet community with whole genomes for GF swine research 3) determine the colonization pattern of the defined piglet community in a GF mouse model. We hypothesized that in the absence of competition from the resident mouse microbiota, the majority of the piglet DC would colonize the GF mouse gut.

2.2 Materials and Methods:

2.2.1 Culture Collection

Sample Collection

Gastrointestinal (GI) content (cecal and fecal) was obtained from pigs ranging from post-natal day (PND) 7 to adulthood housed at the Swine Research and Technology Centre at the University of Alberta (Edmonton, Alberta, Canada). Sow feces were obtained directly from the rectum of each sow into sterile tubes containing the respective pre-reduced broth of the medium that the sample was intended to be plated on (Table 2.2). Samples were immediately processed for plating in an anaerobic chamber. The cecal samples from 7-day old piglets were obtained from a previous study where they were immediately snap frozen and stored at -80°C (Fouhse et al., 2019).

Culture Media

The culture media compositions are listed below (all quantities are per litre of milliQ water unless otherwise indicated). Media was supplemented with 15 g agar when agar plates were made from broth bases. Gut Microbiota Medium (GMM), Laked Blood Agar with Kanamycin and Vancomycin (LKV), Peptone Yeast Glucose agar (PYG), and Yeast Extract, Casitone and Fatty Acid medium (YCFA) media were all pre-reduced in the anaerobic chamber for 24 hrs prior to plating, re-streaking, or broth inoculation steps. Kanamycin and vancomycin when supplemented were added at 100 mg/L and 7.5 mg/L, respectively.

Fastidious Anaerobes Agar (FAA): 46 g (Neogen, ref. NCM0014A).

Difco™ Brain Heart Infusion Agar (BHI): 52 g (BD, ref. 241820).

Columbia Blood Agar Base: 39 g (Oxoid, ref. CM0331); 50 mL sheep blood (Thermo Fischer, ref. R54008).

Difco™ Reinforced Clostridial Medium (RCM): 38 g (BD, ref. 218081).

Difco™ Lactobacilli MRS broth (MRS): 55 g (BD, ref. 288130).

Schaedler broth: 28.4 g (BD, ref. B12191).

Wilkins-Chalgren Agar (WC): 33 g (Oxoid, ref. CM0642).

Brilliant Green Agar (Modified) (BGM): 52 g (Oxoid, ref. CM0329).

BBL™ MacConkey Agar (MAC): 50 g (BD, ref. 211387).

Gut Microbiota Medium (GMM): as previously described by Goodman et al., 2011.

BBL™ Brucella Laked Blood Agar with Kanamycin and Vancomycin (LKV): 10 g casein, 10 g peptic digest of animal tissue, 1 g dextrose, 2 g yeast extract, 5 g sodium chloride, 0.1 g sodium bisulfite, 0.005 g hemin, 0.01 g vitamin K1, 50 mL defibrinated/laked sheep blood (Thermo Fischer, ref. R54008).

Peptone Yeast Glucose agar (PYG): refer to DSMZ 104 PYG Medium (modified).

Yeast Extract, Casitone and Fatty Acid medium (YCFA) (per 100 ml): 1 g casitone, 0.25 g yeast extract, 0.4 g $NaHCO_3$, 0.1 g cysteine, 0.045 g K_2HPO_4, 0.045 g KH_4PO_4, 0.09 g NaCl, 0.009 g $MgSO_4 .7H_2O$, 0.009 g $CaCl_2$, 0.1 mg resazurin, 1 mg haemin, 1 µg biotin, 1 µg cobalamin, 3 µg p-aminobenzoic acid, 5 µg folic acid and 15 µg pyridoxamine. Final concentrations of short-chain fatty acids (SCFA) in the medium were 33 mM acetate, 9 mM propionate and 1 mM each of isobutyrate, isovalerate and valerate.

Bacterial Cultivation and Identification

Samples were homogenized by shaking in a tridimensional motion at 6.0 meters per second for 1 min (FastPrep instrument, MP Biomedicals, Solon, OH, USA). Samples were subsequently transferred to a BACTRON300 Anaerobic Workstation (Sheldon Manufacturing Incorporated; Cornelius, Oregon, USA). Tenfold serial dilutions were carried out to 10^{-6} and 100 µL of each dilution was plated on the respective media and incubated under 1 of 3 gas conditions for 48-72 hrs at 37°C (Table 2.2). Single colonies were selected from plates with distinct separation in each culture experiment. Bacterial cultures were preserved at -80°C in stock medium (broth supplemented with 25% glycerol).

To determine the identity of the bacterial isolate, amplicon polymerase chain reaction (PCR) was performed using 8F (AGAGTTTGATCCTGGCTCAG) and 926R (CCGTCAATTCNTTTRAGT) primers to amplify the 16S ribosomal ribonucleic acid (rRNA) gene (Amann et al., 1995; Lane, 1991). Each 50 µL PCR reaction solution consisted of 2 µL of 10 µM 8F primer, 2 µL of 10 µM 926R primer, 2 µL of 10 mM deoxynucleotide triphosphate mix (Invitrogen, Carlsbad, CA), 5 µL of 10x Taq polymerase buffer (Invitrogen), 2 µL of 50 mM $MgCl_2$ (Invitrogen), 0.5 µL of 1 U/µL Taq polymerase (Invitrogen), and 1 µL of the Nuclease-

free H_2O containing the bacterial colony. The following PCR program was performed: initial 10-min denaturation at 94°C, 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min 40 s, and a final 7-min extension at 72°C. PCR products were run on a 1% agarose gel. Samples with an amplicon of the correct length were cleaned up using a GeneJET Genomic DNA Purification Kit following the manufacturer's protocol (Thermo Scientific, Nepean, ON). Purity and concentration of DNA were determined using a Nanodrop 2000 platform (Thermo Scientific, Wilmington, DE) and Sanger sequencing was carried out. The 16S rRNA sequence was searched against the National Center for Biotechnology Information (NCBI) 16S rRNA sequence and Ribosomal Database Project database (Altschul et al., 1990).

2.2.2 Whole-Genome Sequencing (WGS)

Genomic DNA was extracted from *E. coli* and the isolates in Table 2.1 using a Wizard Genomic DNA Purification kit (Promega Corporation, USA) according to the manufacturer's instructions. Briefly, bacterial cells were collected by centrifugation at 16,000 x g for 2 min. For Gram-positive bacteria, the cell pellet was resuspended in 480 µL of 50 mM ethylenediaminetetraacetic acid (EDTA) and a volume of 120 µL lysozyme solution (10 mg/mL) (Fisher Scientific, Nepean, ON) was subsequently added. After 60 min incubation at 37°C, the suspension was centrifuged at 16,000 x g for 2 min and the pellet was collected for the purification steps as for Gram-negative bacteria. Bacterial cell pellets were re-suspended in 600 µL nuclei lysis solution. The cells were incubated at 80°C for 5 min and 3 µL of RNase solution were added. Following 60 min incubation at 37°C, 200 µL protein precipitation solution was added to the mixture and vortexed vigorously for 30 s. The mixture was incubated on ice for 5 min and centrifuged at 16,000 x g for 3 min. The supernatant was transferred to a clean tube containing 600 µL isopropanol at room temperature and the mixture was centrifuged at 16,000 x

g for 2 min to collect pellet. The pellet was washed with 70% ethanol at room temperature and centrifuged to discard the ethanol. The DNA pellet was air-dried for 10 min and re-suspended in low EDTA-TE buffer (10 mM Tris-HCl, 0.1 mM EDTA). Genomic DNA concentration and quality were measured using a NanoDrop-2000 spectrophotometer and a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). DNA with high purity was used for whole-genome sequencing (WGS).

Whole-genome sequencing was performed using a DNA library preparation kit (New England Biolabs® NEBNext® Ultra™ II DNA Library Prep Kit), followed by paired-end 150 base pair (bp) sequencing on an Illumina NovaSeq 6000 platform (Illumina Inc. San Diego, CA). Sequencing reads were analyzed by FastQC (v 0.11.9) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and adapter sequences were trimmed using Trimmomatic v 0.39 (1). The draft genome was *de novo* assembled using SPAdes assembler v 3.10.1 and Quast v 5.0.2 were used to assess the assembly quality (Bankevich et al., 2012; Gurevich et al., 2013). To confirm species identity, average nucleotide (ANI) was calculated between the contig FASTA file from WGS and NCBI reference genomes of suspected species using the OrthoANIu algorithm at https://www.ezbiocloud.net/tools/ani (Yoon et al., 2017). The ANI cut off was 97% for species identification, but if the reference species all had a 97% ANI the species with the highest percentage was assigned as the species identity (Ciufo et al., 2018). Genome annotation was performed by the Rapid Annotations using Subsystems Technology (RAST) (Aziz et al., 2008). Toxin-related virulence in the whole-genome was predicted with IslandViewer (Dhillon et al., 2015). The predicted amino acid sequences were also annotated against the Comprehensive Antibiotic Resistance Database (CARD) using Resistance Gene Identifier (RGI) with default parameters (Alcock et al., 2020).

2.2.3 Defined Community Inoculum

To create the DC inoculum, 16 isolates were selected from the culture collection and supported by WGS results (Table 2.1). Isolates were streaked onto agar plates of the respective media and incubated for 48-72 hrs at 37°C in a BACTRON300 Anaerobic Workstation (Sheldon Manufacturing Incorporated; Cornelius, Oregon, USA; Table 2.1). Each isolate was subsequently inoculated into 5 mL of the respective broth and incubated for 48-72 hrs at 37°C in 70% N_2, 20% CO_2, 10% H_2 anaerobic gas conditions (Table 2.1). Subsequently, 500 µL of all isolates were combined into a 15 mL falcon tube with 25% glycerol and stored at -80°C (Table 2.1) as a cryostock until mouse inoculation.

Table 2.1 Summary of bacterial isolates in the defined community and culture conditions.

Organism	Media	24 hr pre-reduction of agar plate in anaerobic chamber	Incubation time
Bacteroides eggerthii	FAA	No	48 hrs
Bacteroides thetaiotaomicron	FAA	No	48 hrs
Bacteroides vulgatus	FAA	No	48 hrs
Bacteroides xylaninosolvens	FAA	No	48 hrs
Blautia faecicola	FAA	Yes	72 hrs
Clostridium colicanis	RCM	No	48 hrs
Lactobacillus amylovorus	MRS	No	48 hrs
Lactobacillus delbrueckii	MRS	No	48 hrs
Lactobacillus johnsonii	MRS	No	48 hrs
Lactobacillus mucosae	MRS	No	48 hrs
Limosilactobacillus reuteri	MRS	No	48 hrs
Lactobacillus ruminis	MRS	No	48 hrs
Prevotella copri	Schaedler	Yes	72 hrs
Streptococcus hyointestinalis	FAA	No	48 hrs
Streptococcus pasteurianus	FAA	No	48 hrs
Turicibacter sanguinis	FAA	Yes	72 hrs

Footnote: All isolates were incubated in 70% N_2, 20% CO_2, 10% H_2 anaerobic gas conditions.

2.2.4 Mouse Colonization Experiments

Animal Housing, Colonization, and Sample Collection

Twenty-one- to twenty-four-week-old C57BL/6J (B6) GF male mice (Charles River Laboratories, Wilmington, Massachusetts, USA) were housed in flexible film isolators (Controlled Environment Products, McHenry, Illinois, USA) maintained in the Axenic Mouse Research Unit at the University of Alberta. All mice were housed in sterile cages with aspen wood chip bedding materials mixed with aspen shavings. Nesting materials, paper huts, and nestlets were provided as enrichment. Two cages in separate isolators housed 4 mice each in a room that was environmentally controlled for light cycle (12 hrs light and 12 hrs darkness), temperature (20–22°C), and relative humidity (40%). Mice were fed autoclaved chow diet (Purina Mills, 5010) and autoclaved *ad libitum* water. All mice were orally gavaged once with 100 µL of a thawed cryostock, defrosted on ice, of the DC to assess the colonization ability in a mouse model. One week post oral gavage, fecal pellets were collected, and all mice were euthanized by CO_2 asphyxiation 4 weeks after oral gavage. Content from the ileum, colon, and cecum were collected from each mouse for microbial compositional analysis and total bacterial load. Animal use was approved by the University of Alberta Animal Care and Use Committee (AUP00000671).

DNA Extraction and 16S rRNA Gene Amplicon Sequencing

DNA extraction was performed using the QIAmp Fast DNA Stool Mini Kit (Qiagen Incorporated; Valencia, California, USA) according to manufacturer instructions. The following modifications were made to the protocols: samples in Inhibitex buffer were mixed with 2.0 mm diameter sterile garnet beads (BioSpec Products, Bartlesville, OK) and homogenized by shaking twice in a tridimensional motion at 6.0 meters per second for 30 seconds (FastPrep instrument, MP Biomedicals, Solon, OH, USA). The purity and concentration of extracted DNA were

determined using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE) and a Qubit® dsDNA HS Assay Kits. Amplification of the V3-V4 region of 16S rRNA gene and sequencing library preparation procedures were performed according to Illumina 16S Metagenomic Sequencing Library preparation protocol (#15044223 Rev.B). Paired-end sequencing using 2 x 300 cycles was performed on an Illumina MiSeq Platform (Illumina Inc. San Diego, CA).

16S rRNA Gene Amplicon Sequencing Analysis

Raw sequencing read processing and analyses were performed using Quantitative Insights into Microbial Ecology 2 (QIIME2 v. 2020.2.0) (Bolyen et al., 2019). Forward and reverse reads were trimmed at 270 and 220 bp, respectively. Filtering, dereplication, chimera detection and merging of paired-end reads were performed using DADA2 software (Callahan et al., 2016). Multiple sequence alignment and phylogenetic tree generation were performed using Multiple Alignment using Fast Fourier Transform (MAFFT) (Katoh & Standley, 2013) and FastTree 2 (Price et al., 2010). Taxonomy classification was performed using scikit-learn naive Bayes machine-learning classifier (Bokulich et al., 2018; Pedregosa et al., 2011) and SILVA 99% OTUs (v138) as the reference sequence database. Alpha and beta diversity analyses were performed using the diversity core-metrics function in QIIME2 (Navas-Molina et al., 2013). To ensure that all samples had equal sequencing depth for diversity analyses, reads were normalized to the sample with the lowest number of reads. Linear discriminant analysis Effect Size (LEfSe) was performed to determine which bacterial taxa most likely explain differences between intestinal regions using the microbiomeMarker package in R v4.1.2 (Segata et al., 2011).

Total Bacteria Quantification

Quantification of total bacteria was carried out by quantitative polymerase chain reaction (qPCR) using the StepOnePlusTM Real-Time PCR System (Applied Biosystems by Life

Technologies, Foster City, California, USA) and Fast SYBR green chemistry (Thermo Scientific 4385610). DNA was extracted and assessed from the ileum, cecum, colon, and feces as described above. Briefly, 5 µL of all samples were pooled in each respective assay, to create an amplicon using the forward primer (CGGYCCAGACTCCTACGGG) and the reverse primer (TTACCGCGGCTGCTGGCAC) resulting in a 179-210 bp amplicon of the 16S rRNA gene (Lee et al., 1996). The PCR solution, reaction cycle, and clean up steps were performed as described above in the section on Bacterial Cultivation and Identification. The pooled sample amplicon's purity and concentration were determined using ultraviolet–visible spectroscopy via the Nanodrop 2000 (Thermo Scientific, Wilmington, Deleware, USA) and fluorescence spectroscopy using the Qubit® dsDNA HS Assay Kits, respectively. Subsequently, 1:3 dilutions were made from the pooled sample amplicon to create a standard curve. The samples were normalized to 0.2 ng/µL. The qPCR reaction solution consisted of 5 µL of Fast SYBR green chemistry (Thermo Scientific 4385610), 0.5 µL of forward and reverse primers, 2 µL of nuclease free water (NFH_2O), and 2 µL of DNA template. Each sample was performed in duplicate, including the NFH_2O negative control. The reaction was carried out using the following program: 95°C for 3 min and 40 cycles of 95°C for 10 s, 60°C for 30 s. The amplification efficiencies of all qPCR assays ranged from 91%-97% and there was no detectable amplification from the non-template controls in any of the assays. The bacterial loads were then normalized to the weight of content used to isolate bacterial DNA and log transformed.

Statistical Analysis

All data was tested for normality using Shapiro-Wilk test as each data set was less than 30 samples, and equal standard deviation was tested. The total bacteria abundance (log-transformed) had a Gaussian distribution with unequal standard deviation; thus, a Brown-

Forsythe and Welch analysis of variance (ANOVA) test was performed with Dunnett's T3 multiple comparisons test. The aforementioned statistical analyses were carried out through the software, GraphPad Prism (v9.3.1). Shannon and Chao1 microbial diversity indices for each sample were calculated using the vegan package in R (R v4.1.2). The alpha diversity data set had a non-Gaussian distribution; therefore, a non-parametric Kruskal-Wallis test was performed with the phyloseq package in R v4.1.2. Principle Coordinate Analysis (PCoA) of sequence data to determine compositional differences between regions was performed using the phyloseq package in R (R v4.1.2) using the Bray-Curtis dissimilarity metric. Permutational multivariate analysis of variance (PERMANOVA) and Permutational Analyses of Multivariate Dispersions (PERMDISP) were run on each beta diversity metric using the adonis and betadisper functions in R v4.1.2 (999 permutations). Pairwise comparisons using PERMANOVA (9999 permutations) were subsequently run to determine which regions significantly differed (adonis function, vegan package, R v4.1.2). P-values of less than 0.05 were considered as statistically significant.

2.3 Results and Discussion:

Cultivation of bacteria from the swine intestinal tract has advanced in response to the need to provide access to culture collections for functional studies to examine host-microbe interactions and roles in disease states. Recently, substantial culture efforts have been successfully generated many pure isolates from the swine GI tract (domestic and feral) (Fenske et al., 2020; Moote et al., 2021; Wylensek et al., 2020). These culture collections have revealed novel species and metagenomic investigation has begun to define the functional profiles of specific bacteria, which is important to determining the role of these organisms in the gut. In this study, we isolated 35 species across 15 genera, 12 families, and 6 phyla based on partial 16S rRNA gene sequences from approximately 400 isolates (Table 2.2). The Pig Intestinal Bacterial

Collection (PiBAC), a public library for swine bacterial isolates, consists of 110 species spanning 40 families and 9 phyla with metagenome-assembled genomes. With our culture efforts, we isolated 19 species not available in the PiBAC repository. Most of the gut microbiota is comprised of anaerobic bacteria, and many are fastidious in nature. As such, the vast majority of our culture conditions limited oxygen exposure, provided atmospheres rich in carbon dioxide and complex mediums to select for these microbes (Table 2.2). *P. copri*, an obligate Gram-negative anaerobe of special interest to swine researchers highlights the importance for selective culture conditions (Amat et al., 2020). While *Prevotella* is one of the abundant genera in the large intestine of pigs, the cultivation of this genus does not recapitulate the levels found in samples (Fenske et al., 2020). In the Tamworth pig gut, *Prevotella* was identified as the most abundant taxa, however during culturomic endeavors, this genus was only isolated 7 times from over 1000 purified colonies. *P. copri* depends highly on the availability of CO_2 for biomass formation and carbohydrate substrates (Franke & Deppenmeier, 2018; Hayashi et al., 2007). Thus, to select for *P. copri*, we created specific culture conditions consisting of PYG media supplemented with vancomycin and kanamycin, to prevent overgrowth of other bacteria, and incubated in CO_2 rich atmospheric conditions (80% N_2 and 20% CO_2). Incubation with these conditions in an anerobic chamber for 48 hrs yielded 15 *P. copri* isolates from 25 colonies selected (Table 2.2). It is evident that the generation of culture collections is a laborious pursuit, but such culture dependent methods provide advantages over NGS by allowing for the in *vivo/vitro* and *ex vivo* elucidation of the functions and ecological roles these autochthonous bacteria have in the GI tract.

With the isolation of commensal bacteria from the swine GI tract, the creation of defined bacterial communities for gnotobiotic models was possible. Here, the DC was curated based

upon taxa in the swine core microbiome and prevalent bacteria in pre-weaned piglets (Table 2.1). Multiple studies have begun to determine the core microbiome in pigs by identifying genera that make up over 90% of all GI samples (Holman et al., 2017; Li et al., 2020; Yang et al., 2018). Holman and colleagues (2017) proposed that the core microbiota for commercial swine consists of *Clostridium*, *Blautia*, *Lactobacillus*, *Prevotella*, *Ruminococcus*, *Roseburia*, the RC9 group, *Subdoligranulum*, and *Alloprevotella*. Another study confirmed that *Lactobacillus*, *Clostridium*, *Prevotella*, *Roseburia*, *Ruminococcus*, and *Blautia* were also core microbiota in Jinhua pigs (Yang et al., 2018). In this same study, *Bacteroides*, *Turicibacter*, and *Streptococcus* were identified as other core intestinal microbiota (Yang et al., 2018). However, both studies neglected to include samples prior to weaning, as such we also consulted papers with compositional data from birth to weaning (Frese et al., 2015; Li et al., 2018; Li et al., 2020; Liu et al., 2019; Wang et al., 2019). Many of the same families are present pre and post weaning, however, the dominant families and genera within them change with age (Frese et al., 2015). During weaning, piglets switch from milk rich in lactose to cereal-based diets high in complex carbohydrates and fibre, which drives the shift from high abundances of *Bacteroidaceae* to *Prevotellaceae* largely due to *Prevotella* increasing after weaning compared to *Bacteroides* (Guevarra et al., 2019; Navarro et al., 2019). At the genus level, *Escherichia, Lactobacillus*, *Clostridium*, and *Fusobacterium* are also more abundant in pre-weaned piglets, as they are some of the first colonizers due to their aerotolerance (Li et al., 2018; Yang et al., 2019). It should be noted that while *Fusobacterium* is relatively abundant in piglets, it is a known opportunistic pathogen and has been associated with severe diarrhea in piglets, therefore it was not included in the DC (Huang et al., 2019; Nagaraja et al., 2005). In total, 16 species from the top families making up over 80% of the piglet microbiota (except for *Enterococcaceae* and *Ruminococcaceae*

as the culture collection did not include an isolated representative) were selected for the DC (Frese et al., 2015; Table 2.1). *Enterobacteriaceae*, specifically *E. coli* was not selected to be included in the DC as subsequent studies focused on *E. coli* as an independent variable. To taxonomically characterize the strain of *E. coli* isolated from the predecessor study (Fouhse et al., 2019) and investigate the 16 species included in the DC, WGS was performed (Table 2.3). Upon WGS analysis, no hits of toxin related virulence factors of interest were identified in the genomes of the DC species. Endotoxins and exotoxins were identified for the *E. coli* isolate, some of these included the lipid A and lipopolysaccharide (LPS) endotoxins associated with Gram-negative bacterial cell walls and the exotoxins ShET1B, hemolysin, and colicin. Furthermore, antimicrobial resistance (AMR) genes, which can be directly or indirectly involved in virulence, were analyzed and we identified certain AMR related genes (Fig. 2.1). The *E. coli* isolate had the greatest number of annotated AMR genes, which is unsurprising as *E. coli* is considered a representative indicator of AMR in Gram-negative bacteria (Gregova & Kmet, 2020). Of specific interest, our *E. coli* isolate possesses the ability to produce β-lactamases providing resistance to β-lactam antibiotics.

To assess the colonization success of the swine DC in mice, 8 GF male mice were orally gavaged with freshy defrosted DC cryostock. In the GF mouse gut, 10 out of the 16 species were all detected in the ileum, cecum, colon, and feces (Fig. 2.2). As such, the richness and evenness of the bacterial community, indicated by the alpha diversity indices (Chao1 and Shannon index), did not vary between regions (Adonis $P > 0.05$, Fig. 2.3). However, the community composition based on the Bray-Curtis dissimilarity metric was significantly different between regions, which explained 43.3% variation in bacterial compositions (Adonis $P < 0.05$, Fig. 2.4). No significant differences were detected between the cecum, colon, and feces (Adonis $P > 0.05$). The beta

diversity in the ileum, however, was significantly different from the cecum, colon, and feces (Adonis $P < 0.01$). Typically, the ileal microbiotas cluster away from the cecum, colon, and feces while these lower regions overlap in mice (Anders et al., 2021; Suzuki & Nachman, 2016). This spatial heterogeneity of the gut microbiota is not unique to mice and has also been validated in pigs where small intestinal samples cluster away from large intestinal samples which overlap one another (Crespo-Piazuelo et al., 2018; Holman et al., 2017; Zhao et al., 2015). To explain which taxa drive these differences, LEfSe analysis revealed that the ileum was enriched with *S. pasteurianus*, *L. amylovorus*, *L. johnsonii*, and *L. reuteri* while *B. eggerthii* was enriched in the feces, and *B. thetaiotaomicron* and *B. xylaninosolvens* in the cecum ($P < 0.05$, Fig. 2.5). In line with previous literature, *Streptococcus* and *Lactobacillus* are two of the most abundant taxa in the small intestine, while the large intestine favors *Bacteroides* in both pigs and mice (Anders et al., 2021; Crespo-Piazuelo et al., 2018; Lkhagva et al., 2021). The differences between the dominant taxa in the small intestine versus the large intestine is owed largely to the nutrient profiles and oxygen levels (Donaldson et al., 2015). In the small intestine, microbes including *Lactobacillus* and *Streptococcus* are responsible for simple carbohydrate metabolism, while bacteria such as *Bacteroides* ferment complex polysaccharides (Flint et al., 2012; Zoetendal et al., 2012). Higher levels of oxygen in the small intestine preferentially select for bacteria that can tolerate greater amounts of oxygen, and these facultative anaerobes rapidly utilize oxygen in the small intestine resulting in an environment devoid of oxygen in the large intestine (Lozupone et al., 2012). These conditions are analogous between humans, pigs, and mice, which corroborates the similarities in prominent taxa found along the tract that are conserved amongst these hosts (Gu et al., 2013; Martinez-Guryn et al., 2019; Zhao et al., 2015). Although the majority of the DC colonized the GF mouse gut in the absence of mouse microbiota, many of the obligate

anaerobes did not colonize (Fig. 2.2). The sensitivity of these obligate anaerobes to oxygen may explain the lack of colonization as the inoculum was exposed to oxygen for several minutes during the oral gavaging process. It has been suggested that when dealing with strict anaerobes that exposure to oxygen should be less than 2 minutes (Bellali et al., 2019). Furthermore, in the pursuit of standardization, the inoculum was gavaged from a frozen cyrostock (Eberl et al., 2020). However, many protocols aimed at introducing defined communities to GF mice are generated using fresh bacterial cultures for oral gavage (Desai et al., 2016; Gomes-Neto et al., 2017; Kovatcheva-Datchary et al., 2019). Additionally, the mice in this study were gavaged once with DC inoculum, but other studies have shown that a second exposure a couple days after the first leads to a more stable community composition than a single gavage (Eberl et al., 2020). Therefore, future work involving this swine DC aimed to limit oxygen exposure, provide fresh inocula of the 6 species that did not colonize, and a repeat exposure is warranted.

The total bacteria also differ in the ileum (9.01 colony forming units (CFU)/g ± 0.18, mean ± standard error of the mean (SEM)) compared to the cecum (11.19 CFU/g ± 0.05, mean ± SEM), colon (11.16 CFU/g ± 0.07, mean ± SEM), and feces (11.33 CFU/g ± 0.17, mean ± SEM) (ANOVA $P < 0.0001$, Fig. 2.6). Normally, bacterial populations increase from approximately $10^{7\text{-}8}$ CFU/mL in the ileum to $10^{10\text{-}11}$ CFU/mL in the subsequent regions (Kastl et al., 2020). Again, the drastically different conditions between the upper and lower GI tract provide reasoning for decreased total bacteria in the ileum. The transit rate in the small intestine is much quicker than in the large intestine, and as such bacteria have limited opportunity for colonization in these regions. Furthermore, the small intestine is the primary site for nutrient digestion and absorption where the ileal intestinal epithelial cells are responsible for vitamin B absorption and bile salt reuptake in the liver via enterohepatic cycling. Due to the antimicrobial effects of bile

salts, the abundance and composition of bacteria in the ileum is altered (Urdaneta & Casadesús, 2017). It has been demonstrated that mice lacking the farnesoid X receptor, a bile acid receptor, suffer from ileal bacterial overgrowth and translocation of bacteria across the epithelial barrier due to the obstruction of bile flow, which can be restored by oral bile acid administration (Inagaki et al., 2006; Lorenzo-Zúñiga et al., 2003). To combat such antimicrobial effects, colonizing ileal bacteria like species belonging to the genera *Lactobacillus*, *Clostridium*, *Bacteroides*, and *Bifidobacterium* deconjugate bile acids by producing bile salt hydrolases (Begley et al., 2006; Ridlon et al., 2006; Staley et al., 2018). While bile acid presence in the ileum regulates bacterial colonization, the mucosal immune system in the distal ileum is imperative to bacterial surveillance and homeostasis. As such, the ileum is densely populated by resident phagocytes and lymphocytes, but also antigen sampling and presenting cells associated with Peyer's Patches (da Silva et al., 2017). In the crypts of the lamina propria, Paneth cells produce antimicrobial peptides like defensins, cathelicidins, and c-type lectins to control bacterial populations (Chairatana & Nolan, 2017). For example, to control the abundance of Gram-positive bacteria the C-type lectin, RegIIIγ, exhibits bactericidal effects by disrupting the peptidoglycan layer (Vaishnava et al., 2011). While the mucosal immune system in the ileum mainly targets pathogenic bacteria, it is reasonable to suggest that these dense immune cell populations in combination with the quick transit rate and antimicrobial substances collectively reduce the population of commensal bacteria in the ileum compared to the distal tract.

Table 2.2 Summary of bacterial isolates and culture conditions.

Organism	**Number of isolates**	**Media**	**Antibiotic**	**Gas conditions**	**Source**	**Incubation time**
Bacteroides dorei	9	FAA	None	90% N_2, 5% CO_2, 5% H_2	Frozen sow feces	48 hrs
Bacteroides eggerthii	6	LKV	Vancomycin (7.5 mg/L) & Kanamycin (100 mg/L)	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Bacteroides fragilis	5	LKV	Vancomycin (7.5 mg/L) & Kanamycin (100 mg/L)	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Bacteroides sartorii	4	LKV	Vancomycin (7.5 mg/L) & Kanamycin (100 mg/L)	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Bacteroides stercoris	6	PYG	Vancomycin (7.5 mg/L) & Kanamycin (100 mg/L)	80% N_2, 20% CO_2	Fresh sow feces	48 hrs
Bacteroides thetaiotaomicron	3	WC	None	90% N_2, 5% CO_2, 5% H_2	Frozen PND 7 piglet cecum	48 hrs
Bacteroides vulgatus	5	LKV	Vancomycin (7.5 mg/L) & Kanamycin (100 mg/L)	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Bacteroides xylaninosolvens	11	LKV	Vancomycin (7.5 mg/L) &	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs

			Kanamycin (100 mg/L)			
Blautia faecicola	1	FAA	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Clostridium baratii	12	BA	None	90% N_2, 5% CO_2, 5% H_2	Frozen sow feces	48 hrs
Clostridium colicanis	7	FAA	None	90% N_2, 5% CO_2, 5% H_2	Frozen sow feces	48 hrs
Clostridium perfringens	24	PYG; MRS	Vancomycin (7.5 mg/L) & Kanamycin (100 mg/L); None	80% N_2, 20% CO_2; 90% N_2, 5% CO_2, 5% H_2	Fresh sow feces; frozen PND 7 piglet cecum	48 hrs
Collinsella aerofaciens	1	FAA	None	80% N_2, 20% CO_2; 90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Escherichia coli	159	BGM; MRS; WC; MAC; FAA	None	80% N_2, 20% CO_2; 90% N_2, 5% CO_2, 5% H_2; 100% O_2	Frozen PND 7 piglet cecum; fresh sow feces	24-48 hrs
Fusobacterium mortiferum	1	WC	None	90% N_2, 5% CO_2, 5% H_2	Frozen PND 7 piglet cecum	48 hrs
Hungatella spp.	1	FAA	None	90% N_2, 5% CO_2, 5% H_2	Frozen sow feces	48 hrs
Lachnospiraceae bacterium	1	FAA	None	90% N_2, 5% CO_2, 5% H_2	Frozen sow feces	48 hrs

Lactobacillus amylovorus	14	GMM; PYG; YCFA	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Lactobacillus delbrueckii	5	YCFA	None	90% N_2, 5% CO_2, 5% H_2	Frozen PND 7 piglet cecum	72 hrs
Lactobacillus johnsonii	35	WC; YCFA	None	90% N_2, 5% CO_2, 5% H_2	Frozen PND 7 piglet cecum	48-72 hrs
Lactobacillus mucosae	1	YCFA	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Limosilactobacillus reuteri	14	MRS; BHI; FAA; YCFA, PYG	None	90% N_2, 5% CO_2, 5% H_2	Frozen PND 7 piglet cecum; fresh sow feces	48-72 hrs
Lactobacillus ruminis	6	PYG, YCFA	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Lactobacillus vaginalis	4	YCFA	None	90% N_2, 5% CO_2, 5% H_2	Frozen PND 7 piglet cecum	72 hrs
Prevotella copri	15	PYG	Vancomycin (7.5 mg/L) & Kanamycin (100 mg/L)	80% N_2, 20% CO_2	Fresh sow feces	48 hrs
Sarcina ventriculi	3	BHI	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs

Staphylococcus simulans	17	FAA	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Streptococcus alactolyticus	19	BA	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Streptococcus caballi	1	BA	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Streptococcus hyointestinalis	15	BHI; GMM	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Streptococcus lutetiensis	1	BA	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Streptococcus orisratti	1	BA	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Streptococcus pasteurianus	1	FAA	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Turicibacter sanguinis	2	FAA	None	90% N_2, 5% CO_2, 5% H_2	Fresh sow feces	48 hrs
Victivallis vadensis	7	PYG	Vancomycin (7.5 mg/L) & Kanamycin (100 mg/L)	80% N_2, 20% CO_2	Fresh sow feces	48 hrs

Footnote: All plates were incubated at 37°C.

Table 2.3 Quality metrics of whole-genome sequences.

Bacterial species	Total raw reads	Genome size	GC content	N50	Number of contigs	L50	Predicted genes (unique)
E. coli	4,655,172	5,400,195	50.54%	160,731	139	10	5065
L. johnsonii	6,754,455	1,907,290	34.61%	426,87	89	14	1850
L. delbrueckii	4,706,212	2,170,295	49.82%	354,76	140	19	2122
B. vulgatus	4,165,373	10,740,101	42.90%	498,40	732	66	9407
C. colicanis	4,212,170	6,454,858	41.49%	101,160	417	22	5263
B. eggerthii	4,420,119	4,109,614	44.74%	171,837	68	9	3421
S. hyointestinalis	4,182,667	2,353,274	42.20%	12,833	376	59	2400
P. copri	3,682,662	3,537,572	44.54%	63,961	183	17	3174
B. xylaninosolvens	4,270,655	6,217,039	42.05%	101,160	142	22	4909
T. sanguinis	3,649,848	3,050,728	34.07%	78,628	86	12	2916
L. amylovorus	4,148,825	2,061,597	37.78%	25,962	144	19	2126
L. ruminis	4,146,880	2,116,416	43.43%	56,337	78	11	2087
B. faecicola	2,366,480	3,141,047	44.37%	151,264	60	6	2944
L. mucosae	4,109,536	2,245,560	46.17%	125,915	62	5	2086
B. thetaiotaomicron	3,820,625	5,914,417	42.89%	212,735	63	10	4533
S. pasteurianus	3,635,268	2,276,976	37.09%	48,948	127	16	2319
L. reuteri	3,919,331	2,137,679	38.51%	26,123	167	27	2337

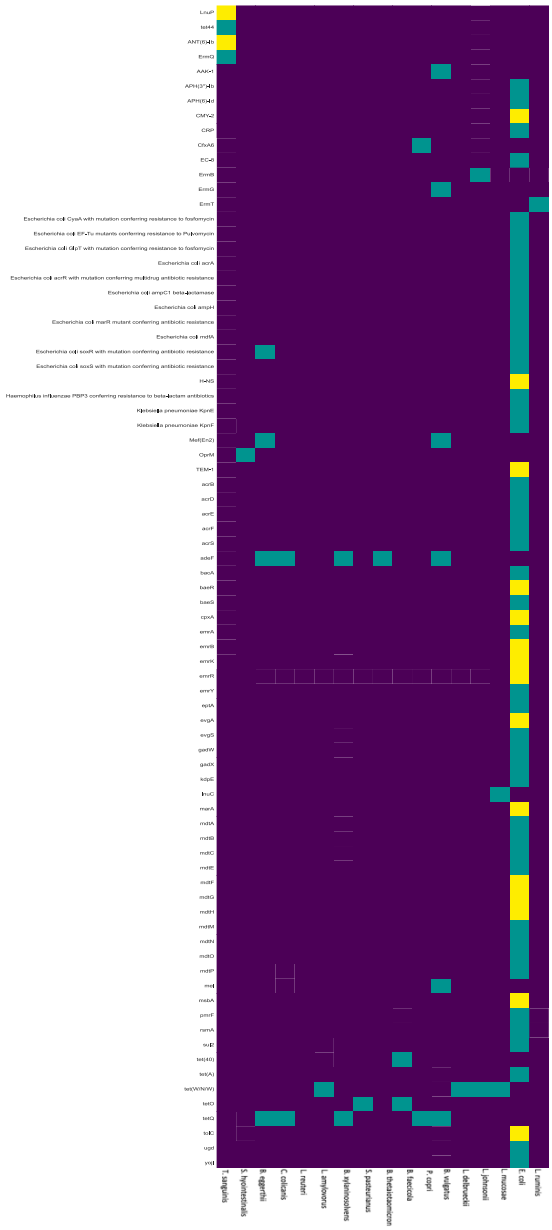


Figure 2.1 Heatmap of RGI antimicrobial resistance genes identified in the DC and *E. coli* isolates. Purple indicates no AMR gene identified, yellow is a perfect hit, and green is a strict hit. Perfect hits match to curated reference sequences and mutation in the CARD, while strict hits detect previously unknown variants of known AMR genes.

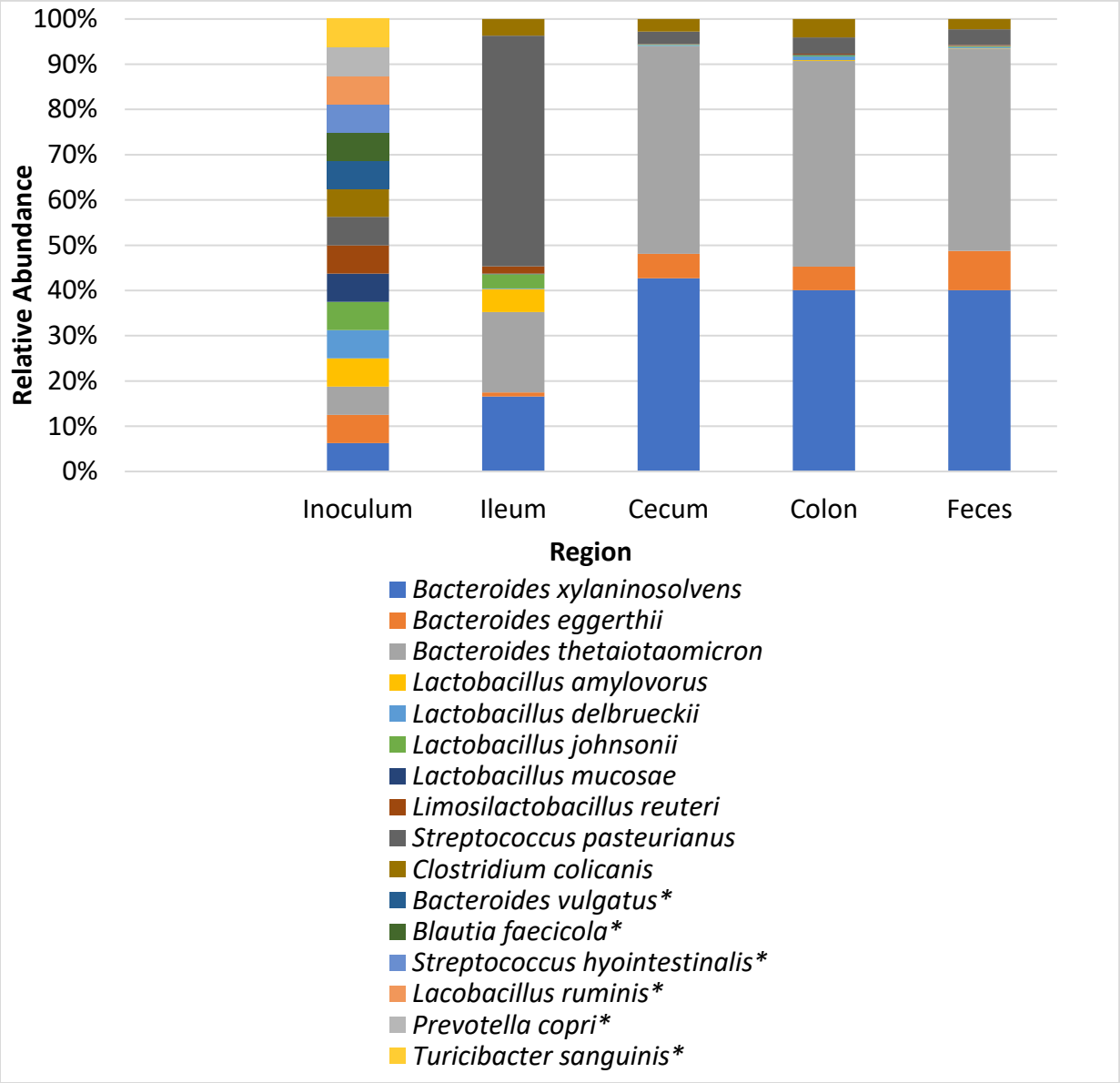


Figure 2.2 Relative abundance of bacteria summarized by region down to species level in male mice according to GI sections (ileum, cecum, colon) at 4 weeks post gavage and feces at week 1 post gavage (N = 8). The inoculum column shows the species included in the defined community. Species that did not colonize are indicated by an asterisk.

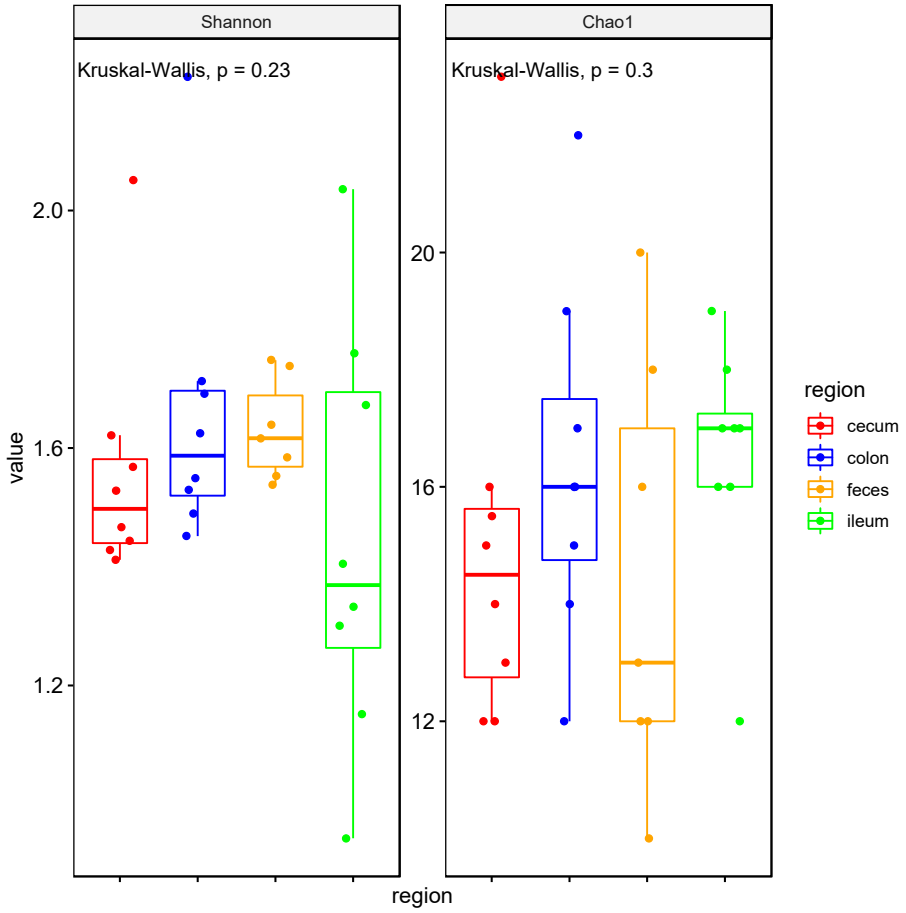


Figure 2.3 Alpha diversity measures from male mice oral gavaged with DC. Ileal, cecal, and colon content were obtained at termination 4 weeks after gavage. Dots represent individual mice and lines depict the mean values with whiskers indicating min to max values. Shannon diversity index (Kruskal Wallis $P = 0.23$, N = 8) and Chao1 index (Kruskal Wallis $P = 0.30$, N = 8). $\alpha = 0.05$.

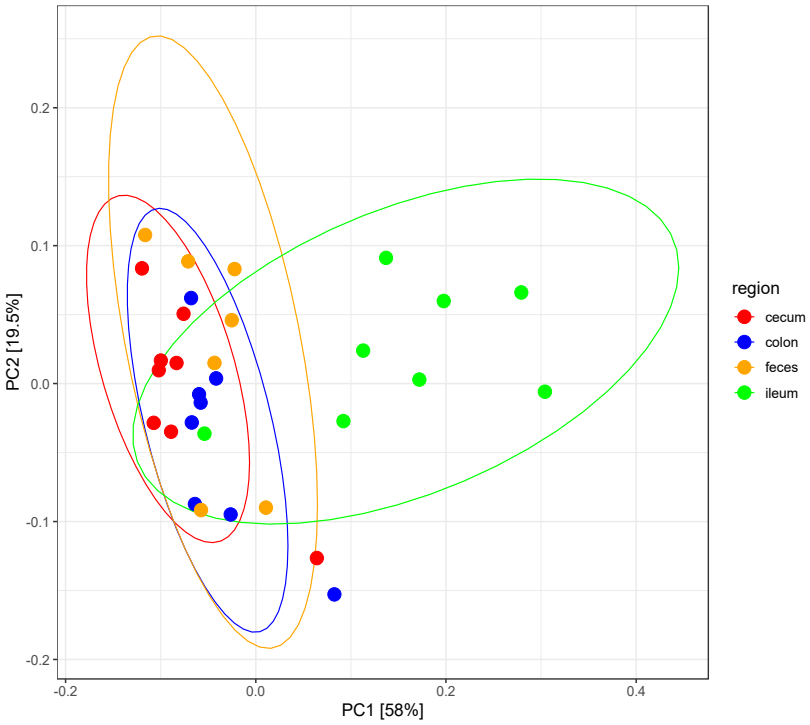


Figure 2.4 Microbial structural analysis of contents collected from different intestinal segments from male mice colonized with DC. Ileal, cecal, and colon content were obtained at termination 4 weeks after gavage while feces were collected 1 week post gavage. Dots represent individual mice. PCoA plots using Bray-Curtis dissimilarity matrix (Adonis $P = 0.001$, Betadispersion $P = 0.02$, $R^2 = 0.43$, N= 8)

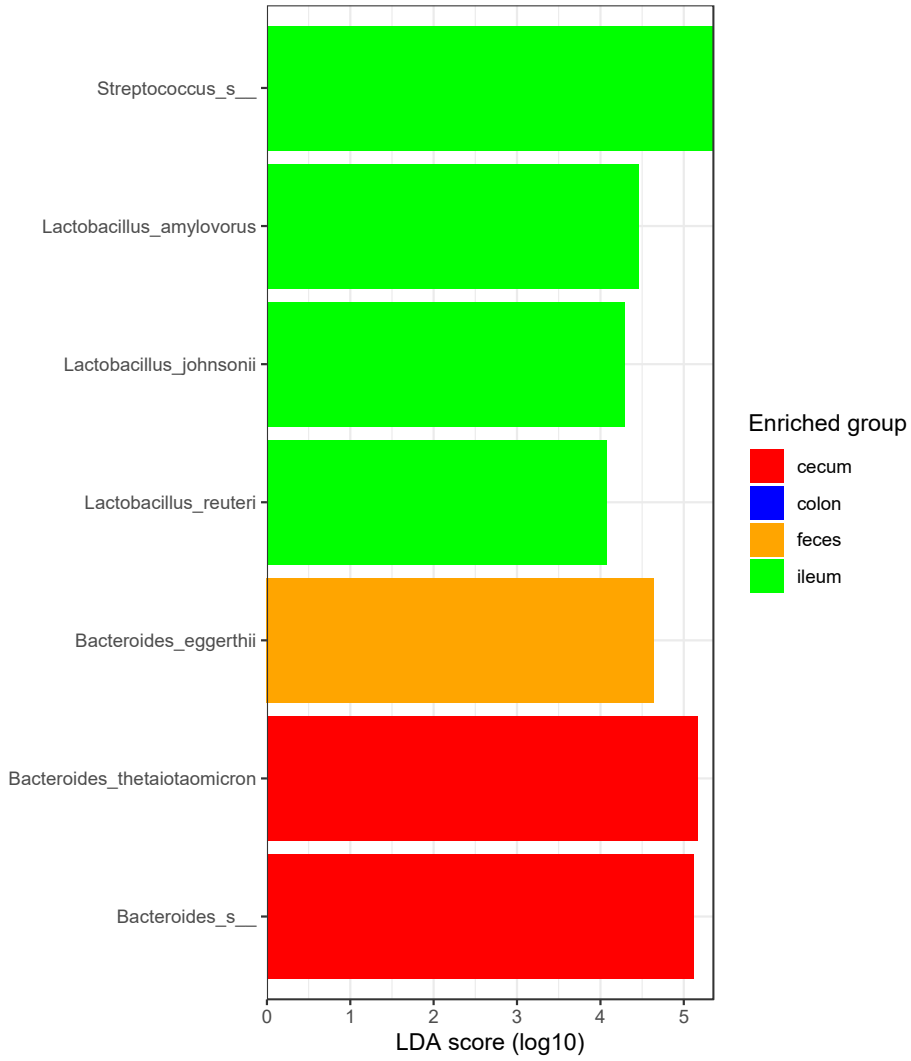


Figure 2.5 LEfSe showing differentially abundant bacterial taxa between the ileum, cecum, colon, and feces of male mice colonized with DC (N = 8). Taxonomic groups significantly enriched ($P < 0.05$) are color coded by GI region. Taxonomy "Streptococcus_s" = *Streptococcus pasteurianus* and "Bacteroides_s" = *Bacteroides xylaninosolvens*. $\alpha = 0.05$.

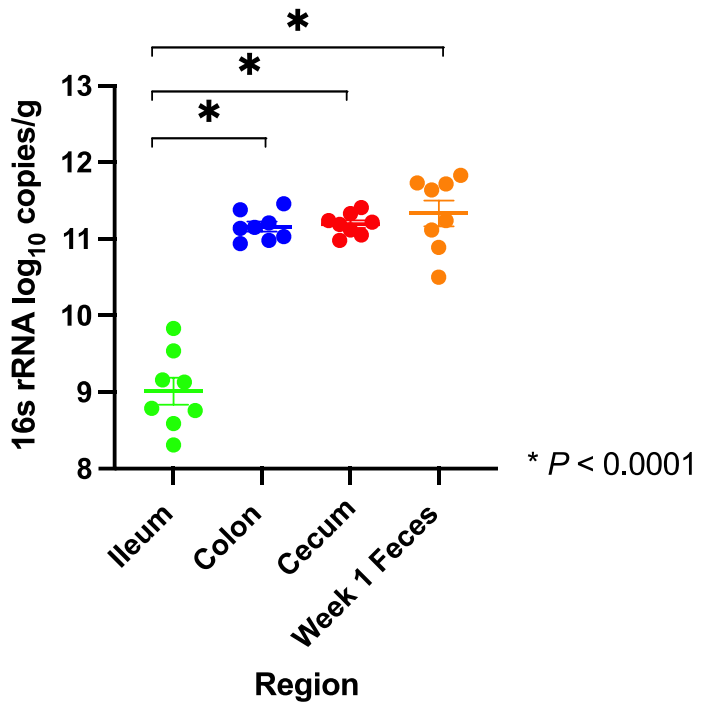


Figure 2.6 Total bacteria (log-transformed) is lower in the ileum than other regions in male mice oral gavaged with DC (Brown-Forsyth and Welch ANOVA with Dunnett's T3 multiple comparisons $P < 0.0001$, N = 8). Data are shown as mean with standard error of the mean (SEM) and dots represent individual mice. $\alpha = 0.05$.

2.4 Conclusion:

In summary, we created a consortium of bacteria isolated from the swine GI tract for the formation of a DC where the majority of species colonized the GF murine gut. Well-characterized genome-sequenced strains are the basis of the assembly of defined bacterial communities and as such WGS of the DC species allowed taxonomic characterization as well as virulence and antimicrobial resistance gene prediction. Further characterization of molecular functions of this consortium should be performed to determine which other species may be warranted in addition to recapitulate the functional capacity of conventionalized piglets. By constructing defined communities that are based on core microbiota that capture the functional capacity of conventionalized piglets, researchers will be able to employ gnotobiotic studies to dissect host-microbe and microbe-microbe relationships under representative conditions. The inoculation of this consortium in the GF mouse gut revealed that the majority of species can colonize in a spatially heterogeneric fashion along the small and large intestine, undoubtedly due to the vastly different environments in these regions. Overall, to our knowledge this study was the first of its kind to introduce and characterise a defined swine bacterial community to GF mice to be able to study pig-related diseases in a “porcinized” mouse model.

Chapter 3: Effects of Early Life *E. coli* and Amoxicillin Administration on Subsequent *Salmonella* Resistance

3.1 Introduction:

Antibiotic exposure in early life has been linked to several disease states later in life including autoimmune disorders, diabetes, obesity, and inflammatory bowel disease (Francino, 2016). Accordingly, researchers and medical professionals commonly discourage over-use of antibiotic treatments. However, there are studies that indicate that early life antibiotic treatment, specifically amoxicillin, may help reduce the severity of *Salmonella* infection later in life and have linked this response to certain bacteria (Costa et al., 2020; Fouhse et al., 2019). Fouhse and colleagues (2019) showed that 49-day old pigs who received amoxicillin during the first 2 weeks of life had more rapid nuclear factor kappa B (NF-kB) translocation in infiltrating leukocytes and increased expression of T helper 1 (Th1) cytokines such as interferon gamma (IFNγ), tumour necrosis factor alpha (TNF-α), and interleukin 2 (IL-2) upon a heat killed-intraperitoneal injection of *Salmonella*. The only appreciable change in the microbiota was a transient 10-fold expansion of *Escherichia coli* during amoxicillin administration that normalized by day 14 (Fouhse et al., 2019). A bloom of *E. coli* has also been observed after antibiotic administration in both pigs and mice (Antonopoulos et al., 2009; Looft et al., 2012). Given the knowledge that *E. coli* can prime the immune system and has been shown to be involved in decreasing *Salmonella* infection severity, further research is needed to characterise the causal role that *E. coli* and amoxicillin play in immune system programming and later life disease resistance (Brugiroux et al., 2016; Chassin et al., 2010; Mcaleer & Vella, 2008; Thiemann et al., 2017; Vatanen et al., 2016).

Lipopolysaccharide (LPS) has been implicated in educating the innate immune system from a young age upon colonization with Gram-negative bacteria like *E. coli* (Vatanen et al., 2016). Toll-like receptor 4 (TLR4) is a pattern recognition receptor that can detect and facilitate host transcriptional responses to LPS, a major component of the Gram-negative bacterial outer cell membrane (Kim et al., 2007). Immunostimulatory LPS plays a role in $CD4^+$ T cell priming by stimulating antigen presenting cells (APCs) causing the APC to facilitate T cell clonal expansion, differentiation, and survival (Mcaleer & Vella, 2008). Furthermore, exposure to LPS in early life has been implicated in conditioning gut epithelial cell responses to subsequent TLR stimulation (Chassin et al., 2010; Lotz et al., 2006). Proper education and activation of the immune system in relation to bacteria is not well understood, although it is well known that without commensal bacteria (germ-free animals) the immune system is not as developed as that of conventional animals and these microbially naïve animals are susceptible to various enteric infections (Round & Mazmanian, 2009). It has also been shown that specific gut microbial signatures are associated with reduced severity of infection with pathogens such as *Salmonella* (Ferreira et al., 2011; Sekirov et al., 2008). Interestingly, transferring these microbial communities, even specific bacterial species, can recapitulate protective phenotypes (Brugiroux et al., 2016; Thiemann et al., 2017). Protection has been linked to specific immune responses and colonization resistance provided by commensal bacteria, including specific strains of *E. coli* (Brugiroux et al., 2016; Hudault et al., 2001; Thiemann et al., 2017; Wotzka et al., 2019). While *E. coli* has been shown to be protective against *Salmonella* infection, it has yet to be explored how early life *E. coli* exposure impacts long-term *Salmonella* infection response.

Non-typhoidal *Salmonella* infections are an important cause of food-borne zoonoses worldwide and are associated with around a half a million human cases a year with 15% of cases

being fatal (Stanaway et al., 2019). Pigs are key hosts for *Salmonella* as they can be chronic carriers and shed the pathogen without clinical signs, however, they can also suffer from diarrhea, dehydration, and fibrinonecrotic enterocolitis (Cameron-Veas et al., 2018; Nollet et al., 2005). As such, it is important to create management strategies to control *Salmonella* loads in swine production. As evidenced by previous research, one way is to control *Salmonella* infection is to develop microbial strategies to shift the composition of microbial populations to recapitulate the previously characterized protective phenotype. To begin to untangle the intricate relationship between early life amoxicillin administration, *E. coli*, and subsequent S. Tm infection we employed a gnotobiotic mouse model colonized with a defined community (DC) of 16 swine bacterial species with or without *E. coli* and/or amoxicillin. The objectives of this study were to determine the colonization of the DC when *E. coli* is present and previously undetectable species are given fresh. Also, to investigate the effects of early life *E. coli* and amoxicillin administration on subsequent disease resistance when challenged with S. Tm later in life. We hypothesized that *E. coli* was required for protection against subsequent *Salmonella* challenge provided by early life amoxicillin exposure.

3.2 Materials and Methods:

3.2.1 *E. coli* Isolation and Amoxicillin Screening

E. coli isolates were cultured from frozen cecal content of a 7-day old male piglet treated with amoxicillin at 30mg/kg/day (Fouhse et al., 2019). Importantly, these *E. coli* isolates were associated with the amoxicillin induced bloom of *E. coli* seen at post-natal day (PND) 7 (Fouhse et al., 2019). Briefly, tenfold serial dilution was performed to 10^{-6} and 100 µL were plated aerobically on MacConkey (MAC) agar (BD, ref. 211387) and incubated aerobically at 37°C for 24 hrs. Sanger sequencing was performed on the 16S ribosomal ribonucleic acid (rRNA) gene

amplicon of each isolate, which was then compared to the 16S rRNA database using Standard Nucleotide BLAST as previously described in Chapter 2.

To select an *E. coli* isolate resistant to amoxicillin treatment, the 12 *E. coli* isolates were plated on amoxicillin (Sigma, ref. A8523-25G) supplemented agar. According to Marion and Siegwart (2015), the minimum inhibitory concentration for almost 70% of the 213 *E. coli* isolates was ≥ 64 μg/mL. Thus, amoxicillin was added to 1 litre of MAC agar at 30 mg/L, 60 mg/L, and 90 mg/L. Tenfold serial dilutions were performed to 10^{-6} in duplicate at each concentration. The plates were incubated at 37°C for 24 hrs aerobically.

Amoxicillin resistance of the *E. coli* isolate was then tested *in vivo*. Ten *E. coli*-free C57BL/6J (B6) female mice (Charles River Laboratories, Wilmington, Massachusetts, USA) were housed in sterile redline cages in the Axenic Mouse Unit at the University of Alberta. These cages contained aspen wood chip bedding materials mixed with aspen shavings; additionally, nesting materials, paper huts, and nestlets were provided as enrichment. Five mice were housed per cage in a room that was environmentally controlled for light cycle (12 hrs light and 12 hrs darkness), temperature (20–22°C), and relative humidity (40%). Mice were fed autoclaved Autoclavable Rodent Laboratory Chow 5010 from Purina Mills and autoclaved *ad libitum* water.

E. coli for the inoculum was plated on MAC agar and incubated aerobically overnight at 37°C. A single colony was then inoculated in 5 mL of Luria-Bertani (LB) broth (BD, ref. 244620) and incubated aerobically overnight at 37°C. All mice received an oral gavage with 100 μL of *E. coli* at 5 x 10^8 colony forming units (CFU). One cage (n = 5) was treated with amoxicillin (0.1667 mg/mL) in sterile drinking water at 25 mg/kg/day one day post gavage for 4 days, while the control group (n = 5) received sterile drinking water for the remainder of the experiment. Fecal pellets were collected from all mice 24 hrs after *E. coli* gavage (prior to amoxicillin administration) then

on days 2 and 4 of amoxicillin administration. Subsequently, fecal pellets suspended in 1 mL of 1 x phosphate-buffered saline (PBS) were weighed and then homogenized by shaking in a tridimensional motion at 6.0 meters per second for 1 min (FastPrep instrument, MP Biomedicals, Solon, OH, USA). Then 100 µL of each sample at 10^0, 10^{-5}, and. 10^{-6} dilutions were plated on MAC agar in duplicate. The plates were incubated at 37°C for 24 hrs aerobically. After 24 hrs, colonies were enumerated and normalized to weight of the sample. Animal use was approved by the University of Alberta Animal Care and Use Committee (AUP00000671).

3.2.2 Defined Community Inoculum, *E. coli* Inoculum, and *S.* Tm Inoculum

Each species in the DC inoculum were initially streaked onto agar plates of the respective media and incubated for 48-72 hrs at 37°C in a BACTRON300 Anaerobic Workstation (Sheldon Manufacturing Incorporated, Cornelius, Oregon, USA; Table 3.1). A single colony from each isolate was subsequently inoculated into 5 mL of the respective broth and incubated for 48-72 hrs at 37°C in 70% N_2, 20% CO_2, 10% H_2 anaerobic gas conditions (Table 3.1). Subsequently, 500 µL of each isolate that was indicated as given frozen was added to a 15 mL falcon tube with 25% glycerol and stored at -80°C (Table 3.1). The species given fresh were grown fresh for each inoculation. Upon inoculation, 500 µL of each of the fresh cultures was added anaerobically to the 15 mL falcon tube and homogenized. *E. coli* prepared as described above. For oral gavage, mice were gavaged with 5 x 10^8 CFU/mL of *E. coli*. *Salmonella enterica* serovar Typhimurium SL1344 stock was plated on Xylose Lysine Deoxycholate (XLD) agar (Oxoid, ref. CM0469) and incubated aerobically at overnight at 37°C. A single colony was then inoculated in 5 mL of LB broth and incubated at 37°C for 16 hrs. For infection, mice were oral gavaged with 7-8 × 10^7 CFU/mL of *S.* Tm SL1344. To confirm bacterial identity, Sanger sequencing was performed on

the 16S rRNA gene, of each species, which was then compared to the 16S rRNA database using Standard Nucleotide BLAST as previously described in Chapter 2.

Table 3.1 Summary of bacterial isolates in the defined community.

Organism	Media	24 hr pre-reduction of agar plate in anaerobic chamber	Incubation time	Fresh or frozen in inoculum
Bacteroides eggerthii	FAA	No	48 hrs	Frozen
Bacteroides thetaiotaomicron	FAA	No	48 hrs	Frozen
Bacteroides vulgatus	FAA	No	48 hrs	Frozen
Bacteroides xylaninosolvens	FAA	No	48 hrs	Frozen
Blautia faecicola	FAA	Yes	72 hrs	Fresh
Clostridium colicanis	RCM	No	48 hrs	Frozen
Lactobacillus amylovorus	MRS	No	48 hrs	Frozen
Lactobacillus delbrueckii	MRS	No	48 hrs	Frozen
Lactobacillus johnsonii	MRS	No	48 hrs	Frozen
Lactobacillus mucosae	MRS	No	48 hrs	Frozen
Limosilactobacillus reuteri	MRS	No	48 hrs	Frozen
Lactobacillus ruminis	MRS	No	48 hrs	Fresh
Prevotella copri	Schaedler	Yes	72 hrs	Fresh
Streptococcus hyointestinalis	FAA	No	48 hrs	Fresh
Streptococcus pasteurianus	FAA	No	48 hrs	Frozen
Turicibacter sanguinis	FAA	Yes	72 hrs	Fresh

3.2.3 *Salmonella* Experiment

C57BL/6J (B6) germ-free mice (Charles River Laboratories, Wilmington, Massachusetts, USA) were housed in flexible film isolators (Controlled Environment Products, McHenry, Illinois, USA) maintained in the Axenic Mouse Unit at the University of Alberta. All mice were housed in sterile cages with aspen wood chip bedding materials mixed with aspen shavings. Nesting

materials, paper huts, and nestlets were provided as enrichment. The room was environmentally controlled as previously described above. Mice were fed autoclaved Autoclavable Rodent Laboratory Chow 5010 from Purina Mills and autoclaved *ad libitum* water. During breeding, 1 week after the second oral gavage, 3 females were housed with 1 male per cage. Once pregnancy was confirmed, females were singly housed until parturition. Pups were weaned and ear notched 3 weeks after birth where they were then housed by sex and treatment group until the end of the experiment.

Breeder females were oral gavaged 100 µL of DC inoculum (N = 21) then 2 days later this gavage was repeated with (n = 11) or without (n = 10) *E. coli*. Fecal pellets were obtained 1 week after the second gavage for *E. coli* plating and 16S rRNA sequencing. Females were bred and 4 litters were produced (N = 29 pups). Each dam and her litter of pups was assigned to 1 of the 4 treatment groups: Amoxicillin (amox, n = 8 pups), *E. coli* (ecoli, n = 8 pups), *E. coli* + Amoxicillin (EA, n = 5 pups), or control (n = 8 pups). On PND 0, females in the amox and EA groups were administered sterile amoxicillin (0.1667 mg/mL: Sigma, ref. A8523-25G) in sterile drinking water at 25 mg/kg/day for 2 weeks and refreshed twice weekly (Marx et al. 2014). Fecal pellets were collected from all pups at 3 weeks of age for 16S rRNA gene amplicon sequencing. To normalize the microbiota after weaning, fecal pellets from all dams were collected, homogenized in 5 mL of sterile water, and 50 µL was syringe fed to each pup. The remaining volume was equally distributed in each cage. At 5 weeks (2 weeks post weaning), fecal pellets were collected for *E. coli* plating, to confirm successful *E. coli* colonization in all mice upon normalization, and for 16S rRNA gene amplicon sequencing. Mice were challenged at 6 weeks of age by oral gavaging 100 µL of S. Tm SL1344. Two days (48 hrs) post challenge, all infected mice were euthanized by CO_2 asphyxiation except for 2 male mice from the control group which had died during the night prior.

Content from the distal ileum, cecum, and the liver was collected from each mouse for plating to determine S. Tm load. Colon content was also collected and stored at -80°C for 16S rRNA gene amplicon sequencing. Cardiac blood was collected via cardiac puncture into ethylenediaminetetraacetic acid (EDTA) tubes and plasma was obtained by centrifuging at 3000 revolutions per minute at 4°C for 10 min for cytokine analysis.

3.2.4 Plating and Enumeration *E. coli*

Fecal samples were weighed and homogenized by shaking in a tridimensional motion at 6.0 meters per second for 1 min (FastPrep instrument, MP Biomedicals, Solon, OH, USA). Tenfold serial dilutions were carried out to 10^{-6}. The neat, 10^{-4}, 10^{-5}, and 10^{-6} were plated on MAC agar in duplicate and aerobically incubated for 24 hrs at 37°C. Colonies were counted and log CFU/g was calculated.

3.2.5 Plating and Enumeration S. Tm

Samples were weighed and ileal and cecal content was homogenized by shaking in a tridimensional motion at 4.0 meters per second for 5 seconds whereas the liver was homogenized in the same fashion for 40 seconds. Tenfold serial dilutions were carried out to 10^{-4} (liver), 10^{-6} (ileum), and 10^{-7} (cecum). Specific dilutions were plated in duplicate from each sample type on XLD agar supplemented with streptomycin (100 µg/mL; Fisher Scientific, ref. BP910-50) and incubated aerobically overnight at 37°C. Colonies were counted and log CFU/g was calculated.

3.2.6 DNA Extraction, 16S rRNA Gene Amplicon Sequencing, and 16S rRNA Gene Amplicon Sequencing Analysis

DNA was extracted from fecal pellets from breeder females, and fecal pellets/cecal content from pups. The DNA extraction, amplicon library construction, paired-end sequencing,

and data analysis were performed using protocols and pipelines previously described in Chapter 2.

3.2.7 Meso Scale Discovery (MSD) Proinflammatory Mouse Cytokine Assay

Blood was collected via cardiac puncture 48 hrs post infection and a MSD multiplex kit (MSD v-plex multi-spot Proinflammatory Panel 1 (mouse) Kit, Meso Scale Discovery, Rockville, MD, USA) was used to measure plasma IFNγ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GRO as per the manufacturer's instructions with an overnight incubation at 4°C shaking at 500 RPM for step 1. Sample dilutions were performed in duplicates for 4-fold dilutions and single samples for 2-fold dilutions.

3.2.8 Statistical Analysis

Data was tested for normality using either Shapiro-Wilk test or the Kolmogorov-Smirnov test depending on sample size, with the latter used for groups larger than 30 samples. To compare the enumeration of *E. coli* (log-transformed) at baseline and day 4 for the amoxicillin pilot, a two-tailed Mann-Whitney U-test and a two-tailed unpaired T-test were performed, respectively. *E. coli* enumeration on log transformed data from pups at 5 weeks of age was analysed using a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons for the four treatment groups, while the sex difference was analysed using a two-tailed unpaired T-test. These statistical measures were all run in GraphPad Prism (v9.3.1). To compare S. Tm loads (log-transformed) after *Salmonella* challenge in the ileum, cecum, and liver of the four treatment groups, a mixed effect model with a Tukey *post-hoc* test was run in SAS (v9.04.01, SAS Inst. Inc. Cary, NC). To determine which treatments groups had a significant interaction between treatment and sex, a Šídák's multiple comparisons test was performed and significance was set at $\alpha = 0.05$. Results from analysis in SAS were visualised in GraphPad Prism (v9.3.1). Microbial

diversity indices (Shannon and Chao1) for each sample were calculated using the vegan package in R (R v4.1.2). To assess bacterial community compositional differences, Principal Coordinate Analysis (PCoA) of sequence data was carried out through the phyloseq package in R v4.1.2 using Bray-Curtis dissimilarity. Permutational multivariate analysis of variance (PERMANOVA) and Permutational Analyses of Multivariate Dispersions (PERMDISP) were performed on each beta diversity metric using the adonis and betadisper functions in R (999 permutations). Pairwise comparisons using permutational multivariate analysis of variance (9999 permutations) were subsequently run to determine which groups significantly differed (adonis function, vegan package, R v4.1.2). Significance was set at $P < 0.05$. To compare the 10 cytokines in plasma, 48 hrs after S. Tm challenge, two-way ANOVAs were performed and visualized in GraphPad Prism (v9.3.1).

3.3 Results:

3.3.1 *E. coli* Isolation, *Ex Vivo*, and *E. coli* and Amoxicillin Pilot Mouse Study

All 12 *E. coli* isolates were resistant to the 3 concentrations of amoxicillin and at the highest concentration all grew to 10^{10} CFU/mL. Thus, one isolate was chosen for whole-genome sequencing (Chapter 2) and was used for all the following experiments using *E. coli*. In the *in vivo* pilot trial, one day post *E. coli* gavage fecal *E. coli* loads did not differ ($P > 0.99$, Fig. 3.1). After 4 days of amoxicillin treatment, the mean *E. coli* load in the amox group was more than a log higher than the control group ($P = 0.0052$, Fig. 3.1).

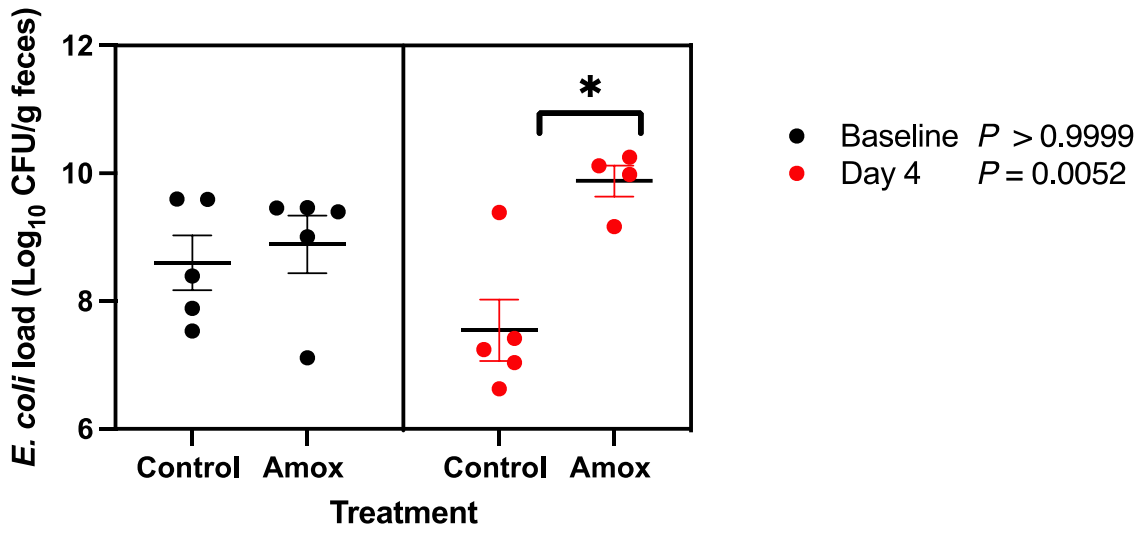
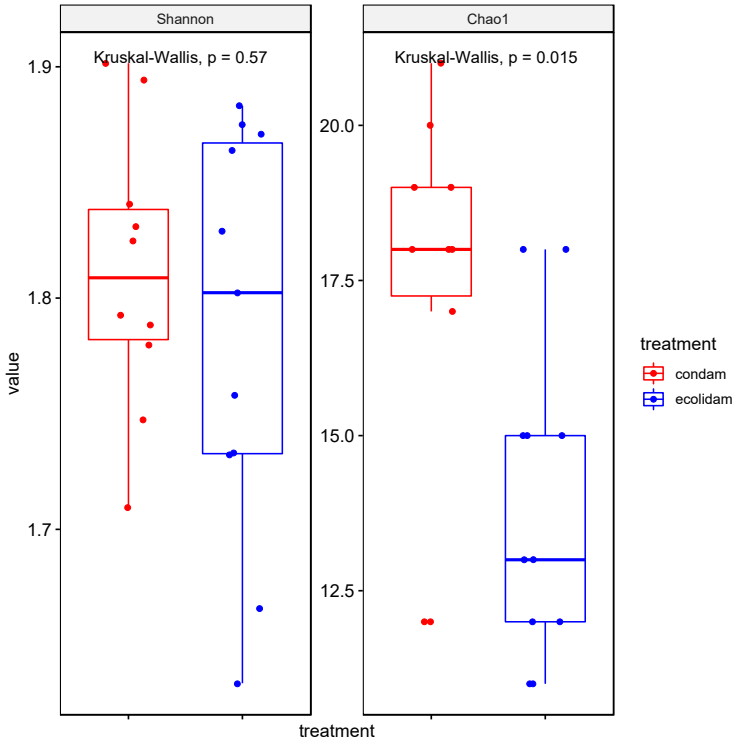


Figure 3.1 Enumeration of *E. coli* in female mouse feces before amoxicillin treatment (baseline = 1 day post *E. coli* colonization) and 4 days after amoxicillin (amox) administration at 25mg/kg/day or water (control). Two-tailed Mann-Whitney U-test on log-transformed baseline data ($P > 0.99$; n = 5/group). Two-tailed unpaired T-test on log-transformed day 4 data ($P = 0.0052$; n = 4-5/group). Dots represent individual mice and lines depict the mean values and error bars indicate standard error of the mean (SEM). $\alpha = 0.05$.

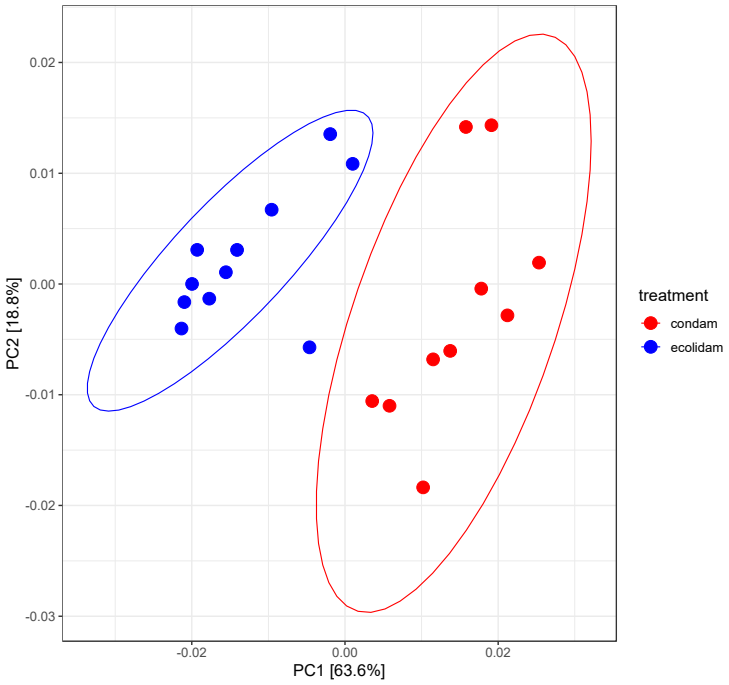
3.3.2 *Salmonella* Trial: Breeder Females *E. coli* vs. No *E. coli*

One week after the second oral gavage of the DC with (ecolidam) or without *E. coli* (condam) the number of species in the feces differed (Chao1 $P = 0.015$), while the Shannon diversity did not (Shannon $P = 0.57$, Fig. 3.2A). The Chao1 index was significantly higher in the condam group, indicating a lower number of species when *E. coli* was not present (Fig. 3.2). To determine if the diversity between the two groups differed, PCoA based on the Bray-Curtis dissimilarity metric revealed distinct clustering (Fig. 3.2B). The permutational multivariate analysis of variance (adonis) exhibited the separation of the condam and ecolidam groups ($P =$

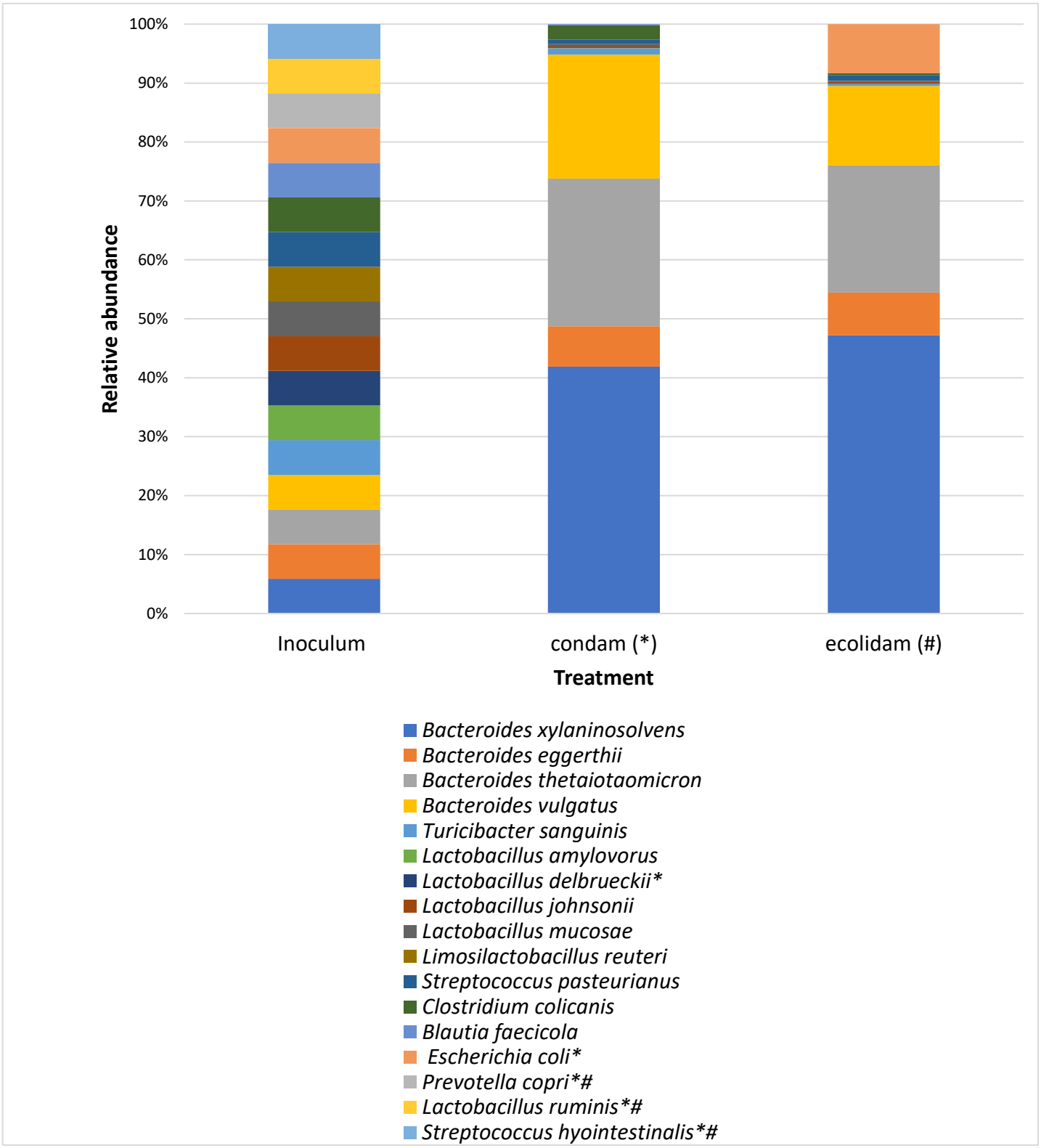
0.001, $R^2 = 0.52$, Fig. 3.2B). The DC consisted of 16 species, 13 of which were present in both groups. The species that were not present in the sequencing data at this time point in either of the groups were *P. copri*, *L. ruminis*, and *S. hyointestinalis* (Fig. 3.2C). The condams lacked 1 species, *L. delbrueckii*, which was present in the ecolidam group (Fig. 3.2C). *Bacteroides* accounted for on average 94.8% of the condam microbial community and 89.5% in the ecolidam group (Fig. 3.2C). On average, *E. coli* accounted for 8.3% of the microbial community in the ecolidam group (Fig. 3.2C). To determine the bacterial species driving the differences between groups Linear discriminant analysis Effect Size (LEfSe) analysis revealed that *C. colicanis* was significantly enriched (2.5% vs. 0.3%, $P < 0.05$, Fig. 3.2D) in the condam group while, as expected, *E. coli* was enriched in the ecolidam group ($P < 0.05$, Fig. 3.2D).


Shannon
Chao1
Kruskal-Wallis, p = 0.57
Kruskal-Wallis, p = 0.015
value
1.9
1.8
1.7
20.0
17.5
15.0
12.5
treatment
treatment
condam
ecolidam

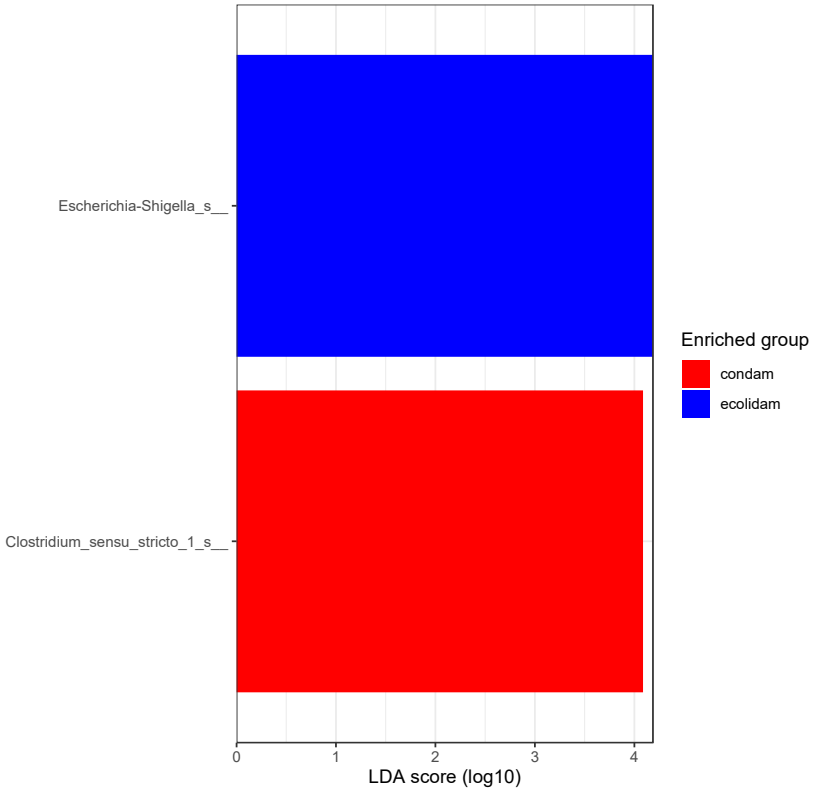
A)


PC2 [18.8%]
PC1 [63.6%]
0.02
0.01
0.00
-0.01
-0.02
-0.03
-0.02
0.00
0.02
treatment
condam
ecolidam

B)


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance
Inoculum
condam (*)
ecolidam (#)
Treatment
Bacteroides xylaninosolvens
Bacteroides eggerthii
Bacteroides thetaiotaomicron
Bacteroides vulgatus
Turicibacter sanguinis
Lactobacillus amylovorus
Lactobacillus delbrueckii*
Lactobacillus johnsonii
Lactobacillus mucosae
Limosilactobacillus reuteri
Streptococcus pasteurianus
Clostridium colicanis
Blautia faecicola
Escherichia coli*
Prevotella copri*#
Lactobacillus ruminis*#
Streptococcus hyointestinalis*#

C)



D)

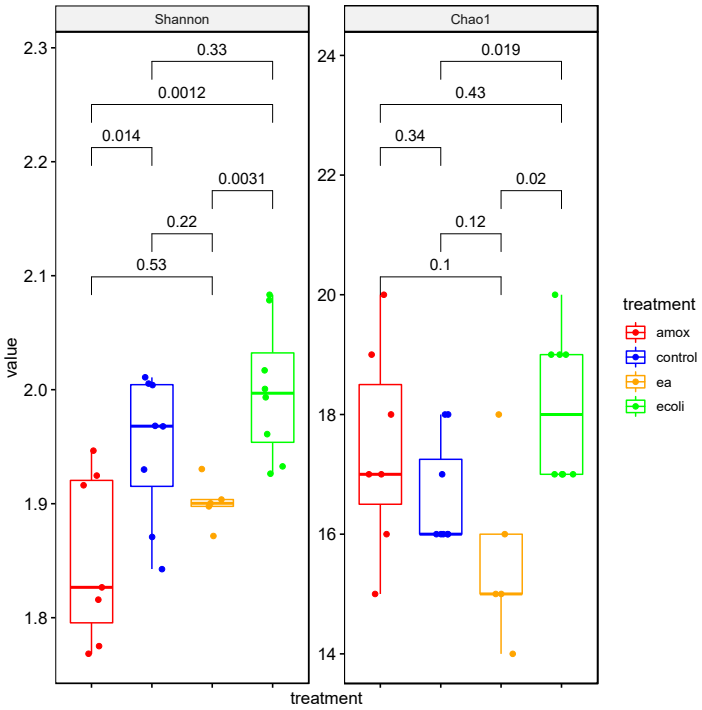
Figure 3.2 Microbial structural analysis of breeder female mice feces 1 week post DC oral gavage with (ecolidam) or without *E. coli* (condam). **A)** Alpha diversity analysis of bacterial communities. Shannon diversity index (Kruskal Wallis $P = 0.57$; n = 10-11/group) and Chao1index (Kruskal Wallis $P = 0.015$; n = 10-11/group). Dots represent individual mice and lines depict the mean values with whiskers indicating min to max values. **B)** PCoA plot of bacterial communities based on Bray-Curtis dissimilarity matrix (Adonis $P = 0.001$; Betadispersion $P = 0.94$; $R^2 = 0.52$; n = 10-11/group). Dots represent individual mice. **C)** Relative abundance of bacteria summarized by treatment group down to species level (n = 10-11/group). The inoculum column shows the species included in the DC – *E. coli* not administered to the condam females. Species that did not colonize are indicated by an asterisk for

the condam group and a pound sign for the ecolidam group. **D)** LEfSe showing differentially abundant bacterial taxa between the no *E. coli* (condam) and *E. coli* positive (ecolidam) females (n = 10-11/group). Taxonomic groups significantly enriched ($P < 0.05$) are color coded by treatment group. "Escherichia_shigella_s" = *E. coli* and "Clostridium_sensu_stricto_1_s" = *Clostridium colicanis*. $\alpha = 0.05$.

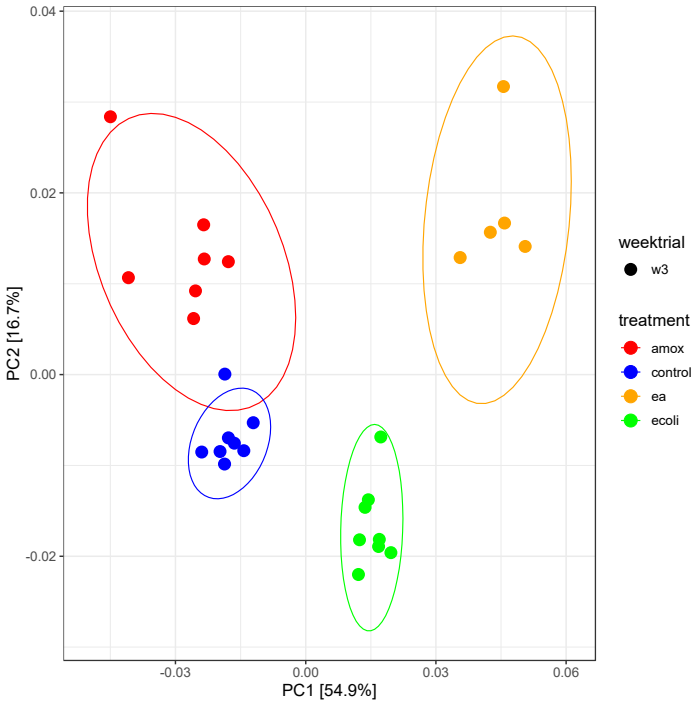
3.3.3 Community Composition and Fecal Microbiota Structural Changes in 3-week-old Pups

To evaluate phylogenetic richness and evenness of the 3-week-old pup feces in each of the four treatment groups, one week after amoxicillin withdrawal, Chao1 diversity index and Shannon index were calculated in each sample. Significant differences were observed in Chao1 ($P = 0.027$) and Shannon indexes ($P = 0.0036$) for treatment, however, there was no difference in alpha diversity between males and females (Fig. 3.3A). The Chao1 index of the control and EA groups were significantly lower than that of the ecoli group, indicating higher species richness ($P < 0.05$, Fig. 3.3A). When both richness and evenness was considered, the amox group had a significantly lower Shannon index than the ecoli group, the control group had a significantly lower Shannon index than the control group, and the EA group was significantly lower than the ecoli group indicating a higher alpha diversity ($P < 0.05$, Fig. 3.3A). To assess the microbial community structure, PCoA based on the β-diversity metric of Bray-Curtis dissimilarity was performed and showed distinct separation between groups (Adonis $P = 0.001$, Fig. 3.3B). The PERMANOVA of the Bray-Curtis dissimilarity metric was used to determine the difference in overall bacterial composition across treatments and revealed that each group was significantly different from one another at 3 weeks PND (Adonis $P < 0.01$). This analysis showed that

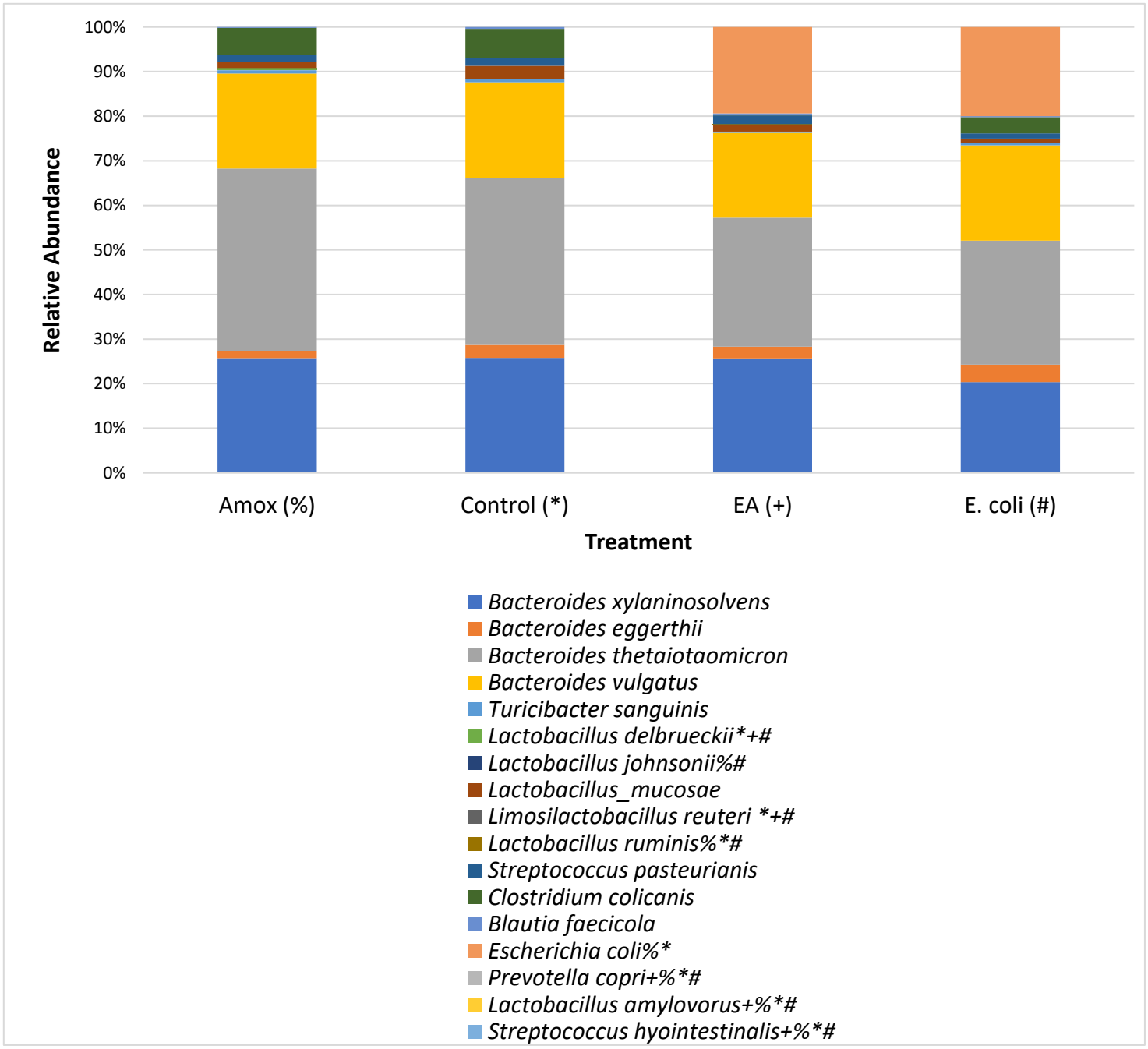
treatment could explain 74.4% of the overall variance (Adonis $P = 0.001$, Fig. 3.3B) and there was no difference between sexes. The species present in each group differed at 3 weeks, mainly between the *Lactobacillus* species. The amox group lacked detection of *L. johnsonii* and *L. ruminis*, while in the EA group *L. delbrueckii* and *L. reuteri* were not detected (Fig. 3.3C). *L. delbreuckii, L. reuteri*, and *L. ruminis* were not detected in the control group (Fig. 3.3C). The ecoli group lacked *L. delbreuckii*, *L. johnsonii*, *L. reuteri*, and *L. ruminis* (Fig. 3.3C). *L. amylovorus*, *P. copri*, and *S. hyointestinalis* were not detected in any of the groups and the control and amox group remained *E. coli*-free as intended (Fig. 3.3C). The LEfSe analysis revealed the microbial species that most likely explained the differences between groups. Species that were significantly enriched in the control group were *B. xylaninosolvens* and *L. mucosae*, *B. thetaiotaomicron* and *C. colicanis* were enriched in the amox group, while *B. eggerthii* and *E. coli* were enriched in the ecoli group ($P < 0.05$, Fig 3D). However, the mean relative abundance of *E. coli* in the ecoli group was 20.0% and 19.4% in the EA group (Fig. 3.3C).


Shannon
Chao1
value
treatment
0.33
0.0012
0.014
0.0031
0.22
0.53
0.019
0.43
0.34
0.02
0.12
0.1
treatment
amox
control
ea
ecoli

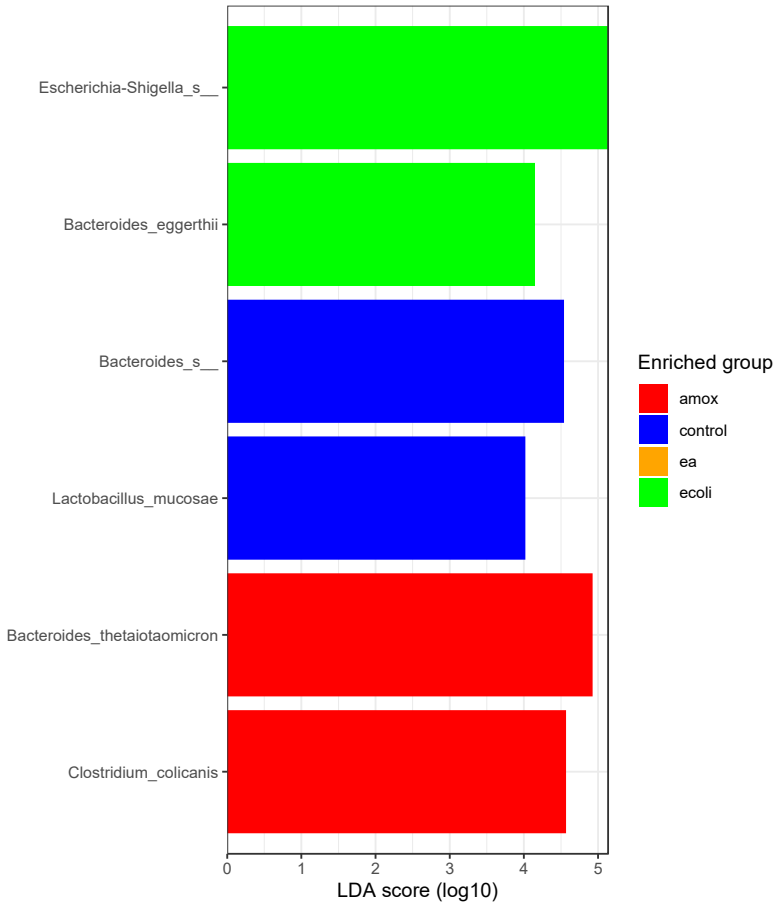
A)


PC2 [16.7%]
PC1 [54.9%]
weektrial
w3
treatment
amox
control
ea
ecoli

B)


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Abundance
Amox (%)
Control (*)
EA (+)
E. coli (#)
Treatment
Bacteroides xylaninosolvens
Bacteroides eggerthii
Bacteroides thetaiotaomicron
Bacteroides vulgatus
Turicibacter sanguinis
Lactobacillus delbrueckii*+#
Lactobacillus johnsonii%#
Lactobacillus_mucosae
Limosilactobacillus reuteri *+#
Lactobacillus ruminis%*#
Streptococcus pasteurianis
Clostridium colicanis
Blautia faecicola
Escherichia coli%*
Prevotella copri+%*#
Lactobacillus amylovorus+%*#
Streptococcus hyointestinalis+%*#

C)



D)

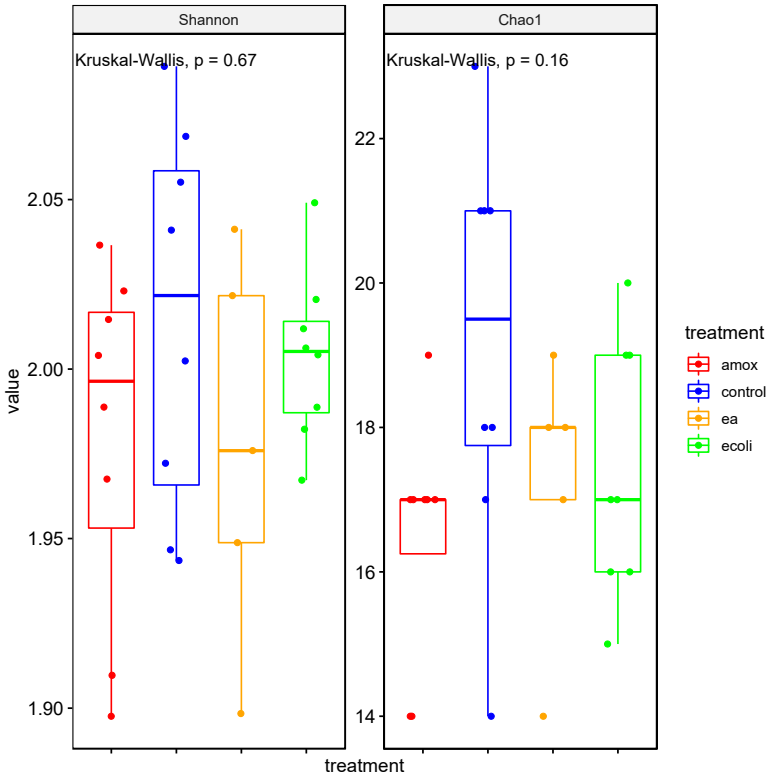
Figure 3.3 Microbial structural analysis of 3-week pup feces from the four treatment groups (N = 29). Amox and EA groups received amoxicillin through their dam's milk for the first 2 weeks of life. These dams were administered sterile amoxicillin drinking water at 25 mg/kg/day. EA and *E. coli* groups were born to dams administered *E. coli* with the DC. The dam of the control group did not receive amoxicillin or *E. coli*, just the DC and sterile water. All treatment groups n = 8 except for EA (n = 5). **A)** Alpha diversity analysis of bacterial communities. Shannon diversity index (Kruskal Wallis treatment $P = 0.0036$, sex $P = 0.39$; n = 5-8/group, n = 16 females/13 males) and Chao1 metric (Kruskal Wallis treatment $P = 0.027$; n = 5-8/group). Dots represent individual mice and lines depict the mean values with whiskers indicating min to max

values. **B)** PCoA plot of bacterial communities based on Bray-Curtis dissimilarity matrix (Adonis treatment $P = 0.001$, Betadispersion treatment $P = 0.009$; Adonis sex $P = 0.62$, Betadispersion sex $P = 0.94$; $R^2 = 0.74$; n = 5-8/group; n = 16 females/13 males). Dots represent individual mice. **C)** Relative abundance of bacteria summarized by treatment group down to species level (n = 5-8/group). Species that are absent in each group are indicated by + (EA), % (amox), * (control), and # (E. coli) **D)** LEfSe showing differentially abundant bacterial taxa between the four treatment groups (n = 5-8/group). Taxonomic groups significantly enriched ($P < 0.05$) are color coded by treatment group. Taxonomy "Escherichia-shigella_s" = *E. coli*, and "Bacteroides_s" = *Bacteroides xylaninosolvens*. $\alpha = 0.05$.

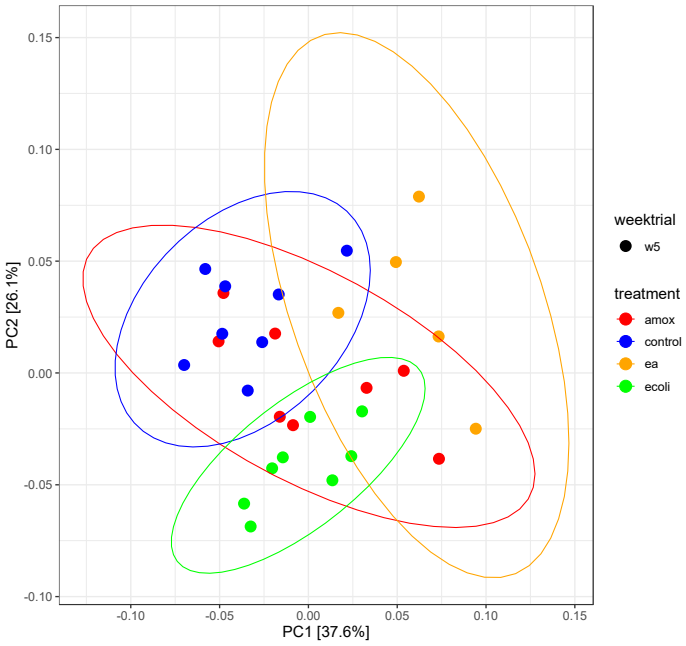
3.3.4 Community Composition and Fecal Microbiota Structural Changes in 5-week-old Pups

At 5 weeks of age, the phylogenetic richness and evenness of the bacterial communities for the four treatments were evaluated by Chao1 diversity index and Shannon index. There was no significant difference in either metric for treatment or sex (Fig. 3.4A). β-diversity based on Bray-Curtis dissimilarity was significantly different and showed separation on the PCoA plot (Adonis $P = 0.001$, Fig. 3.4B). Pairwise comparison MANOVA of the four treatments revealed that even after "normalization" of the microbiota, the overall composition differed between all groups (Adonis $P < 0.05$, Fig. 3.4B). This analysis showed that treatment could explain 41.2% of the overall variance (Fig. 3.4B). Just as in the 3-week-old data, β-diversity did not differ between sexes. The species that were not detected at 3 weeks (*L. amylovorus*, *P. copri*, and *S. hyointestinalis*) remained undetected at 5 weeks (Fig. 3.4C). The *Lactobacillus* species absent in the groups at this time point were different from that of 3 weeks. In all of the groups, *L.*

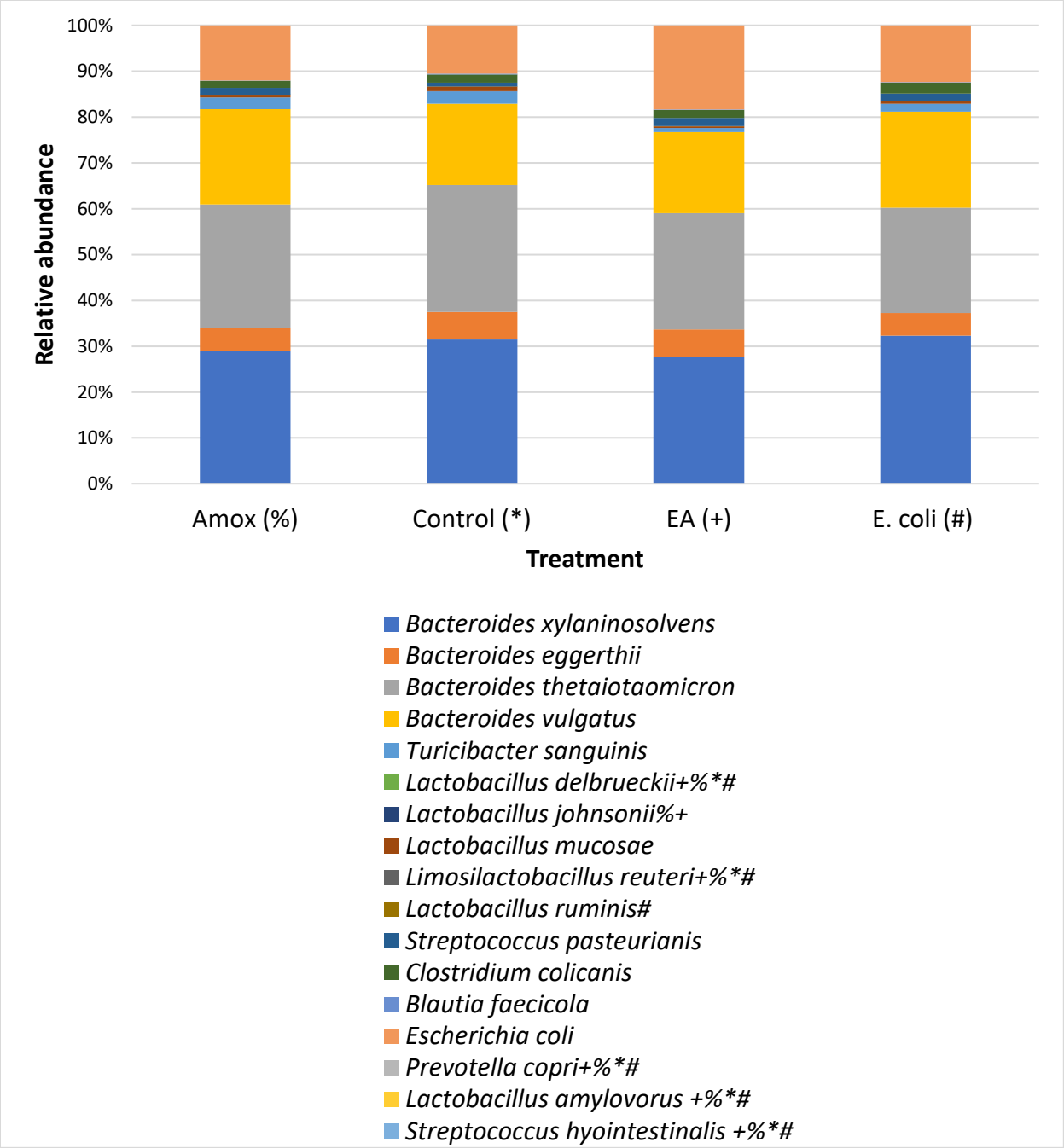
delbreuckii was not present (Fig. 3.4C). The amox group also did not have detectible *L. johnsonii* and *L. reuteri*; the control group did not show any *L. reuteri*; the EA group lacked the same species as the amox group; and *L. reuteri* and *L. ruminis* were not present in the ecoli group (Fig. 3.4C). The amox and control groups were successfully colonized with *E. coli* (Fig. 3.4C and 4D). The species that were identified as enriched by LEfSe analysis were *B. xylaninosolvens* and *B. vulgatus* in the ecoli group; *B. thetaioataomicron* and *T. sanguinis* in the control group; and *E. coli* in the EA group ($P < 0.05$, Fig. 3.4E). However, to determine the live *E. coli* load in the feces of each group, *E. coli* was enumerated from MAC agar plates (Fig. 3.4D). The *E. coli* load was not different between groups (Fig. 3.4D). The *E. coli* load between sexes was also not different (Fig. 3.4D).


Shannon
Chao1
Kruskal-Wallis, p = 0.67
Kruskal-Wallis, p = 0.16
value
treatment
2.05
2.00
1.95
1.90
22
20
18
16
14
treatment
amox
control
ea
ecoli

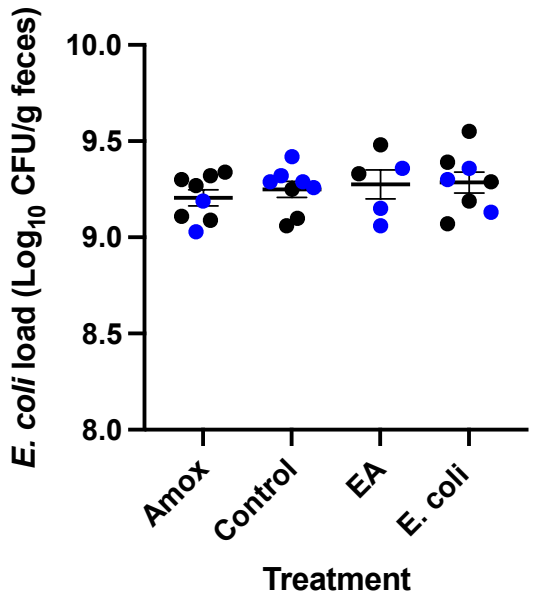
A)


PC2 [26.1%]
PC1 [37.6%]
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.10
-0.05
0.00
0.05
0.10
0.15
weektrial
w5
treatment
amox
control
ea
ecoli

B)

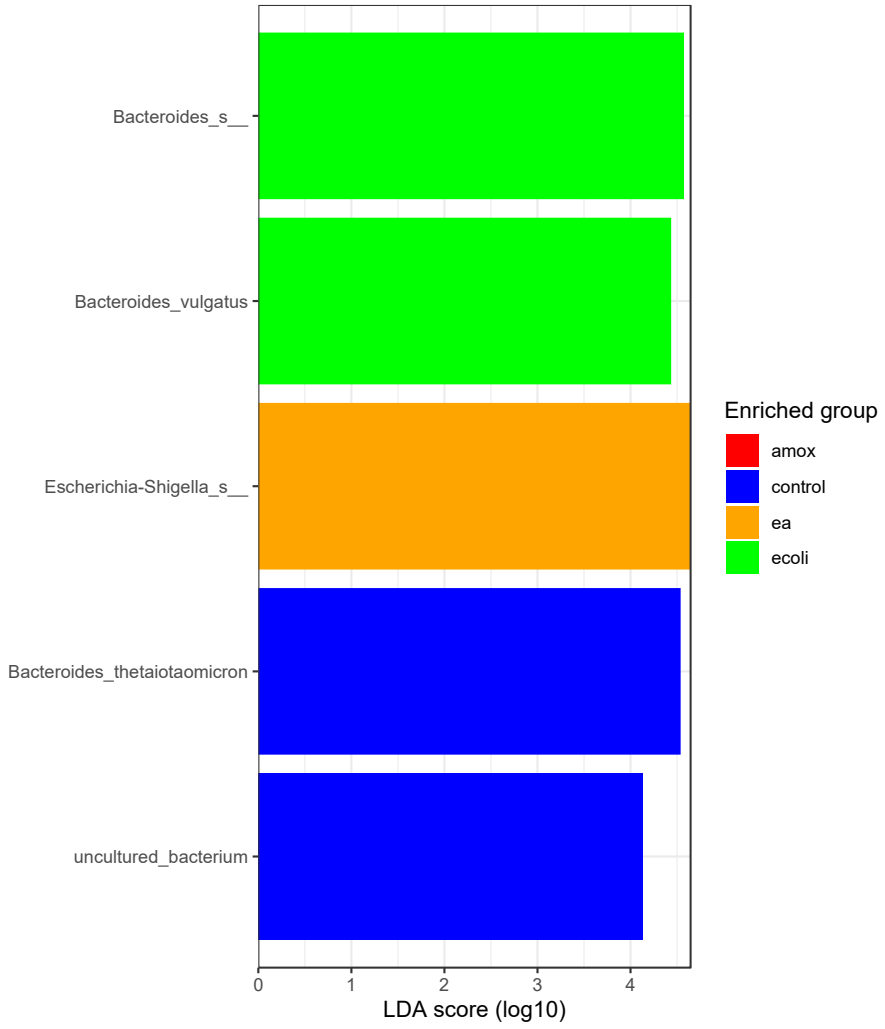

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance
Amox (%)
Control (*)
EA (+)
E. coli (#)
Treatment
Bacteroides xylaninosolvens
Bacteroides eggerthii
Bacteroides thetaiotaomicron
Bacteroides vulgatus
Turicibacter sanguinis
Lactobacillus delbrueckii+%*#
Lactobacillus johnsonii%+
Lactobacillus mucosae
Limosilactobacillus reuteri+%*#
Lactobacillus ruminis#
Streptococcus pasteurianis
Clostridium colicanis
Blautia faecicola
Escherichia coli
Prevotella copri+%*#
Lactobacillus amylovorus +%*#
Streptococcus hyointestinalis +%*#

C)



Treatment $P = 0.7877$
Sex $P = 0.8019$

D)



E)

Figure 3.4 Microbial structural analysis and *E. coli* enumeration of 5-week pup feces from the four treatment groups post microbiota normalization (N = 29). All treatment groups n = 8 except for EA (n = 5). **A)** Alpha diversity analysis of bacterial communities. Shannon diversity index (Kruskal Wallis treatment $P = 0.67$, sex $P = 0.60$; n = 5-8/group, n = 16 females/13 males) and Chao1 index (Kruskal Wallis treatment $P = 0.16$; n = 5-8/group). Dots represent individual mice and lines depict the mean values with whiskers indicating min to max values. **B)** PCoA plot of bacterial communities based on Bray-Curtis dissimilarity matrix (Adonis treatment $P = 0.001$,

Betadispersion treatment $P = 0.41$; Adonis sex $P = 0.084$, Betadispersion sex $P = 0.74$; $R^2 = 0.41$; n = 5-8/group, n = 16 females/13 males). Dots represent individual mice. **C)** Relative abundance of bacteria summarized by treatment group down to species level (n = 5-8/group). Species that are absent in each group are indicated by + (EA), % (amox), * (control), and # (E. coli) **D)** Enumeration of *E. coli* (log-transformed) in feces 5 weeks PND (One-way ANOVA with Tukey's multiple comparison test treatment $P = 0.79$, n = 5-8/group; two-tailed unpaired T-test sex $P = 0.80$; n = 16 females/13 males). Dots represent individual mice and lines depict the mean values and error bars indicate SEM. Black, blue, and red dots represent females, males, and deceased males (after 24 hrs of challenge), respectively. **E)** LEfSe showing differentially abundant bacterial taxa between the four treatment groups (n = 5-8/group). Taxonomic groups significantly enriched ($P < 0.05$) are color coded by treatment group. Taxonomy "Bacteroides_s" = *Bacteroides xylaninosolvens*, "Escherichia-shigella_s" = *E. coli*, and "uncultured_bacterium" = *Turicibacter sanguinis*. $\alpha = 0.05$.

3.3.5 S. Tm Loads in the Ileum, Cecum, and Liver

After 48 hrs of S. Tm challenge, the S. Tm load was determined by plating content from the ileum, cecum, and liver. In the ileum both treatment and sex had a significant effect ($P < 0.05$, Fig. 3.5). Specifically, the EA group had lower S. Tm loads than the control group (6.80 $\log_{10}$ CFU/g ± 0.89 vs 8.87 $\log_{10}$ CFU/g ± 0.47, mean ± SEM) and females had lower S. Tm loads than males (7.01 $\log_{10}$ CFU/g ± 0.30 vs 8.71 $\log_{10}$ CFU/g ± 0.42, mean ± SEM). In the cecum, the treatment effect was not significant, however the sex effect was significant and there was an interaction effect between treatment and sex ($P < 0.05$, Fig. 3.5). Females had significantly lower S. Tm loads in the cecum compared to males (8.66 $\log_{10}$ CFU/g ± 0.10 vs

9.91 $\log_{10}$ CFU/g ± 0.095 ± SEM, mean ± SEM). The effect of sex differed across treatment in the cecum in the ecoli, amox, and control groups with the females having less S. Tm than males ($P < 0.0001$, n = 5 females/3males; $P = 0.039$, n = 6 females/2 males; $P < 0.0001$, n = 2 females/5 males; Fig. 3.5). There was a significant effect of treatment, sex, and the interaction in the liver ($P < 0.01$, Fig 5). The EA group had less S. Tm (4.20 $\log_{10}$ CFU/g ± 0.65, mean ± SEM) than the ecoli group (5.79 $\log_{10}$ CFU/g ± 0.55, mean ± SEM). The amox group also had less S. Tm than the ecoli group (4.66 $\log_{10}$ CFU/g ± 0.23 vs 5.79 $\log_{10}$ CFU/g ± 0.55, mean ± SEM). Again, the S. Tm load in the females (4.32 $\log_{10}$ CFU/g ± 0.22, mean ± SEM) was lower than the males (6.35 $\log_{10}$ CFU/g ± 0.43, mean ± SEM). Similar to the cecum, the effect of sex differed across treatment in the liver with females having less S. Tm loads in the ecoli and control groups than males ($P = 0.0012$, n = 5 females/3males; $P < 0.0001$, n = 3 females/5 males; Fig. 3.5).

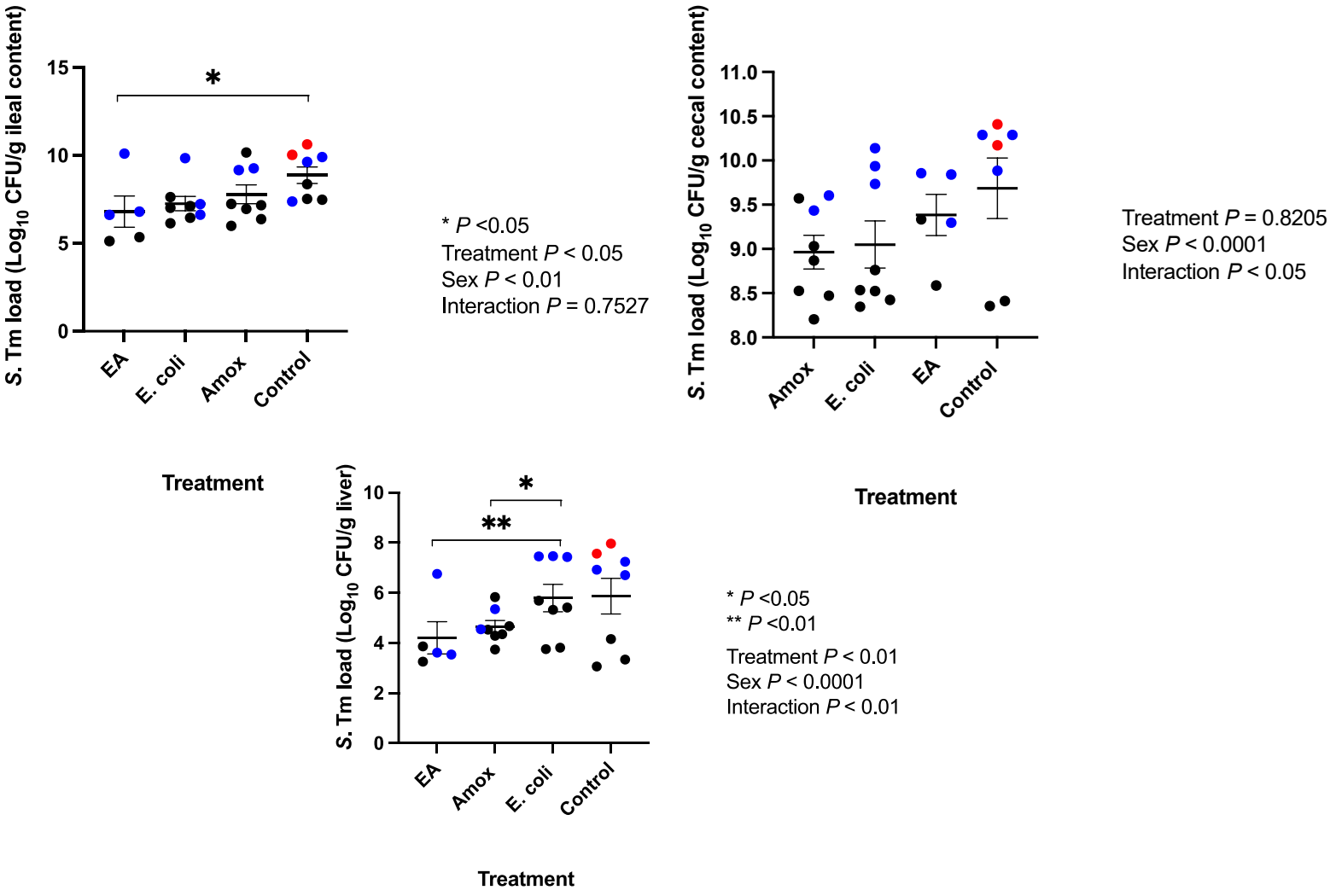
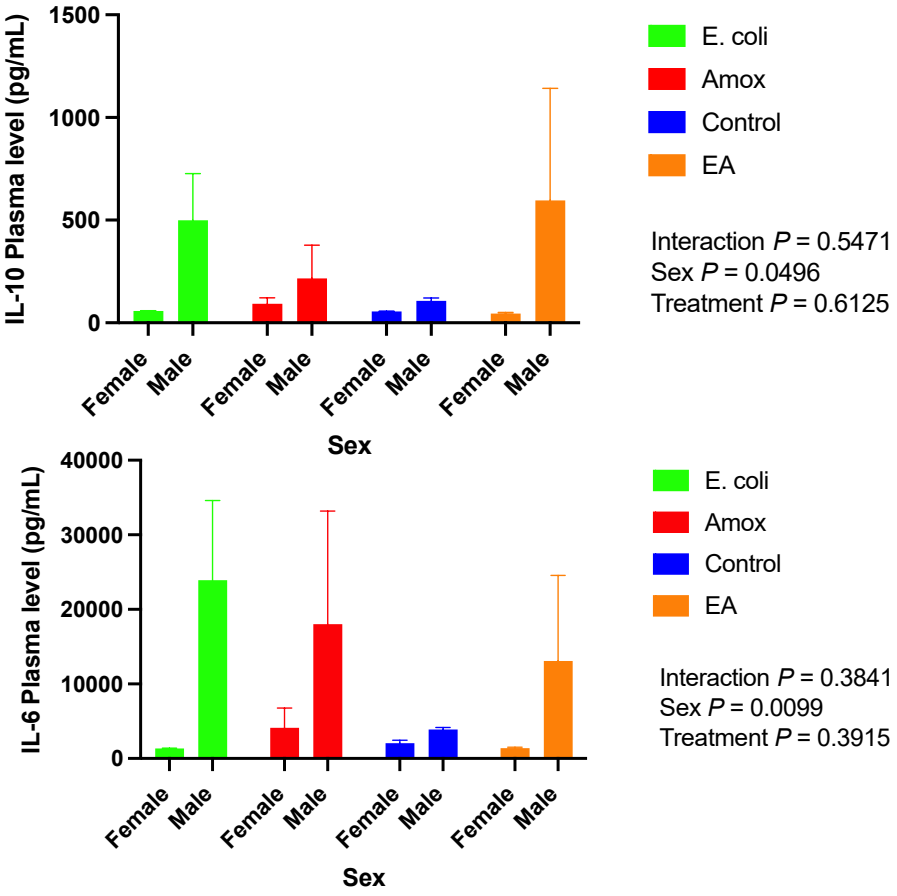


Figure 3.5 S. Tm loads (log-transformed) in the ileum, cecum, and liver of 6-week-old pups 48 hrs after infection with 7-8 x 10^7 CFU/mL S. Tm. All treatment groups n = 8 except for *E. coli* + Amox (n = 5); females n = 16 (except for cecum n = 15) and males n = 13. Dots represent individual mice and lines depict the mean values and error bars indicate SEM. Black, blue, and red dots represent females, males, and deceased males, respectively. Mixed effect model with Tukey's multiple comparisons. $\alpha = 0.05$.

3.3.6 Cytokine Response to S. Tm Challenge

Plasma cytokine levels were analyzed 48 hrs after S. Tm infection to determine if the differences in S. Tm loads could be explained by differential cytokine responses. The cytokines IFNγ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, and the chemokine KC/GRO (CXCL1) were analyzed and there were no differences between treatment groups. However, IL-10 (69.18 pg/mL ± 11.52, 367.50 pg/mL ± 154.10, mean ± SEM), IL-6 (2522 pg/mL ± 986.70, 14425 pg/mL ± 4833, mean ± SEM), and KC/GRO (3027 pg/mL ± 386.30, 6546 pg/mL ± 715.80, mean ± SEM) were significantly lower in females than males ($P < 0.05$, n = 16 females/11 males, Fig. 3.6).



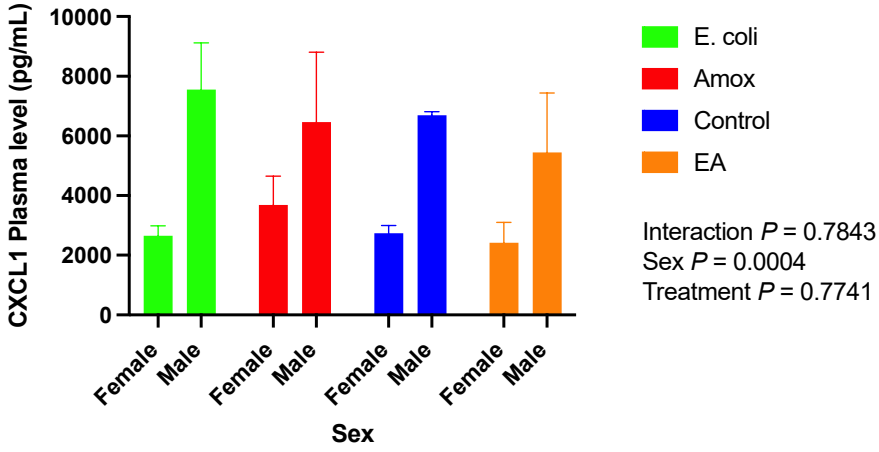


Figure 3.6 Cytokine levels in plasma after 48 hrs of infection by S. Tm. Each treatment group is presented by sex. Data was analyzed using a Two-way ANOVA where $\alpha = 0.05$. Lines depict the mean values and error bars indicate SEM.

3.4 Discussion:

Here, we investigated the influence of early life *E. coli* and amoxicillin treatment on the severity of infection by S. Tm in a mouse model with a defined swine bacterial community. To date, there is limited research examining how early life *E. coli* colonization and amoxicillin administration modulates immune system development and subsequent disease resistance. While determining the mechanisms behind this interaction between *E. coli* and amoxicillin on immune system development was beyond the scope of this paper, the data supports the idea that early life *E. coli* and amoxicillin administration influences subsequent S. Tm infection later in life.

In the current study, we found that the majority of the swine DC did colonize the dams and could be vertically transmitted to their pups. Here, 14/16 species, compared to 10/16 species in Chapter 2, colonized when species previously undetected were given fresh in duplicate gavages. *P. copri* and *S. hyointestinalis* were not present at any of the time points in either study,

therefore these species may be host adapted or require specific conditions to colonize. *P. copri* is an important strict anaerobe in the human and swine gut, and as such has gained research interest. All of the research in mice has used the human isolate of *P. copri* and while studies claim colonization of this microbe, these studies implement regular oral gavages and lack plating methods to confirm colonization (Chen et al., 2021; Huang et al., 2020; Pareek et al., 2019). One study by Kovatcheva-Datchary and colleagues (2019) presents compelling evidence that the human strain of P. copri can colonize over long periods of time after initially reducing the gut with *B. thetaiotaomicron*. Although our inoculum contained *B. thetaiotaomicron* and repeated gavages were performed, our pig *P. copri* isolate did not colonize. While the sequencing data from the pups showed that most of the species were present at all sample time points, we noted variation in the detection of some the *Lactobacillus* between the weeks 3 and 5. These results may have been due to sequencing depth issues and highlights the fact that fecal samples are not representative of the overall microbial colonization in the gastrointestinal tract as *Lactobacillus* species are known to preferentially colonize the small intestine (Walter, 2008).

While we could not confirm a bloom of *E. coli* in response to amoxicillin treatment previously at the early post-natal time point seen in other works, we did observe higher *E. coli* loads in female mice on day 4 of amoxicillin treatment compared to the control group. Moreover, the normalization of the microbiota of the 5-week-old pups successfully colonized all groups with equal loads of *E. coli* as evidenced by plating data. It is important to note that plating data is often more reliable as live bacteria are quantified. Whereas sequencing methods sequence the total DNA in a sample and may incorrectly present significant differences like in this study where the sequencing data suggested that the EA group had significantly more *E. coli* going into the S. Tm challenge than the other groups. While the microbial composition was different

between groups going into the S. Tm challenge and may have contributed to the variation in S. Tm infection, the differences remained subtle.

To investigate the long-term implications of the effects of early life *E. coli* and amoxicillin on disease resistance, the pups were challenged with S. Tm at 6 weeks of age. In line with the hypothesis, the EA group had the lowest S. Tm loads in multiple sites and the control group consistently had the highest numerical load of S. Tm in all sites. Furthermore, the significant additive effect of *E. coli* and amoxicillin in the ileum and liver suggests that early life treatment may contribute to a less severe local and systemic S. Tm infection later in life. This difference in S. Tm load may be associated with the more robust Th1 response as observed in the pig studies (Fouhse et al., 2019), however, there were no differences between any groups for the 10 cytokines at 48 hrs post infection. An earlier sampling point may be warranted as certain important Th1 cytokines like IFNγ, TNF-α, and IL-2 have been shown to peak within the first 24 hrs in response to S. Tm (Dar et al., 2019; Foster et al., 2003; McSorley et al., 2002; Perkins et al., 2015).

Interestingly, males consistently had a significantly higher load of S. Tm in the ileum, cecum, and liver. This phenotype linked to sex has been underexplored in relation to *Salmonella* infection, but also in immunological research as approximately only 10% of articles analyze data by sex (Klein & Flanagan, 2016). A few studies have found differences between sexes in both humans and mice. In humans, men were found to clear *Salmonella* infection slower than their female counterparts and young males under 15 have a higher Salmonellosis incidence compared to women and older men (Lonnermark et al., 2014; Peer et al., 2021). Caron and colleagues (2002) also found that male mice challenged with *S. enteritidis* suffered from higher splenic *Salmonella* loads than female mice. This discrepancy between sexes in infectious diseases has

been noted in other enteric infections such as *Campylobacter jejuni*, *Helicobacter pylori*, *Clostridium difficile*, and *Yersinia enterocolitica* (Vázquez-Martínez et al., 2018). Many researchers attribute the sexual dimorphism associated with pathogen loads to sex hormone differences and the fact that several genes involved in the innate and adaptive immune responses are located on the X chromosome (Elderman et al., 2016; Fish, 2008; Klein & Flanagan, 2016; Sankaran-Walters et al., 2013; Vázquez-Martínez et al., 2018). It has been suggested that since estrogen can regulate the human gut microbiota, that the estrogen driven microbiota differences may be a factor of sexual dimorphism in *Salmonella* infection (Peer et al., 2021). However, in our model, we did not observe any significant microbiota related differences between the sexes at any time point. It has been further suggested that since estrogen-regulated cytokines provide a favorable environment for the differentiation of $CD4^+$ T cells toward both Th1 and Th17 cells that females are more efficient at clearing intracellular pathogens (Jaillon et al., 2019; Klein & Flanagan, 2016). In this study, females did not have higher levels of Th1 cytokines 48 hrs post infection, but males did have significantly more IL-6 and IL-10. IL-6 is a proinflammatory cytokine that has been repeatedly associated with poor prognosis, especially in males which have greater IL-6 plasma levels during sepsis (Frink et al., 2007; Oberholzer et al., 2005; Remick et al., 2002). Higher levels of IL-10 have also been correlated with worse outcomes in septic patients due to tolerogenic mechanisms provided by this anti-inflammatory cytokine (Frink et al., 2007; Parsons et al., 2005). This tolerogenic effect of IL-10 has been demonstrated multiple times in IL-10 deficient/knockout mice in response to S. Tm (Arai et al., 1995; Neves et al., 2010; Salazar et al., 2017). Most recently, an IL-10 knockout model demonstrated that mice lacking IL-10 had significantly less S. Tm in the ileum, spleen, and liver and a significantly higher percent survival rate (Salazar et al., 2017). As such, we propose that significantly higher

IL-10 and IL-6 in males could be responsible for greater local loads and systemic dissemination of S. Tm.

Resistance to S. Tm is clearly multifactorial, thus we postulate that the difference in pathogen loads between sexes could also be due to different phagocytic cell populations, and a greater capacity to detect pathogens through higher TLR4 expression in females driven by 17β-oestradiol. Comparatively, females have greater phagocytic activity of neutrophils, macrophages, and greater $CD4^+$ T cells than males, all of which are important to clear S. Tm infections (Klein & Flanagan, 2016; Scotland et al., 2011). In 2011, Scotland and colleagues showed that resident leukocytes in C57BL/6 mice and rats were more numerous in females than males. Furthermore, resident macrophages expressed higher levels of TLR4, which implies that these cells have a greater capacity to detect Gram-negative pathogens and eliminate them. Of note, excessive cytokine production was also not found in females, however, chemokines that recruit monocytes, macrophages, and lymphocytes were significantly higher in mesenteric tissue than males. These findings suggest that following enteric infections that females can mount a greater innate immune response via specific cell subsets without excessive production of cytokines.

3.5 Conclusion:

To conclude, we showed that the majority (14/16) of the swine DC colonize the mouse gut when the previously undetected species were administered fresh. Furthermore, the presence of *E. coli* did not preclude any species from colonizing this mouse model. This limited swine consortium mouse model serves as a model for detailed investigation of host-microbe interactions in the context of swine enteric infections. We clarified that early life *E. coli* colonization and amoxicillin does have long lasting impacts on S. Tm load in both local and

systemic sites. Additionally, males suffered from higher loads of S. Tm in the ileum, cecum, and liver, which may be driven by excess production of cytokines that allow for greater systemic dissemination and exacerbate sepsis. This study is the first step in developing management strategies to recapitulate this protective phenotype and highlights the importance of sexual dimorphism in enteric infections. Besides replication of the study, the next step is to define the effects that early life *E. coli* and amoxicillin have on immune system development to develop non-antibiotic-based strategies to reduce Salmonellosis.

Chapter 4: General Discussion

4.1 Summary and Conclusions:

The call for mechanistic studies in gut microbiome research has spurred the cultivation of bacteria derived from various hosts (Fischbach, 2018). While high-throughput sequencing combined with subsequent bioinformatic and omics analyses generate copious amounts of data, pure isolates are imperative to test correlative predictions. To test these predictions and gain insight into the roles specific members of the gut microbiota play in host health and disease, gnotobiotic animal models are the gold standard. Gnotobiotic animals allow researchers to tease apart the relationships between microbes and host-microbe interactions (Fiebiger et al., 2016). The first step in creating a gnotobiotic animal model is to select appropriate strains of bacterial species, from culture collections, to include in a defined community (DC) that the animals will be administered. As such, the foundation of this principle is to generate culture collections and characterize isolates. In this work, bacteria were isolated from conventional pig gastrointestinal samples to curate a swine-microbe derived culture collection. Then, based on proposed swine core microbiomes and prevalent bacteria in piglets, a DC with whole genomes was curated.

With the intent of first employing a gnotobiotic mouse model to test findings associated with *Escherichia coli* and amoxicillin administration in early life and subsequent disease resistance in pigs from a previous study by Fouhse et al (2019), the DC was introduced to germ-free (GF) mice to assess colonization patterns. At the phylum level, both mice and pigs are dominated by Firmicutes and Bacteroidetes which was observed in our swine DC mouse model even when *E. coli* was introduced in the third chapter; however, the relative abundance of Bacteroidetes in our model is higher than what it typically seen in wild mice and conventional pigs, but comparable to gnotobiotic mice colonized with both mouse and human microbiota

(Chung et al., 2012; Holman et al., 2017; Lkhagva et al., 2021). In chapter 2, our swine DC mouse model exhibited specific gut microbial community structures and total bacteria loads that are conserved between pigs and mice (Anders et al., 2021; Crespo-Piazuelo et al., 2018; Holman et al., 2017; Suzuki & Nachman, 2016; Zhao et al., 2015). Specifically, a high degree of overlap in the community structure between the cecum, colon, and feces, but distinct separation in the ileum was observed. This result can be explained by the enrichment of *Lactobacillus* and *Streptococcus* species in the ileum and *Bacteroides* species in the lower gastrointestinal tract, which were presumably due to the differences in substrate and oxygen availability that is similar between mammalian hosts (Anders et al., 2021; Crespo-Piazuelo et al., 2018; Lkhagva et al., 2021). These conserved results suggest that this limited swine consortium may colonize in a similar fashion when introduced to GF piglets and lends evidence for the use of a swine microbe derived gnotobiotic mouse model to serve as a basis to test associations from pig studies when GF pig work is not feasible or warranted. Having said this, similar models like humanized mice have come under scrutiny as specific members of the mouse microbiota are essential for the maturation of the immune system and confer better protection against certain enteric pathogens (Chung et al., 2012). Consideration of potentially different interactions resulting from a foreign host microbial community should be taken into account when conducting such studies. Furthermore, the functional potential of this DC should be assessed as the hallmarks of a suitable gnotobiotic model are not limited to membership, composition, and structure if the intent is to mimic “normal” conditions in the gut. For example, a recent genome-guided study conducted in gnotobiotic mice harboring a mouse DC analyzed the functional potential of each strain and compared them to the fraction of KEGG modules of a conventional mouse microbiota (Brugiroux et al., 2016). With this knowledge, they identified conserved modules between the

two consortia and were able to add facultative anaerobes that covered missing aerobic respiration functions to restore colonization resistance of S. Tm to comparable levels observed in conventional mice (Brugiroux et al., 2016). Functional redundancy within the gut microbiome is well known and if the members of a DC can cover the major functions as well as mimic the community composition of the donor, gnotobiotic models can be improved and shift towards a healthy representative microbiome.

Given the ability of the swine defined community to colonize in a gnotobiotic mouse model, we were able to test the effects of early life pig *E. coli* and amoxicillin treatment on subsequent resistance to S. Tm infection in our swine DC mouse model. When the members of the DC that did not colonize in chapter 2 were given fresh in duplicate exposures of the DC in chapter 3, 13/16 species colonized in the DC dams while 14/16 species colonized in the DC + *E. coli* dams. This shows that the addition of *E. coli* does not reduce the DC colonization ability. Additionally, the majority of the DC was vertically transmitted to the pups of all groups with only the differences spanning the *Lactobacillus* genera, which are known to be influenced by antibiotic administration (Graversen et al., 2020). Upon S. Tm challenge, the EA group showed the lowest S. Tm loads in the ileum and liver. Importantly, the S. Tm loads in this study were comparable to other S. Tm models in C57BL/6J mice 48 hrs post infection (Nilsson et al., 2019). While the mechanism behind this finding is yet to be elucidated, we confirmed previous results suggesting that the administration of *E. coli* and amoxicillin during the first two weeks of life does have lasting impacts on disease resistance (Fouhse et al., 2019). Many studies focussing on the role of *E. coli* on subsequent S. Tm resistance suggest that the mere presence of *E. coli* provides colonization resistance (Brugiroux et al., 2016; Furter et al., 2019; Thiemann et al., 2017; Wotzka et al., 2019). However, at the time of challenge in this study, the *E. coli* counts did

not significantly differ, yet there were significant differences in S. Tm loads between treatment groups. Therefore, we postulate that colonization resistance is not the only mechanism by which *E. coli* can decrease S. Tm loads. It was then hypothesized that given the significant increase of T helper 1 (Th1) mediated cytokines in pigs previously characterized with amoxicillin induced *E. coli* blooms in early life, that the combination of *E. coli* and amoxicillin primes the immune system towards a more proinflammatory response which may be beneficial in clearing intracellular pathogens like S. Tm later in life (Fouhse et al., 2019). However, no significant differences in proinflammatory cytokines were observed between groups at 48 hrs post infection. Interestingly, there were significantly higher S. Tm loads in the ileum, cecum, and liver in male mice that was accompanied by increased cytokines, IL-6 and IL-10, which are implicated in sepsis and increased systemic dissemination of S. Tm (Arai et al., 1995; Frink et al., 2007; Neves et al., 2010; Oberholzer et al., 2005; Remick et al., 2002; Salazar et al., 2017). Sexual dimorphism, while largely underreported in S. Tm infection, is a major factor in immunological responses to various disease states (Lonnermark et al., 2014; Peer et al., 2021). Human females account for about 80% of autoimmune disorders whereas males are typically afflicted with infectious diseases and non-reproductive cancers (Klein & Flanagan, 2016). Sex differences in immune function are not limited to mammals. In fruit flies, genes encoding for innate signalling proteins on the X chromosome have been found to be responsible for the reduction of bacterial loads in females (Hill-Burns & Clark, 2009). Sex hormones can bind to immune cells and alter immune responses by acting as transcription factors to modify gene expression have also been shown to influence immune responses in birds and lizards (Fox et al., 1991; Kovats, 2015; vom Steeg & Klein, 2016). Male birds, especially during mating season when testosterone is the highest, have lower antibody and cell-mediated immune responses; and male lizards show less

phagocytic activity of macrophages than females resulting from suppressive androgens (Mondal & Rai, 1999; Pap et al., 2010). In mammals, the primary principles driving sexual dimorphism in immunity predictably stem back to these two principles – immune functions encoded on the X chromosome and sex hormones (Klein & Flanagan, 2016). Briefly, the immune functions encoded on the X chromosome include those implicating toll-like receptors (TLR7 and TLR9), various cytokine receptors, and cellular and humoral mediated immunity (Fish, 2008). The effects of sex hormones on innate and adaptive immune responses are complex and are not all synonymous between species nor throughout life (Klein & Flanagan, 2016). Furthermore, the levels of estrogen, progesterone and androgens differentially affect immune cell functions but in large progesterone and androgens suppress proinflammatory responses, whereas estrogen in higher levels promote proinflammatory responses (Gay et al., 2021). Due to the importance of cell-mediated pro-inflammatory Th1 mediated responses in clearing S. Tm, it is relevant to note that in general, females display greater cell-mediated immune responses characterized by higher naïve and helper T cell counts, especially Th1 cells, and cytotoxic T cell activity (Abdullah et al., 2012; Amadori et al., 1995; Villacres et al., 2004). Estrogen in mice has repeatedly been shown to increase expression of TLR4 on macrophages, which correlated with higher inflammatory cytokine levels and reduced lipopolysaccharide (LPS) endotoxin-associated morbidity (Rettew et al., 2008, 2009; Scotland et al., 2011). Additionally, females show higher phagocytic activity of macrophages and increased NETosis of neutrophils, as well as antigen presentation (Klein & Flanagan, 2016; Lu et al., 2021; Scotland et al., 2011). Taken together, while females did not show increased levels of proinflammatory cytokines at 48 hrs post infection they may have had differential activation of cell populations involved in innate and cell-mediated immunity that controlled S. Tm loads without excessive cytokine production.

4.2 Limitations:

It is valuable to acknowledge the limitations of this thesis for both the interpretation of results and to improve future work. Gnotobiotic mouse models are an incredibly important tool to study the gut microbiome, thus the protocols for raising mice in germ-free isolators are established and rigorously validated. Mice are by far the most popular gnotobiotic animal due to these established protocols, cost, and ease of rearing. In this thesis, gnotobiotic mice were used to test previous effects of amoxicillin and *E. coli* in early life on subsequent disease resistance later in life in pigs. Gnotobiotic pig models are far less widespread in gut microbiome research due to their incredibly demanding rearing, cost, and largely unvalidated protocols. As such, the numbers of piglets realistically raised at one time is reduced in comparison to mice. While the sample size was limited in this study due to cannibalization of litters, leaving one remaining litter per treatment group, it remained larger than many gnotobiotic pig trials (Huang et al., 2018; Kozakova et al., 2006; Zhou et al., 2021). Cannibalization was a limiting factor in this study, and we tried to reduce the stress these dams were facing from loud construction occurring next to the unit these animals were housed in by reducing handling during the first three weeks of life. Unfortunately, this limited our ability to mix litters, sex match, and take important samples. To investigate an amoxicillin induced *E. coli* bloom, samples throughout the first two weeks would have been ideal to confirm this previous result during the first week of amoxicillin treatment in piglets from Fouhse et al (2019). Although amoxicillin induced *E. coli* blooms are characteristic of this particular antibiotic treatment and our pilot *E. coli* plus amoxicillin trial revealed that amoxicillin increased *E. coli* compared to control mice, it is important to definitively observe a bloom in this early life study to claim causality (Antonopoulos et al., 2009; Fouhse et al., 2019; Looft et al., 2012). Antibiotic administration in this trial was facilitated through sterile

amoxicillin supplemented water and thereby milk from dams to pups. Small mouse pups cannot be gavaged daily, which is not the case for piglets as they can be directly administered antibiotics. While gnotobiotic mouse models possess clear benefits over gnotobiotic piglets, sampling and sexing piglets is not typically an issue as they are larger, not raised by their sow, and can be sexed at birth to balance treatments, which is important in disease challenge models due to sexual dimorphism (Klein & Flanagan, 2016). Furthermore, blood samples were not feasible from these animals prior to challenge, which prevented baseline cytokine characterization to determine the magnitude of the difference between the sexes. Finally, if the intent of a study is to investigate the effect of early life bacterial constituents (*E. coli*) on pathogen resistance, it is imperative to normalize the microbiota across treatment groups well in advance of infection. Here, we fed back fecal slurries composed of combined fecal pellets from each of the treatment group dams to the pups at weaning. Although there were still small beta diversity differences between every group due to minutely enriched species in certain treatment groups, each group had equal *E. coli* loads. To rectify such differences, replication studies could oral gavage the DC and *E. coli* in the same fashion as the dams to test if beta diversity differences still result. Normalization between groups is also an issue when using gnotobiotic pigs for this study as they come out of isolators at weaning due to size restrictions of the isolators. Since these piglets are exposed to a wide variety of microbes when transitioning from sterile isolators to conventional settings, it is fair to presume that these piglets would exhibit differences in diversity measures between treatments. In large many of the limitations in this study can be mitigated by increasing sample size by repeating this study in gnotobiotic mice and then confirming results in gnotobiotic pigs.

4.3 Future Directions:

In pig production, opportunistic and pathogenic *E. coli* are a major health concern which results in management practices based on controlling *E. coli* populations (Luppi, 2017). While research is obviously lacking regarding the effects of the presence of *E. coli* in early life on immune system development, it has been suggested that *E. coli* derived LPS may be one mechanism of educating the immune system by priming antigen presenting cells to facilitate T cell clonal expansion, differentiation, and survival as well as conditioning gut epithelial cell responses to subsequent TLR stimulation (Chassin et al., 2010; Lotz et al., 2006; Mcaleer & Vella, 2008). Thus, it is possible that further research will support the administration of beneficial probiotic *E. coli* or *E. coli* derivates to facilitate enhanced resistance to enteric pathogens such as S. Tm.

It is important to acknowledge that the overwhelming body of research involving neonatal antibiotic administration shows that antibiotic induced microbial dysbiosis can differentially direct immune development resulting in increased risk of allergies, asthma, irritable bowel disease, and various infections (Neuman et al., 2018; Vangay et al., 2015). As such, this body of work is not suggesting the unnecessary administration of amoxicillin in early life, but rather intended to confirm that early life antibiotic and microbial interventions are responsible for S. Tm resistance later in life. It is appropriate for future studies to repeat this model, and then investigate in gnotobiotic pigs, to characterize immune cell subtypes and cytokine profiles during the two-week administration of amoxicillin and during S. Tm challenge at various time points. This information will lend evidence to how these treatments modulate immune system development and confer increased S. Tm resistance. If an additive effect of *E. coli* and amoxicillin is still observed, the follow up research would be to pinpoint the mechanism

facilitating this differential immune response to recapitulate the previously characterized protective phenotype without using antibiotic administration. Clearly, this field is in its infancy, but with continued research there is great potential to employ the largely underutilized gut microbiota as a tool to improve disease resistance.

References:

Chapter 1

1. Aagaard, K., Riehle, K., Ma, J., Segata, N., & Mistretta, T.-A. (2012). A Metagenomic Approach to Characterization of the Vaginal Microbiome Signature in Pregnancy. *PLoS ONE*, *7*(6), 36466. https://doi.org/10.1371/journal.pone.0036466
2. Albenberg, L., Esipova, T. v., Judge, C. P., Bittinger, K., Chen, J., Laughlin, A., Grunberg, S., Baldassano, R. N., Lewis, J. D., Li, H., Thom, S. R., Bushman, F. D., Vinogradov, S. A., & Wu, G. D. (2014). Correlation between intraluminal oxygen gradient and radial partitioning of intestinal microbiota. *Gastroenterology*, *147*(5), 1055-1063.e8. https://doi.org/10.1053/j.gastro.2014.07.020
3. Alhanout, K., Malesinki, S., Vidal, N., Peyrot, V., Rolain, J. M., & Brunel, J. M. (2010). New insights into the antibacterial mechanism of action of squalamine. *Journal of Antimicrobial Chemotherapy*, *65*(8), 1688–1693. https://doi.org/10.1093/JAC/DKQ213
4. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2
5. Aluthge, N. D., Tom, W. A., Bartenslager, A. C., Burkey, T. E., Miller, P. S., Heath, K. D., Kreikemeier-Bower, C., Kittana, H., Schmaltz, R. J., Ramer-Tait, A. E., & Fernando, S. C. (2020). Differential longitudinal establishment of human fecal bacterial communities in germ-free porcine and murine models. *Communications Biology*, *3*(1). https://doi.org/10.1038/s42003-020-01477-0
6. Amann, R. I., Ludwig, W., & Schleifer, K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, *59*(1), 143–169. https://doi.org/10.1128/MR.59.1.143-169.1995
7. Amies, C. R. (1967). A Modified Formula for the Preparation of Stuart's Transport Medium. In *Journal of Public Health / Revue Canadienne de Santé Publique* (Vol. 58, Issue 7).
8. Antonopoulos, D. A., Huse, S. M., Morrison, H. G., Schmidt, T. M., Sogin, M. L., & Young, V. B. (2009). Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. *Infection and Immunity*, *77*(6), 2367–2375. https://doi.org/10.1128/IAI.01520-08
9. Arai, T., Hiromatsu, K., Nishimura, H., Kimura, Y., Kobayashi, N., Ishida, H., Nimurat, Y., & Yoshikai, Y. (1995). Effects of in vivo administration of anti-IL-10 monoclonal antibody on the host defence mechanism against murine Salmonella infection. *Immunology*, *85*, 381–388.
10. Aranki, A., & Freter, R. (1972). Use of anaerobic glove boxes for the cultivation of strictly anaerobic bacteria. *The American Journal of Clinical Nutrition*, *25*(12), 1329–1334. https://doi.org/10.1093/ajcn/25.12.1329
11. Arrieta, M.-C., Stiemsma, L. T., Amenyogbe, N., Brown, E. M., Finlay, B., Massari, P., & Strunk, T. (2014). The intestinal microbiome in early life: health and disease. *Frontiers in Immunology*, *5*, 427. https://doi.org/10.3389/fimmu.2014.00427
12. Avershina, E., Storrø, O., Øien, T., Johnsen, R., Pope, P., & Rudi, K. (2014). Major faecal microbiota shifts in composition and diversity with age in a geographically

restricted cohort of mothers and their children. *FEMS Microbiology Ecology*, *87*(1), 280–290. https://doi.org/10.1111/1574-6941.12223

13. Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., Li, Y., Xia, Y., Xie, H., Zhong, H., Khan, M. T., Zhang, J., Li, J., Xiao, L., Al-Aama, J., Zhang, D., Lee, Y. S., Kotowska, D., Colding, C., … Jun, W. (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host and Microbe*, *17*(5), 690–703. https://doi.org/10.1016/j.chom.2015.04.004
14. Baron, E. J. (2015). Specimen Collection, Transport, and Processing: Bacteriology. In *Manual of Clinical Microbiology*. ASM Press. https://doi.org/10.1128/9781555817381.CH18
15. Barthel, M., Hapfelmeier, S., Quintanilla-Martínez, L., Kremer, M., Rohde, M., Hogardt, M., Pfeffer, K., Rüssmann, H., & Hardt, W. D. (2003). Pretreatment of mice with streptomycin provides a Salmonella enterica serovar Typhimurium colitis model that allows analysis of both pathogen and host. *Infection and Immunity*, *71*(5), 2839–2858. https://doi.org/10.1128/IAI.71.5.2839-2858.2003
16. Baümler, A. J., & Sperandio, V. (2016). Interactions between the microbiota and pathogenic bacteria in the gut. *Nature*, *535*(7610), 85–93. https://doi.org/10.1038/nature18849
17. Bellali, S., Lagier, J. C., Raoult, D., & Khalil, J. B. (2019). Among live and dead bacteria, the optimization of sample collection and processing remains essential in recovering gut microbiota components. *Frontiers in Microbiology*, *10*(JULY). https://doi.org/10.3389/fmicb.2019.01606
18. Bennett, J., Dolin, R., & Blaser, M. (2014). *Mandell, douglas, and bennett's principles and practice of infectious diseases: 2-volume set*. https://books.google.ca/books?hl=en&lr=&id=BseNCgAAQBAJ&oi=fnd&pg=PP1&dq=Bennett+JE,+Dolin+R,+Blaser+MJ.+Mandell,+Douglas,+and+Bennett%E2%80%99s+prin-+ciples+and+practice+of+infectious+diseases.+8th+ed.,+vol.+1.+Philadelphia:+Elsevier+Health+Sciences%3B+2014.&ots=0LJxq3e9VT&sig=lRk8HMoyJHzuuRg-fMHgLzGQ62Y
19. Bergström, A., Skov, T. H., Bahl, M. I., Roager, H. M., Christensen, L. B., Ejlerskov, K. T., Mølgaard, C., Michaelsen, K. F., & Licht, T. R. (2014). Establishment of intestinal microbiota during early life: A longitudinal, explorative study of a large cohort of Danish infants. *Applied and Environmental Microbiology*, *80*(9), 2889–2900. https://doi.org/10.1128/AEM.00342-14/SUPPL_FILE/ZAM999105322SO1.PDF
20. Bian, G., Ma, S., Zhu, Z., Su, Y., Zoetendal, E. G., Mackie, R., Liu, J., Mu, C., Huang, R., Smidt, H., & Zhu, W. (2016). Age, introduction of solid feed and weaning are more important determinants of gut bacterial succession in piglets than breed and nursing mother as revealed by a reciprocal cross-fostering model. In *Environmental Microbiology* (Vol. 18, Issue 5, pp. 1566–1577). https://doi.org/10.1111/1462-2920.13272
21. Bilen, M., Dufour, J. C., Lagier, J. C., Cadoret, F., Daoud, Z., Dubourg, G., & Raoult, D. (2018). The contribution of culturomics to the repertoire of isolated human bacterial and archaeal species. In *Microbiome* (Vol. 6, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s40168-018-0485-5

22. Bircher, L., Geirnaert, A., Hammes, F., Lacroix, C., & Schwab, C. (2018). Effect of cryopreservation and lyophilization on viability and growth of strict anaerobic human gut microbes. *Microbial Biotechnology*, *11*(4), 721–733. https://doi.org/10.1111/1751-7915.13265
23. Blanchette, J., Jaramillo, M., & Olivier, M. (2003). Signalling events involved in interferon-c-inducible macrophage nitric oxide generation. *Immunology*, *108*(4), 513–522.
24. Bonnet, M., Lagier, J. C., Raoult, D., & Khelaifia, S. (2020). Bacterial culture through selective and non-selective conditions: the evolution of culture media in clinical microbiology. In *New Microbes and New Infections* (Vol. 34). Elsevier Ltd. https://doi.org/10.1016/j.nmni.2019.100622
25. Breitwieser, F. P., Pertea, M., Zimin, A. v., & Salzberg, S. L. (2019). Human contamination in bacterial genomes has created thousands of spurious proteins. *Genome Research*, *29*(6), 954–960. https://doi.org/10.1101/gr.245373.118
26. Brown, A. F., Leech, J. M., Rogers, T. R., & McLoughlin, R. M. (2013). Staphylococcus aureus colonization: Modulation of host immune response and impact on human vaccine design. *Frontiers in Immunology*, *4*(DEC), 1–20. https://doi.org/10.3389/fimmu.2013.00507
27. Browne, H. P., Forster, S. C., Anonye, B. O., Kumar, N., Neville, B. A., Stares, M. D., Goulding, D., & Lawley, T. D. (2016). Culturing of "unculturable" human microbiota reveals novel taxa and extensive sporulation. *Nature*, *533*(7604), 543–546. https://doi.org/10.1038/nature17645
28. Browne, P. D., Nielsen, T. K., Kot, W., Aggerholm, A., Gilbert, M. T. P., Puetz, L., Rasmussen, M., Zervas, A., & Hansen, L. H. (2020). GC bias affects genomic and metagenomic reconstructions, underrepresenting GC-poor organisms. *GigaScience*, *9*(2). https://doi.org/10.1093/gigascience/giaa008
29. Broz, P., Newton, K., Lamkanfi, M., Mariathasan, S., Dixit, V. M., & Monack, D. M. (2010). Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. *Journal of Experimental Medicine*, *207*(8), 1745–1755. https://doi.org/10.1084/jem.20100257
30. Brugiroux, S., Beutler, M., Pfann, C., Garzetti, D., Ruscheweyh, H. J., Ring, D., Diehl, M., Herp, S., Lötscher, Y., Hussain, S., Bunk, B., Pukall, R., Huson, D. H., Münch, P. C., McHardy, A. C., McCoy, K. D., MacPherson, A. J., Loy, A., Clavel, T., … Stecher, B. (2016). Genome-guided design of a defined mouse microbiota that confers colonization resistance against Salmonella enterica serovar Typhimurium. *Nature Microbiology*, *2*(2), 1–12. https://doi.org/10.1038/nmicrobiol.2016.215
31. Brusa, T., Canzi, E., Pacini, N., Zanchi, R., & Ferrari, A. (1989). Oxygen Tolerance of Anaerobic Bacteria Isolated from Human Feces. In *CURRENT MICROBIOLOGY* (Vol. 19).
32. Bryukhanov, A. L., & Netrusov, A. I. (2006). Long-term storage of obligate anaerobic microorganisms in glycerol. *Applied Biochemistry and Microbiology*, *42*(2), 177–180. https://doi.org/10.1134/S0003683806020116
33. Buerger, S., Spoering, A., Gavrish, E., Leslin, C., Ling, L., & Epstein, S. S. (2012). Microbial scout hypothesis, stochastic exit from dormancy, and the nature of slow growers. *Applied and Environmental Microbiology*, *78*(9), 3221–3228.

https://doi.org/10.1128/AEM.07307-11/ASSET/24ED206C-4273-4B6F-BBBC-A0172C5C56DD/ASSETS/GRAPHIC/ZAM9991031990005.JPEG
34. Cabreiro, F., & Gems, D. (2013). Worms need microbes too: microbiota, health and aging in Caenorhabditis elegans. *EMBO Molecular Medicine*, *5*(9), 1300–1310. https://doi.org/10.1002/EMMM.201100972
35. Caldwell, D. R., & Bryant', M. P. (1966). Medium Without Rumen Fluid for Nonselective Enumeration and Isolation of Rumen Bacteria. In *APPLIED MICROBIOLOGY, Sept* (Vol. 14, Issue 5).
36. Cary, S. G., & Blair', E. B. (1964). New transport medium for shipment of clinical specimens i. Fecal specimens. In *Journal of Bacteriology* (Vol. 88, Issue 1). http://jb.asm.org/
37. Chassin, C., Kocur, M., Pott, J., Duerr, C. U., Gütle, D., Lotz, M., & Hornef, M. W. (2010). MiR-146a mediates protective innate immune tolerance in the neonate intestine. *Cell Host and Microbe*, *8*(4), 358–368. https://doi.org/10.1016/j.chom.2010.09.005
38. Che, C., Pang, X., Hua, X., Zhang, B., Shen, J., Zhu, J., Wei, H., Sun, L., Chen, P., Cui, L., Zhao, L., & Yang, Q. (2009). Effects of human fecal flora on intestinal morphology and mucosal immunity in human flora-associated piglet. *Scandinavian Journal of Immunology*, *69*(3), 223–233. https://doi.org/10.1111/j.1365-3083.2008.02211.x
39. Chijiiwa, R., Hosokawa, M., Kogawa, M., Nishikawa, Y., Ide, K., Sakanashi, C., Takahashi, K., & Takeyama, H. (2020). Single-cell genomics of uncultured bacteria reveals dietary fiber responders in the mouse gut microbiota. *Microbiome*, *8*(1), 1–14. https://doi.org/10.1186/s40168-019-0779-2
40. Chowdhury, S. R., King, D. E., Willing, B. P., Band, M. R., Beever, J. E., Lane, A. B., Loor, J. J., Marini, J. C., Rund, L. A., Schook, L. B., van Kessel, A. G., & Rex, H. R. (2007). Transcriptome profiling of the small intestinal epithelium in germfree versus conventional piglets. *BMC Genomics*, *8*. https://doi.org/10.1186/1471-2164-8-215
41. Chu, D. M., Ma, J., Prince, A. L., Antony, K. M., Seferovic, M. D., & Aagaard, K. M. (2017). Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. *Nature Medicine*, *23*(3), 314–326. https://doi.org/10.1038/nm.4272
42. Chung, H., Pamp, S. J., Hill, J. A., Surana, N. K., Edelman, S. M., Troy, E. B., Reading, N. C., Villablanca, E. J., Wang, S., Mora, J. R., Umesaki, Y., Mathis, D., Benoist, C., Relman, D. A., & Kasper, D. L. (2012). Gut immune maturation depends on colonization with a host-specific microbiota. *Cell*, *149*(7), 1578–1593. https://doi.org/10.1016/j.cell.2012.04.037
43. Clark, M. A., Jepson, M. A., Simmons, N. L., & Hirst, B. H. (1994). Preferential interaction of Salmonella typhimurium with mouse Peyer's patch M cells. *Res. Microbiol*, *145*, 543–552.
44. Conlan, J. W. (1996). Neutrophils Prevent Extracellular Colonization of the Liver Microvasculature by Salmonella typhimurium. *Infection and Immunity*, *64*(3), 1043–1047. https://journals.asm.org/journal/iai
45. Costa, M. O., Fouhse, J., Silva, A. P. P., Willing, B., & Harding, J. C. S. (2020). Putting the microbiota to work: Epigenetic effects of early life antibiotic treatment are associated with immune-related pathways and reduced epithelial necrosis following Salmonella

Typhimurium challenge in vitro. *PLoS ONE*, *15*(4). https://doi.org/10.1371/journal.pone.0231942

46. Costea, P. I., Zeller, G., Sunagawa, S., Pelletier, E., Alberti, A., Levenez, F., Tramontano, M., Driessen, M., Hercog, R., Jung, F. E., Kultima, J. R., Hayward, M. R., Coelho, L. P., Allen-Vercoe, E., Bertrand, L., Blaut, M., Brown, J. R. M., Carton, T., Cools-Portier, S., … Bork, P. (2017). Towards standards for human fecal sample processing in metagenomic studies. *Nature Biotechnology*, *35*(11), 1069–1076. https://doi.org/10.1038/nbt.3960
47. Creevey, C. J., Kelly, W. J., Henderson, G., & Leahy, S. C. (2014). Determining the culturability of the rumen bacterial microbiome. *Microbial Biotechnology*, *7*(5), 467–479. https://doi.org/10.1111/1751-7915.12141
48. Crhanova, M., Karasova, D., Juricova, H., Matiasovicova, J., Jahodarova, E., Kubasova, T., Seidlerova, Z., Cizek, A., & Rychlik, I. (2019). Systematic culturomics shows that half of chicken caecal microbiota members can be grown in vitro except for two lineages of Clostridiales and a single lineage of Bacteroidetes. *Microorganisms*, *7*(11). https://doi.org/10.3390/microorganisms7110496
49. Curran, M., Boothe, D. M., Hathcock, T. L., & Lee-Fowler, T. (2020). Analysis of the effects of storage temperature and contamination on aerobic bacterial culture results of bronchoalveolar lavage fluid. *Journal of Veterinary Internal Medicine*, *34*(1), 160–165. https://doi.org/10.1111/jvim.15686
50. Curtis, T. P., Sloan, W. T., & Scannell, J. W. (2002). *Estimating prokaryotic diversity and its limits*. www.pnas.orgcgidoi10.1073pnas.142680199
51. Dawson, H. D., Reece, J. J., & Urban, J. F. (2011). A comparative assessment of the pig, mouse and human genomes. *The Minipig in Biomedical Research*, *128*(1–3), 323–342. https://doi.org/10.1016/j.vetimm.2008.10.211
52. de Filippo, C., Cavalieri, D., di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., Collini, S., Pieraccini, G., & Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(33), 14691–14696. https://doi.org/10.1073/pnas.1005963107
53. de Meester, L., Vanoverbeke, J., Kilsdonk, L. J., & Urban, M. C. (2016). Evolving Perspectives on Monopolization and Priority Effects. *Trends in Ecology & Evolution*, *31*(2), 136–146. https://doi.org/10.1016/J.TREE.2015.12.009
54. de Rodas, B., Youmans, B. P., Danzeisen, J. L., Tran, H., & Johnson, T. J. (2018). Microbiome profiling of commercial pigs from farrow to finish. *Journal of Animal Science*, *96*(5), 1778–1794. https://doi.org/10.1093/JAS/SKY109
55. del Chierico, F., Vernocchi, P., Petrucca, A., Paci, P., Fuentes, S., Praticò, G., Capuani, G., Masotti, A., Reddel, S., Russo, A., Vallone, C., Salvatori, G., Buffone, E., Signore, F., Rigon, G., Dotta, A., Miccheli, A., de Vos, W. M., Dallapiccola, B., & Putignani, L. (2015). Phylogenetic and Metabolic Tracking of Gut Microbiota during Perinatal Development. *PLoS ONE*, *10*(9), 0137347. https://doi.org/10.1371/journal.pone.0137347
56. DeMarco, A. L., Rabe, L. K., Austin, M. N., Stoner, K. A., Avolia, H. A., Meyn, L. A., & Hillier, S. L. (2017). Survival of vaginal microorganisms in three commercially available transport systems. *Anaerobe*, *45*, 44–49. https://doi.org/10.1016/j.anaerobe.2017.02.019

57. Deriu, E., Liu, J. Z., Pezeshki, M., Edwards, R. A., Ochoa, R. J., Contreras, H., Libby, S. J., Fang, F. C., & Raffatellu, M. (2013). Probiotic bacteria reduce salmonella typhimurium intestinal colonization by competing for iron. *Cell Host and Microbe*, *14*(1), 26–37. https://doi.org/10.1016/j.chom.2013.06.007
58. Derrien, M., Vaughan, E. E., Plugge, C. M., & de Vos, W. M. (2004). Akkermansia municiphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology*, *54*(5), 1469–1476. https://doi.org/10.1099/ijs.0.02873-0
59. Diakite, A., Dubourg, G., Dione, N., Afouda, P., Bellali, S., Ngom, I. I., Valles, C., Tall, M. L., Lagier, J. C., & Raoult, D. (2020). Optimization and standardization of the culturomics technique for human microbiome exploration. *Scientific Reports*, *10*(1), 9674. https://doi.org/10.1038/s41598-020-66738-8
60. Dione, N., Khelaifia, S., la Scola, B., Lagier, J. C., & Raoult, D. (2016). A quasi-universal medium to break the aerobic/anaerobic bacterial culture dichotomy in clinical microbiology. *Clinical Microbiology and Infection*, *22*(1), 53–58. https://doi.org/10.1016/j.cmi.2015.10.032
61. Duar, R. M., Frese, S. A., Lin, X. B., Fernando, S. C., Burkey, T. E., Tasseva, G., Peterson, D. A., Blom, J., Wenzel, C. Q., Szymanski, C. M., & Walter, J. (2017). Experimental evaluation of host adaptation of Lactobacillus reuteri to different vertebrate species. *Applied and Environmental Microbiology*, *83*(12). https://doi.org/10.1128/AEM.00132-17
62. Dubourg, G., Lagier, J. C., Armougom, F., Robert, C., Hamad, I., Brouqui, P., & Raoult, D. (2013). The gut microbiota of a patient with resistant tuberculosis is more comprehensively studied by culturomics than by metagenomics. *European Journal of Clinical Microbiology and Infectious Diseases*, *32*(5), 637–645. https://doi.org/10.1007/s10096-012-1787-3
63. Dubourg, G., Lagier, J. C., Robert, C., Armougom, F., Hugon, P., Metidji, S., Dione, N., Dangui, N. P. M., Pfleiderer, A., Abrahao, J., Musso, D., Papazian, L., Brouqui, P., Bibi, F., Yasir, M., Vialettes, B., & Raoult, D. (2014). Culturomics and pyrosequencing evidence of the reduction in gut microbiota diversity in patients with broad-spectrum antibiotics. *International Journal of Antimicrobial Agents*, *44*(2), 117–124. https://doi.org/10.1016/j.ijantimicag.2014.04.020
64. Duncan, S. H., Aminov, R. I., Scott, K. P., Louis, P., Stanton, T. B., & Flint, H. J. (2006). Proposal of Roseburia faecis sp. nov., Roseburia hominis sp. nov. and Roseburia inulinivorans sp. nov., based on isolates from human faeces. *International Journal of Systematic and Evolutionary Microbiology*, *56*(10), 2437–2441. https://doi.org/10.1099/ijs.0.64098-0
65. Duncan, S. H., Hold, G. L., Harmsen, H. J. M., Stewart, C. S., & Flint, H. J. (2002). Growth requirements and fermentation products of Fusobacterium prausnitzii, and a proposal to reclassify it as Faecalibacterium prausnitzii gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, *52*(6), 2141–2146. https://doi.org/10.1099/ijs.0.02241-0
66. Erkosar, B., Storelli, G., Defaye, A., & Leulier, F. (2013). Host-Intestinal Microbiota Mutualism: "Learning on the Fly." *Cell Host & Microbe*, *13*(1), 8–14. https://doi.org/10.1016/J.CHOM.2012.12.004

67. Esteban, M., Marcos, P., Horna, C., Galan-Malo, P., Mata, L., Pérez, M. D., Calvo, M., & Sánchez, L. (2020). Evaluation of methods for DNA extraction from Clostridium tyrobutyricum spores and its detection by qPCR. *Journal of Microbiological Methods*, *169*. https://doi.org/10.1016/j.mimet.2019.105818
68. Everest, P., Allen, J., Papakonstantinopoulou, A., Mastroeni, P., Roberts, M., & Dougan, G. (2021). IL-4 in infection-associated pathology. deficient in interleukin-4 production: role of Salmonella typhimurium infections in mice. *The Journal of Immunology*, *159*(4), 1820–1827. http://www.jimmunol.org/content/159/4/
69. Fallani, M., Amarri, S., Uusijarvi, A., Adam, R., Khanna, S., Aguilera, M., Gil, A., Vieites, J. M., Norin, E., Young, D., Scott, J. A., Doré, J., & Edwards, C. A. (2011). Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. *Microbiology*, *157*(5), 1385–1392. https://doi.org/10.1099/MIC.0.042143-0/CITE/REFWORKS
70. Falony, G., Joossens, M., Vieira-Silva, S., Wang, J., Darzi, Y., Faust, K., Kurilshikov, A., Bonder, M. J., Valles-Colomer, M., Vandeputte, D., Tito, R. Y., Chaffron, S., Rymenans, L., Verspecht, C., Sutter, L. de, Lima-Mendez, G., D'hoe, K., Jonckheere, K., Homola, D., … Raes, J. (2016). Population-level analysis of gut microbiome variation. *Science*, *352*(6285), 560–564. https://doi.org/10.1126/SCIENCE.AAD3503/SUPPL_FILE/TABLE_S9.XLSX
71. Farris, M. H., & Olson, J. B. (2007). Detection of Actinobacteria cultivated from environmental samples reveals bias in universal primers. *Letters in Applied Microbiology*, *45*(4), 376–381. https://doi.org/10.1111/j.1472-765X.2007.02198.x
72. Fenske, G. J., Ghimire, S., Antony, L., Christopher-Hennings, J., & Scaria, J. (2020). Integration of culture-dependent and independent methods provides a more coherent picture of the pig gut microbiome. *FEMS Microbiology Ecology*, *96*(3). https://doi.org/10.1093/femsec/fiaa022
73. Forster, S. C., Kumar, N., Anonye, B. O., Almeida, A., Viciani, E., Stares, M. D., Dunn, M., Mkandawire, T. T., Zhu, A., Shao, Y., Pike, L. J., Louie, T., Browne, H. P., Mitchell, A. L., Neville, B. A., Finn, R. D., & Lawley, T. D. (2019a). A human gut bacterial genome and culture collection for improved metagenomic analyses. *Nature Biotechnology*, *37*(2), 186–192. https://doi.org/10.1038/s41587-018-0009-7
74. Forster, S. C., Kumar, N., Anonye, B. O., Almeida, A., Viciani, E., Stares, M. D., Dunn, M., Mkandawire, T. T., Zhu, A., Shao, Y., Pike, L. J., Louie, T., Browne, H. P., Mitchell, A. L., Neville, B. A., Finn, R. D., & Lawley, T. D. (2019b). A human gut bacterial genome and culture collection for improved metagenomic analyses. *Nature Biotechnology*, *37*(2), 186–192. https://doi.org/10.1038/s41587-018-0009-7
75. Fouhse, J. M., Yang, K., More-Bayona, J., Gao, Y., Goruk, S., Plastow, G., Field, C. J., Barreda, D. R., & Willing, B. P. (2019). Neonatal Exposure to Amoxicillin Alters Long-Term Immune Response Despite Transient Effects on Gut-Microbiota in Piglets. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.02059
76. Fouhse, J. M., Zijlstra, R. T., & Willing, B. P. (2016). The role of gut microbiota in the health and disease of pigs. *Animal Frontiers*, *6*(3), 30–36. https://doi.org/10.2527/af.2016-0031
77. Fouhy, F., Deane, J., Rea, M. C., O'Sullivan, Ó., Ross, R. P., O'Callaghan, G., Plant, B. J., & Stanton, C. (2015). The effects of freezing on faecal microbiota as determined using

miseq sequencing and culture-based investigations. *PLoS ONE*, *10*(3). https://doi.org/10.1371/journal.pone.0119355
78. Franchi, L., Eigenbrod, T., Muñoz-Planillo, R., & Nuñez, G. (2009). The inflammasome: A caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nature Immunology*, *10*(3), 241–247. https://doi.org/10.1038/ni.1703
79. Franke, T., & Deppenmeier, U. (2018). Physiology and central carbon metabolism of the gut bacterium Prevotella copri. *Molecular Microbiology*, *109*(4), 528–540. https://doi.org/10.1111/MMI.14058
80. Frese, S. A., Parker, K., Calvert, C. C., & Mills, D. A. (2015). Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome*, *3*(1), 1–10. https://doi.org/10.1186/s40168-015-0091-8
81. Fukami, T. (2015). Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects. *Http://Dx.Doi.Org/10.1146/Annurev-Ecolsys-110411-160340*, *46*, 1–23. https://doi.org/10.1146/ANNUREV-ECOLSYS-110411-160340
82. Furter, M., Sellin, M. E., Hansson, G. C., & Hardt, W. D. (2019). Mucus Architecture and Near-Surface Swimming Affect Distinct Salmonella Typhimurium Infection Patterns along the Murine Intestinal Tract. *Cell Reports*, *27*(9), 2665-2678.e3. https://doi.org/10.1016/j.celrep.2019.04.106
83. Furuse, M., & Okumura, J. (1994). Nutritional and physiological characteristics in germ-free chickens. *Comparative Biochemistry and Physiology Part A: Physiology*, *109*(3), 547–556. https://doi.org/10.1016/0300-9629(94)90193-7
84. Gal-Mor, O., Boyle, E. C., & Grassl, G. A. (2014). Same species, different diseases: How and why typhoidal and non-typhoidal Salmonella enterica serovars differ. *Frontiers in Microbiology*, *5*(AUG). https://doi.org/10.3389/fmicb.2014.00391
85. Gogoi, M., Shreenivas, M. M., & Chakravortty, D. (2019). Hoodwinking the Big-Eater to Prosper: The Salmonella -Macrophage Paradigm. *Journal of Innate Immunity*, *11*(3), 289–299. https://doi.org/10.1159/000490953
86. Gonzalez, L. M., Moeser, A. J., & Blikslager, A. T. (2015). Porcine models of digestive disease: the future of large animal translational research. *Translational Research*, *166*(1), 12–27. https://doi.org/10.1016/J.TRSL.2015.01.004
87. Goodman, A. L., Kallstrom, G., Faith, J. J., Reyes, A., Moore, A., Dantas, G., & Gordon, J. I. (2011). Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(15), 6252–6257. https://doi.org/10.1073/pnas.1102938108
88. Govoni, G., Vidal, S., Gauthier, S., Skamene, E., Malo, D., & Gros, P. (1996). The Bcg/Ity/Lsh Locus: Genetic Transfer of Resistance to Infections in C57BL/6J Mice Transgenic for the Nramp1 Gly169 Allele. In *Infection and Immunity* (Vol. 64, Issue 8).
89. Greub, G. (2012). Culturomics: A new approach to study the human microbiome. In *Clinical Microbiology and Infection* (Vol. 18, Issue 12, pp. 1157–1159). Blackwell Publishing Ltd. https://doi.org/10.1111/1469-0691.12032
90. Guevarra, R. B., Lee, J. H., Lee, S. H., Seok, M. J., Kim, D. W., Kang, B. N., Johnson, T. J., Isaacson, R. E., & Kim, H. B. (2019). Piglet gut microbial shifts early in life: Causes

and effects. *Journal of Animal Science and Biotechnology*, *10*(1), 1–10. https://doi.org/10.1186/s40104-018-0308-3
91. Guilhot, E., Khelaifia, S., la Scola, B., Raoult, Di., & Dubourg, G. (2018). Methods for culturing anaerobes from human specimen. *Future Microbiology*, *13*(3), 369–381. https://doi.org/10.2217/fmb-2017-0170
92. Gulig, P. A., Doyle, T. J., Clare-Salzler, M. J., Maiese, R. L., And, †, & Matsui, H. (1997). Systemic Infection of Mice by Wild-Type but Not Spv Salmonella typhimurium Is Enhanced by Neutralization of Gamma Interferon and Tumor Necrosis Factor Alpha. *Infection and Immunity*, *65*(12), 5191–5197. https://journals.asm.org/journal/iai
93. Gupta, S., Mortensen, M. S., Schjørring, S., Trivedi, U., Vestergaard, G., Stokholm, J., Bisgaard, H., Krogfelt, K. A., & Sørensen, S. J. (2019). Amplicon sequencing provides more accurate microbiome information in healthy children compared to culturing. *Communications Biology*, *2*(1). https://doi.org/10.1038/s42003-019-0540-1
94. Hansen, A. K., Hansen, C. H. F., Krych, L., & Nielsen, D. S. (2014). Impact of the gut microbiota on rodent models of human disease. *World Journal of Gastroenterology*, *20*(47), 17727–17736. https://doi.org/10.3748/wjg.v20.i47.17727
95. Haverson, K., Rehakova, Z., Sinkora, J., Sver, L., & Bailey, M. (2007). Immune development in jejunal mucosa after colonization with selected commensal gut bacteria: A study in germ-free pigs. *Veterinary Immunology and Immunopathology*, *119*(3–4), 243–253. https://doi.org/10.1016/j.vetimm.2007.05.022
96. Hayashi, H., Shibata, K., Sakamoto, M., Tomita, S., & Benno, Y. (2007). Prevotella copri sp. nov. and Prevotella stercorea sp. nov., isolated from human faeces. *International Journal of Systematic and Evolutionary Microbiology*, *57*(5), 941–946. https://doi.org/10.1099/ijs.0.64778-0
97. Hess, J., Ladel, C., Miko, D., & Kaufmann, S. H. (1996). independent of intracellular location. IFN-gamma in bacterial clearance role of CD4+ TCR-alpha beta cells and gene-targeted immunodeficient mice: major Salmonella typhimurium aroA-infection in. *The Journal of Immunology*, *156*(9), 3321–3326. http://www.jimmunol.org/content/156/9/3321
98. Hoefman, S., van Hoorde, K., Boon, N., Vandamme, P., de Vos, P., & Heylen, K. (2012). Survival or revival: Long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria. *PLoS ONE*, *7*(4). https://doi.org/10.1371/journal.pone.0034196
99. Holman, D. B., Brunelle, B. W., Trachsel, J., & Allen, H. K. (2017). Meta-analysis To Define a Core Microbiota in the Swine Gut. *MSystems*, *2*(3), 1–14. https://doi.org/10.1128/msystems.00004-17
100. Hopkins, S. A., Niedergang, F., Corthesy-Theulaz, I. E., & Kraehenbuhl, J.-P. (2000). A recombinant Salmonella typhimurium vaccine strain is taken up and survives within murine Peyer's patch dendritic cells. *Cellular Microbiology*, *2*(1), 59–68.
101. Hörmannsperger, G., Schaubeck, M., & Haller, D. (2015). Intestinal microbiota in animal models of inflammatory diseases. *ILAR Journal*, *56*(2), 179–191. https://doi.org/10.1093/ilar/ilv019
102. Hsieh, C.-S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'garra, A., Murphy, K. M., Hsieh, C.-S., Tripp, C. S., Murphy, K. M., Macatonia, S. E., O'garra, A., Research, D., & Wolf, S. F. (1993). Development of TH1 CD4' T Cells Through IL-12 Produced by

Listeria-lnduced Macrophages. *Science*, *260*(5107), 547–549. http://science.sciencemag.org/
103. Huang, T., Huang, X., Shi, B., Wang, F., Feng, W., & Yao, M. (2018). Regulators of Salmonella-host interaction identified by peripheral blood transcriptome profiling: Roles of TGFB1 and TRP53 in intracellular Salmonella replication in pigs. *Veterinary Research*, *49*(1). https://doi.org/10.1186/s13567-018-0616-9
104. Hubálek, Z. (2003). Protectants used in the cryopreservation of microorganisms. In *Cryobiology* (Vol. 46, Issue 3, pp. 205–229). Academic Press Inc. https://doi.org/10.1016/S0011-2240(03)00046-4
105. Hudault, S., Guignot, J., & Servin, A. L. (2001). Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection. *Gut*, *49*(1), 47–55. https://doi.org/10.1136/gut.49.1.47
106. Hugon, P., Lagier, J. C., Robert, C., Lepolard, C., Papazian, L., Musso, D., Vialettes, B., & Raoult, D. (2013). Molecular studies neglect apparently gram-negative populations in the human gut microbiota. *Journal of Clinical Microbiology*, *51*(10), 3286–3293. https://doi.org/10.1128/JCM.00473-13
107. Hungate, R. E., Smith, W., & Clarke, R. T. J. (1966). Suitability of Butyl Rubber Stoppers for Closing Anaerobic Roll Culture Tubes Reciprocal Recombination of Chromosome and F- Merogenote in Escherichia coli Methyl Dipicolinate Monoester from Spores of Bacillus cereus var. glob igii. *Microbiology*, *91*(2), 908–909.
108. Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J. H., Chinwalla, A. T., Creasy, H. H., Earl, A. M., Fitzgerald, M. G., Fulton, R. S., Giglio, M. G., Hallsworth-Pepin, K., Lobos, E. A., Madupu, R., Magrini, V., Martin, J. C., Mitreva, M., Muzny, D. M., Sodergren, E. J., … White, O. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–214. https://doi.org/10.1038/nature11234
109. Imaoka, A., Setoyama, H., Takagi, A., Matsumoto, S., & Umesaki, Y. (2004). Improvement of human faecal flora-associated mouse model for evaluation of the functional foods. *Journal of Applied Microbiology*, *96*(4), 656–663. https://doi.org/10.1111/j.1365-2672.2004.02189.x
110. Ito, T., Sekizuka, T., Kishi, N., Yamashita, A., & Kuroda, M. (2019). Conventional culture methods with commercially available media unveil the presence of novel culturable bacteria. *Gut Microbes*, *10*(1), 77–91. https://doi.org/10.1080/19490976.2018.1491265/SUPPL_FILE/KGMI_A_1491265_SM8264.ZIP
111. Jakobsson, H. E., Rodríguez-Piñeiro, A. M., Schütte, A., Ermund, A., Boysen, P., Bemark, M., Sommer, F., Bäckhed, F., Hansson, G. C., & Johansson, M. E. (2015). The composition of the gut microbiota shapes the colon mucus barrier. *EMBO Reports*, *16*(2), 164–177. https://doi.org/10.15252/embr.201439263
112. Johanns, T. M., Ertelt, J. M., Rowe, J. H., & Way, S. S. (2010). Regulatory t cell suppressive potency dictates the balance between bacterial proliferation and clearance during persistent Salmonella infection. *PLoS Pathogens*, *6*(8), 31–32. https://doi.org/10.1371/journal.ppat.1001043
113. Kaeberlein, T., Lewis, K., & Epstein, S. S. (2002). Isolating “Uncultivable” Microorganisms in Pure Culture in a Simulated Natural Environment. *Science*, *296*(5570), 1127–1129. www.sciencemag.org/cgi/content/

114. Kennedy, E. A., King, K. Y., & Baldridge, M. T. (2018). Mouse microbiota models: Comparing germ-free mice and antibiotics treatment as tools for modifying gut bacteria. *Frontiers in Physiology*, *9*(OCT). https://doi.org/10.3389/fphys.2018.01534
115. Kenny, D. J., Plichta, D. R., Shungin, D., Koppel, N., Hall, A. B., Fu, B., Vasan, R. S., Shaw, S. Y., Vlamakis, H., Balskus, E. P., & Xavier, R. J. (2020). Cholesterol Metabolism by Uncultured Human Gut Bacteria Influences Host Cholesterol Level. *Cell Host and Microbe*, *28*(2), 245-257.e6. https://doi.org/10.1016/j.chom.2020.05.013
116. Kim, H. B., Borewicz, K., White, B. A., Singer, R. S., Sreevatsan, S., Tu, Z. J., & Isaacson, R. E. (2011). Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Veterinary Microbiology*, *153*(1–2), 124–133. https://doi.org/10.1016/J.VETMIC.2011.05.021
117. Kim, H. B., & Isaacson, R. E. (2015). The pig gut microbial diversity: Understanding the pig gut microbial ecology through the next generation high throughput sequencing. *Veterinary Microbiology*, *177*(3–4), 242–251. https://doi.org/10.1016/j.vetmic.2015.03.014
118. Knight, R., Vrbanac, A., Taylor, B. C., Aksenov, A., Callewaert, C., Debelius, J., Gonzalez, A., Kosciolek, T., McCall, L. I., McDonald, D., Melnik, A. v., Morton, J. T., Navas, J., Quinn, R. A., Sanders, J. G., Swafford, A. D., Thompson, L. R., Tripathi, A., Xu, Z. Z., … Dorrestein, P. C. (2018). Best practices for analysing microbiomes. In *Nature Reviews Microbiology* (Vol. 16, Issue 7, pp. 410–422). Nature Publishing Group. https://doi.org/10.1038/s41579-018-0029-9
119. Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., Angenent, L. T., & Ley, R. E. (2011). Succession of microbial consortia in the developing infant gut microbiome. *Proceedings of the National Academy of Sciences*, *108*, 4578–4585. https://doi.org/10.1073/PNAS.1000081107
120. Krych, L., Hansen, C. H. F., Hansen, A. K., van den Berg, F. W. J., & Nielsen, D. S. (2013). Quantitatively Different, yet Qualitatively Alike: A Meta-Analysis of the Mouse Core Gut Microbiome with a View towards the Human Gut Microbiome. *PLoS ONE*, *8*(5). https://doi.org/10.1371/journal.pone.0062578
121. Kubasova, T., Davidova-Gerzova, L., Merlot, E., Medvecky, M., Polansky, O., Gardan-Salmon, D., Quesnel, H., & Rychlik, I. (2017). Housing Systems Influence Gut Microbiota Composition of Sows but Not of Their Piglets. *PLoS ONE*, *12*(1), e0170051. https://doi.org/10.1371/journal.pone.0170051
122. Kukurba, K. R., & Montgomery, S. B. (2016). RNA sequencing and analysis. *Cold Spring Harbor Protocols*, *2015*(11), 951–969. https://doi.org/10.1101/pdb.top084970
123. Kupz, A., Scott, T. A., Belz, G. T., Andrews, D. M., Greyer, M., Lew, A. M., Brooks, A. G., Smyth, M. J., Curtiss, R., Bedoui, S., & Strugnell, R. A. (2013). Contribution of Thy1+ NK cells to protective IFN- γ production during Salmonella Typhimurium infections. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(6), 2252–2257. https://doi.org/10.1073/pnas.1222047110
124. Kurtz, J. R., Goggins, J. A., & McLachlan, J. B. (2017). Salmonella infection: Interplay between the bacteria and host immune system. *Immunology Letters*, *190*, 42–50. https://doi.org/10.1016/j.imlet.2017.07.006
125. la Scola, B., Khelaifia, S., Lagier, J. C., & Raoult, D. (2014). Aerobic culture of anaerobic bacteria using antioxidants: a preliminary report. *European Journal of Clinical*

Microbiology and Infectious Diseases, *33*(10), 1781–1783. https://doi.org/10.1007/s10096-014-2137-4
126. Lagier, J. C., Armougom, F., Million, M., Hugon, P., Pagnier, I., Robert, C., Bittar, F., Fournous, G., Gimenez, G., Maraninchi, M., Trape, J. F., Koonin, E. v., la Scola, B., & Raoult, D. (2012). Microbial culturomics: Paradigm shift in the human gut microbiome study. *Clinical Microbiology and Infection*, *18*(12), 1185–1193. https://doi.org/10.1111/1469-0691.12023
127. Lagier, J. C., el Karkouri, K., Mishra, A. K., Robert, C., Raoult, D., & Fournier, P. E. (2013). Non contiguous-finished genome sequence and descriptttion of enterobacter massiliensis sp. nov. *Standards in Genomic Sciences*, *7*(3), 399–412. https://doi.org/10.4056/sigs.3396830
128. Lagier, J. C., Hugon, P., Khelaifia, S., Fournier, P. E., la Scola, B., & Raoult, D. (2015). The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. In *Clinical Microbiology Reviews* (Vol. 28, Issue 1, pp. 237–264). American Society for Microbiology. https://doi.org/10.1128/CMR.00014-14
129. Lagier, J. C., Khelaifia, S., Alou, M. T., Ndongo, S., Dione, N., Hugon, P., Caputo, A., Cadoret, F., Traore, S. I., Seck, E. H., Dubourg, G., Durand, G., Mourembou, G., Guilhot, E., Togo, A., Bellali, S., Bachar, D., Cassir, N., Bittar, F., … Raoult, D. (2016). Culture of previously uncultured members of the human gut microbiota by culturomics. *Nature Microbiology*, *1*. https://doi.org/10.1038/nmicrobiol.2016.203
130. Lagkouvardos, I., Lesker, T. R., Hitch, T. C. A., Gálvez, E. J. C., Smit, N., Neuhaus, K., Wang, J., Baines, J. F., Abt, B., Stecher, B., Overmann, J., Strowig, T., & Clavel, T. (2019). Sequence and cultivation study of Muribaculaceae reveals novel species, host preference, and functional potential of this yet undescribed family. *Microbiome*, *7*(1). https://doi.org/10.1186/s40168-019-0637-2
131. Lagkouvardos, I., Overmann, J., & Clavel, T. (2017). Cultured microbes represent a substantial fraction of the human and mouse gut microbiota. *Gut Microbes*, *8*(5), 493–503. https://doi.org/10.1080/19490976.2017.1320468
132. Lagkouvardos, I., Pukall, R., Abt, B., Foesel, B. U., Meier-Kolthoff, J. P., Kumar, N., Bresciani, A., Martínez, I., Just, S., Ziegler, C., Brugiroux, S., Garzetti, D., Wenning, M., Bui, T. P. N., Wang, J., Hugenholtz, F., Plugge, C. M., Peterson, D. A., Hornef, M. W., … Clavel, T. (2016). The Mouse Intestinal Bacterial Collection (miBC) provides host-specific insight into cultured diversity and functional potential of the gut microbiota. *Nature Microbiology*, *1*. https://doi.org/10.1038/nmicrobiol.2016.131
133. Lalmanach, A.-C., & Lantier, F. (1999). Host cytokine response and resistance to Salmonella infection. *Microbes and Infection*, *1*(9), 719–726.
134. Lamendella, R., Santo Domingo, J. W., Ghosh, S., Martinson, J., & Oerther, D. B. (2011). Comparative fecal metagenomics unveils unique functional capacity of the swine gut. *BMC Microbiology*, *11*(1), 1–17. https://doi.org/10.1186/1471-2180-11-103/FIGURES/6
135. Lau, J. T., Whelan, F. J., Herath, I., Lee, C. H., Collins, S. M., Bercik, P., & Surette, M. G. (2016). Capturing the diversity of the human gut microbiota through culture-enriched molecular profiling. *Genome Medicine*, *8*(1). https://doi.org/10.1186/s13073-016-0327-7
136. Laursen, M. F., Andersen, L. B. B., Michaelsen, K. F., Mølgaard, C., Trolle, E., Bahl, M. I., & Licht, T. R. (2016). Infant Gut Microbiota Development Is Driven by Transition to

Family Foods Independent of Maternal Obesity. *MSphere*, *1*(1), 00069–15. https://doi.org/10.1128/msphere.00069-15
137. Lawley, T. D., Bouley, D. M., Hoy, Y. E., Gerke, C., Relman, D. A., & Monack, D. M. (2008). Host transmission of Salmonella enterica serovar Typhimurium is controlled by virulence factors and indigenous intestinal microbiota. *Infection and Immunity*, *76*(1), 403–416. https://doi.org/10.1128/IAI.01189-07
138. Lawley, T. D., & Walker, A. W. (2013). Intestinal colonization resistance. *Immunology*, *138*(1), 1–11. https://doi.org/10.1111/J.1365-2567.2012.03616.X
139. Lee, S. J., Dunmire, S., & McSorley, S. J. (2012). MHC class-I-restricted CD8 T cells play a protective role during primary Salmonella infection. *Immunology Letters*, *148*(2), 138–143. https://doi.org/10.1016/j.imlet.2012.10.009
140. Ley, R. E., Bä Ckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., & Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences*, *102*(31), 11070–11075. www.arb-home.de
141. Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., & Gordon, J. I. (2008). Worlds within worlds: Evolution of the vertebrate gut microbiota. *Nature Reviews Microbiology*, *6*(10), 776–788. https://doi.org/10.1038/nrmicro1978
142. Li, F., Hitch, T. C. A., Chen, Y., Creevey, C. J., & Guan, L. L. (2019). Comparative metagenomic and metatranscriptomic analyses reveal the breed effect on the rumen microbiome and its associations with feed efficiency in beef cattle 06 Biological Sciences 0604 Genetics 06 Biological Sciences 0605 Microbiology. *Microbiome*, *7*(6), 1–21. https://doi.org/10.1186/s40168-019-0618-5
143. Lim, E. S., Wang, D., & Holtz, L. R. (2016). The Bacterial Microbiome and Virome Milestones of Infant Development. *Trends in Microbiology*, *24*(10), 801–810. https://doi.org/10.1016/J.TIM.2016.06.001
144. Liu, Y., Zheng, Z., Yu, L., Wu, S., Sun, L., Wu, S., Xu, Q., Cai, S., Qin, N., & Bao, W. (2019). Examination of the temporal and spatial dynamics of the gut microbiome in newborn piglets reveals distinct microbial communities in six intestinal segments. *Scientific Reports*, *9*(1), 1–8. https://doi.org/10.1038/s41598-019-40235-z
145. Lloyd, K. G., Steen, A. D., Ladau, J., Yin, J., & Crosby, L. (2018). Phylogenetically Novel Uncultured Microbial Cells Dominate Earth Microbiomes. *MSystems*, *3*(5), e00055-18. https://doi.org/10.1128/msystems.00055-18
146. Lo, W., Ong, H., … E. M.-T. J. of, & 1999, U. (1999). T cell responses to Gram-negative intracellular bacterial pathogens: a role for CD8+ T cells in immunity to Salmonella infection and the involvement of MHC class Ib. *The Journal of Immunology*, *162*(9), 5398–5406. https://www.jimmunol.org/content/162/9/5398.short
147. Locey, K. J., & Lennon, J. T. (2016). Scaling laws predict global microbial diversity. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(21), 5970–5975. https://doi.org/10.1073/pnas.1521291113
148. Loesche', W. J. (1969). Oxygen Sensitivity of Various Anaerobic Bacteria. In *Applied Microbiology* (Vol. 18, Issue 5).
149. Loman, N. J., Constantinidou, C., Christner, M., Chan, J. Z. M., Quick, J., Weir, J. C., Quince, C., Smith, G. P., Betley, J. R., Aepfelbacher, M., & Pallen, M. J. (2013). A culture-independent sequence-based metagenomics approach to the investigation of an

outbreak of Shiga-toxigenic Escherichia coli O104:H4. *JAMA*, *309*(14), 1502–1510. https://doi.org/10.1001/JAMA.2013.3231
150. Looft, T., Johnson, T. A., Allen, H. K., Bayles, D. O., Alt, D. P., Stedtfeld, R. D., Sul, W. J., Stedtfeld, T. M., Chai, B., Cole, J. R., Hashsham, S. A., Tiedje, J. M., & Stanton, T. B. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(5), 1691–1696. https://doi.org/10.1073/pnas.1120238109
151. Loomis, W. P., Delaney, M. A., Johnson, M. L., & Cookson, B. T. (2021). Failure of CD4 T cell-deficient hosts to control chronic nontyphoidal salmonella infection leads to exacerbated inflammation, chronic anemia, and altered myelopoiesis. *Infection and Immunity*, *89*(1). https://doi.org/10.1128/IAI.00417-20
152. Lotz, M., Gütle, D., Walther, S., Ménard, S., Bogdan, C., & Hornef, M. W. (2006). Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. *Journal of Experimental Medicine*, *203*(4), 973–984. https://doi.org/10.1084/jem.20050625
153. Mach, N; Berri, M; Estellé, J; Levenez, F; Lemonnier, G; Denis, C; Leplat, J.J; Chevaleyre, C; Billon, Y; Doré, J; Rogel-Gaillard, C. (2015). Mach et al., 2015 Early-life establishment of the swine gut microbiome and impact on host phenotypes.pdf. *Environmental Microbiology Reports*, *7*(3), 554–569.
154. Maclennan, C., Fieschi, C., Lammas, D. A., Picard, C., Dorman, S. E., Sanal, O., Maclennan, J. M., Holland, S. M., Ottenhoff, T. H. M., Casanova, J.-L., & Kumararatne, D. S. (2004). Interleukin (IL)-12 and IL-23 Are Key Cytokines for Immunity against Salmonella in Humans. *The Journal of Infectious Diseases*, *190*, 1755–1762. https://academic.oup.com/jid/article/190/10/1755/2191509
155. Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J., & Woese, C. R. (1997). The RDP (Ribosomal Database Project). *Nucleic Acids Research*, *25*(1), 109–110. https://doi.org/10.1093/NAR/25.1.109
156. Manetti, R., Parronchi, P., Giudizi, M. G., Piccinni, M.-P., Maggi, E., Trinchieri, G., & Omagnani, S. P. (1993). Natural Killer Cell Stimulatory Factor (Interleukin 12 [11,12]) Induces T Helper Type 1 (Thl)-specific Immune Responses and Inhibits the Development of ID4-producing Th Cells. *The Journal of Experimental Medicine*, *177*(4), 1199–1204. http://rupress.org/jem/article-pdf/177/4/1199/1103637/1199.pdf
157. Marcobal, A., Barboza, M., Sonnenburg, E. D., Pudlo, N., Martens, E. C., Desai, P., Lebrilla, C. B., Weimer, B. C., Mills, D. A., German, J. B., & Sonnenburg, J. L. (2011). Bacteroides in the Infant Gut Consume Milk Oligosaccharides via Mucus-Utilization Pathways. *Cell Host & Microbe*, *10*(5), 507–514. https://doi.org/10.1016/J.CHOM.2011.10.007
158. Mastroeni, P., Grant, A., Restif, O., & Maskell, D. (2009). A dynamic view of the spread and intracellular distribution of Salmonella enterica. *Nature Reviews Microbiology 2009 7:1*, *7*(1), 73–80. https://doi.org/10.1038/nrmicro2034
159. Mastroeni, P., Simmons, C., Fowler, R., Hormaeche, C. E., & Dougan, G. (2000). Igh-6 / (B-Cell-Deficient) Mice Fail To Mount Solid Acquired Resistance to Oral Challenge with Virulent Salmonella enterica Serovar Typhimurium and Show Impaired Th1 T-Cell Responses to Salmonella Antigens. *Infection and Immunity*, *68*(1), 46–53.
160. Mata, L. J., Carrillo, C., & Villatoro, E. (1969). Fecal Microflora in Healthy Persons in a Preindustrial Region. In *Applied Microbiology* (Vol. 17, Issue 4).

161. Matamoros, S., Gras-Leguen, C., le Vacon, F., Potel, G., & de La Cochetiere, M. F. (2013). Development of intestinal microbiota in infants and its impact on health. *Trends in Microbiology*, *21*(4), 167–173. https://doi.org/10.1016/J.TIM.2012.12.001
162. McAleer, J. P., & Vella, A. T. (2008). Understanding how lipopolysaccharide impacts CD4 T-cell immunity. *Critical Reviews™ in Immunology*, *28*(4).
163. McGeachy, M. J., & McSorley, S. J. (2012). Microbial-Induced Th17: Superhero or Supervillain? *The Journal of Immunology*, *189*(7), 3285–3291. https://doi.org/10.4049/jimmunol.1201834
164. Mcsorley, S. J., & Jenkins, M. K. (2000). Antibody Is Required for Protection against Virulent but Not Attenuated Salmonella enterica Serovar Typhimurium. *Infection and Immunity*, *68*(6), 3344–3348.
165. Melancon, E., Gomez De La Torre Canny, S., Sichel, S., Kelly, M., Wiles, T. J., Rawls, J. F., Eisen, J. S., & Guillemin, K. (2017). Best practices for germ-free derivation and gnotobiotic zebrafish husbandry. *Methods in Cell Biology*, *138*, 61–100. https://doi.org/10.1016/BS.MCB.2016.11.005
166. Merrifield, C. A., Lewis, M. C., Berger, B., Cloarec, O., Heinzmann, S. S., Charton, F., Krause, L., Levin, N. S., Duncker, S., Mercenier, A., Holmes, E., Bailey, M., & Nicholson, J. K. (2016). Neonatal environment exerts a sustained influence on the development of the intestinal microbiota and metabolic phenotype. *The ISME Journal*, *10*, 145–157. https://doi.org/10.1038/ismej.2015.90
167. Metzker, M. L. (2005). Emerging technologies in DNA sequencing. *Genome Research*, *15*(12), 1767–1776. https://doi.org/10.1101/gr.3770505
168. Miki, T., Goto, R., Fujimoto, M., Okada, N., & Hardt, W. D. (2017). The Bactericidal Lectin RegIIIβ Prolongs Gut Colonization and Enteropathy in the Streptomycin Mouse Model for Salmonella Diarrhea. *Cell Host & Microbe*, *21*(2), 195–207. https://doi.org/10.1016/J.CHOM.2016.12.008
169. Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., Belzer, C., Delgado Palacio, S., Arboleya Montes, S., Mancabelli, L., Lugli, G. A., Rodriguez, J. M., Bode, L., de Vos, W., Gueimonde, M., Margolles, A., van Sinderen, D., & Ventura, M. (2017). The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiology and Molecular Biology Reviews*, *81*(4), 1–67. https://doi.org/10.1128/mmbr.00036-17
170. Mittrucker, H. W., & Kaufmann, S. H. E. (2000). Immune response to infection with Salmonella typhimurium in mice. *Journal of Leukocyte Biology*, *67*(4), 457–463. https://doi.org/10.1002/jlb.67.4.457
171. Mittrücker, H.-W., Raupach, B., Köhler, A., & Kaufmann, S. H. E. (2000). Cutting Edge: Role of B Lymphocytes in Protective Immunity Against Salmonella typhimurium Infection. *The Journal of Immunology*, *164*(4), 1648–1652. https://doi.org/10.4049/jimmunol.164.4.1648
172. Moeller, A. H., Caro-Quintero, A., Mjungu, D., Georgiev, A. v., Lonsdorf, E. v., Muller, M. N., Pusey, A. E., Peeters, M., Hahn, B. H., & Ochman, H. (2016). Cospeciation of gut microbiota with hominids. *Science*, *353*(6297), 380–382. https://doi.org/10.1126/science.aaf3951
173. Monack, D. M., Bouley, D. M., & Falkow, S. (2004). Salmonella typhimurium Persists within Macrophages in the Mesenteric Lymph Nodes of Chronically Infected Nramp1+/+

Mice and Can Be Reactivated by IFNγ Neutralization. *Journal of Experimental Medicine*, *199*(2), 231–241. https://doi.org/10.1084/jem.20031319
174. Moote, P. E., Bescucci, D. M., Polo, R. O., Uwiera, R. R. E., & Douglas Inglis, G. (2021). Comparison of Strategies for Isolating Anaerobic Bacteria from the Porcine Intestine. *Applied and Environmental Microbiology*, *87*(9), e00088-21. https://doi.org/10.1128/AEM
175. Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., & Erlich, H. (1992). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Biotechnology (Reading, Mass.)*, *24*, 17–27.
176. Musser, J. M. B., & Gonzalez, R. (2011). Efficacy of an anaerobic swab transport system to maintain aerobic and anaerobic microorganism viability after storage at -80°C. *Journal of Veterinary Diagnostic Investigation*, *23*(1), 95–99. https://doi.org/10.1177/104063871102300115
177. Nagpal, R., Wang, S., Solberg Woods, L. C., Seshie, O., Chung, S. T., Shively, C. A., Register, T. C., Craft, S., McClain, D. A., & Yadav, H. (2018). Comparative microbiome signatures and short-chain fatty acids in mouse, rat, non-human primate, and human feces. *Frontiers in Microbiology*, *9*(NOV). https://doi.org/10.3389/fmicb.2018.02897
178. Nairz, M., Fritsche, G., Crouch, M. L. v., Barton, H. C., Fang, F. C., & Weiss, G. (2009). Slc11a1 limits intracellular growth of Salmonella enterica sv. Typhimurium by promoting macrophage immune effector functions and impairing bacterial iron acquisition. *Cellular Microbiology*, *11*(9), 1365–1381. https://doi.org/10.1111/j.1462-5822.2009.01337.x
179. Nance, F. C., & Cain, J. L. (1968). Studies of Hemorrhagic Pancreatitis in Germ-Free Dogs. *Gastroenterology*, *55*(3), 368–374. https://doi.org/10.1016/S0016-5085(19)34046-6
180. Nanton, M. R., Way, S. S., Shlomchik, M. J., & McSorley, S. J. (2012). Cutting Edge: B Cells Are Essential for Protective Immunity against Salmonella Independent of Antibody Secretion. *The Journal of Immunology*, *189*(12), 5503–5507. https://doi.org/10.4049/jimmunol.1201413
181. Nauciel, C., & Espinasse-Maes, F. (1992). Role of Gamma Interferon and Tumor Necrosis Factor Alpha in Resistance to Salmonella typhimurium Infection. *Infection and Immunity*, *60*(2), 450–454. https://journals.asm.org/journal/iai
182. Navarro, D. M. D. L., Abelilla, J. J., & Stein, H. H. (2019). Structures and characteristics of carbohydrates in diets fed to pigs: A review. *Journal of Animal Science and Biotechnology*, *10*(1), 1–17. https://doi.org/10.1186/s40104-019-0345-6
183. Nayfach, S., Rodriguez-Mueller, B., Garud, N., & Pollard, K. S. (2016). An integrated metagenomics pipeline for strain profiling reveals novel patterns of bacterial transmission and biogeography. *Genome Research*, *26*(11), 1612–1625. https://doi.org/10.1101/gr.201863.115
184. Nemergut, D. R., Schmidt, S. K., Fukami, T., O'Neill, S. P., Bilinski, T. M., Stanish, L. F., Knelman, J. E., Darcy, J. L., Lynch, R. C., Wickey, P., & Ferrenberg, S. (2013). Patterns and Processes of Microbial Community Assembly. *Microbiology and Molecular Biology Reviews*, *77*(3), 342–356. https://doi.org/10.1128/MMBR.00051-12/FORMAT/EPUB

185. Neves, P., Lampropoulou, V., Calderon-Gomez, E., Roch, T., Stervbo, U., Shen, P., Kühl, A. A., Loddenkemper, C., Haury, M., Nedospasov, S. A., Kaufmann, S. H. E., Steinhoff, U., Calado, D. P., & Fillatreau, S. (2010). Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during salmonella typhimurium infection. *Immunity*, *33*(5), 777–790. https://doi.org/10.1016/j.immuni.2010.10.016
186. Nguyen, T. L. A., Vieira-Silva, S., Liston, A., & Raes, J. (2015). How informative is the mouse for human gut microbiota research? *DMM Disease Models and Mechanisms*, *8*(1), 1–16. https://doi.org/10.1242/dmm.017400
187. Nilsson, O. R., Kari, L., & Steele-Mortimer, O. (2019). Foodborne infection of mice with Salmonella Typhimurium. *PLoS ONE*, *14*(8). https://doi.org/10.1371/journal.pone.0215190
188. Olsen, G. J., Lane, D. J., Giovannoni, S. J., Pace, N. R., & Stahl, D. A. (1986). Microbial Ecology and Evolution: A Ribosomal RNA Approach. *Annual Reviews in Microbiology*, *40*(1), 337–365. https://doi.org/10.1146/ANNUREV.MI.40.100186.002005
189. Ottman, N., Smidt, H., de Vos, W. M., & Belzer, C. (2012). The function of our microbiota: who is out there and what do they do? *Frontiers in Cellular and Infection Microbiology*, *2*, 104. https://doi.org/10.3389/fcimb.2012.00104
190. Pabst, R. (2020). The pig as a model for immunology research. *Cell and Tissue Research*, *380*(2), 287–304. https://doi.org/10.1007/s00441-020-03206-9
191. Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. v., Braden, C. R., Angulo, F. J., & Griffin, P. M. (2013). Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerging Infectious Diseases*, *19*(3), 407–415. https://doi.org/10.3201/eid1903.111866
192. Pajarillo, E. A. B., Chae, J. P., Balolong, M. P., Kim, H. B., & Kang, D. K. (2014). Assessment of fecal bacterial diversity among healthy piglets during the weaning transition. *Journal of General and Applied Microbiology*, *60*(4), 140–146. https://doi.org/10.2323/jgam.60.140
193. Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A., & Brown, P. O. (2007). Development of the human infant intestinal microbiota. *PLoS Biol*, *5*(7), 177. https://doi.org/10.1371/journal.pbio
194. Pang, X., Hua, X., Yang, Q., Ding, D., Che, C., Cui, L., Jia, W., Bucheli, P., & Zhao, L. (2007). Inter-species transplantation of gut microbiota from human to pigs. *ISME Journal*, *1*(2), 156–162. https://doi.org/10.1038/ismej.2007.23
195. Park, D., Lara-Tejero, M., Waxham, M. N., Li, W., Hu, B., Galá, J. E., & Liu, J. (2018). Visualization of the type III secretion mediated Salmonella-host cell interface using cryo-electron tomography. *Elife*, *7*, e39514. https://doi.org/10.7554/eLife.39514.001
196. Park, J. C., & Im, S. H. (2020). Of men in mice: the development and application of a humanized gnotobiotic mouse model for microbiome therapeutics. In *Experimental and Molecular Medicine* (Vol. 52, Issue 9, pp. 1383–1396). Springer Nature. https://doi.org/10.1038/s12276-020-0473-2
197. Park, S.-C., & Won, S. (2018). Evaluation of 16S rRNA Databases for Taxonomic Assignments Using a Mock Community. *Genomics & Informatics*, *16*(4), e24. https://doi.org/10.5808/GI.2018.16.4.E24
198. Pasolli, E., Asnicar, F., Manara, S., Zolfo, M., Karcher, N., Armanini, F., Beghini, F., Manghi, P., Tett, A., Ghensi, P., Collado, M. C., Rice, B. L., DuLong, C., Morgan, X. C.,

Golden, C. D., Quince, C., Huttenhower, C., & Segata, N. (2019). Extensive Unexplored Human Microbiome Diversity Revealed by Over 150,000 Genomes from Metagenomes Spanning Age, Geography, and Lifestyle. *Cell*, *176*(3), 649-662.e20. https://doi.org/10.1016/j.cell.2019.01.001

199. Patel, R., & Sad, S. (2016). Transcription factor Batf3 is important for development of CD8+ T-cell response against a phagosomal bacterium regardless of the location of antigen. *Immunology and Cell Biology*, *94*(4), 378–387. https://doi.org/10.1038/icb.2015.98
200. Pereira, A. C., & Cunha, M. v. (2020). An effective culturomics approach to study the gut microbiota of mammals. *Research in Microbiology*, *171*(8), 290–300. https://doi.org/10.1016/j.resmic.2020.09.001
201. Petersen, L. M., Martin, I. W., Moschetti, W. E., Kershaw, C. M., & Tsongalis, G. J. (2020). Third-Generation Sequencing in the Clinical Laboratory: Sequencing. *Journal of Clinical Microbiology*, *58*(1), e01315-19.
202. Pfleiderer, A., Lagier, J. C., Armougom, F., Robert, C., Vialettes, B., & Raoult, D. (2013). Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample. *European Journal of Clinical Microbiology and Infectious Diseases*, *32*(11), 1471–1481. https://doi.org/10.1007/s10096-013-1900-2
203. Prakash, O., Nimonkar, Y., & Desai, D. (2020). A Recent Overview of Microbes and Microbiome Preservation. In *Indian Journal of Microbiology* (Vol. 60, Issue 3). Springer. https://doi.org/10.1007/s12088-020-00880-9
204. Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., … Zoetendal, E. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*, *464*(7285), 59–65. https://doi.org/10.1038/nature08821
205. Qin, J., Li, R., Raes, J., Arumugam, M., Solvsten Burgdorf, K., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., … Wang, J. (2010). Additional MetaHIT Consortium members Maria Antolin 5 , François Artiguenave 10 , Hervé Blottiere 6 , Natalia Borruel 5 , Thomas Bruls 10 , Francesc Casellas 5 , Christian Chervaux 15 , Antonella Cultrone 6 , Christine Delorme 6 , Gérard Denariaz 15 , Roz. *Michiel Kleerebezem*, *6*(7285), 59–65. https://doi.org/10.1038/nature08821.A
206. Rajilić-Stojanović, M., & de Vos, W. M. (2014). The first 1000 cultured species of the human gastrointestinal microbiota. *FEMS Microbiology Reviews*, *38*(5), 996–1047. https://doi.org/10.1111/1574-6976.12075
207. Rajilić-Stojanović, M., Smidt, H., & de Vos, W. M. (2007). Diversity of the human gastrointestinal tract microbiota revisited. In *Environmental Microbiology* (Vol. 9, Issue 9, pp. 2125–2136). https://doi.org/10.1111/j.1462-2920.2007.01369.x
208. Ramasamy, D., Lagier, J. C., Gorlas, A., Raoult, D., & Fournier, P. E. (2013). Non contiguous-finished genome sequence and description of Bacillus massiliosenegalensis sp. nov. *Standards in Genomic Sciences*, *8*(2), 264–278. https://doi.org/10.4056/sigs.3496989
209. Ravindran, R., Foley, J., Stoklasek, T., Glimcher, L. H., & McSorley, S. J. (2005). Expression of T-bet by CD4 T Cells Is Essential for Resistance to Salmonella Infection.

The Journal of Immunology, *175*(7), 4603–4610. https://doi.org/10.4049/JIMMUNOL.175.7.4603
210. Rebuffat, S. (2012). Microcins in action: Amazing defence strategies of Enterobacteria. *Biochemical Society Transactions*, *40*(6), 1456–1462. https://doi.org/10.1042/BST20120183
211. Reimer, L. C., Vetcininova, A., Carbasse, J. S., Söhngen, C., Gleim, D., Ebeling, C., & Overmann, J. (2019). BacDive in 2019: Bacterial phenotypic data for High-throughput biodiversity analysis. *Nucleic Acids Research*, *47*(D1), D631–D636. https://doi.org/10.1093/nar/gky879
212. Ren, Z., Gay, R., Thomas, A., Pae, M., Wu, D., Logsdon, L., Mecsas, J., & Meydani, S. N. (2009). Effect of age on susceptibility to Salmonella Typhimurium infection in C57BL/6 mice. *Journal of Medical Microbiology*, *58*(12), 1559–1567. https://doi.org/10.1099/jmm.0.013250-0
213. Rettedal, E. A., Gumpert, H., & Sommer, M. O. A. (2014). Cultivation-based multiplex phenotyping of human gut microbiota allows targeted recovery of previously uncultured bacteria. *Nature Communications*, *5*. https://doi.org/10.1038/ncomms5714
214. Rivera-Chávez, F., Zhang, L. F., Faber, F., Lopez, C. A., Byndloss, M. X., Olsan, E. E., Xu, G., Velazquez, E. M., Lebrilla, C. B., Winter, S. E., & Bäumler, A. J. (2016). Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of Salmonella. *Cell Host and Microbe*, *19*(4), 443–454. https://doi.org/10.1016/j.chom.2016.03.004
215. Rodríguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., Avershina, E., Rudi, K., Narbad, A., Jenmalm, M. C., Marchesi, J. R., & Collado, M. C. (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. *Microbial Ecology in Health and Disease*, *26*(1), 26050. https://doi.org/10.3402/mehd.v26.26050
216. Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., Costea, P., Godneva, A., Kalka, iris N., Bar, N., Shilo, S., Lador, D., Vich Vila, A., Zmora, N., Pevsner-Fischer, M., Israeli, D., Kosower, N., Malka, G., chen Wolf, B., … Segal, E. (2018). Environment dominates over host genetics in shaping human gut microbiota. *Nature Publishing Group*, *555*(7695), 210–215. https://doi.org/10.1038/nature25973
217. Rydström, A., & Wick, M. J. (2007). Monocyte Recruitment, Activation, and Function in the Gut-Associated Lymphoid Tissue during Oral Salmonella Infection. *The Journal of Immunology*, *178*(9), 5789–5801. https://doi.org/10.4049/jimmunol.178.9.5789
218. Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., & Arnheim, N. (1992). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Biotechnology (Reading, Mass.)*, *24*, 476–480. https://doi.org/10.1007/BF00985904
219. Salazar, G. A., Peñaloza, H. F., Pardo-Roa, C., Schultz, B. M., Muñoz-Durango, N., Gómez, R. S., Salazar, F. J., Pizarro, D. P., Riedel, C. A., González, P. A., Alvarez-Lobos, M., Kalergis, A. M., & Bueno, S. M. (2017). Interleukin-10 production by T and B cells is a key factor to promote systemic Salmonella enterica serovar typhimurium infection in mice. *Frontiers in Immunology*, *8*(AUG). https://doi.org/10.3389/fimmu.2017.00889

220. Sanger, F., & Coulson, A. R. (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Journal of Molecular Biology*, *94*(3), 441–448. https://doi.org/10.1016/0022-2836(75)90213-2
221. Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, *74*(12), 5463–5467. https://doi.org/10.1073/PNAS.74.12.5463
222. Santiago, A., Panda, S., Mengels, G., Martinez, X., Azpiroz, F., Dore, J., Guarner, F., & Manichanh, C. (2014). Processing faecal samples: A step forward for standards in microbial community analysis. *BMC Microbiology*, *14*(1). https://doi.org/10.1186/1471-2180-14-112
223. Sassone-Corsi, M., Chairatana, P., Zheng, T., Perez-Lopez, A., Edwards, R. A., George, M. D., Nolan, E. M., & Raffatellu, M. (2016). Siderophore-based immunization strategy to inhibit growth of enteric pathogens. *Proceedings of the National Academy of Sciences*, *113*(47), 13462–13467. https://doi.org/10.1073/pnas.1606290113/-/DCSupplemental
224. Sassone-Corsi, M., & Raffatellu, M. (2015). No Vacancy: How Beneficial Microbes Cooperate with Immunity To Provide Colonization Resistance to Pathogens. *The Journal of Immunology*, *194*(9), 4081–4087. https://doi.org/10.4049/jimmunol.1403169
225. Schaedler, R. W., Dubos, R., & Costello, R. (1965). The development of the bacterial flora in the gastrointestinal tract of mice. *Journal of Experimental Medicine*, *122*(1), 59–66. https://doi.org/10.1084/JEM.122.1.59
226. Schmidt, B., Mulder, I. E., Musk, C. C., Aminov, R. I., Lewis, M., Stokes, C. R., Bailey, M., Prosser, J. I., Gill, B. P., Pluske, J. R., & Kelly, D. (2011). Establishment of Normal Gut Microbiota Is Compromised under Excessive Hygiene Conditions. *PloS One*, *6*(12), e28284. https://doi.org/10.1371/journal.pone.0028284
227. Schokker, D., Zhang, J., Zhang, L.-L., & Vastenhouw, S. A. (2014). Early-Life Environmental Variation Affects Intestinal Microbiota and Immune Development in New-Born Piglets. *PLoS ONE*, *9*(6), e100040. https://doi.org/10.1371/journal.pone.0100040
228. Sellin, M. E., Müller, A. A., Felmy, B., Dolowschiak, T., Diard, M., Tardivel, A., Maslowski, K. M., & Hardt, W. D. (2014). Epithelium-intrinsic NAIP/NLRC4 inflammasome drives infected enterocyte expulsion to restrict salmonella replication in the intestinal mucosa. *Cell Host and Microbe*, *16*(2), 237–248. https://doi.org/10.1016/j.chom.2014.07.001
229. Sender, R., Fuchs, S., & Milo, R. (2016). Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. *Cell*, *164*(3), 337–340. https://doi.org/10.1016/J.CELL.2016.01.013
230. Seng, P., Drancourt, M., Gouriet, F., Scola, B. la, Fournier, P. E., Rolain, J. M., & Raoult, D. (2009). Ongoing revolution in bacteriology: Routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clinical Infectious Diseases*, *49*(4), 543–551. https://doi.org/10.1086/600885
231. Shade, A., Peter, H., Allison, S. D., Baho, D. L., Berga, M., Bürgmann, H., Huber, D. H., Langenheder, S., Lennon, J. T., Martiny, J. B. H., Matulich, K. L., Schmidt, T. M., & Handelsman, J. (2012). Fundamentals of microbial community resistance and resilience. *Frontiers in Microbiology*, *3*, 417. https://doi.org/10.3389/FMICB.2012.00417/BIBTEX

232. Shapiro, B. J., Friedman, J., Cordero, O. X., Preheim, S. P., Timberlake, S. C., Szabó, G., Polz, M. F., & Alm, E. J. (2012). Population genomics of early events in the ecological differentiation of bacteria. *Science*, *335*(6077), 48–51. https://doi.org/10.1126/SCIENCE.1218198/SUPPL_FILE/SHAPIRO_SOM.PDF
233. Shirkey, T. W., Siggers, R. H., Goldade, B. G., Marshall, J. K., Drew, M. D., Laarveld, B., & van Kessel, A. G. (2006). Effects of Commensal Bacteria on Intestinal Morphology and Expression of Proinflammatory Cytokines in the Gnotobiotic Pig. *Experimental Biology and Medicine*, *231*(8), 1333–1345.
234. Sokatch, J. (2014). *Bacterial physiology and metabolism*. https://books.google.ca/books?hl=en&lr=&id=pftfAwAAQBAJ&oi=fnd&pg=PP1&dq=Sokatch+JR.+Bacterial+Physiology+and+Metabolism.+Academic+Press,+London,+UK+(2014).&ots=O4qDz2PBKP&sig=ACfw2ynpvIPMmtUl2BJopOXSIr8
235. Song, D., Peng, Q., Chen, Y., Zhou, X., Zhang, F., Li, A., Huang, D., Wu, Q., Ye, Y., He, H., Wang, L., & Tang, Y. (2017). Altered Gut Microbiota Profiles in Sows and Neonatal Piglets Associated with Porcine Epidemic Diarrhea Virus Infection. *Scientific Reports*, *7*(1), 1–10. https://doi.org/10.1038/s41598-017-17830-z
236. Sorbara, M. T., & Pamer, E. G. (2019). Interbacterial mechanisms of colonization resistance and the strategies pathogens use to overcome them. *Mucosal Immunology*, *12*(1). https://doi.org/10.1038/s41385-018-0053-0
237. Spees, A. M., Kingsbury, D. D., Wangdi, T., Xavier, M. N., Tsolis, R. M., & Bäumler, A. J. (2014). Neutrophils are a source of gamma interferon during acute Salmonella enterica serovar typhimurium colitis. *Infection and Immunity*, *82*(4), 1692–1697. https://doi.org/10.1128/IAI.01508-13
238. Srinivasan, A., Salazar-Gonzalez, R.-M., Jarcho, M., Sandau, M. M., Lefrancois, L., & McSorley, S. J. (2007). Innate Immune Activation of CD4 T Cells in Salmonella - Infected Mice Is Dependent on IL-18 . *The Journal of Immunology*, *178*(10), 6342–6349. https://doi.org/10.4049/jimmunol.178.10.6342
239. Staley, J. T., & Konopka, A. (1985). Measurement of in situ acti\tities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annual Review of Microbiology*, *39*, 321–346. www.annualreviews.org
240. Stanaway, J. D., Parisi, A., Sarkar, K., Blacker, B. F., Reiner, R. C., Hay, S. I., Nixon, M. R., Dolecek, C., James, S. L., Mokdad, A. H., Abebe, G., Ahmadian, E., Alahdab, F., Alemnew, B. T. T., Alipour, V., Allah Bakeshei, F., Animut, M. D., Ansari, F., Arabloo, J., … Crump, J. A. (2019). The global burden of non-typhoidal salmonella invasive disease: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Infectious Diseases*, *19*(12), 1312–1324. https://doi.org/10.1016/S1473-3099(19)30418-9
241. Steele-Mortimer, O., Brumell, J. H., Knodler, L. A., Méresse, S., Lopez, A., & Finlay, B. B. (2002). The invasion-associated type III secretion system of Salmonella enterica serovar Typhimurium is necessary for intracellular proliferation and vacuole biogenesis in epithelial cells. *Cellular Microbiology*, *4*(1), 43–54. https://doi.org/10.1046/J.1462-5822.2002.00170.X
242. Stuart, R. D., Toshach, S. R., & Patsula, T. M. (1954). The Problem of Transport of Specimens for Culture of Gonococci. In *Journal of Public Health / Revue Canadienne de Santé Publique* (Vol. 45, Issue 2).

243. Suzuki, T. A., & Nachman, M. W. (2016). Spatial Heterogeneity of Gut Microbial Composition along the Gastrointestinal Tract in Natural Populations of House Mice. *PLoS ONE*, *11*(9), e0163720. https://doi.org/10.1371/journal.pone.0163720
244. Swart, A., & Hensel, M. (2012). Interactions of Salmonella enterica with dendritic cells. *Virulence*, *3*(7), 660–667. https://doi.org/10.4161/viru.22761
245. Tam, M. A., Rydström, A., Sundquist, M., & Wick, M. J. (2008). Early cellular responses to Salmonella infection: Dendritic cells, monocytes, and more. *Immunological Reviews*, *225*(1), 140–162. https://doi.org/10.1111/J.1600-065X.2008.00679.X
246. Thiemann, S., Smit, N., Roy, U., Lesker, T. R., Gálvez, E. J. C., Helmecke, J., Basic, M., Bleich, A., Goodman, A. L., Kalinke, U., Flavell, R. A., Erhardt, M., & Strowig, T. (2017). Enhancement of IFNγ Production by Distinct Commensals Ameliorates Salmonella-Induced Disease. *Cell Host and Microbe*, *21*(6), 682-694.e5. https://doi.org/10.1016/j.chom.2017.05.005
247. Tierney, B. T., Yang, Z., Luber, J. M., Beaudin, M., Wibowo, M. C., Baek, C., Mehlenbacher, E., Patel, C. J., & Kostic, A. D. (2019). The Landscape of Genetic Content in the Gut and Oral Human Microbiome. *Cell Host & Microbe*, *26*(2), 283-295.e8. https://doi.org/10.1016/J.CHOM.2019.07.008
248. Tramontano, M., Andrejev, S., Pruteanu, M., Klünemann, M., Kuhn, M., Galardini, M., Jouhten, P., Zelezniak, A., Zeller, G., Bork, P., Typas, A., & Patil, K. R. (2018). Nutritional preferences of human gut bacteria reveal their metabolic idiosyncrasies. *Nature Microbiology*, *3*(4), 514–522. https://doi.org/10.1038/s41564-018-0123-9
249. Traore, S. I., Brah, S., Delerce, J., Fournier, P. E., Raoult, D., & Edouard, S. (2016). "Nigerium massiliense" gen. nov., sp. nov., a new bacterium isolated from the gut from a patient with acute malnutrition. *Human Microbiome Journal*, *1*, 12–13. https://doi.org/10.1016/j.humic.2016.08.001
250. Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I. (2007). The Human Microbiome Project. In *Nature* (Vol. 449, Issue 7164, pp. 804–810). Nature Publishing Group. https://doi.org/10.1038/nature06244
251. Uchida-Fujii, E., Niwa, H., Kinoshita, Y., & Nukada, T. (2020). Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) for Identification of Bacterial Isolates From Horses. *Journal of Equine Veterinary Science*, *95*, 103276. https://doi.org/10.1016/J.JEVS.2020.103276
252. Vandeputte, D., Tito, R. Y., Vanleeuwen, R., Falony, G., & Raes, J. (2017). Practical considerations for large-scale gut microbiome studies. In *FEMS microbiology reviews* (Vol. 41, Issue 1, pp. S154–S167). https://doi.org/10.1093/femsre/fux027
253. Vatanen, T., Kostic, A. D., D'Hennezel, E., Siljander, H., Franzosa, E. A., Yassour, M., Kolde, R., Vlamakis, H., Arthur, T. D., Hämäläinen, A. M., Peet, A., Tillmann, V., Uibo, R., Mokurov, S., Dorshakova, N., Ilonen, J., Virtanen, S. M., Szabo, S. J., Porter, J. A., … Xavier, R. J. (2016). Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. *Cell*, *165*(4), 842–853. https://doi.org/10.1016/j.cell.2016.04.007
254. Vellend, M. (2010). Conceptual Synthesis in Community Ecology. *The Quarterly Review of Biology*, *85*(2), 183–206.
255. Venter, C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R.

M., Huson, D. H., Wortman, J. R., Zhang, Q., Kodira, C. D., Zheng, X. H., Chen, L., … Zhu, X. (2001). The sequence of the human genome. *Science*, *291*(5507), 1304–1351. https://doi.org/10.1126/science.1058040

256. Videnska, P., Smerkova, K., Zwinsova, B., Popovici, V., Micenkova, L., Sedlar, K., & Budinska, E. (2019). Stool sampling and DNA isolation kits affect DNA quality and bacterial composition following 16S rRNA gene sequencing using MiSeq Illumina platform. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-49520-3
257. Vlasova, A. N., Rajashekara, G., & Saif, L. J. (2018). Interactions between human microbiome, diet, enteric viruses and immune system: Novel insights from gnotobiotic pig research. In *Drug Discovery Today: Disease Models* (Vol. 28, pp. 95–103). Elsevier Ltd. https://doi.org/10.1016/j.ddmod.2019.08.006
258. Voedisch, S., Koenecke, C., David, S., Herbrand, H., Förster, R., Rhen, M., & Pabst, O. (2009). Mesenteric lymph nodes confine dendritic cell-mediated dissemination of Salmonella enterica serovar typhimurium and limit systemic disease in mice. *Infection and Immunity*, *77*(8), 3170–3180. https://doi.org/10.1128/IAI.00272-09
259. Vogt, N. M., Kerby, R. L., Dill-McFarland, K. A., Harding, S. J., Merluzzi, A. P., Johnson, S. C., Carlsson, C. M., Asthana, S., Zetterberg, H., Blennow, K., Bendlin, B. B., & Rey, F. E. (2017). Gut microbiome alterations in Alzheimer's disease. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-13601-y
260. Walker, A. W., Martin, J. C., Scott, P., Parkhill, J., Flint, H. J., & Scott, K. P. (2015). 16S rRNA gene-based profiling of the human infant gut microbiota is strongly influenced by sample processing and PCR primer choice. *Microbiome*, *3*(1). https://doi.org/10.1186/s40168-015-0087-4
261. Wang, X., Howe, S., Wei, X., Deng, F., Tsai, T., Chai, J., Xiao, Y., Yang, H., Maxwell, C. v, Li, Y., Zhao, J., & Wang, C. X. (2021). *Comprehensive Cultivation of the Swine Gut Microbiome Reveals High Bacterial Diversity and Guides Bacterial Isolation in Pigs* (Vol. 6). https://doi.org/10
262. Wang, X., Tsai, T., Deng, F., Wei, X., Chai, J., Knapp, J., Apple, J., Maxwell, C. v., Lee, J. A., Li, Y., & Zhao, J. (2019). Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. *Microbiome*, *7*(1), 1–18. https://doi.org/10.1186/s40168-019-0721-7
263. Ward, D. M., Weller, R., & Bateson, M. M. (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature 1990 345:6270*, *345*(6270), 63–65. https://doi.org/10.1038/345063a0
264. Warriss, P. D., Knowles, T. G., Brown, S. N., Edwards, J. E., Kettlewell, P. J., Mitchell, M. A., & Baxter, C. A. (1999). Effects of lairage time on body temperature and glycogen reserves of broiler chickens held in transport modules. *Veterinary Record*, *145*(8), 218–222. https://doi.org/10.1136/vr.145.8.218
265. Winter, S. E., Thiennimitr, P., Winter, M. G., Butler, B. P., Huseby, D. L., Crawford, R. W., Russell, J. M., Bevins, C. L., Adams, L. G., Tsolis, R. M., Roth, J. R., & Bäumler, A. J. (2010). Gut inflammation provides a respiratory electron acceptor for Salmonella. *Nature*, *467*(7314), 426–429. https://doi.org/10.1038/nature09415
266. Woese, C. R., & Fox, G. E. (1977). Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proceedings of the National Academy of Sciences of the United States of America*, *74*(11), 5088–5090. https://doi.org/10.1073/pnas.74.11.5088

267. Wotzka, S. Y., Kreuzer, M., Maier, L., Arnoldini, M., Nguyen, B. D., Brachmann, A. O., Berthold, D. L., Zünd, M., Hausmann, A., Bakkeren, E., Hoces, D., Gül, E., Beutler, M., Dolowschiak, T., Zimmermann, M., Fuhrer, T., Moor, K., Sauer, U., Typas, A., … Hardt, W. D. (2019). Escherichia coli limits Salmonella Typhimurium infections after diet shifts and fat-mediated microbiota perturbation in mice. *Nature Microbiology*, *4*(12), 2164–2174. https://doi.org/10.1038/s41564-019-0568-5
268. Wylensek, D., Hitch, T. C. A., Riedel, T., Afrizal, A., Kumar, N., Wortmann, E., Liu, T., Devendran, S., Lesker, T. R., Hernández, S. B., Heine, V., Buhl, E. M., M. D'Agostino, P., Cumbo, F., Fischöder, T., Wyschkon, M., Looft, T., Parreira, V. R., Abt, B., … Clavel, T. (2020). A collection of bacterial isolates from the pig intestine reveals functional and taxonomic diversity. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-19929-w
269. Xiao, L., Estellé, J., Kiilerich, P., Ramayo-Caldas, Y., Xia, Z., Feng, Q., Liang, S., Pedersen, A., Kjeldsen, N. J., Liu, C., Maguin, E., Doré, J., Pons, N., le Chatelier, E., Prifti, E., Li, J., Jia, H., Liu, X., Xu, X., … Wang, J. (2016). A reference gene catalogue of the pig gut microbiome. *Nature Microbiology*, *1*(December), 1–6. https://doi.org/10.1038/nmicrobiol.2016.161
270. Xiao, Y., Kong, F., Xiang, Y., Zhou, W., Wang, J., Yang, H., Zhang, G., & Zhao, J. (2018). Comparative biogeography of the gut microbiome between Jinhua and Landrace pigs. *Scientific Reports*, *8*(1), 1–10. https://doi.org/10.1038/s41598-018-24289-z
271. Yang, Q., Huang, X., Wang, P., Yan, Z., Sun, W., Zhao, S., & Gun, S. (2019). Longitudinal development of the gut microbiota in healthy and diarrheic piglets induced by age-related dietary changes. *MicrobiologyOpen*, *8*(12), e923. https://doi.org/10.1002/MBO3.923
272. Yarza, P., Yilmaz, P., Pruesse, E., Glöckner, F. O., Ludwig, W., Schleifer, K. H., Whitman, W. B., Euzéby, J., Amann, R., & Rosselló-Móra, R. (2014). Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nature Reviews Microbiology*, *12*(9), 635–645. https://doi.org/10.1038/nrmicro3330
273. Yassour, M., Vatanen, T., Siljander, H., Hämäläinen, A. M., Härkönen, T., Ryhänen, S. J., Franzosa, E. A., Vlamakis, H., Huttenhower, C., Gevers, D., Lander, E. S., Knip, M., & Xavier, R. J. (2016). Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. *Science Translational Medicine*, *8*(343), 343ra81-343ra81. https://doi.org/10.1126/SCITRANSLMED.AAD0917/SUPPL_FILE/8-343RA81_TABLE_S1.ZIP
274. Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Gloria Dominguez-Bello, M., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P., Heath, A. C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J. G., Lozupone, C. A., Lauber, C., Clemente, J. C., Knights, D., … Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature*, *486*(7402), 222–227. https://doi.org/10.1038/nature11053
275. Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., & Glöckner, F. O. (2014). The SILVA and "all-species Living

Tree Project (LTP)" taxonomic frameworks. *Nucleic Acids Research*, *42*(D1). https://doi.org/10.1093/nar/gkt1209
276. Yousi, F., Kainan, C., Junnan, Z., Chuanxing, X., Lina, F., Bangzhou, Z., Jianlin, R., & Baishan, F. (2019). Evaluation of the effects of four media on human intestinal microbiota culture in vitro. *AMB Express*, *9*(1). https://doi.org/10.1186/s13568-019-0790-9
277. Zehavi, T., Probst, M., & Mizrahi, I. (2018). Insights into culturomics of the rumen microbiome. *Frontiers in Microbiology*, *9*(AUG). https://doi.org/10.3389/fmicb.2018.01999
278. Zhang, H., Wang, H., Shepherd, M., Wen, K., Li, G., Yang, X., Kocher, J., Giri-Rachman, E., Dickerman, A., Settlage, R., & Yuan, L. (2014). Probiotics and virulent human rotavirus modulate the transplanted human gut microbiota in gnotobiotic pigs. *Gut Pathogens*, *6*(1). https://doi.org/10.1186/s13099-014-0039-8
279. Zhang, Q., Widmer, G., & Tzipori, S. (2013). A pig model of the human gastrointestinal tract. *Gut Microbes*, *4*(3), 193–200. https://doi.org/10.4161/gmic.23867
280. Zheng, L., Kelly, C. J., & Colgan, S. P. (2015). Physiologic hypoxia and oxygen homeostasis in the healthy intestine. A review in the theme: Cellular responses to hypoxia. *American Journal of Physiology - Cell Physiology*, *309*(6), C350–C360. https://doi.org/10.1152/ajpcell.00191.2015
281. Zhou, H., Sun, J., Yu, B., Liu, Z., Chen, H., He, J., Mao, X., Zheng, P., Yu, J., Luo, J., Luo, Y., Yan, H., Ge, L., & Chen, D. (2021). Gut microbiota absence and transplantation affect growth and intestinal functions: An investigation in a germ-free pig model. *Animal Nutrition*, *7*(2), 295–304. https://doi.org/10.1016/j.aninu.2020.11.012
282. Zhou, J., & Ning, D. (2017). Stochastic Community Assembly: Does It Matter in Microbial Ecology? *Microbiology and Molecular Biology Reviews*, *81*(4), 1–32.

Chapter 2

1. Alcock, B. P., Raphenya, A. R., Lau, T. T. Y., Tsang, K. K., Bouchard, M., Edalatmand, A., Huynh, W., Nguyen, A. L. v., Cheng, A. A., Liu, S., Min, S. Y., Miroshnichenko, A., Tran, H. K., Werfalli, R. E., Nasir, J. A., Oloni, M., Speicher, D. J., Florescu, A., Singh, B., … McArthur, A. G. (2020). CARD 2020: Antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic Acids Research*, *48*(D1), D517–D525. https://doi.org/10.1093/nar/gkz935
2. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2
3. Amann, R. I., Ludwig, W., & Schleifer, K.-H. (1995). Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. In *Microbiological Reviews* (Vol. 59, Issue 1).
4. Amat, S., Lantz, H., Munyaka, P. M., & Willing, B. P. (2020). Prevotella in pigs: The positive and negative associations with production and health. In *Microorganisms* (Vol. 8, Issue 10, pp. 1–27). MDPI AG. https://doi.org/10.3390/microorganisms8101584
5. Anders, J. L., Moustafa, M. A. M., Mohamed, W. M. A., Hayakawa, T., Nakao, R., & Koizumi, I. (2021). Comparing the gut microbiome along the gastrointestinal tract of

three sympatric species of wild rodents. *Scientific Reports*, *11*(1). https://doi.org/10.1038/s41598-021-99379-6

6. Aziz, R. K., Bartels, D., Best, A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., … Zagnitko, O. (2008). The RAST Server: Rapid annotations using subsystems technology. *BMC Genomics*, *9*. https://doi.org/10.1186/1471-2164-9-75
7. Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. v., Sirotkin, A. v., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology*, *19*(5), 455–477. https://doi.org/10.1089/cmb.2012.0021
8. Becker, N., Kunath, J., Loh, G., & Blaut, M. (2011). Human intestinal microbiota: Characterization of a simplified and stable gnotobiotic rat model. *Gut Microbes*, *2*(1), 25–33. https://doi.org/10.4161/gmic.2.1.14651
9. Begley, M., Hill, C., & Gahan, C. G. M. (2006). Bile salt hydrolase activity in probiotics. In *Applied and Environmental Microbiology* (Vol. 72, Issue 3, pp. 1729–1738). https://doi.org/10.1128/AEM.72.3.1729-1738.2006
10. Bellali, S., Lagier, J. C., Raoult, D., & Khalil, J. B. (2019). Among live and dead bacteria, the optimization of sample collection and processing remains essential in recovering gut microbiota components. *Frontiers in Microbiology*, *10*(JULY). https://doi.org/10.3389/fmicb.2019.01606
11. Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., & Gregory Caporaso, J. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*, *6*(1). https://doi.org/10.1186/s40168-018-0470-z
12. Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170
13. Bolyen, E. et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. In *Nature Biotechnology* (Vol. 37, Issue 8, pp. 850–852). Nature Publishing Group. https://doi.org/10.1038/s41587-019-0190-3
14. Brugiroux, S., Beutler, M., Pfann, C., Garzetti, D., Ruscheweyh, H. J., Ring, D., Diehl, M., Herp, S., Lötscher, Y., Hussain, S., Bunk, B., Pukall, R., Huson, D. H., Münch, P. C., McHardy, A. C., McCoy, K. D., MacPherson, A. J., Loy, A., Clavel, T., … Stecher, B. (2016). Genome-guided design of a defined mouse microbiota that confers colonization resistance against Salmonella enterica serovar Typhimurium. *Nature Microbiology*, *2*. https://doi.org/10.1038/nmicrobiol.2016.215
15. Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869
16. Chairatana, P., & Nolan, E. M. (2017). Defensins, lectins, mucins, and secretory immunoglobulin A: microbe-binding biomolecules that contribute to mucosal immunity in the human gut. In *Critical Reviews in Biochemistry and Molecular Biology* (Vol. 52,

Issue 1, pp. 45–56). Taylor and Francis Ltd. https://doi.org/10.1080/10409238.2016.1243654
17. Ciufo, S., Kannan, S., Sharma, S., Badretdin, A., Clark, K., Turner, S., Brover, S., Schoch, C. L., Kimchi, A., & DiCuccio, M. (2018). Using average nucleotide identity to improve taxonomic assignments in prokaryotic genomes at the NCBI. *International Journal of Systematic and Evolutionary Microbiology*, *68*(7), 2386–2392. https://doi.org/10.1099/ijsem.0.002809
18. Crespo-Piazuelo, D., Estellé, J., Revilla, M., Criado-Mesas, L., Ramayo-Caldas, Y., Óvilo, C., Fernández, A. I., Ballester, M., & Folch, J. M. (2018). Characterization of bacterial microbiota compositions along the intestinal tract in pigs and their interactions and functions. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-30932-6
19. da Silva, C., Wagner, C., Bonnardel, J., Gorvel, J. P., & Lelouard, H. (2017). The Peyer's patch mononuclear phagocyte system at steady state and during infection. In *Frontiers in Immunology* (Vol. 8, Issue OCT). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2017.01254
20. Darnaud, M., de Vadder, F., Yansouni, J., Schwarzer, M., Leulier, F., & Tamellini, A. (2019). *A standardized gnotobiotic mouse model harboring a minimal 15-member mouse gut microbiota recapitulates SOPF phenotype 2*. https://doi.org/10.1101/2019.12.30.890954
21. Desai, M. S., Seekatz, A. M., Koropatkin, N. M., Kamada, N., Hickey, C. A., Wolter, M., Pudlo, N. A., Kitamoto, S., Terrapon, N., Muller, A., Young, V. B., Henrissat, B., Wilmes, P., Stappenbeck, T. S., Núñez, G., & Martens, E. C. (2016). A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. *Cell*, *167*(5), 1339-1353.e21. https://doi.org/10.1016/j.cell.2016.10.043
22. Dhillon, B. K., Laird, M. R., Shay, J. A., Winsor, G. L., Lo, R., Nizam, F., Pereira, S. K., Waglechner, N., McArthur, A. G., Langille, M. G. I., & Brinkman, F. S. L. (2015). IslandViewer 3: More flexible, interactive genomic island discovery, visualization and analysis. *Nucleic Acids Research*, *43*(W1), W104–W108. https://doi.org/10.1093/nar/gkv401
23. Donaldson, G. P., Lee, S. M., & Mazmanian, S. K. (2015). Gut biogeography of the bacterial microbiota. In *Nature Reviews Microbiology* (Vol. 14, Issue 1, pp. 20–32). Nature Publishing Group. https://doi.org/10.1038/nrmicro3552
24. Eberl, C., Ring, D., Münch, P. C., Beutler, M., Basic, M., Slack, E. C., Schwarzer, M., Srutkova, D., Lange, A., Frick, J. S., Bleich, A., & Stecher, B. (2020). Reproducible Colonization of Germ-Free Mice With the Oligo-Mouse-Microbiota in Different Animal Facilities. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.02999
25. Fenske, G. J., Ghimire, S., Antony, L., Christopher-Hennings, J., & Scaria, J. (2020). Integration of culture-dependent and independent methods provides a more coherent picture of the pig gut microbiome. *FEMS Microbiology Ecology*, *96*(3). https://doi.org/10.1093/femsec/fiaa022
26. Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P., & Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. In *Gut Microbes* (Vol. 3, Issue 4). https://doi.org/10.4161/gmic.19897
27. Fouhse, J. M., Yang, K., More-Bayona, J., Gao, Y., Goruk, S., Plastow, G., Field, C. J., Barreda, D. R., & Willing, B. P. (2019). Neonatal Exposure to Amoxicillin Alters Long-

Term Immune Response Despite Transient Effects on Gut-Microbiota in Piglets. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.02059
28. Franke, T., & Deppenmeier, U. (2018). Physiology and central carbon metabolism of the gut bacterium Prevotella copri. *Molecular Microbiology*, *109*(4), 528–540. https://doi.org/10.1111/mmi.14058
29. Frese, S. A., Parker, K., Calvert, C. C., & Mills, D. A. (2015). Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome*, *3*(1). https://doi.org/10.1186/s40168-015-0091-8
30. Freter, R., & Abrams, G. D. (1972). Function of Various Intestinal Bacteria in Converting Germfree Mice to the Normal State. In *Atnerican Society for Microbiology* (Vol. 6, Issue 2).
31. Gomes-Neto, J. C., Kittana, H., Mantz, S., Segura Munoz, R. R., Schmaltz, R. J., Bindels, L. B., Clarke, J., Hostetter, J. M., Benson, A. K., Walter, J., & Ramer-Tait, A. E. (2017). A gut pathobiont synergizes with the microbiota to instigate inflammatory disease marked by immunoreactivity against other symbionts but not itself. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-18014-5
32. Gregova, G., & Kmet, V. (2020). Antibiotic resistance and virulence of Escherichia coli strains isolated from animal rendering plant. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-72851-5
33. Gu, S., Chen, D., Zhang, J. N., Lv, X., Wang, K., Duan, L. P., Nie, Y., & Wu, X. L. (2013). Bacterial Community Mapping of the Mouse Gastrointestinal Tract. *PLoS ONE*, *8*(10). https://doi.org/10.1371/journal.pone.0074957
34. Guevarra, R. B., Lee, J. H., Lee, S. H., Seok, M. J., Kim, D. W., Kang, B. N., Johnson, T. J., Isaacson, R. E., & Kim, H. B. (2019). Piglet gut microbial shifts early in life: Causes and effects. In *Journal of Animal Science and Biotechnology* (Vol. 10, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s40104-018-0308-3
35. Gurevich, A., Saveliev, V., Vyahhi, N., & Tesler, G. (2013). QUAST: Quality assessment tool for genome assemblies. *Bioinformatics*, *29*(8), 1072–1075. https://doi.org/10.1093/bioinformatics/btt086
36. Hayashi, H., Shibata, K., Sakamoto, M., Tomita, S., & Benno, Y. (2007). Prevotella copri sp. nov. and Prevotella stercorea sp. nov., isolated from human faeces. *International Journal of Systematic and Evolutionary Microbiology*, *57*(5), 941–946. https://doi.org/10.1099/ijs.0.64778-0
37. Hibberd, M. C., Wu, M., Rodionov, D. A., Li, X., Cheng, J., Griffin, N. W., Barratt, M. J., Giannone, R. J., Hettich, R. L., Osterman, A. L., & Gordon, J. I. (2017). *The effects of micronutrient deficiencies on bacterial species from the human gut microbiota*. http://stm.sciencemag.org/
38. Holman, D. B., Brunelle, B. W., Trachsel, J., & Allen, H. K. (2017). Meta-analysis To Define a Core Microbiota in the Swine Gut. *MSystems*, *2*(3). https://doi.org/10.1128/msystems.00004-17
39. Huang, A., Cai, R., Wang, Q., Shi, L., Li, C., & Yan, H. (2019). Dynamic change of gut microbiota during porcine epidemic Diarrhea virus infection in suckling piglets. *Frontiers in Microbiology*, *10*(FEB). https://doi.org/10.3389/fmicb.2019.00322
40. Inagaki, T., Moschetta, A., Lee, Y.-K., Peng, L., Zhao, G., Downes, M., Yu, R. T., Shelton, J. M., Richardson, J. A., Repa, J. J., Mangelsdorf, D. J., Kliewer, S. A., &

O'malley, B. W. (2006). *Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor*. https://www.pnas.org
41. Kastl, A. J., Terry, N. A., Wu, G. D., & Albenberg, L. G. (2020). The Structure and Function of the Human Small Intestinal Microbiota: Current Understanding and Future Directions. In *CMGH* (Vol. 9, Issue 1, pp. 33–45). Elsevier Inc. https://doi.org/10.1016/j.jcmgh.2019.07.006
42. Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, *30*(4), 772–780. https://doi.org/10.1093/molbev/mst010
43. Kho, Z. Y., & Lal, S. K. (2018). The human gut microbiome - A potential controller of wellness and disease. In *Frontiers in Microbiology* (Vol. 9, Issue AUG). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2018.01835
44. Kovatcheva-Datchary, P., Shoaie, S., Lee, S., Wahlström, A., Nookaew, I., Hallen, A., Perkins, R., Nielsen, J., & Bäckhed, F. (2019). Simplified Intestinal Microbiota to Study Microbe-Diet-Host Interactions in a Mouse Model. *Cell Reports*, *26*(13), 3772-3783.e6. https://doi.org/10.1016/j.celrep.2019.02.090
45. Lane, D. J. 1991. 16S/23S rRNA sequencing, p. 115-176. In E. Stackebrandt and M. Goodfellow (ed.), *Nucleic acid techniques in bacterial systematics*. John Wiley and Sons, New York, N.Y.
46. Laycock, G., Sait, L., Inman, C., Lewis, M., Smidt, H., van Diemen, P., Jorgensen, F., Stevens, M., & Bailey, M. (2012). A defined intestinal colonization microbiota for gnotobiotic pigs. *Veterinary Immunology and Immunopathology*, *149*(3–4), 216–224. https://doi.org/10.1016/j.vetimm.2012.07.004
47. Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M., & Moøller, K. (2002). Culture-independent analysis of gut bacteria: The pig gastrointestinal tract microbiota revisited. *Applied and Environmental Microbiology*, *68*(2), 673–690. https://doi.org/10.1128/AEM.68.2.673-690.2002
48. Li, N., Huang, S., Jiang, L., Wang, W., Li, T., Zuo, B., Li, Z., & Wang, J. (2018). Differences in the gut microbiota establishment and metabolome characteristics between low- and normal-birth-weight piglets during early-life. *Frontiers in Microbiology*, *9*(SEP). https://doi.org/10.3389/fmicb.2018.01798
49. Li, Y., Wang, X., Wang, X. Q., Wang, J., & Zhao, J. (2020). Life-long dynamics of the swine gut microbiome and their implications in probiotics development and food safety. *Gut Microbes*, *11*(6), 1824–1832. https://doi.org/10.1080/19490976.2020.1773748
50. Liu, Y., Zheng, Z., Yu, L., Wu, S., Sun, L., Wu, S., Xu, Q., Cai, S., Qin, N., & Bao, W. (2019). Examination of the temporal and spatial dynamics of the gut microbiome in newborn piglets reveals distinct microbial communities in six intestinal segments. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-40235-z
51. Lkhagva, E., Chung, H. J., Hong, J., Tang, W. H. W., Lee, S. il, Hong, S. T., & Lee, S. (2021). The regional diversity of gut microbiome along the GI tract of male C57BL/6 mice. *BMC Microbiology*, *21*(1). https://doi.org/10.1186/s12866-021-02099-0
52. Lorenzo-Zúñiga, V., Bartolí, R., Planas, R., Hofmann, A. F., Viñado, B., Hagey, L. R., Hernández, J. M., Mañé, J., Alvarez, M. A., Ausina, V., & Gassull, M. A. (2003). Oral bile acids reduce bacterial overgrowth, bacterial translocation, and endotoxemia in cirrhotic rats. *Hepatology*, *37*(3), 551–557. https://doi.org/10.1053/jhep.2003.50116

53. Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. In *Nature* (Vol. 489, Issue 7415, pp. 220–230). https://doi.org/10.1038/nature11550
54. Martinez-Guryn, K., Leone, V., & Chang, E. B. (2019). Regional Diversity of the Gastrointestinal Microbiome. In *Cell Host and Microbe* (Vol. 26, Issue 3, pp. 314–324). Cell Press. https://doi.org/10.1016/j.chom.2019.08.011
55. Moote, P. E., Bescucci, D. M., Polo, R. O., Uwiera, R. R. E., & Douglas Inglis, G. (2021). Comparison of Strategies for Isolating Anaerobic Bacteria from the Porcine Intestine. *Applied and Environmental Microbiology*, *87*(9), e00088-21. https://doi.org/10.1128/AEM
56. Nagaraja, T. G., Narayanan, S. K., Stewart, G. C., & Chengappa, M. M. (2005). Fusobacterium necrophorum infections in animals: Pathogenesis and pathogenic mechanisms. *Anaerobe*, *11*(4), 239–246. https://doi.org/10.1016/j.anaerobe.2005.01.007
57. Navarro, D. M. D. L., Abelilla, J. J., & Stein, H. H. (2019). Structures and characteristics of carbohydrates in diets fed to pigs: A review. In *Journal of Animal Science and Biotechnology* (Vol. 10, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s40104-019-0345-6
58. Navas-Molina, J. A., Peralta-Sánchez, J. M., González, A., McMurdie, P. J., Vázquez-Baeza, Y., Xu, Z., Ursell, L. K., Lauber, C., Zhou, H., Song, S. J., Huntley, J., Ackermann, G. L., Berg-Lyons, D., Holmes, S., Caporaso, J. G., & Knight, R. (2013). Advancing our understanding of the human microbiome using QIIME. In *Methods in Enzymology* (Vol. 531, pp. 371–444). Academic Press Inc. https://doi.org/10.1016/B978-0-12-407863-5.00019-8
59. Norin, E., & Midtvedt, T. (2010). Intestinal microflora functions in laboratory mice claimed to harbor a "normal" intestinal microflora. Is the SPF concept running out of date? *Anaerobe*, *16*(3), 311–313. https://doi.org/10.1016/j.anaerobe.2009.10.006
60. Orcutt, R. P., Gianni, F. J., & Judge, R. J. (1987). Development of an "altered Schaedler flora" for NCI gnotobiotic rodents. *Microecol Ther*, *17*(59).
61. Pedregosa, F., Michel, V., Grisel, O., Blondel, M., Prettenhofer, P., Weiss, R., Vanderplas, J., Cournapeau, D., Pedregosa, F., Varoquaux, G., Gramfort, A., Thirion, B., Grisel, O., Dubourg, V., Passos, A., Brucher, M., Perrot andÉdouardand, M., Duchesnay, andÉdouard, & Duchesnay, Fré. (2011). Scikit-learn: Machine Learning in Python Gaël Varoquaux Bertrand Thirion Vincent Dubourg Alexandre Passos, et al. Matthieu Perrot. In *Journal of Machine Learning Research* (Vol. 12). http://scikit-learn.sourceforge.net.
62. Pickard, J. M., Zeng, M. Y., Caruso, R., & Núñez, G. (2017). Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. In *Immunological Reviews* (Vol. 279, Issue 1, pp. 70–89). Blackwell Publishing Ltd. https://doi.org/10.1111/imr.12567
63. Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2 - Approximately maximum-likelihood trees for large alignments. *PLoS ONE*, *5*(3). https://doi.org/10.1371/journal.pone.0009490
64. Ridlon, J. M., Kang, D. J., & Hylemon, P. B. (2006). Bile salt biotransformations by human intestinal bacteria. In *Journal of Lipid Research* (Vol. 47, Issue 2, pp. 241–259). https://doi.org/10.1194/jlr.R500013-JLR200

65. Schaedler, R. W., Dubos, R., & Costello, R. (1965). The development of the bacterial flora in the gastrointestinal tract of mice. *Journal of Experimental Medicine*, *122*(1), 59–66. https://doi.org/10.1084/JEM.122.1.59
66. Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6). https://doi.org/10.1186/gb-2011-12-6-r60
67. Staley, C., Weingarden, A. R., Khoruts, A., & Sadowsky, M. J. (2017). Interaction of gut microbiota with bile acid metabolism and its influence on disease states. *Applied microbiology and biotechnology*, *101*(1), 47-64.
68. Suzuki, T. A., & Nachman, M. W. (2016). Spatial heterogeneity of gut microbial composition along the gastrointestinal tract in natural populations of house mice. *PLoS ONE*, *11*(9). https://doi.org/10.1371/journal.pone.0163720
69. Syed, S. A., Abrams, G. D., & Freter, R. (1970). *Efficiency of Various Intestinal Bacteria in Assuming Normal Functions of Enteric Flora After Association with Germ-Free Mice* (Vol. 2, Issue 4).
70. Urdaneta, V., & Casadesús, J. (2017). Interactions between bacteria and bile salts in the gastrointestinal and hepatobiliary tracts. In *Frontiers in Medicine* (Vol. 4, Issue OCT). Frontiers Media S.A. https://doi.org/10.3389/fmed.2017.00163
71. Vaishnava, S., Yamamoto, M., Severson, K. M., Ruhn, K. A., Yu, X., Koren, O., Ley, R., Wakeland, E. K., & Hooper, L. v. (2011). The antibacterial lectin RegIIIγ promotes the spatial segregation of microbiota and host in the intestine. *Science*, *334*(6053), 255–258. https://doi.org/10.1126/science.1209791
72. Wang, X., Tsai, T., Deng, F., Wei, X., Chai, J., Knapp, J., Apple, J., Maxwell, C. v., Lee, J. A., Li, Y., & Zhao, J. (2019). Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. *Microbiome*, *7*(1). https://doi.org/10.1186/s40168-019-0721-7
73. Wylensek, D., Hitch, T. C. A., Riedel, T., Afrizal, A., Kumar, N., Wortmann, E., Liu, T., Devendran, S., Lesker, T. R., Hernández, S. B., Heine, V., Buhl, E. M., M. D'Agostino, P., Cumbo, F., Fischöder, T., Wyschkon, M., Looft, T., Parreira, V. R., Abt, B., … Clavel, T. (2020). A collection of bacterial isolates from the pig intestine reveals functional and taxonomic diversity. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-19929-w
74. Yang, Q., Huang, X., Wang, P., Yan, Z., Sun, W., Zhao, S., & Gun, S. (2019). Longitudinal development of the gut microbiota in healthy and diarrheic piglets induced by age-related dietary changes. *Microbiologyopen*, *8*(12), e923.
75. Yang, H., Xiao, Y., Wang, J., Xiang, Y., Gong, Y., Wen, X., & Li, D. (2018). Core gut microbiota in Jinhua pigs and its correlation with strain, farm and weaning age. *Journal of Microbiology*, *56*(5), 346–355. https://doi.org/10.1007/s12275-018-7486-8
76. Yoon, S. H., Ha, S. min, Lim, J., Kwon, S., & Chun, J. (2017). A large-scale evaluation of algorithms to calculate average nucleotide identity. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *110*(10), 1281–1286. https://doi.org/10.1007/s10482-017-0844-4
77. Zhao, W., Wang, Y., Liu, S., Huang, J., Zhai, Z., He, C., Ding, J., Wang, J., Wang, H., Fan, W., Zhao, J., & Meng, H. (2015). The dynamic distribution of porcine microbiota

across different ages and gastrointestinal tract segments. *PLoS ONE*, *10*(2). https://doi.org/10.1371/journal.pone.0117441
78. Zoetendal, E. G., Raes, J., van den Bogert, B., Arumugam, M., Booijink, C. C., Troost, F. J., Bork, P., Wels, M., de Vos, W. M., & Kleerebezem, M. (2012). The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. *ISME Journal*, *6*(7), 1415–1426. https://doi.org/10.1038/ismej.2011.212

Chapter 3

1. Antonopoulos, D. A., Huse, S. M., Morrison, H. G., Schmidt, T. M., Sogin, M. L., & Young, V. B. (2009). Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. *Infection and Immunity*, *77*(6), 2367–2375. https://doi.org/10.1128/IAI.01520-08
2. Arai, T., Hiromatsu, K., Nishimura, H., Kimura, Y., Kobayashi, N., Ishida, H., Nimurat, Y., & Yoshikai, Y. (1995). Effects of in vivo administration of anti-IL-10 monoclonal antibody on the host defence mechanism against murine Salmonella infection. In *Immunology* (Vol. 85).
3. Brugiroux, S., Beutler, M., Pfann, C., Garzetti, D., Ruscheweyh, H. J., Ring, D., Diehl, M., Herp, S., Lötscher, Y., Hussain, S., Bunk, B., Pukall, R., Huson, D. H., Münch, P. C., McHardy, A. C., McCoy, K. D., MacPherson, A. J., Loy, A., Clavel, T., … Stecher, B. (2016). Genome-guided design of a defined mouse microbiota that confers colonization resistance against Salmonella enterica serovar Typhimurium. *Nature Microbiology*, *2*. https://doi.org/10.1038/nmicrobiol.2016.215
4. Cameron-Veas, K., Fraile, L., Napp, S., Garrido, V., Grilló, M. J., & Migura-Garcia, L. (2018). Multidrug resistant Salmonella enterica isolated from conventional pig farms using antimicrobial agents in preventative medicine programmes. *Veterinary Journal*, *234*, 36–42. https://doi.org/10.1016/j.tvjl.2018.02.002
5. Caron, J., Loredo-Osti, J. C., Laroche, L., Skamene, E., Morgan, K., & Malo, D. (2002). Identification of genetic loci controlling bacterial clearance in experimental Salmonella enteritidis infection: An unexpected role of Nramp1 (Slc11a1) in the persistence of infection in mice. *Genes and Immunity*, *3*(4), 196–204. https://doi.org/10.1038/sj.gene.6363850
6. Chassin, C., Kocur, M., Pott, J., Duerr, C. U., Gütle, D., Lotz, M., & Hornef, M. W. (2010). MiR-146a mediates protective innate immune tolerance in the neonate intestine. *Cell Host and Microbe*, *8*(4), 358–368. https://doi.org/10.1016/j.chom.2010.09.005
7. Chen, C., Fang, S., Wei, H., He, M., Fu, H., Xiong, X., Zhou, Y., Wu, J., Gao, J., Yang, H., & Huang, L. (2021). Prevotella copri increases fat accumulation in pigs fed with formula diets. *Microbiome*, *9*(1). https://doi.org/10.1186/s40168-021-01110-0
8. Costa, M. O., Fouhse, J., Silva, A. P. P., Willing, B., & Harding, J. C. S. (2020). Putting the microbiota to work: Epigenetic effects of early life antibiotic treatment are associated with immune-related pathways and reduced epithelial necrosis following Salmonella Typhimurium challenge in vitro. *PLoS ONE*, *15*(4). https://doi.org/10.1371/journal.pone.0231942
9. Dar, M. A., Urwat, U., Ahmad, S. M., Ahmad, R., Kashoo, Z. A., Dar, T. A., Bhat, S. A., Mumtaz, P. T., Shabir, N., Shah, R. A., & Heidari, M. (2019). Gene expression and

antibody response in chicken against Salmonella Typhimurium challenge. *Poultry Science*, *98*(5), 2008–2013. https://doi.org/10.3382/ps/pey560
10. Elderman, M., van Beek, A., Brandsma, E., de Haan, B., Savelkoul, H., de Vos, P., & Faas, M. (2016). Sex impacts Th1 cells, Tregs, and DCs in both intestinal and systemic immunity in a mouse strain and location-dependent manner. *Biology of Sex Differences*, *7*(1). https://doi.org/10.1186/s13293-016-0075-9
11. Ferreira, R. B. R., Gill, N., Willing, B. P., Antunes, L. C. M., Russell, S. L., Croxen, M. A., & Finlay, B. B. (2011). The intestinal microbiota plays a role in salmonella-induced colitis independent of pathogen colonization. *PLoS ONE*, *6*(5). https://doi.org/10.1371/journal.pone.0020338
12. Fish, E. N. (2008). The X-files in immunity: sex-based differences predispose immune responses. *Nature Reviews Immunology*, *8*(9), 737-744.
13. Foster, N., Hulme, S. D., & Barrow, P. A. (2003). Induction of antimicrobial pathways during early-phase immune response to Salmonella spp. in murine macrophages: Gamma interferon (IFN-γ) and upregulation of IFN-γ receptor alpha expression are required for NADPH phagocytic oxidase gp91-stimulated oxidative burst and control of virulent Salmonella spp. *Infection and Immunity*, *71*(8), 4733–4741. https://doi.org/10.1128/IAI.71.8.4733-4741.2003
14. Fouhse, J. M., Yang, K., More-Bayona, J., Gao, Y., Goruk, S., Plastow, G., Field, C. J., Barreda, D. R., & Willing, B. P. (2019). Neonatal Exposure to Amoxicillin Alters Long-Term Immune Response Despite Transient Effects on Gut-Microbiota in Piglets. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.02059
15. Francino, M. P. (2016). Antibiotics and the human gut microbiome: Dysbioses and accumulation of resistances. In *Frontiers in Microbiology* (Vol. 6, Issue JAN). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2015.01543
16. Frink, M., Pape, H. C., van Griensven, M., Krettek, C., Chaudry, I. H., & Hildebrand, F. (2007). Influence of sex and age on mods and cytokines after multiple injuries. *Shock*, *27*(2), 151–156. https://doi.org/10.1097/01.shk.0000239767.64786.de
17. Huang, Y., Tang, J., Cai, Z., Zhou, K., Chang, L., Bai, Y., & Ma, Y. (2020). Prevotella Induces the Production of Th17 Cells in the Colon of Mice. *Journal of Immunology Research*, *2020*. https://doi.org/10.1155/2020/9607328
18. Hudault, S., Guignot, J., & Servin, A. L. (2001). Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection. *Gut*, *49*(1), 47–55. https://doi.org/10.1136/gut.49.1.47
19. Jaillon, S., Berthenet, K., & Garlanda, C. (2019). Sexual Dimorphism in Innate Immunity. In *Clinical Reviews in Allergy and Immunology* (Vol. 56, Issue 3, pp. 308–321). Humana Press Inc. https://doi.org/10.1007/s12016-017-8648-x
20. Kim, H. M., Park, B. S., Kim, J. I., Kim, S. E., Lee, J., Oh, S. C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O. J., & Lee, J. O. (2007). Crystal Structure of the TLR4-MD-2 Complex with Bound Endotoxin Antagonist Eritoran. *Cell*, *130*(5), 906–917. https://doi.org/10.1016/j.cell.2007.08.002
21. Klein, S. L., & Flanagan, K. L. (2016). Sex differences in immune responses. In *Nature Reviews Immunology* (Vol. 16, Issue 10, pp. 626–638). Nature Publishing Group. https://doi.org/10.1038/nri.2016.90

22. Kovatcheva-Datchary, P., Shoaie, S., Lee, S., Wahlström, A., Nookaew, I., Hallen, A., Perkins, R., Nielsen, J., & Bäckhed, F. (2019). Simplified Intestinal Microbiota to Study Microbe-Diet-Host Interactions in a Mouse Model. *Cell Reports*, *26*(13), 3772-3783.e6. https://doi.org/10.1016/j.celrep.2019.02.090
23. Lonnermark, E., Lappas, G., Friman, V., Wold, A. E., Backhaus, E., & Adlerberth, I. (2014). *Effects of Probiotic Intake and Gender on Nontyphoid Salmonella Infection*. www.jcge.com
24. Looft, T., Johnson, T. A., Allen, H. K., Bayles, D. O., Alt, D. P., Stedtfeld, R. D., Sul, W. J., Stedtfeld, T. M., Chai, B., Cole, J. R., Hashsham, S. A., Tiedje, J. M., & Stanton, T. B. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(5), 1691–1696. https://doi.org/10.1073/pnas.1120238109
25. Lotz, M., Gütle, D., Walther, S., Ménard, S., Bogdan, C., & Hornef, M. W. (2006). Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. *Journal of Experimental Medicine*, *203*(4), 973–984. https://doi.org/10.1084/jem.20050625
26. Marion, H., & Siegwart, E. (2015). Quotient Bio Analytical Sciences, Final Analytical Report –MIC determination of the VetPath III collection of veterinary bacterial pathogens from Europe.
27. Marx, J. O., Vudathala, D., Murphy, L., Rankin, S., & Hankenson, F. C. (2014). Antibiotic administration in the drinking water of mice. *Journal of the American Association for Laboratory Animal Science*, *53*(3), 301-306.
28. McAleer, J. P., & Vella, A. T. (2008). Understanding how lipopolysaccharide impacts CD4 T-cell immunity. *Critical Reviews™ in Immunology*, *28*(4).
29. McSorley, S. J., Asch, S., Costalonga, M., Lee Reinhardt, R., & Jenkins, M. K. (2002). Tracking Salmonella-Specific CD4 T Cells In Vivo Reveals a Local Mucosal Response to a Disseminated Infection 2000). A more detailed understanding of CD4 T cell biology during Development of an Adoptive Transfer System to Track Salmonella-Specific CD4 T Cells In Vivo. In *Immunity* (Vol. 16). McSorley and Jenkins.
30. Neves, P., Lampropoulou, V., Calderon-Gomez, E., Roch, T., Stervbo, U., Shen, P., Kühl, A. A., Loddenkemper, C., Haury, M., Nedospasov, S. A., Kaufmann, S. H. E., Steinhoff, U., Calado, D. P., & Fillatreau, S. (2010). Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during salmonella typhimurium infection. *Immunity*, *33*(5), 777–790. https://doi.org/10.1016/j.immuni.2010.10.016
31. Nollet, N., Maes, D., Duchateau, L., Hautekiet, V., Houf, K., van Hoof, J., de Zutter, L., de Kruif, A., & Geers, R. (2005). Discrepancies between the isolation of Salmonella from mesentric lymph nodes and the results of serological screening in slaughter pigs. *Veterinary Research*, *36*(4), 545–555. https://doi.org/10.1051/vetres:2005014
32. Oberholzer, A., Souza, S. M., Tschoeke, S. K., Oberholzer, C., Abouhamze, A., Pribble, J. P., & Moldawer, L. L. (2005). Plasma cytokine measurements augment prognostic scores as indicators of outcome in patients with severe sepsis. *Shock*, *23*(6), 488–493. https://doi.org/10.1097/01.shk.0000163802.46355.59
33. Pareek, S., Kurakawa, T., Das, B., Motooka, D., Nakaya, S., Rongsen-Chandola, T., Goyal, N., Kayama, H., Dodd, D., Okumura, R., Maeda, Y., Fujimoto, K., Nii, T., Ogawa, T., Iida, T., Bhandari, N., Kida, T., Nakamura, S., Nair, G. B., & Takeda, K. (2019). Comparison of Japanese and Indian intestinal microbiota shows diet-dependent

interaction between bacteria and fungi. *Npj Biofilms and Microbiomes*, *5*(1). https://doi.org/10.1038/s41522-019-0110-9
34. Parsons, P. E., Eisner, M. D., Thompson, B. T., Matthay, M. A., Ancukiewicz, M., Bernard, G. R., ... & NHLBI Acute Respiratory Distress Syndrome Clinical Trials Network. (2005). Lower tidal volume ventilation and plasma cytokine markers of inflammation in patients with acute lung injury. *Critical care medicine*, *33*(1), 1-6.
35. Peer, V., Schwartz, N., & Green, M. S. (2021). Sex differences in salmonellosis incidence rates—an eight-country national data-pooled analysis. *Journal of Clinical Medicine*, *10*(24). https://doi.org/10.3390/jcm10245767
36. Perkins, D. J., Rajaiah, R., Tennant, S. M., Ramachandran, G., Higginson, E. E., Dyson, T. N., & Vogel, S. N. (2015). Salmonella Typhimurium Co-Opts the Host Type I IFN System To Restrict Macrophage Innate Immune Transcriptional Responses Selectively. *The Journal of Immunology*, *195*(5), 2461–2471. https://doi.org/10.4049/jimmunol.1500105
37. Remick, D. G., Bolgos, G. R., Siddiqui, J., Shin, J., & Nemzek, J. A. (2002). Six at six: interleukin-6 measured 6 h after the initiation of sepsis predicts mortality over 3 days. *Shock*, *17*(6), 463-467.
38. Round, J. L., & Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. In *Nature Reviews Immunology* (Vol. 9, Issue 5, pp. 313–323). https://doi.org/10.1038/nri2515
39. Salazar, G. A., Peñaloza, H. F., Pardo-Roa, C., Schultz, B. M., Muñoz-Durango, N., Gómez, R. S., Salazar, F. J., Pizarro, D. P., Riedel, C. A., González, P. A., Alvarez-Lobos, M., Kalergis, A. M., & Bueno, S. M. (2017). Interleukin-10 production by T and B cells is a key factor to promote systemic Salmonella enterica serovar typhimurium infection in mice. *Frontiers in Immunology*, *8*(AUG). https://doi.org/10.3389/fimmu.2017.00889
40. Sankaran-Walters, S., Macal, M., Grishina, I., Nagy, L., Goulart, L., Coolidge, K., Li, J., Fenton, A., Williams, T., Miller, M. K., Flamm, J., Prindiville, T., George, M., & Dandekar, S. (2013). *Sex differences matter in the gut: effect on mucosal immune activation and inflammation*. http://www.bsd-journal.com/content/4/1/10
41. Scotland, R. S., Stables, M. J., Madalli, S., Watson, P., & Gilroy, D. W. (2011). Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. *Blood*, *118*(22), 5918–5927. https://doi.org/10.1182/blood-2011-03-340281
42. Sekirov, I., Tam, N. M., Jogova, M., Robertson, M. L., Li, Y., Lupp, C., & Finlay, B. B. (2008). Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. *Infection and Immunity*, *76*(10), 4726–4736. https://doi.org/10.1128/IAI.00319-08
43. Stanaway, J. D., Parisi, A., Sarkar, K., Blacker, B. F., Reiner, R. C., Hay, S. I., Nixon, M. R., Dolecek, C., James, S. L., Mokdad, A. H., Abebe, G., Ahmadian, E., Alahdab, F., Alemnew, B. T. T., Alipour, V., Allah Bakeshei, F., Animut, M. D., Ansari, F., Arabloo, J., … Crump, J. A. (2019). The global burden of non-typhoidal salmonella invasive disease: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Infectious Diseases*, *19*(12), 1312–1324. https://doi.org/10.1016/S1473-3099(19)30418-9

44. Thiemann, S., Smit, N., Roy, U., Lesker, T. R., Gálvez, E. J. C., Helmecke, J., Basic, M., Bleich, A., Goodman, A. L., Kalinke, U., Flavell, R. A., Erhardt, M., & Strowig, T. (2017). Enhancement of IFNγ Production by Distinct Commensals Ameliorates Salmonella-Induced Disease. *Cell Host and Microbe*, *21*(6), 682-694.e5. https://doi.org/10.1016/j.chom.2017.05.005
45. Vatanen, T., Kostic, A. D., D'Hennezel, E., Siljander, H., Franzosa, E. A., Yassour, M., Kolde, R., Vlamakis, H., Arthur, T. D., Hämäläinen, A. M., Peet, A., Tillmann, V., Uibo, R., Mokurov, S., Dorshakova, N., Ilonen, J., Virtanen, S. M., Szabo, S. J., Porter, J. A., … Xavier, R. J. (2016). Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. *Cell*, *165*(4), 842–853. https://doi.org/10.1016/j.cell.2016.04.007
46. Vázquez-Martínez, E. R., García-Gómez, E., Camacho-Arroyo, I., & González-Pedrajo, B. (2018). Sexual dimorphism in bacterial infections. In *Biology of Sex Differences* (Vol. 9, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s13293-018-0187-5
47. Walter, J. (2008). Ecological role of lactobacilli in the gastrointestinal tract: Implications for fundamental and biomedical research. In *Applied and Environmental Microbiology* (Vol. 74, Issue 16, pp. 4985–4996). American Society for Microbiology. https://doi.org/10.1128/AEM.00753-08
48. Wotzka, S. Y., Kreuzer, M., Maier, L., Arnoldini, M., Nguyen, B. D., Brachmann, A. O., Berthold, D. L., Zünd, M., Hausmann, A., Bakkeren, E., Hoces, D., Gül, E., Beutler, M., Dolowschiak, T., Zimmermann, M., Fuhrer, T., Moor, K., Sauer, U., Typas, A., … Hardt, W. D. (2019). Escherichia coli limits Salmonella Typhimurium infections after diet shifts and fat-mediated microbiota perturbation in mice. In *Nature Microbiology* (Vol. 4, Issue 12, pp. 2164–2174). Nature Research. https://doi.org/10.1038/s41564-019-0568-5

Chapter 4:

1. Abdullah, M., Chai, P. S., Chong, M. Y., Tohit, E. R. M., Ramasamy, R., Pei, C. P., & Vidyadaran, S. (2012). Gender effect on in vitro lymphocyte subset levels of healthy individuals. *Cellular Immunology*, *272*(2), 214–219. https://doi.org/10.1016/j.cellimm.2011.10.009
2. Amadori, A., Zamarchi, R., De Silvestro, G., Forza, G., Cavatton, G., Danieli, G. A., ... & Chieco-Bianchi, L. (1995). Genetic control of the CD4/CD8 T-cell ratio in humans. *Nature medicine*, *1*(12), 1279-1283.
3. Anders, J. L., Moustafa, M. A. M., Mohamed, W. M. A., Hayakawa, T., Nakao, R., & Koizumi, I. (2021). Comparing the gut microbiome along the gastrointestinal tract of three sympatric species of wild rodents. *Scientific Reports*, *11*(1). https://doi.org/10.1038/s41598-021-99379-6
4. Antonopoulos, D. A., Huse, S. M., Morrison, H. G., Schmidt, T. M., Sogin, M. L., & Young, V. B. (2009). Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. *Infection and Immunity*, *77*(6), 2367–2375. https://doi.org/10.1128/IAI.01520-08
5. Arai, T., Hiromatsu, K., Nishimura, H., Kimura, Y., Kobayashi, N., Ishida, H., Nimurat, Y., & Yoshikai, Y. (1995). Effects of in vivo administration of anti-IL-10 monoclonal

antibody on the host defence mechanism against murine Salmonella infection. In *Immunology* (Vol. 85).
6. Brugiroux, S., Beutler, M., Pfann, C., Garzetti, D., Ruscheweyh, H. J., Ring, D., Diehl, M., Herp, S., Lötscher, Y., Hussain, S., Bunk, B., Pukall, R., Huson, D. H., Münch, P. C., McHardy, A. C., McCoy, K. D., MacPherson, A. J., Loy, A., Clavel, T., … Stecher, B. (2016). Genome-guided design of a defined mouse microbiota that confers colonization resistance against Salmonella enterica serovar Typhimurium. *Nature Microbiology*, *2*. https://doi.org/10.1038/nmicrobiol.2016.215
7. Chassin, C., Kocur, M., Pott, J., Duerr, C. U., Gütle, D., Lotz, M., & Hornef, M. W. (2010). MiR-146a mediates protective innate immune tolerance in the neonate intestine. *Cell Host and Microbe*, *8*(4), 358–368. https://doi.org/10.1016/j.chom.2010.09.005
8. Chung, H., Pamp, S. J., Hill, J. A., Surana, N. K., Edelman, S. M., Troy, E. B., Reading, N. C., Villablanca, E. J., Wang, S., Mora, J. R., Umesaki, Y., Mathis, D., Benoist, C., Relman, D. A., & Kasper, D. L. (2012). Gut immune maturation depends on colonization with a host-specific microbiota. *Cell*, *149*(7), 1578–1593. https://doi.org/10.1016/j.cell.2012.04.037
9. Crespo-Piazuelo, D., Estellé, J., Revilla, M., Criado-Mesas, L., Ramayo-Caldas, Y., Óvilo, C., Fernández, A. I., Ballester, M., & Folch, J. M. (2018). Characterization of bacterial microbiota compositions along the intestinal tract in pigs and their interactions and functions. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-30932-6
10. Fiebiger, U., Bereswill, S., & Heimesaat, M. M. (2016). Dissecting the interplay between intestinal microbiota and host immunity in health and disease: Lessons learned from germfree and gnotobiotic animal models. *European Journal of Microbiology and Immunology*, *6*(4), 253–271. https://doi.org/10.1556/1886.2016.00036
11. Fischbach, M. A. (2018). Microbiome: Focus on Causation and Mechanism. In *Cell* (Vol. 174, Issue 4, pp. 785–790). Cell Press. https://doi.org/10.1016/j.cell.2018.07.038
12. Fish, E. N. (2008). The X-files in immunity: sex-based differences predispose immune responses. *Nature Reviews Immunology*, *8*(9), 737-744.
13. Fouhse, J. M., Yang, K., More-Bayona, J., Gao, Y., Goruk, S., Plastow, G., Field, C. J., Barreda, D. R., & Willing, B. P. (2019). Neonatal Exposure to Amoxicillin Alters Long-Term Immune Response Despite Transient Effects on Gut-Microbiota in Piglets. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.02059
14. Fox, H. S., Bond, B. L., & Parslow+, T. G. (1991). ESTROGEN REGULATES THE IFN-7 PROMOTER'. In *Association of Immunologists* (Vol. 146, Issue 12). http://www.jimmunol.org/
15. Frink, M., Pape, H. C., van Griensven, M., Krettek, C., Chaudry, I. H., & Hildebrand, F. (2007). Influence of sex and age on mods and cytokines after multiple injuries. *Shock*, *27*(2), 151–156. https://doi.org/10.1097/01.shk.0000239767.64786.de
16. Furter, M., Sellin, M. E., Hansson, G. C., & Hardt, W. D. (2019). Mucus Architecture and Near-Surface Swimming Affect Distinct Salmonella Typhimurium Infection Patterns along the Murine Intestinal Tract. *Cell Reports*, *27*(9), 2665-2678.e3. https://doi.org/10.1016/j.celrep.2019.04.106
17. Gay, L., Melenotte, C., Lakbar, I., Mezouar, S., Devaux, C., Raoult, D., Bendiane, M. K., Leone, M., & Mège, J. L. (2021). Sexual Dimorphism and Gender in Infectious Diseases.

In *Frontiers in Immunology* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2021.698121
18. Graversen, K. B., Bahl, M. I., Larsen, J. M., Ballegaard, A. S. R., Licht, T. R., & Bøgh, K. L. (2020). Short-Term Amoxicillin-Induced Perturbation of the Gut Microbiota Promotes Acute Intestinal Immune Regulation in Brown Norway Rats. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.00496
19. Hill-Burns, E. M., & Clark, A. G. (2009). X-linked variation in immune response in Drosophila melanogaster. *Genetics*, *183*(4), 1477–1491. https://doi.org/10.1534/genetics.108.093971
20. Holman, D. B., Brunelle, B. W., Trachsel, J., & Allen, H. K. (2017). Meta-analysis To Define a Core Microbiota in the Swine Gut. *MSystems*, *2*(3). https://doi.org/10.1128/msystems.00004-17
21. Huang, H. C., Vlasova, A. N., Kumar, A., Kandasamy, S., Fischer, D. D., Deblais, L., Paim, F. C., Langel, S. N., Alhamo, M. A., Rauf, A., Shao, L., Saif, L. J., & Rajashekara, G. (2018). Effect of antibiotic, probiotic, and human rotavirus infection on colonisation dynamics of defined commensal microbiota in a gnotobiotic pig model. *Beneficial Microbes*, *9*(1), 71–86. https://doi.org/10.3920/BM2016.0225
22. Klein, S. L., & Flanagan, K. L. (2016). Sex differences in immune responses. In *Nature Reviews Immunology* (Vol. 16, Issue 10, pp. 626–638). Nature Publishing Group. https://doi.org/10.1038/nri.2016.90
23. Kovats, S. (2015). Estrogen receptors regulate innate immune cells and signaling pathways. *Cellular Immunology*, *294*(2), 63–69. https://doi.org/10.1016/j.cellimm.2015.01.018
24. Kozakova, H., Kolinska, J., Lojda, Z., Rehakova, Z., Sinkora, J., Zakostelecka, M., Splichal, I., & Tlaskalova-Hogenova, H. (2006). Effect of bacterial monoassociation on brush-border enzyme activities in ex-germ-free piglets: comparison of commensal and pathogenic Escherichia coli strains. *Microbes and Infection*, *8*(11), 2629–2639. https://doi.org/10.1016/j.micinf.2006.07.008
25. Lkhagva, E., Chung, H. J., Hong, J., Tang, W. H. W., Lee, S. il, Hong, S. T., & Lee, S. (2021). The regional diversity of gut microbiome along the GI tract of male C57BL/6 mice. *BMC Microbiology*, *21*(1). https://doi.org/10.1186/s12866-021-02099-0
26. Lonnermark, E., Lappas, G., Friman, V., Wold, A. E., Backhaus, E., & Adlerberth, I. (2014). *Effects of Probiotic Intake and Gender on Nontyphoid Salmonella Infection*. www.jcge.com
27. Looft, T., Johnson, T. A., Allen, H. K., Bayles, D. O., Alt, D. P., Stedtfeld, R. D., Sul, W. J., Stedtfeld, T. M., Chai, B., Cole, J. R., Hashsham, S. A., Tiedje, J. M., & Stanton, T. B. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(5), 1691–1696. https://doi.org/10.1073/pnas.1120238109
28. Lotz, M., Gütle, D., Walther, S., Ménard, S., Bogdan, C., & Hornef, M. W. (2006). Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. *Journal of Experimental Medicine*, *203*(4), 973–984. https://doi.org/10.1084/jem.20050625
29. Lu, R. J., Taylor, S., Contrepois, K., Kim, M., Bravo, J. I., Ellenberger, M., Sampathkumar, N. K., & Benayoun, B. A. (2021). Multi-omic profiling of primary

mouse neutrophils predicts a pattern of sex- and age-related functional regulation. *Nature Aging*, *1*(8), 715–733. https://doi.org/10.1038/s43587-021-00086-8
30. Luppi, A. (2017). Swine enteric colibacillosis: Diagnosis, therapy and antimicrobial resistance. In *Porcine Health Management* (Vol. 3). BioMed Central Ltd. https://doi.org/10.1186/s40813-017-0063-4
31. McAleer, J. P., & Vella, A. T. (2008). Understanding how lipopolysaccharide impacts CD4 T-cell immunity. *Critical Reviews™ in Immunology*, *28*(4).
32. Mondal, S., & Rai, U. (1999). *Sexual Dimorphism in Phagocytic Activity of Wall Lizard's Splenic Macrophages and Its Control by Sex Steroids*. http://www.idealibrary.com
33. Neuman, H., Forsythe, P., Uzan, A., Avni, O., & Koren, O. (2018). Antibiotics in early life: dysbiosis and the damage done. In *FEMS microbiology reviews* (Vol. 42, Issue 4, pp. 489–499). NLM (Medline). https://doi.org/10.1093/femsre/fuy018
34. Neves, P., Lampropoulou, V., Calderon-Gomez, E., Roch, T., Stervbo, U., Shen, P., Kühl, A. A., Loddenkemper, C., Haury, M., Nedospasov, S. A., Kaufmann, S. H. E., Steinhoff, U., Calado, D. P., & Fillatreau, S. (2010). Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during salmonella typhimurium infection. *Immunity*, *33*(5), 777–790. https://doi.org/10.1016/j.immuni.2010.10.016
35. Nilsson, O. R., Kari, L., & Steele-Mortimer, O. (2019). Foodborne infection of mice with Salmonella Typhimurium. *PLoS ONE*, *14*(8). https://doi.org/10.1371/journal.pone.0215190
36. Oberholzer, A., Souza, S. M., Tschoeke, S. K., Oberholzer, C., Abouhamze, A., Pribble, J. P., & Moldawer, L. L. (2005). Plasma cytokine measurements augment prognostic scores as indicators of outcome in patients with severe sepsis. *Shock*, *23*(6), 488–493. https://doi.org/10.1097/01.shk.0000163802.46355.59
37. Pap, P. L., Czirják, G. Á., Vágási, C. I., Barta, Z., & Hasselquist, D. (2010). Sexual dimorphism in immune function changes during the annual cycle in house sparrows. *Naturwissenschaften*, *97*(10), 891–901. https://doi.org/10.1007/s00114-010-0706-7
38. Peer, V., Schwartz, N., & Green, M. S. (2021). Sex differences in salmonellosis incidence rates—an eight-country national data-pooled analysis. *Journal of Clinical Medicine*, *10*(24). https://doi.org/10.3390/jcm10245767
39. Remick, D. G., Bolgos, G. R., Siddiqui, J., Shin, J., & Nemzek, J. A. (2002). Six at six: interleukin-6 measured 6 h after the initiation of sepsis predicts mortality over 3 days. *Shock*, *17*(6), 463-467.
40. Rettew, J. A., Huet, Y. M., & Marriott, I. (2009). Estrogens augment cell surface TLR4 expression on murine macrophages and regulate sepsis susceptibility in vivo. *Endocrinology*, *150*(8), 3877–3884. https://doi.org/10.1210/en.2009-0098
41. Rettew, J. A., Huet-Hudson, Y. M., & Marriott, I. (2008). Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. *Biology of Reproduction*, *78*(3), 432–437. https://doi.org/10.1095/biolreprod.107.063545
42. Salazar, G. A., Peñaloza, H. F., Pardo-Roa, C., Schultz, B. M., Muñoz-Durango, N., Gómez, R. S., Salazar, F. J., Pizarro, D. P., Riedel, C. A., González, P. A., Alvarez-Lobos, M., Kalergis, A. M., & Bueno, S. M. (2017). Interleukin-10 production by T and B cells is a key factor to promote systemic Salmonella enterica serovar typhimurium

infection in mice. *Frontiers in Immunology*, *8*(AUG). https://doi.org/10.3389/fimmu.2017.00889
43. Scotland, R. S., Stables, M. J., Madalli, S., Watson, P., & Gilroy, D. W. (2011). Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. *Blood*, *118*(22), 5918–5927. https://doi.org/10.1182/blood-2011-03-340281
44. Suzuki, T. A., & Nachman, M. W. (2016). Spatial heterogeneity of gut microbial composition along the gastrointestinal tract in natural populations of house mice. *PLoS ONE*, *11*(9). https://doi.org/10.1371/journal.pone.0163720
45. Thiemann, S., Smit, N., Roy, U., Lesker, T. R., Gálvez, E. J. C., Helmecke, J., Basic, M., Bleich, A., Goodman, A. L., Kalinke, U., Flavell, R. A., Erhardt, M., & Strowig, T. (2017). Enhancement of IFNγ Production by Distinct Commensals Ameliorates Salmonella-Induced Disease. *Cell Host and Microbe*, *21*(6), 682-694.e5. https://doi.org/10.1016/j.chom.2017.05.005
46. Vangay, P., Ward, T., Gerber, J. S., & Knights, D. (2015). Antibiotics, pediatric dysbiosis, and disease. In *Cell Host and Microbe* (Vol. 17, Issue 5, pp. 553–564). Cell Press. https://doi.org/10.1016/j.chom.2015.04.006
47. Villacres, M. C., Longmate, J., Auge, C., & Diamond, D. J. (2004). Predominant type 1 CMV-specific memory T-helper response in humans: Evidence for gender differences in cytokine secretion. *Human Immunology*, *65*(5), 476–485. https://doi.org/10.1016/j.humimm.2004.02.021
48. vom Steeg, L. G., & Klein, S. L. (2016). SeXX Matters in Infectious Disease Pathogenesis. In *PLoS Pathogens* (Vol. 12, Issue 2). Public Library of Science. https://doi.org/10.1371/journal.ppat.1005374
49. Wotzka, S. Y., Kreuzer, M., Maier, L., Arnoldini, M., Nguyen, B. D., Brachmann, A. O., Berthold, D. L., Zünd, M., Hausmann, A., Bakkeren, E., Hoces, D., Gül, E., Beutler, M., Dolowschiak, T., Zimmermann, M., Fuhrer, T., Moor, K., Sauer, U., Typas, A., … Hardt, W. D. (2019). Escherichia coli limits Salmonella Typhimurium infections after diet shifts and fat-mediated microbiota perturbation in mice. In *Nature Microbiology* (Vol. 4, Issue 12, pp. 2164–2174). Nature Research. https://doi.org/10.1038/s41564-019-0568-5
50. Zhao, W., Wang, Y., Liu, S., Huang, J., Zhai, Z., He, C., Ding, J., Wang, J., Wang, H., Fan, W., Zhao, J., & Meng, H. (2015). The dynamic distribution of porcine microbiota across different ages and gastrointestinal tract segments. *PLoS ONE*, *10*(2). https://doi.org/10.1371/journal.pone.0117441
51. Zhou, H., Sun, J., Yu, B., Liu, Z., Chen, H., He, J., Mao, X., Zheng, P., Yu, J., Luo, J., Luo, Y., Yan, H., Ge, L., & Chen, D. (2021). Gut microbiota absence and transplantation affect growth and intestinal functions: An investigation in a germ-free pig model. *Animal Nutrition*, *7*(2), 295–304. https://doi.org/10.1016/j.aninu.2020.11.012