Development of the cockroach as a human gut microbiome model

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Abstract

The human gut microbiome is a noteworthy area of interest due to the potential beneficial functions involving human health, fitness, and immunity. Cockroaches are omnivorous insects with a diverse microbial population that could be suggested as an alternate model for investigating the effect of the microbiome on the human gut. The aim of this project was to explore whether broader knowledge and understanding of the human gut microbiota could be achieved by studying cockroaches as a model. The microbiota of a total of 693 publicly accessible samples (345 humans, 86 mice, and 262 cockroaches) were analysed. *Firmicutes*, *Bacteroidota*, and *Proteobacteria* are predominant phyla across all samples, which led us to pursue further analyses of the cockroach microbiome, as a potential model. Colonization of a commensal *Escherichia coli* (*E. coli*), and response to antibiotics were studied in the adult female American cockroaches (*Periplaneta americana*). Kanamycin treatment on the cockroach gut microbiome showed similarities with the control group but lower community abundance. In contrast, bacterial communities fed ampicillin exhibited significant shifts and were less abundant than those fed kanamycin or control. In the following experiment with *E. coli* inoculation, the findings demonstrated that despite our application of antibiotics for ten days prior to the introduction of *E. coli*, the invading bacterium could not be established in the cockroach's digestive organ. By treating cockroaches with increased concentrations of 0.1% and 0.2% sulfate, the impact of a sulfate diet was investigated. According to the study, there were no significant variations in the abundance of *Desulfovibrio* and bacterial communities between the diet groups. Additionally, the dissimilatory sulfate reduction genes, including *dsrA* and *aprA* genes, did not significantly alter expression between treatments, but they did appear to be expressed slightly higher than the control.

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Author’s Declaration

I hereby declare that I am the sole author of this thesis and that it is my own original work. This thesis has not previously been presented to the University of York or other institutions for consideration for a degree or other qualification elsewhere. Every debate and review of the literature is cited.

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Chapter 1: General Introduction

1.1 Human gut microbiome

The human gut microbiome accounted for about 10^{11}-10^{12} cells per gram of faeces (Langendijk et al., 1995; Suau et al., 1999). It was found that distinct bacterial communities are found in different parts of the gastrointestinal sections, including the stomach, duodenum, jejunum, ileum, and colon (Sekirov et al., 2010). More significantly, the quantity and variety of bacteria increase along the longitudinal tract, from 10^{1} to 10^{3} in the stomach to 10^{11} to 10^{12} bacterial cells per gram of contents in the colon (Sekirov et al., 2010).

In the first era of microbiome study, the study in body habitats in healthy adults from Westerners found that human microbes occur in several human body parts, including the oral cavity, skin, nasal cavity, vagina, and stool samples (Turnbaugh et al., 2007). The Human Genome Project (HMP) was first initiated and completed in 2003. It was discovered that the human genome contains only approximately 20,000 genes, which is far fewer genes than we expected (Moraes and Góes, 2016). While the human microbiome revealed that the human microbiome genome contains about 3.3 million genes, or roughly 150 times the number of the human genome (Zhu, Wang and Li, 2010).

A study of the healthy human gut microbiome of all ages across several countries found that there are five predominant enterosignatures that are common to populations. These are *Bacteroides, Firmicutes*, *Prevotella, Bifidobacterium,* and *Escherichia* (Frioux et al., 2023a). The study of gut microbiomes in 20 cohorts from different human populations found that the core microbiota consists of several genera, with the main predominant ones being *Bacteroides, Faecalibacterium, Blautia, Alistipes,* and *Subdoligranulum* (Lim et al., 2020).

Distinct bacterial communities coexist in the host's gastrointestinal tract. These microorganisms can compete directly or indirectly through the production of antimicrobial peptides, such as bacteriocins or bacteriophages, which can harm or kill competitors, as well as through nutritional competition (Martinson and Walk, 2020). The human commensal microbiome is crucial in guarding against opportunistic infections. On the other hand, an unstable or unhealthy gut microbiome can result in adverse gut disorders, including weakened immune systems and malfunctions of metabolic pathways (Dey, 2024). The dysbiosis of the gut microbiome can be caused by a number of illnesses that affect people, such as Crohn's disease and ulcerative colitis (Carding et al., 2015).

1.1.1 Factors influencing the human gut microbiome.

1.1.1.1 Stages of life

One of the factors that influence our gut microbiome is ageing (Mariat et al., 2009; Yatsunenko et al., 2012; Bradley and Haran, 2024). The human gut microbiota seems to vary depending on the life stage. Overall, alpha diversity varied with human age; infants had the least amount of variety, while adolescents and young adults had a greater increase in alpha diversity (Badal et al., 2020). Infants' gut microbiomes appear to be impacted by their mothers' diets. High levels of oligosaccharides found in human breast milk are primarily responsible for mediating early immune responses in infants, including allergies and asthma (Hegar et al., 2019). In the first year of life, *Bifidobacterium longum* is the predominant strain; nevertheless, as an increase in age, their abundance declined in both the US urban areas and Amazonas of Venezuela and rural Malawi (Yatsunenko et al., 2012). Moreover, the gut microbiome of infants is impacted by different infant delivery methods, either cesarean section or vaginal birth (Lundgren et al., 2018). The gut microbiota of newborns can be transferred from their mothers. However, not all maternal gut microbiome can be colonized in infant guts; only some bacterial strains can colonize the infant guts, such as *Bifidobacteria* and *Bacteroides* (Ferretti et al., 2018). These bacterial-transmitted strains appear to be more stable in the infant's gut later on, in contrast to non-maternal microorganisms, which are more unstable (Ferretti et al., 2018). Research on the gut microbiome of healthy Japanese revealed that *Firmicutes* was the predominant phylum following weaning, while *Actinobacteria* declined after weaning and continued to decline with age (Odamaki et al., 2016).

Firmicutes, *Bacteroides*, and *Actinobacteria* are the primary bacterial phyla in healthy adult gut microbiomes (Vaiserman et al., 2020; Claesson et al., 2011). The human gut microbiome can change with age; there are some bacterial strains that predominantly differ between young adults and the elderly (Rampelli et al., 2013).

There are significant changes across age groups in the gut microbiome of healthy people (Vaiserman et al., 2020). The *Firmicute*/*Bacteroides* ratio increases with age (Vaiserman et al., 2020). More *Bacteroidetes* than *Firmicutes* were found in the elderly over 79 years old, but more *Firmicutes* were found in younger individuals (Claesson et al., 2011). Furthermore, older adults over the age of 70 have higher levels of *Bacteroidetes* and *Proteobacteria* (Odamaki et al., 2016). In elderly populations, the diversity of bacterial species was lower in numerous anaerobic bacterial groups, such as *Bacteroides, Prevotella, and Bifidobacteria* (Woodmansey et al., 2004). These were also associated with a decline in amylolytic activities and a decrease in total short-chain fatty acids (SCFAs) in the elderly (Woodmansey et al., 2004). There are differences in the amount of SCFA genes across age

groups. Proteolytic genes were more prevalent in the elderly than in adults, but saccharolytic genes were less common (Rampelli et al., 2013).

1.1.1.2 Diet

Several diets influence the human gut microbiome. Fed breastfed and commercial formulas with components that closely resemble breast milk, the infant gut microbiomes are distinct (Baumann-Dudenhoeffer et al., 2018). The Mediterranean diet is renowned for being a nutritious diet that can change the human gut flora and has positive effects on gut health (Krznarić, Vranešić Bender and Meštrović, 2019). The Mediterranean diet was applied to various Western populations for one year; specifically, volunteers consumed more whole grains, fish, legumes, and nuts. The outcomes indicated that these diets could change the microbiome in their guts and lower blood cholesterol (Meslier et al., 2020). In addition, a yearlong Mediterranean diet combined with exercise indicated that patients who were overweight or obese lost weight, improved their risk factors for cardiovascular disease, and changed their gut microbiome (Muralidharan et al., 2021).

Prevotella is known as a beneficial bacterium that aids in the digestion of foods high in fibre. This bacterium is typically prevalent in persons who consume a healthy diet rich in carbohydrates and fibre (Yatsunenko et al., 2012; De Filippis et al., 2019; Prasoodanan P. K. et al., 2021). High-calorie, high-animal protein, high-sugar, high-saturated fat, low-fibre, fast food, and highly processed foods are Western diets (Zinöcker and Lindseth, 2018; Newsome, Yang and Jobin, 2023). In contrast to the Western population, which is enriched with inflammatory *Prevotella* strains, *P. copri* is more common in healthy Indian and non-Western populations (Prasoodanan P. K. et al., 2021). Moreover, *Bacteroides* are associated with Western diets (Frioux et al., 2023a).

1.1.1.3 Role of the human gut microbiome

Human health and diseases are impacted by metabolic pathways, which are significantly influenced by the human gut microbiome. The primary short-chain fatty acids (SCFAs) found in the human gut are butyrate, propionate, and acetate. These are byproducts of the breakdown of polysaccharides by gut microbiota bacteria such as *Firmicutes* and *Bacteroidetes* (Macfarlane, Gibson and Cummings, 1992). Changes in the composition of the microbiota can affect the synthesis of SCFAs, which can cause a number of diseases, including diabetes, obesity (Pascale et al., 2018), and brain disorders (Silva, Bernardi and Frozza, 2020).

The gut microbiota is crucial to human health and performs a variety of activities that promote gut fitness. Some specific bacterial genera could be used as markers to distinguish type 1 diabetes (T1D) in infants as their alpha diversity diverges compared with infants who did not progress to T1D (Kostic

et al., 2015). *Lactobacillus* is a lactic acid bacterium. This bacterium has been used as a probiotic that can improve intestinal health from intestinal diseases such as inflammatory and infectious gut diseases (Huang et al., 2022). Intestinal bacteria contribute to human immunity. For example, *Bacteroides fragilis* prevented *Helicobacter hepaticus*, harmful bacteria, from causing intestinal inflammation (Mazmanian, Round and Kasper, 2008).

1.1.1.4 Human diseases

Several diseases compared to healthy humans showed that their gut microbiome is altered by several diseases in humans, including type 1 or 2 diabetes (Kostic et al., 2015; Larsen et al., 2010), inflammatory bowel diseases (IBDs) (Halfvarson et al., 2017a; Loubinoux et al., 2002a), and neuropathological disorders (Borrego-Ruiz and Borrego, 2024).

IBD is associated with several diseases, such as ulcerative colitis and Crohn's disease (Halfvarson et al., 2017a). The gut microbiota can shift significantly in response to inflammation. Specifically, there was a decrease in the quantity of advantageous bacterial strains in IBD patients, including *Prevotella copri* and *Faecalibacterium prauznitzii* (Halfvarson et al., 2017a).

Alzheimer's disease, Parkinson's disease, dementia, and mild cognitive impairment are among the neuropathological conditions that affect elderly people. In contrast to senior people in good health, several studies on the human gut microbiome revealed that various illnesses could change the gut microbiome of the elderly (Borrego-Ruiz and Borrego, 2024).

Patients with type 2 diabetes had a lower abundance of *Bacteroides vulgatus* and *Bifidobacterium* than healthy individuals (Wu et al., 2010). Patients with type 2 diabetes had higher levels of *Firmicutes* and the class *Clostridia* compared to the healthy (Larsen et al., 2010). Understanding the interactions between gut microbiota and diseases is crucial for developing effective therapeutic strategies for managing human diseases.

1.1.1.5 Host genetics

The impact of host genetics on the human gut microbiome has been determined through many studies. A study including identical and fraternal twins from the United Kingdom revealed that the identical twins had a more comparable gut microbiome than the fraternal twins (Goodrich et al., 2016). Moreover, some taxa in *Firmicutes* had a heritable ability, such as family *Christensenellaceae* and genus *Turicibacter* from the study of twins in the UK (Goodrich et al., 2016). The gut microbiome of twins is expected to be similar. While unrelated twins exposed to the same type of soy formula had highly comparable gut microbiomes, related twins exposed to different types of soy formula had distinct microbiomes (Baumann-Dudenhoeffer et al., 2018). It has been shown that the gut

microbiota may change with socioeconomic status, including factors like income, education, and criminality (Bowyer et al., 2019). Specifically, bacterial alpha diversity decreased with decreasing individual income in the discordant UK twins (Bowyer et al., 2019).

Overall, the human gut microbiota can be altered by a variety of causes. While some research indicated that host genetics had a major impact on the human gut microbiome, other investigations only revealed slight effects. More and more further studies are required to be investigated.

1.1.1.6 Geographical locations and environmental exposure

Several studies have found that distinct bacterial species are typically found in geographical areas. The core bacterial genera microbiota could be different across different human populations (Falony et al., 2016). The relative abundance of major phyla *Firmicutes* and *Bacteroides* is affected by latitude. *Firmicutes* and latitude showed a strong positive correlation, whereas *Bacteroides* and latitude showed a negative correlation (Suzuki and Worobey, 2014). Rural populations had higher richness of gut microbiome than in urban-industrial populations (Obregon-Tito et al., 2015). The gut microbial communities of indigenous people were shown to be richer than those of industrialized populations (Obregon-Tito et al., 2015). Specifically, *Proteobacteria* and *Spirochaetes* were predominant in the rural populations of Matses and Tunapuco, while *Actinobacteria* was more abundant in the urban-industrialized population of Norman (Obregon-Tito et al., 2015). The gut microbiomes of US residents differed and were altered from those of rural residents in Malawi and Venezuela (Moeller et al., 2014). Specifically, compared to populations in rural areas, the intestinal tract of US populations showed a five-fold increase in *Bacteroides* (Moeller et al., 2014).

It has been controversial whether the environment or host genetics have a greater impact on the gut microbiome. Some studies showed that the gut microbiome is more influenced by environmental factors than genetic factors (Rothschild et al., 2018; Scepanovic et al., 2019). There was no substantial microbiome similarity among relatives who do not live together, while unrelated people living together showed significant species-level similarities in their gut microbiomes (Rothschild et al., 2018). A greater understanding of how the gut microbiome is influenced by genetic and environmental variables assists us in paying more attention to and being more cautious about our habits in order to become healthier.

1.1.1.7 Medication

A variety of medications, including antibiotics and nonantibiotics, affect gut microbiota and metabolism in Japanese populations (Nagata et al., 2022). In particular, compared to other pharmaceutical categories, diabetes, and alimentary tract drugs showed a greater correlation with

the gut microbiome's composition (Nagata et al., 2022). Moreover, multiple drugs could be associated with an increase in antimicrobial resistance potential (Nagata et al., 2022). The study of the bidirectional interaction between medication and the gut microbiome showed that the gut microbiome could influence drug efficiency in the human gut (Weersma, Zhernakova and Fu, 2020). In general, research on the effects of drug use is crucial for gaining a better knowledge of how medications affect human health and gut flora. In order to obtain more knowledge, more detailed research in this field about their consequences and appropriate utilization is required.

The effects of different antibiotics can influence the gut microbiome in different ways, and the same antibiotics influence differently depending on individuals (Zaura et al., 2015). Furthermore, the effectiveness of any antibiotic can vary based on a number of variables, including the type of antibiotics (Zaura et al., 2015), the patient's condition, the dosage and duration of treatment, and the delivery method.

Short-term antibiotic treatment can significantly alter the human gut microbiota for several days, months, or years. Furthermore, a number of studies have discovered that the effects of antibiotics are long-lasting, including antimicrobial resistance being one of the effects (Jernberg et al., 2010; Francino, 2016). For example, substantial amounts of antibiotic-resistance genes were found a year after a week of ampicillin treatment, and the gene was still detectable four years later (Jakobsson et al., 2010). Following a week of clindamycin treatment for *Bacteroides* infections, the number of *Bacteroides* decreased, and this effect lasted for up to two years (Jernberg et al., 2007). While ciprofloxacin differed from the placebo for up to one month, clindamycin had a strong significant impact on the gut microbiome for up to four months (Zaura et al., 2015). Oral clindamycin treatment for one week had a long-lasting effect on intestinal enterococci species for more than nine months, and resistance gene increase for up to twelve months (Lindgren et al., 2009).

An increasing number of studies have focused on repeated exposure to antibiotics. After being altered by antibiotics, the gut microbiomes require time to return to their normal flora, but if the same medications are used again, the recovery may be incomplete (Dethlefsen and Relman, 2011). Research on antibiotic exposure in the early stages of life and repetitive antibiotic use may contribute to the development of pediatric Crohn's disease in children (Virta et al., 2012). Infants exposed to repeated antibiotic courses during their first year of life can develop disruptions in their gut flora, which raises the risk of childhood obesity and increases adiposity (Chen et al., 2020a).

1.1.2 Functional redundancy of the gut microbiome

Functional redundancy refers to different microorganisms that are capable of carrying out the same function (Louca et al., 2018). More specifically, each person's gut microbiome has a different taxonomic composition; yet their functional genes are similar (Tian et al., 2020). The investigation of bile salt hydrolase activity is conserved throughout the microbiota of individual human hosts and contributed across major bacterial taxa (Jones et al., 2008). Gaining further insight into the functional redundancy within the human gut microbiome is crucial, as it is essential to maintaining the stability and resilience of the microbiome and can contribute to our understanding of it.

1.1.3 Human gut microbiome models

The human gut microbiota is a dynamic and diverse microbial community that is challenging to study in the host organism. The human gut microbiome needs to be more explored for its interaction with the microbes and several factors that can alter the compositions and functions of the microbiome. These lead to research on the human gut microbiome using several animal models. Nonhuman primates were used to study the gut microbiome. The study demonstrated that after being kept in captivity, monkeys lose their natural microbiota and develop a higher prevalence of *Prevotella* and *Bacteroides* (Clayton et al., 2016), the two bacterial species that predominate in industrialized human populations. The gut microbiomes of nonhuman primates and humans were similar, with *Firmicutes* and *Bacteroidetes* predominating, but *Bacteroidetes* is more common in mice and rats (Nagpal et al., 2018).

1.1.3.1 Simple animal models

A simple animal model that includes basal vertebrate and invertebrate species has grown more interest in order to gain more fundamental knowledge and understanding. *Drosophila* are an effective model due to their easy rearing, short lifespan, and simple commensal gut. Their physiology is influenced by both nutrition and microbiota (Lee, Lee and Lee, 2020). Its simpler and less diverse microbial communities can be used to study the functions of particular taxa of microorganisms and the fundamental processes (Douglas, 2019). Overall, the majority of research on insects or animals' microbiome models has been focused on how the microbiome affects host fitness. These foundational insights can also help us comprehend the intricate human gut microbiota better. A disadvantage of *Drosophila* is that its actual microbial community is not similar to that of mammals, in terms of taxonomic composition (Chandler et al., 2011).

1.1.3.2 Mouse model

Mouse (*Mus musculus*) model has been massively used in the human gut microbiome models. The most prevalent strain, C57BL/6, is further subdivided into C57BL/6J and C57BL/6N, two significant substrains (Zurita et al., 2011). Mice models are being used more and more in gut microbiota research to investigate the functions and roles of the gut microbiota as well as its relationship to human diseases. *Firmicutes* and *Bacteroidetes* are predominant bacterial phyla in both humans and mice. However, compared to the three laboratory mouse strains, including NOD, BALB/c, and B6 strains, humans harbored comparatively more *Firmicutes* and fewer *Bacteroidetes* (Krych et al., 2013).

1.1.3.2.1 Disadvantages of mouse model

We cannot dispute that the mouse model has been massively used in representative human gut microbiome models. Given that the mouse model can act as a standard model for some investigations on the human gut microbiota. But mouse and human conditions differ in a number of ways. Several factors could lead to different results between human and mouse models, including diets and genotype.

Mice are herbivores, while humans consume a greater variety of foods. Moreover, the main difference is the physiology of the intestinal organs between mice and humans (Kararli, 1995). The difference in pH in the stomach is very low in humans about 1.0-1.6, while it is relatively higher in mice about 3.1-4.5 (Kararli, 1995). Bile acid composition is also different between humans and mice (Takahashi et al., 2016).

Gut transit time plays an essential role in either shaping gut microbiome composition or human health conditions (Procházková et al., 2023). While the average overall transit times in both humanised and conventional mice were significantly shorter than that of humans, at 4.75 hours (Kashyap et al., 2013), the median whole gut transit time in humans was approximately 28.7 hours (Asnicar et al., 2021). The transit time depends on several factors, including gender (Fischer and Fadda, 2016) and type of food (Fadda et al., 2009). Comparing the genomic responses of inflammatory diseases between human and mouse models indicated that the responses were different from human conditions and also varied between murine strains (Seok et al., 2013).

There are several reasons to critically consider the mouse model because of the different conditions between mice and humans. It is crucial to have accurate and effective experiments that take these variations into account. However, as we know mouse models cannot be representative of all human microbiome studies. Therefore, it is worth searching for a new alternative model for some specific

questions. While the majority of insects are poorly studied, some, such as *Apis mellifera* and *Drosophila melanogaster*, are well-known organisms that have been studied for several years, including symbiotic relationships, their immune systems, microorganisms' influence on their health and fitness, pathogenic bacterial vectors, and so on. The Insecta contain the greatest species richness of any animal clade on the earth. It is worth considering insects as an alternative.

1.1.3.3 Insect immunity

To protect the organism from infections, the immune system is crucial. Since humans are extremely complicated organisms, there is no ideal model that could be used as completely representative. However, understanding the fundamental biological concepts of humans can be greatly improved by studying several organism models altogether, such as invertebrates, vertebrates, and non-human primates. The study of insect immunity provides an understanding of the fundamental characteristics of innate immune systems and how they react against various infections.

Insects possess solely the innate immune system (Ali Mohammadie Kojour, Han and Jo, 2020). Insects contain multiple types of immune cells and tissues, including hemocytes, midgut, fat body, salivary glands, and other tissues (Hillyer, 2016). Physical barriers provide the first defense against host invasion, such as the epidermis and cuticle (Manniello et al., 2021). Humoral and cellular responses are the two primary responses of innate immunity when pathogens can pass the physical barriers (Royet, Meister and Ferrandon, 2003). Immune cells typically respond to infections initially after they enter the host. The primary immune cells in insects are the hemocytes in the hemocoel, which can respond with cellular processes such as phagocytosis (Hillyer and Strand, 2014). Melanisation and the release of antimicrobial peptides (AMPs) are examples of the humoral reaction (Sheehan et al., 2018a). The invasive pathogen may be enclosed in a proteinaceous capsule of melanisation, which can cause them to die (Dolezal, 2023). A variety of antimicrobial peptides (AMPs) are produced mostly in the fat body of insects and are part of the humoral response. These peptides protect against pathogens by breaking microbial cellular membranes of invasive microorganisms, which eventually results in their death (Wu, Patočka and Kuča, 2018).

Additionally, there are three main immune signaling pathways, namely Toll, IMD (immune deficiency), and JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathways in insects (Tzou, 2002). Gram-positive bacteria and fungi trigger the Toll pathway, whereas Gram-negative bacteria activate the IMD pathway (Lemaitre and Hoffmann, 2007).

1.1.3.4 The differences between insect and human immunity

The innate immune system serves as the initial line of defense against invasive infections in both insects and humans. However, a complex system of the immune system in humans includes both innate and adaptive immune systems, the latter of which is absent in insects (Sheehan et al., 2018a). Some differences in the humoral immune response between insects and humans have been observed.

Toll and Toll-like receptors are found in insects and mammals, respectively. However, differences in the structure and function of Toll receptors and genes are observed between insects and mammals (Sheehan et al., 2018a). For example, the Toll pathway functions in both development and immunity in insects, in which AMPs and other immune effector genes are released in insect immunity (Lindsay and Wasserman, 2014). On the other hand, cytokines and co-stimulatory molecules are produced by mammalian Toll-like genes (Sheehan et al., 2018a). While AMPs act directly in the innate system in insects, cytokines can indirectly activate the adaptive system in mammals (Stokes et al., 2015).

The insect IMD pathway is highly similar to the tumor necrosis factor (TNF) pathway in mammals. The IMD pathway eradicates bacteria in insects (Tzou, 2002), while the TNF pathway induces intermediate inflammatory immune responses and prepares antibodies for the adaptive immune response (Ting and Bertrand, 2016).

The JAK/STAT pathway is involved in the immune system, development, and insect interactions with pathogens (Zhou and Agaisse, 2012). For example, the JAK/STAT pathway signaling plays a part in *Drosophila* wing development (Johnstone et al., 2013) and maintaining intestinal gut microbiota balance (Zhou and Agaisse, 2012). In mammals, this pathway is a signaling mechanism for different types of cytokines and growth factors (Rawlings, Rosler and Harrison, 2004).

Although the human and insect immune systems differ, there are also structural and functional analogies in the defense mechanisms against infections. Further study into these analogies and differences between insects and human beings may provide significant data on the development of immunity and innovative treatment approaches.

1.1.3.5 The differences between innate and adaptive immune systems

The initial line of defense against pathogens is the innate immune system. It is characterized by its rapid reaction to infections and elimination of a broad range of pathogenic organisms (Dempsey, Vaidya and Cheng, 2003). There are several factors that mediate this response, including cellular and humoral responses. Innate immune cells, including macrophages, eosinophils, mast cells, natural killer cells, dendritic cells, neutrophils, and natural killer T cells, are observed in cellular response,

while the humoral components, which consist of AMPs, mannose-binding lectin, complement proteins, and lipopolysaccharide-binding protein (Turvey and Broide, 2010).

The adaptive system develops an immunological memory of antigens, which is more specific than the innate immunity. The adaptive immunity may take several days (Huang and Chen, 2016). The adaptive immunity receptors are members of the immunoglobulin family and are expressed on B cells and T cells, the two primary lymphocytes. Antibodies, which are special immunoglobulin molecules, mediate the humoral response, while T lymphocytes mediate cellular immunity, which eradicates infected or dysfunctional cells (Bonilla and Oettgen, 2010).

1.1.3.6 The advantages and limitations of invertebrate models

Complete genomes of certain laboratory organisms, such as cockroaches, honeybees, and *Drosophilas*, provide enough conserved and genomic genes that have some human homologs (Wang et al., 2023; The Honeybee Genome Sequencing Consortium, 2006; Reiter et al., 2001). Both insects and mammals have a number of conserved genes and pathways, such as the humoral and cellular responses of their innate immune systems (Sheehan et al., 2018a).

Insects possess a diverse gut microbiota, some of which are similar to the gut microbiota of humans. Insects are an effective model system for studying symbiotic associations between animals and their symbiosis (Douglas, 2014). The gut microbiome's dysbiosis typically plays a role in pathogenesis (Carding et al., 2015). Additionally, certain insects harbour harmful microbes like viruses, bacteria, and fungi (Gurung, Wertheim and Falcao Salles, 2019). These harmful microorganisms also interfere with the host's defenses against them. This could provide the fundamentals of how the host reacts to infections. More significantly, insect models have the ability to perform research with large sample sizes, low costs, rapid generation times, and a decreased need for vertebrate models like mice or rats.

Although there are some benefits to using invertebrate models for investigating human immunity, such as genetic similarities and the evolutionary conservation of immune pathways, some drawbacks cannot be ignored, including the intricacy of human diseases and complicated human physiology. Insect models are mainly useful for studying innate immunity. Insects lack adaptive immunity, while humans possess both the innate and adaptive immune systems.

Studying the innate immune response to certain bacterial infections in insects, there may be restrictions on the choice of bacterial administration. When non-pathogenic bacteria infect *Drosophila*, the injection method used to research Gram-positive bacterial infections causes mortality (Jensen et al., 2007). This might produce inaccurate results if a poor technique is applied.

1.1.3.6.1 Cockroaches in comparison to other invertebrate models

Drosophila and honeybees are two important invertebrate models that have been used in human research for a very long time. These invertebrates have been used in studies of innate immunity, insect behaviours, gut microbiome homeostasis, and bacterial or viral infections (Igboin, Griffen and Leys, 2012; Wang et al., 2018b). However, they do have certain disadvantages, though, which make other invertebrates worth considering.

For a humanized gut microbiome model, *Drosophila* might not be an appropriate candidate. Fly gut microbiota diversity is lower than that of human gut microbiota (Broderick and Lemaitre, 2012). *Acetobacter* and *Lactobacillus* contribute to the vast majority (over 90%) of bacterial communities in *Drosophila* (Wong, Ng and Douglas, 2011). Additionally, obligate anaerobes, which predominate in the guts of humans (Eckburg et al., 2005; Andrade et al., 2020) and cockroaches (Bracke, Cruden and Markovetz, 1979), appear to be lacking from the gut microbiome of flies (Erkosar et al., 2013).

Given that cockroaches typically reside close to humans, it appears that they consume a greater variety of foods and have a connection with human diets. In contrast to cockroaches, flies and honeybees consume a limited variety of foods. Higher nutritional quality was related to greater gut microbial diversity in humans, which included more fiber, vegetables, fruits, berries, and dairy (Laitinen and Mokkala, 2019). Cockroaches may be a better option for researching how diets affect gut microbiota and could provide important insights for mammals research.

Cockroaches are commonly exposed to human pathogenic bacteria because of their tendency to reside in unhygienic environments and human habitats (Al-bayati, Al-Ubaidi and Al-Ubaidi, 2011). The cockroaches usually live and thrive in environments where they are exposed to a range of harmful compounds, such as pesticides and microbiological toxins. Compared to *Drosophila* and honeybees, the cockroach may be a more efficient way to study microbes and diseases and how effectively their commensal bacteria work against pathogens. Recent genetic and functional studies on American cockroaches (Li et al., 2018) have provided remarkable insights into their gut microbiota, which will enable further investigation of them.

1.2 Cockroach

Cockroaches have inhabited the world for about 300 million years. Approximately 4,600 species have been found worldwide (Guzman and Vilcinskas, 2020). The greatest three superfamilies within the *Blattodea* order are *Blaberoidea, Blattoidea,* and *Corydiodea*, respectively (Guzman and Vilcinskas, 2020). Most of them live in forests. They are crucial to the breakdown of organic materials in forests, including wood and leaf litter (Guzman and Vilcinskas, 2020). A small number of species are recognised as pests that affect people and homes. The American cockroach (*Periplaneta americana*) and the German cockroach (*Blattella germanica*) are the two most well-known pests (Gondhalekar et al., 2021). Cockroaches have been considered to be harmful to humans because they are typically found in homes and hospital settings. Numerous investigations revealed that the cockroaches' bodies or intestinal organs contain some pathogenic microbes (Nasirian, 2019; Mehainaoui et al., 2020). There are several nosocomial pathogens that can be isolated from several cockroach species in hospital areas, such as *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli,* and *Klebsiella pneumoniae* (Donkor, 2019).

1.2.1 American cockroach

The American cockroach is in the Blattidae family. The three phases of the growth and developmental process are the egg, nymph, and adult (Figure 1) (Lin et al., 2022a). The majority of cockroaches are oviparous, meaning that their young develop in oothecae outside of their mother's body (Schal, Gautier and Bell, 1984). The American cockroach is one of them. The development time from eggs to develop into adults can vary, ranging from 150 to 830 days (Gould and Deay, 1940). The adult females' life duration also differed based on whether they were mated or not (XIAN, 1998). The rate of ootheca hatching depends either on mated (gamogenesis) or unmated (parthenogenesis) females; the mated rate of reproduction is higher (80%) than the unmated rate (45%) (Lin et al., 2022a). The American cockroach has 14 molts that are raised at 29°C and 70% relative humidity with a light and dark photoperiod of 12 hours each (Lin et al., 2022a).

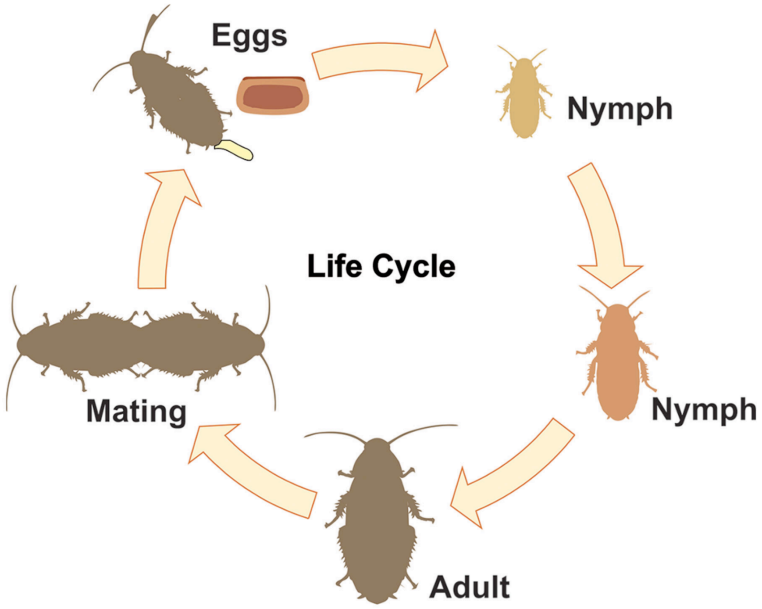


Figure 1 The life cycle of the cockroaches consists of three main stages, including egg, nymph, and adult (Adedara et al., 2022).

Blattabacterium, an obligatory bacterial symbiont, resides in the fat bodies of American cockroaches. This is the major symbiont present in the ootheca of cockroaches and is vertically transferred into their progeny (Carrasco and Pérez-Cobas, 2014; Chen et al., 2023). The majority of cockroaches contain this bacterium. According to research on *Blattabacterium*'s genome sequencing, the bacterium is crucial to the nitrogen metabolism of the American cockroaches. It is associated with several amino acid biosynthesis as well as nitrogen recycling from urea and ammonia (Cazzaniga et al., 2023).

1.2.2 Gut microbiome of the American cockroach

Many years of research on the microbiome of cockroaches using both culture-dependent and culture-independent methods have shown that their digestive organs contain diverse gut microbiota. The four most predominant bacterial phyla in the intestinal organs of the American cockroaches are *Bacteroidetes*, *Firmicutes*, *Desulfobacterota*, and *Proteobacteria* (Tinker and Ottesen, 2016a; Chen et al., 2023). Moreover, as the life stages develop, the diversity of bacteria increases, with nymphs and adults possessing higher diversity than ootheca (Guzman and Vilcinskas, 2020; Chen et al., 2023). It is interesting to note that research on the impacts of synthetic food, particularly when xylan is fed, could alter the gut microbiome of the American cockroaches, causing them to harbor fewer *Desulfobacterota* and more *Firmicutes* (Dockman and Ottesen, 2024b).

Because the intestines of American cockroaches are home to a wide variety of microorganisms. Their interactions with their gut flora and how those interactions affect their roles in growth and

development have been the main topics of numerous research. By developing gnotobiotic cockroaches, their ootheca is surface sterile and incubated under sterile conditions (Dukes, Dyer and Ottesen, 2021a). However, the vertically transmitted primary endosymbiont *Blattabacterium* is still contained (Jahnes, Herrmann and Sabree, 2019; Dukes, Dyer and Ottesen, 2021a). According to research on germ-free American cockroaches, their body size decreased and their growth and development time extended (Jahnes, Herrmann and Sabree, 2019; Vera-Ponce De León et al., 2021). Their growth and development responded negatively to the absence of their gut microbiota (Vera-Ponce De León et al., 2021). It has been proven that feeding the stools of conspecifics and siblings to young offspring can transfer their gut microorganisms to them (Jahnes, Herrmann and Sabree, 2019). Moreover, cockroaches that are exposed to coprophagy throughout their early life are more likely to develop normally than gnotobiotic ones that live in sterile conditions (Jahnes, Herrmann and Sabree, 2019). It is important to note that a number of factors that can also affect the human gut microbiome can also affect the cockroach's gut microbiome, including genetics, diet, life stage, and environment. It is very interesting to consider the cockroach as one of the alternative models in studying the human gut microbiome.

1.3 Intestinal colonization resistance

A stable and diverse microbiota in hosts can effectively prevent invading microbes from colonizing the host intestinal organs. This phenomenon is called “colonization resistance” (Lawley and Walker, 2013; Horrocks et al., 2023). The colonization resistance is very important to host health. It can support the host in maintaining their fitness or metabolism and preventing viruses or other invasive pathogens from colonizing the host (Ducarmon et al., 2019).

Escherichia coli growth and colonization can be inhibited by the human gut microbiome when the bacteria come into contact with indigenous microorganisms. It was also unable to develop antibiotic resistance when exposed to the beta-lactam antibiotic ampicillin (Baumgartner et al., 2020). The colonization resistance assists hosts in preventing the colonization of pathogenic bacteria. For example, pathogenic *E. coli* EDL933 colonization was inhibited by two commensal *E. coli* strains (*E. coli* HS and Nissle 1917) (Maltby et al., 2013).

Numerous techniques, such as germ-free animals, gnotobiotic animals, and antibiotic treatments, have been developed and applied in animal models to explore the functions or interactions among microbiota in host guts. These methods can eradicate microorganisms or at least reduce their number before we introduce specific microbes into hosts. Animals that are germ-free are those that do not contain any microorganisms in their hosts that would enable the inoculation with specific microbes. The animal needed to be kept germ-free and raised in a sterile storage location, fed only sterile food and water. Both the Gram-stained and the culture methods are used to detect bacteria in their faeces (Arvidsson, Hallén and Bäckhed, 2012). Mice designated as specific pathogen-free (SPF) are produced using the Caesarean aseptic method or germ-free animals, and they lack common pathogens (Dobson et al., 2019).

However, during treatment, both gnotobiotic and germ-free animals require specialized equipment, an axenic environment, and potentially routine checks for sterilization using both molecular and cultural methods. Furthermore, some bacteria are essential to the host's early stages of development. If these bacteria are eradicated at an early age that can lead to incomplete or delayed host development. The fact that not all microorganisms can also be isolated is a drawback. Later on, however, a number of approaches have been developed to identify microbes in the hosts that are more sensitive and effective than the culture-dependent method, such as PCR and qPCR (Fontaine et al., 2015) and next-generation sequencing (Van Dijk et al., 2014).

1.4 Antibiotic treatment

Human infectious diseases have been treated with antibiotics, which also extend human life (Fishbein, Mahmud and Dantas, 2023). On the other hand, when the gut microbiota becomes unstable after receiving antibiotics, it may develop dysbiosis. Moreover, using antibiotics can disrupt the gut flora, which promotes the growth of resistant bacteria and affects the immune system (Ramirez et al., 2020). Therefore, it allows either colonization or proliferation of invasive microbes to be studied for their functions in the gut microbiome.

In cockroaches, both American (*Periplaneta americana*) and German (*Blattella germanica*) cockroaches are the main species that have been used in scientific research (Akbari et al., 2015; Tinker and Ottesen, 2016a; Nasirian, 2019; Domínguez-Santos et al., 2021). The primary goals of antibiotic use in the cockroach are to examine the composition, function, and ecology of the gut bacterial communities (Ayayee et al., 2020; Tinker and Ottesen, 2021; Dukes, Dyer and Ottesen, 2021a). Table 1 displays the cockroaches' studies that have involved antibiotic treatment.

Cockroaches, however, cannot be reared in a germ-free environment because they carry and transmit on their primary endosymbiont, *Blattabacterium*, vertically to their progeny. This suggests that even after surface sterilising their eggs and raising them in a sterile environment, the *Blattabacterium* is still present in the bacteriocytes in their fat bodies. This bacterium is vital to the cockroaches as it plays an important role in nitrogen metabolism (Latorre et al., 2022). Cockroaches have a crucial endosymbiont and an abundant diversity of bacteria in their digestive organs (Guzman and Vilcinskas, 2020). Therefore, in our study, the antibiotics that have an impact on the endosymbiont are not selected, and rifampicin is excluded.

Table 1 Antibiotics treated in cockroaches.

Cockroaches	**Antibiotic concentration**	**Antibiotic**	**Duration time**	**Reference**
German cockroach	0.2 mg·ml^{-1} in water	Kanamycin	G1:G2* (30: 34 +30 days)	(Domínguez-Santos et al., 2021)
German cockroach	0.02% w/v in water (0.2 mg·ml^{-1})	1. Vancomycin 2. Ampicillin	G1:G2 (30: 34 +30 days)	(Domínguez-Santos et al., 2020)
German cockroach	0.02% w/v in water (0.2 mg·ml^{-1})	Rifampicin	G1:G2 (30: nymphal stage + 10 days)	(Rosas et al., 2018)
insecticide-resistant and insecticide-susceptible of German cockroaches	5% w/v in water	Kanamycin	72 hours	(Wolfe and Scharf, 2021)
German cockroach	0.025% in tap water (0.25 mg·ml^{-1})	Ampicillin	After hatching + adult + 21 days after	(Lee et al., 2021)
German cockroach	0.1 mg·ml^{-1} in water	Rifampicin	12 days in adult (G1:G2:G3)	(Muñoz-Benavent et al., 2021a)
American cockroach	0.025, 0.25 mg·ml^{-1} in water	Chloramphenicol	7 days	(Ayayee et al., 2018)

*G represents generation.

1.5 *Escherichia coli*

Escherichia coli (*E. coli*) is a Gram-negative and facultative anaerobe that is a predominant aerobic bacterium in the intestinal tract in humans and other animals, including chickens, cows, goats, pigs, and sheep (Carlos et al., 2010). Moreover, *E. coli* is frequently found in environmental habitats such as terrestrial and aquatic habitats (Van Elsas et al., 2011a). *E. coli* is often used as an indicator of faecal contamination in nature since it can be contaminated in the environment (Ishii and Sadowsky, 2008).

E. coli is one of the bacteria groups that colonizes after birth; they could proliferate to approximately 10^9 CFU per gram of faeces (Penders et al., 2006). *E. coli* is a facultative anaerobe; it can reduce oxygen levels to generate an environment that is favorable for strict anaerobes (Mueller et al., 2015; Secher, Brehin and Oswald, 2016). As a result, bacteria like *Bacteroides*, *Bifidobacterium*, and *Clostridium* which are obligate anaerobes are able to thrive and reside in the digestive tracts (Secher, Brehin and Oswald, 2016). However, *E. coli* density is approximately 10^9 CFU in neonates, decreases to 10^8 CFU in infants one-year-old, and then gradually decreases with age. Its prevalence and quantity in humans (10^7-10^9 CFU) are higher than in other animals (10^4-10^6) (Secher, Brehin and Oswald, 2016). The prevalence is approximately 88% of infants at one month old in the Netherlands (Penders et al., 2006).

E. coli is the most abundant facultative anaerobic bacterium in the human gut. It is considered a commensal and harmless bacteria with the host guts (Conway and Cohen, 2015a). In addition, using phylogenetic groups, *E. coli* can be categorized into several main groups, including A, B1, B2, and D (Herzer et al., 1990; Tenaillon et al., 2010). The main phylogroup distribution across adult humans is group A, followed by groups D, B2, and B1, respectively (Stoppe et al., 2017). Moreover, there was no variation in the phylogroup among adults based on sex, age, and body mass index (Stoppe et al., 2017). However, the two phylogroups that were most prevalent in the *E. coli* phylogroups from Indian neonates were B1 and A (Das et al., 2013). Phylogenetic groups B2 and D are associated with pathogenic strains that carry multiple virulence factors, whereas phylogenetic groups A and B1 are often identified in commensal strains and include a few virulence factors (Clermont, Bonacorsi and Bingen, 2000). Isolation of commensal *E. coli* from different human populations, including France, Croatia, and Mali, revealed the phylogenetic groups A, B1, and B2 were distributed differently across populations, while group B2 was uncommon in these populations (Duriez et al., 2001). Moreover, in comparison to the other phylogenetic groups, the phylogenetic group B2 has the greatest number of virulence factors (Duriez et al., 2001; Das et al., 2013).

Commensal *E. coli* from healthy adults displayed antibiotic resistance to several antibiotics (Bailey et al., 2010). Isolated commensal *E. coli* was about 10^6 CFU per gram of faeces in healthy adults (Bailey et al., 2010). Several pathogenic *E. coli* strains carry virulence factors that enable them to colonize and perform their pathogenic circumstances on their hosts (Mainil, 2013; Richter et al., 2018). These can cause several diseases in humans, either extraintestinal or intestinal infections (Bekal et al., 2003). Since antibiotic discovery, they have been used to treat or prevent a variety of infectious diseases in humans. This is one of the factors that leads to the development of antibiotic resistance in a number of bacteria, and *E. coli* is also a major issue globally (Prestinaci, Pezzotti and Pantosti, 2015).

Mice were pre-colonized with commensal *E. coli* strains, such as *E. coli* HS and *E. coli* Nissle 1917, which prevented and eradicated pathogenic *E. coli* O157:H7 from colonizing in the intestinal organs (Maltby et al., 2013). Furthermore, pre-colonization with three different commensal *E. coli* strains, including *E. coli* MG1655, *E. coli* HS, and *E. coli* Nissle 1917, was even more successful than pre-colonization with a single commensal strain at a time in preventing the pathogenic *E. coli* EDL933 from colonizing in the mouse intestine (Leatham et al., 2009). Moreover, in germ-free mice, *E. coli* could provide protection against *Salmonella typhimurium* infection (Hudault, 2001).

Different *E. coli* strains contain several virulence factors (Mainil, 2013). It is of some interest to establish a model for colonizing cockroaches with pathogenic *E. coli*, as this would be an experimental tool for understanding their virulence factors, commensal-pathogen interactions, colonization process, and host response. These might be helpful in our comprehension of how invasive microorganisms are able to inhabit and flourish within the host. Obtaining a basic understanding of this could allow us to conduct on mammal models or humans.

1.6 *Desulfovibrio*

Desulfovibrio typically lives in natural environments, but they can also be isolated from humans (Chen et al., 2019b, 2021a) and animal digestive tracts, such as the rumen of sheep (Huisingh, McNeill and Matrone, 1974). *Desulfovibrio* spp. are sulfate-reducing anaerobic bacteria. They can perform dissimilatory sulfate reduction, which can reduce sulfate to sulfide by receiving electrons from several organic substrates, including lactate, propionate, and butyrate (Muyzer and Stams, 2008). The sulfate reduction produces hydrogen sulfide as a final product. Since hydrogen sulfide is more soluble in lipophilic solvents than in water and can penetrate cell membranes (Fiorucci et al., 2006), it can harm the intestinal mucosa (Rowan et al., 2009).

Several species, including *D. desulfuricans, D. piger,* and *D. legallii*, have been isolated from human faeces (Chen et al., 2019b). In addition, several species of *Desulfovibrio*, including *D. piger, D. fairfielddensis,* and *D. desulfuricans* have been isolated from patients suffering from digestive tract diseases. Interestingly, compared to patients with other non-inflammatory bowel disorders or those with healthy ones, the prevalence of *D. piger* was higher in patients with inflammatory bowel disease (Loubinoux et al., 2002a). In a mouse model, the study of sulfide ion production by *Desulfovibrio* was higher in rodents with ulcerative colitis than in *Desulfovibrio* from healthy rodents (Kováč, Vítězová and Kushkevych, 2018).

It is also a controversial topic about their roles in human health, as they can be isolated from both healthy and unhealthy humans (Loubinoux et al., 2002a). There could potentially be some benefits of hydrogen sulfide for humans. There are several studies found that hydrogen sulfide functions as a mediator in the liver and gastrointestinal mucosa, contributing to tissue damage or anti-inflammation (Fiorucci et al., 2006). Alteration of hydrogen sulfide production can lead to gastrointestinal tract and liver disorders (Fiorucci et al., 2006) as well as inflammation in several animal models (Szabó, 2007).

Several foods, including processed and dried fruits, brassica vegetables, commercial bread and cereal products, beer, and red wines, are rich in sulfate (Florin et al., 1993). Food consumption, particularly the consumption of animal-based proteins, can increase the production of hydrogen sulfide in the human gut (Teigen et al., 2022) which is heavily consumed in Western society. This resulted in an increase in the production of hydrogen sulfide, which damaged the stomach (Dostal Webster et al., 2019). Hydrogen sulfide can damage the intestinal mucosa. However, it is unclear how *Desulfovibrio* or hydrogen sulfide is related to inflammatory bowel conditions.

1.6.1 Dissimilatory and assimilatory sulfate reduction

Dissimilatory sulfate reduction (DSR) is a process that uses sulfate as the terminal electron acceptor to generate sulfide. Sulfate-reducing bacteria groups are divided into five Bacteria and two Archaeal phylogenetic lineages, including *Deltaproteobacteria*, *Clostridia*, *Nitrospirae*, *Thermodesulfobacteria*, and *Thermodesulfobiaceae* from the Bacteria, and *Euryarchaeota* and *Crenarchaeota* from the Archaea (Muyzer and Stams, 2008).

Compared to DSR, assimilatory sulfate reduction (ASR) is more complex (Figure 2). ASR uses sulfate; however, the final product is cysteine, which can occur in both bacteria and plants (Schiff, 1980). The ASR occurs in prokaryotes such as *Escherichia coli* that contain up to seven enzymes (Kushkevych et al., 2020a).

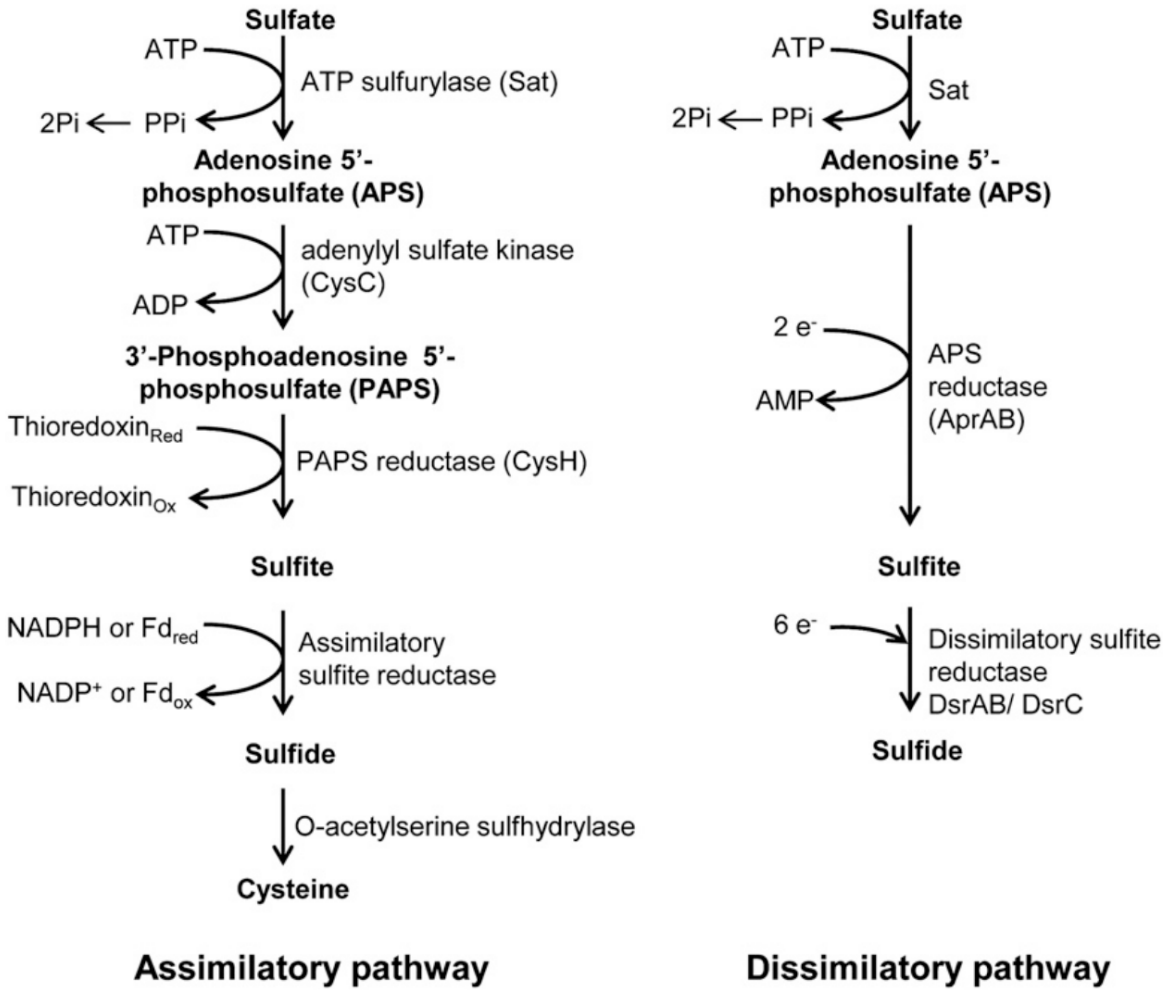


Figure 2 The assimilatory and dissimilatory sulfate reduction pathways (Grein et al., 2013).

1.7 Methodologies to study human gut microbiome

1.7.1 Culture-dependent technique

Theodor Escherich initially isolated *Bacterium coli commune* and *Bacterium lactis aërogenes* from human stools in 1885. These microorganisms were subsequently *Escherichia coli* and *Klebsiella pneumoniae*, respectively (Shulman, Friedmann and Sims, 2007). After that, there were several studies focused on culturable human gut microbiome. Sanborn discovered that the majority of the gut microbiome is anaerobic after isolating the faecal flora in both aerobic and anaerobic conditions using several media (Sanborn, 1931). Anaerobic culturing was developed further by Hungate (Hungate, 1969), and various microorganisms are being studied using this technique to this day.

For many decades, the traditional culture-dependent technique has been used for the research of microbiomes. The culture technique requires providing several media for each specific bacterial group. Various media consist of different nutrients and additives that are necessary for particular

microorganisms. Furthermore, a wide variety of bacteria can grow in a variety of environments with appropriate nutrition, oxygen, and temperature (Vartoukian, Palmer and Wade, 2010). The majority of microbes found in the human gut microbiome are anaerobes (Mata, Carrillo and Villatoro, 1969), meaning they require specific equipment to survive in an anaerobic environment. Culture techniques provide a number of benefits that have been used, such as the ability to further our knowledge of bacterial diversity, metabolism, and functional potential. However, the majority of the gut microbiome cannot thrive on solid medium, just as in host habitats, which are more complex environments (Vartoukian, Palmer and Wade, 2010).

Of 10,000 isolates from a sample of human faeces, approximately 400 different species could be identified in a culturing study (Liu et al., 2021a). Additionally, cultivated bacteria were classified into 527 species from eight predominant bacterial phyla from healthy Chinese (Lin et al., 2023). Overall, the culture-dependent method is very laborious and costly and may require various nutrients for certain types of bacteria.

1.7.2 Culture-independent technique

Molecular methods have been developed and used based on amplification and sequencing of the small subunit ribosomal RNA which can distinguish and divide along with a phylogenetic tree into three separate domains, including Bacteria, Archaea, and Eucarya (Woese, Kandler and Wheelis, 1990). The two ribosomal subunit types present in prokaryotic ribosomal RNA are the large (50S) and small (30S) subunits. In prokaryotes, the conserved 16S ribosomal RNA (16S rRNA) gene exists in a small subunit. The 16S rRNA gene in bacteria provides sufficient information to identify and categorize bacteria at the species and strain levels. This gene is about 1.5 kb (Wang and Qian, 2009). References to 16S rRNA gene sequence databases are available to assist with the taxonomy classification of microorganisms, including GenBank (Benson et al., 2018), RDP (Maidak, 2000), SILVA (Quast et al., 2012), and Greengenes (DeSantis et al., 2006). The molecular method allows us to identify more bacterial communities of both culturable and unculturable bacteria. These provide a better understanding of bacteria in many ways such, as their taxonomic classifications, metabolic processes, functional analyses, and so on.

The 16S rRNA gene can be amplified using the polymerase chain reaction (PCR) approach (Zhu et al., 2020). The specificity of sample collection from various sources, the DNA extraction process, and the chemicals used during the extraction, or the commercial kits specialized for each sample, are some of the method's challenges (Prosdocimi et al., 2015; Song, Lee and Nam, 2018). Specific primers are utilized to target the microorganisms. Furthermore, if there is no information available on unknown species, it is difficult to identify them (Fraher, O'Toole and Quigley, 2012).

1.7.3 Sanger sequencing

The bacterial 16S rRNA gene contains nine variable regions that have been used in sequencing and bacterial amplification (Yang, Wang and Qian, 2016). Sanger sequencing is full-length 16S rRNA sequencing. There is sufficient information in this method to distinguish between mostly bacterial communities. Using the 16S rRNA sequencing could define about 13,355 prokaryotic rRNA gene sequences from the human gut microbiome (Eckburg et al., 2005), a significantly higher number than those found using the culturable approach. This allows us to discover more uncultivated and novel microorganisms in the human gut microbiome.

However, 16S rRNA sequencing still has many drawbacks and biases. It is unable to discriminate between two closely related species (Almeida and Araujo, 2013). Additionally, the results of microbial abundance and diversity are influenced by various factors such as sample collection and preservation, DNA extraction techniques, and the selection of hypervariable area targets and primers (Yang, Wang and Qian, 2016; Chen et al., 2019c).

1.7.4 Quantitative PCR (qPCR)

A target gene's expression can be measured more precisely quantitatively using qPCR. Furthermore, the use of fluorescence detection makes it possible to quantify the increase in the number of amplified genes in each PCR cycle (Kubista et al., 2006). Primers used in qPCR can be designed for qPCR that are unique to particular bacteria or their functional genes (Bustin and Huggett, 2017). The targeted nucleic acid is measured in absolute quantification compared to a set of nucleic acid standards that are used to generate a standard curve (Svec et al., 2015). It is acknowledged that this technique is the most sensitive for measuring very small amounts of nucleic acids. Amplicon sequencing, on the other hand, provides a proportion of microorganisms that frequently cannot represent their actual abundance (Tkacz, Hortala and Poole, 2018a). When compared to next-generation sequencing, qPCR provides quantitative precision, high specificity, and relatively low costs.

Moreover, the use of reverse transcription-qPCR (RT-qPCR) is increasing in a number of scientific studies, particularly those focusing on the human gut microbiota. This method uses RNA isolation from samples directly and the RNA template is free from contaminating DNA. The reverse transcriptase enzyme then synthesizes complementary DNA (cDNA) from RNA, which can be utilised as a template for qPCR amplification. Standard curves made from cDNA or comparisons with reference genes can be used to determine the absolute numbers of cDNA (Bustin et al., 2005). With a limited number of templates, this strategy is very sensitive. One of qPCR's drawbacks is that, in order to generate a standard curve, it requires an indigenous gene or reference gene, which can become challenging when working with new strains.

1.7.5 Next-generation sequencing

The high-throughput sequencing techniques based on the amplification of the 16S rRNA gene can detect more unculturable microorganisms (Dave et al., 2012). Next-generation sequencing allows greater depth and resolution to identify microorganisms' taxa. There are numerous commercial platforms for next-generation sequencing available including Illumina sequencing (Van Dijk et al., 2014). Numerous techniques have been used to study the ecology of microbial communities, including DNA-based analyses for taxonomic diversity (metabarcoding), gene expression (metatranscriptomics), and the synthesis of various compounds (metaproteomics, metabolomics) (Lobanov, Gobet and Joyce, 2022). Metaproteomics, which more precisely determines which genes are functional, might differ from the predictions provided by bacterial taxonomy. (Verberkmoes et al., 2009).

However, there are some limitations to this technique. As it is a short sequence with about 250 -300 bp, it can be used to categorize microorganisms mainly at the genus level and is generally unable to discriminate between closely related species. Additionally, the sensitivity of the nine hypervariable areas (V1-V9) of the 16S rRNA gene can be problematic due to the need for separate primers for each region. Six amplicons of V2, V3, V4, V6-V7, and V9 of the 16S rRNA gene were amplified. However, several regions were obviously different from what they were supposed to be, especially V9 region (Barb et al., 2016). Additionally, the investigation of all V1–V9 sections revealed that, while V2 and V8 were the least reliable regions in the phylogenetic analysis, V4–V6 were suggested to be representative full-length 16S rRNA sequences with the most reliable regions (Yang, Wang and Qian, 2016). For several bacterial species, distinct primer sets from different regions proved to be more sensitive and specific (Chen et al., 2019c).

1.8 Thesis objectives

The general aim is to develop the cockroach as a human gut microbiome model. In order to investigate the cockroach, we examined its gut microbiota and how it has changed, which can be divided into four separate chapters.

Chapter 2: Data analysis was carried out on the gut microbiota of humans, mice, and cockroaches. Using the 16S rRNA gene of bacteria, this data was obtained from the GenBank database. This would provide us with more knowledge about the prevalent taxonomy of bacteria in each organism. This may enable us to determine whether the cockroach and human bacterial communities are similar, and the cockroach may also be an alternate model of the human gut.

Chapter 3: The gut microbiome of the cockroaches was eliminated by antibiotic treatment. The cockroaches were treated with two different antibiotics, ampicillin and kanamycin, with a concentration of 0.3 $mg \cdot ml^{-1}$. Both Illumina sequencing and quantitative polymerase chain reaction (qPCR) were used to identify the bacterial populations in the cockroach guts.

Chapter 4: It was proven in Chapter 3 that some bacterial populations in the cockroach gut could be eradicated by both antibiotics. The aim was to introduce commensal *E. coli* into the cockroach's digestive organ. Prior to the inoculation of *E. coli*, the adult female American cockroaches were treated with either ampicillin or kanamycin. The bacterial communities in the cockroach gut were determined using the 16S rRNA gene by Illumina sequencing.

Chapter 5: In order to determine whether cockroaches can serve as models for the inflammatory gut of humans, this chapter aimed to investigate the relationship between the sulfate supplement in drinking water to *Desulfovibro* and the bacterial communities in cockroach guts using qPCR and Illumina sequencing of the 16S rRNA gene. Furthermore, the expression of two sulfate-reducing bacteria genes, *dsrA* and *aprA*, was assessed by quantitative reverse transcription-PCR (qRT-PCR) in order to validate the activity of dissimilatory sulfate reduction.

Chapter 2: Bacterial communities compared among human, mouse, and cockroach guts

2.1 Introduction

Microbial communities are most usually found in intestinal systems rather than other internal organs (Turnbaugh et al., 2007). The human gut microbiome is of particular interest, and other models that simulate this setting are thus also of interest. In addition, microbial communities associated with hosts have an impact on human health, fitness, and immunity (Cho and Blaser, 2012). For example, some of them are beneficial for digesting oligosaccharides in breast milk from mothers, such as *Bifidobacterium* genus (Lugli et al., 2020). They have also been linked to improving infant health, including boosting the immune system and metabolic pathway (Korpela and de Vos, 2018; Mancabelli et al., 2020). Microbial species, in particular, can be classified into three different enterotypes of the healthy human gut (Costea et al., 2018). However, variations in microbiota composition have been associated with diseases or abnormal conditions in humans (Cho and Blaser, 2012). Moreover, the human gastrointestinal tract produces a variety of metabolic products as a result of its microbial symbiosis (Van Treuren and Dodd, 2020). These findings indicate that the human gut microbiome is influenced by a variety of factors throughout our lives, including where we live and what we eat (Mancabelli et al., 2017). By examining bacterial composition and functions, a deeper knowledge of the human gut microbiota can be developed. However, due to the intricacies of the human gut and ethical problems, the human gut is extremely difficult to examine and cannot be evaluated easily. Human gut microbiome models rely on either in vitro or in vivo models. Several other animals have been used as human gut microbiome models, such as pigs (Heinritz, Mosenthin and Weiss, 2013), rats (Becker et al., 2011), non-mammalian species such as zebrafish for autism spectrum disorder (Rea and Van Raay, 2020) or liver disease (Rea and Van Raay, 2020; Katoch and Patial, 2021), *Drosophila melanogaster* for human gut diseases (Apidianakis and Rahme, 2011) and also study ageing in *Hydra* (Tomczyk et al., 2015). Every approach offers benefits and drawbacks of its own, depending on the main objective of each study.

A large number of studies on the human gut have studied the mouse model because of their shared similarity of predominant microbiota phylum (Nguyen et al., 2015). Several studies have used germ-free or gnotobiotic mice, which can involve inoculation of the human gut microbiome or specific bacteria into mouse guts (Mark Welch et al., 2017; Mahowald et al., 2009). Even though this mouse model has been largely introduced over several decades, this also has some constraints: ethical

reasons, complex gut, long periods of experimentation, and low throughput. All these limitations could affect mouse models (Cho and Blaser, 2012; Nguyen et al., 2015). In addition, the research from different studies or locations has produced inconsistent results (Hugenholtz and de Vos, 2018; Spor, 2011). Despite the majority of studies regarding human gut models being associated with rodent models, several constraints should lead to the development of an alternative insect model. Using animal models to research the human gut microbiota is inevitable. Apart from the mouse model, it would be beneficial if we could discover an alternative model that would reduce the demand for vertebrate models. The representative insect model might be useful in at least some circumstances.

The Insecta is one of the greatest species-rich classes in this world (Stork, 2018). Cockroaches are one of the insect groups that are remarkable in a large number of species, with almost 5,000 species of cockroaches reported (Guzman and Vilcinskas, 2020). For a number of years, cockroaches have been studied (Guzman and Vilcinskas, 2020). The majority of studies have focused on their gut microbiota (Guzman and Vilcinskas, 2020) and the harmful bacteria they harbour, which could have an adverse effect on a number of illnesses in humans (Mehainaoui et al., 2020). They usually spread several pathogenic bacteria, especially as they actually survive in unhygienic conditions and have been found to be a contributor towards hospital diseases (Guzman and Vilcinskas, 2020; Mehainaoui et al., 2020). However, the number of studies on the microbiome of cockroaches has recently expanded with cockroach studies (Guzman and Vilcinskas, 2020) and their associations with symbiosis microbiomes (Tinker and Ottesen, 2020; Bauer et al., 2015). Cockroaches have been investigated previously, leading to the conclusion that this insect has a diverse microbial community (Bracke, Cruden and Markovetz, 1979; Tinker and Ottesen, 2021; Chen et al., 2023), is easy to rear, and is an omnivorous insect (Guzman and Vilcinskas, 2020). Similar to humans, cockroaches are omnivorous, indicating they consume a wide variety of foods depending on several criteria such as eating habits and geographic regions (Schal, Gautier and Bell, 1984; Bell, 1982); this is not that distinctive from human behaviour.

Insects have more complexity in their gastrointestinal systems than several other human models, including *Hydra* (Douglas, 2019). Cockroaches have three main intestinal sections: the foregut, midgut, and hindgut. (Cruden and Markovetz, 1987). Their hindgut hosts more microorganisms than the other sections (Vicente, Ozawa and Hasegawa, 2016). Their life-cycle consists of 3 stages: embryonic, nymphal, and adult stages. At 29°C, it takes about 35 days for the 20 embryonic development stages and 14 moults to reach adulthood (Lin et al., 2022b). The length and variability of the nymphal development period, however, are dependent on a number of variables, including

temperature, diet, and egg from different genders (Bell, 1982; Lin et al., 2022b). Additionally, they can reproduce ootheca, each of which contains 12-16 eggs (Lin et al., 2022b).

Cockroaches have been spreading across several continents (Schal, Gautier and Bell, 1984). Cockroaches can also be found in households, human areas, and hospital areas (Syamsuar Manyullei et al., 2022). We have studied insect models for several years and are knowledgeable about their morphological, physiological (Chown, Addo-Bediako and Gaston, 2002), and metabolic features (Muñoz-Benavent et al., 2021b). Based on a number of earlier studies, cockroaches have been developed as a germ-free model to study microbiomes associated with their intestinal organs such as *Shelfordella lateralis* (Mikaelyan et al., 2016; Tegtmeier et al., 2016). Additionally, it is worth considering using insects as a model, as they would be less expensive than mice or other animals. In particular, insects require less space for experiments and can be kept in great numbers with lower food intake.

This project's purpose is to evaluate the potential of the cockroaches as a model for the human gut microbiome model by comparing the gut microbiota in humans, mice, and cockroaches. This data is obtained from the publicly accessible Sequence Read Archive (SRA) in the GenBank database. The data was analysed using DADA2, which is running on RStudio software on the Viking2 supercomputer platform, the University of York. Overall, the taxonomy of bacteria is compared, and the microbial communities of cockroaches with the most similarities are considered for further investigation.

2.2 Materials and Methodologies

2.2.1 Bacterial communities compared among human guts, mouse guts, and cockroach guts

The Sequence Read Archive (SRA) publicly available data from the GenBank database was chosen to compare bacterial communities found in human, mouse, and cockroach guts. For our comparison study, we only included healthy humans, control mice, and cockroaches. A comparison was made only between fecal samples of healthy humans and mice and the dissected guts of cockroaches. The drawback of fecal microbiota is that it is unlikely for the fecal microbiome to completely represent the mucosal microbiome in humans (Marteau et al., 2001; Vasapolli et al., 2019). However, in the German cockroach, 80 to 90% of the gut microbiome is shared between the intestinal tract and feces (Kakumanu et al., 2018). All sequence data, including the amplicon on the V4 region and Illumina sequencing, are handled using the same protocols in order to provide comparable sequence information. The SRA-Toolkit version 2.10.9 was used to download the sequences of the 16S rRNA gene. All the studies shown in Table 2, only the samples undergoing the control treatment from each dataset were included. Healthy adults balanced with ages, genders, and geographical locations were taken into the data analysis. The data analysis also included the commonly used laboratory mouse strains C57BL/6, C57BL/6J, and B6D2F1. Due to how frequently they are used as human models and the abundance of publicly available data in the database, laboratory mice were selected for our comparative analysis. A total of 693 samples were included in the data analysis. These data are divided into three different groups: 345 samples of human guts, 86 samples of mouse guts, and 262 samples of cockroach guts.

Table 2 Public data of 16S rRNA gene sequencing from the GenBank database.

BIOPROJECT	HOST	NO OF SAMPLES	GENDERS	COUNTRIES	REFERENCES
HUMAN					
PRJNA381395	Human	38	-	Germany	(Heintz-Buschart et al., 2018)
PRJEB38491	Human	37	Female: Male (22:15)	France	(Lecomte et al., 2020)
PRJEB34168	Human	38	Female: Male (26:12)	USA	(Choileáin et al., 2020)
PRJEB30836	Human	135	-	Africa	(Lokmer et al., 2020)

PRJDB9608	Human	97	Female: Male (41:56)	Japan	(Hashimoto et al., 2020)
COCKROACH					
PRJNA320551	American cockroach	23	Female: Male (15:8)	USA	(Tinker and Ottesen, 2016b)
PRJNA433530	Cockroaches (19 species)	239	-	USA	(Tinker and Ottesen, 2018)
MOUSE					
PRJNA429444	C57BL/6J	12	-	Italy	(Ciciliot et al., 2018)
PRJNA729715	C57BL/6	8	-	USA	(Tao et al., 2022)
PRJNA557826	C57BL/6	9	Female	USA	(Younge et al., 2019)
PRJNA636288	C57BL/6J	12	Male	China	(Li et al., 2020b)
PRJNA647919	C57BL/6J × DBA2/J (B6D2F1)	24	Female: Male (12:12)	USA	(Raber et al., 2020)
PRJNA659149	C57BL/6	6	Male	Australia	(Gubert et al., 2020)
PRJNA532979	C57BL/6J	10	Male	Australia	(Gamage et al., 2020)
PRJNA650014	C57BL/6J	5	Female	China	(Pan et al., 2021)

2.2.2 Data Analysis

All of the data were sequenced using Illumina sequencing methodology with an amplicon of the V4 regions of the 16S rRNA gene that were amplified using different primers. The dataset was analysed using DADA2 (Callahan et al., 2016) in the RStudio programme (Allaire, 2012) running on the Viking2 supercomputer platform, the University of York. The steps are started with paired-end fastq files from each sample deposited into the DADA2 R package. The ambiguous bases (Ns) were excluded from the sequences, and then their primers were removed separately using Cutadapt (v3.5) (Martin, 2011). Those sequences that showed a quality value of less than 25 were eliminated. Filtering quality and denoising were applied to all set runs, and multiple runs were merged altogether using mergeSequenceTables in the DADA2 package. Chimeras were eliminated using BimeraDenovo after merging both paired ends, and the remaining sequences were classified taxonomically using the Silva reference database (v138.1) (Quast et al., 2012). Sequences belonging to unclassified Kingdom and Phylum, Eukaryota, Chloroplasts, Mitochondria, and *Blattabacterium* (a cockroach endosymbiont) were removed from the study of microbial communities in human, mouse, and cockroach guts. The amplicon sequence variant (ASV) (Callahan, McMurdie and Holmes, 2017) abundance table was used

to group the remaining samples. However, some data sets failed to meet quality conditions and were not included in our analysis, including the ASV table missing in comparison to the original data.

2.2.3 Statistical analysis

The bacterial taxonomic levels, the bacterial abundance, and the metadata were gathered into a single Excel file and subjected into phyloseq format using phyloseq package (v1.48.0) (McMurdie and Holmes, 2013) on RStudio. Rarefy was used to normalise the samples without replacing any of them, based on 90 percent of the minimum sample depth. Venn diagrams of bacterial taxa were visualized using microeco package (v1.7.1) (Liu et al., 2021b). The Shannon and Chao1 indices were determined using Kruskal-Wallis test with a pairwise Dunn test between human, cockroach, and mouse samples using microeco package. The relative abundances of bacterial communities were calculated using MicrobiotaProcess package (v1.16.0) (Xu et al., 2023). A heatmap of bacterial genera was generated by microeco package. A two-dimensional principal coordinate analysis (PCoA) was performed based on Bray-Curtis distances using the MicrobiotaProcess package. The statistical test of bacterial communities across samples was assessed with permutational multivariate analysis of variance (PERMANOVA) with Bray-Curtis distance in the vegan package (v2.6.6.1) (Oksanen et al., 2018). Based on the 16S rRNA gene Illumina sequencing, the bacterial functions were predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST2) (v2.5.2) (Douglas et al., 2020). The bacterial ASVs file was used to create predicted KO abundances. The microeco programme was used to create a heat map of the predominant KO abundance.

2.3 Results

2.3.1 Publicly available data comparison bacterial communities and diversity analysis

A total of 693 samples, including humans, mice, and cockroaches were analysed using DADA2 running on the R programme. A total of 62,327 amplicon sequence variants (ASVs) were retrieved using a cut-off at the samples containing sequences with more than 10,000 sequence reads. Therefore, a total of 10 samples were excluded. The sample reads had an average of 59,247 reads and ranged from 10,065 to 267,684 reads. Then, a total of 280 ASVs were excluded using the filtering of low relative abundance and low occurrence frequency (microeco package).

62,047 ASVs were left, which were classified across three samples, including human, cockroach, and mouse (Figure 3). The Venn diagrams indicate that cockroach samples had a higher number of bacterial taxa (ASVs) than in humans and mice. Moreover, there was a greater similarity of shared ASVs in the bacterial community between humans and cockroaches than there was between humans and mice.

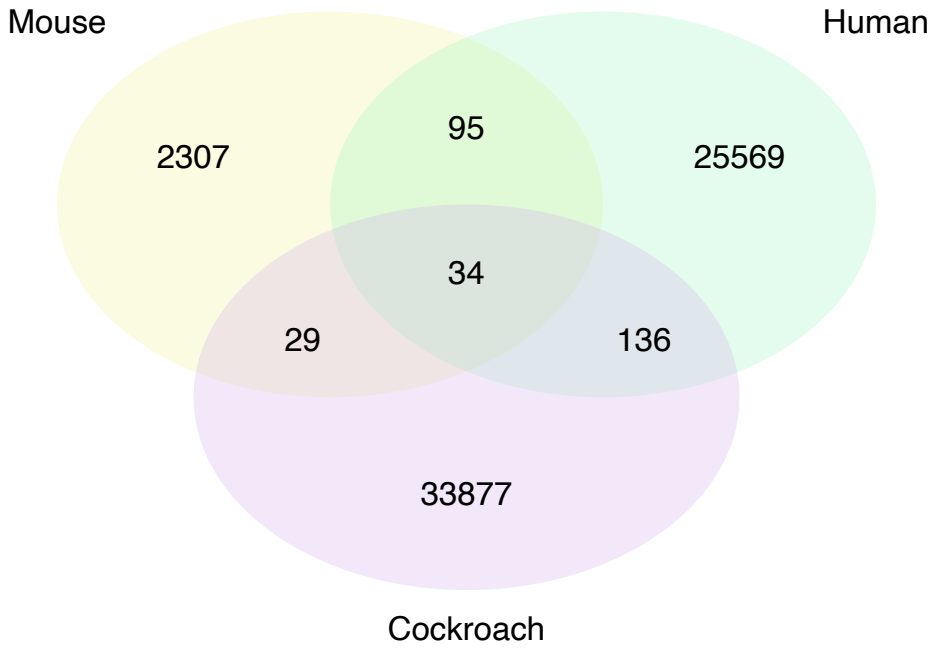


Figure 3 Venn diagrams indicate bacterial taxa (ASVs) across human, cockroach, and mouse samples.

The Shannon and Chao1 indices (Figure 4) were determined for the bacterial communities among the cockroach, mouse, and human guts. There were significant differences in the bacterial diversity among all the samples (Kruskal-Wallis; $P < 0.001$). Specifically, the bacterial diversity assessed both by Shannon and Chao1 was significantly distinct between samples with the Dunn's post-hoc test ($P < 0.001$). Both alpha diversity indices illustrate the same results since the bacterial diversity was highest in the guts of cockroaches, followed by those of human and mouse guts, respectively. The beta diversity was calculated using Bray-Curtis distances and visualized with PCoA (Figure 5). The PCoA indicates that the bacterial communities were grouped differently in human, cockroach, and mouse samples confirming with a PERMANOVA with R2 = 0.1421 (P = 1e-04). It was discovered that there were significant differences between the three samples (pairwise ADONIS, mouse vs. human, R2 = 0.1190, $P = 0.001$; mouse vs. cockroach, R2 = 0.1269, $P = 0.001$; human vs. cockroach, R2 = 0.0907, $P = 0.001$). The first three principal component scores accounted for 11.81%, 6.5%, and 5.32%, respectively. The first two PCoA1 and PCoA2 indicated that bacterial communities were grouped differently across all samples. While PCoA1 and PCoA3 indicate some bacterial taxa were clustered closely between cockroaches and mice, the bacterial communities of humans separated into two different groups.

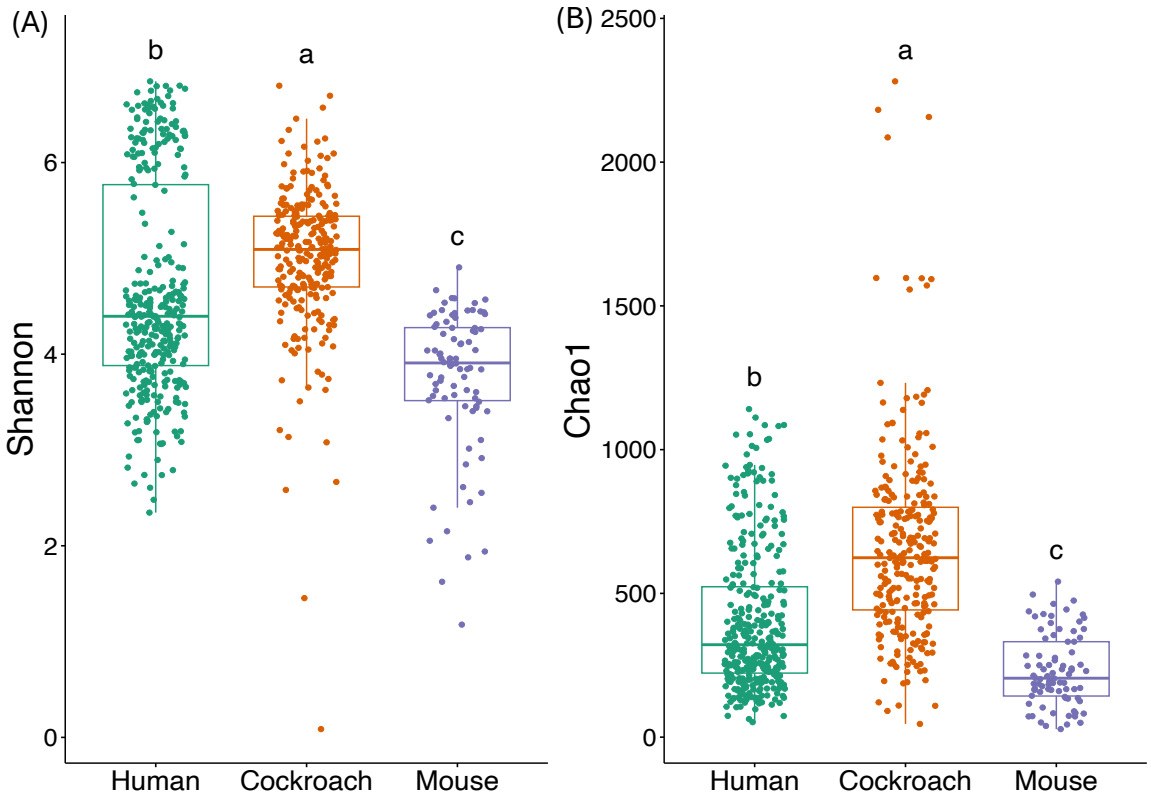


Figure 4 Alpha diversity indices among cockroach, human, and mouse gut samples. Shannon (A) and Chao1 (B) indices show in a box plot. The Kruskal-Wallis test with Dunn's test ($P < 0.05$) was used, and the letters a, b, and c represent statistically different values. The horizontal bars indicate the median for each box, while the hinges represent the lower and the higher quartiles.

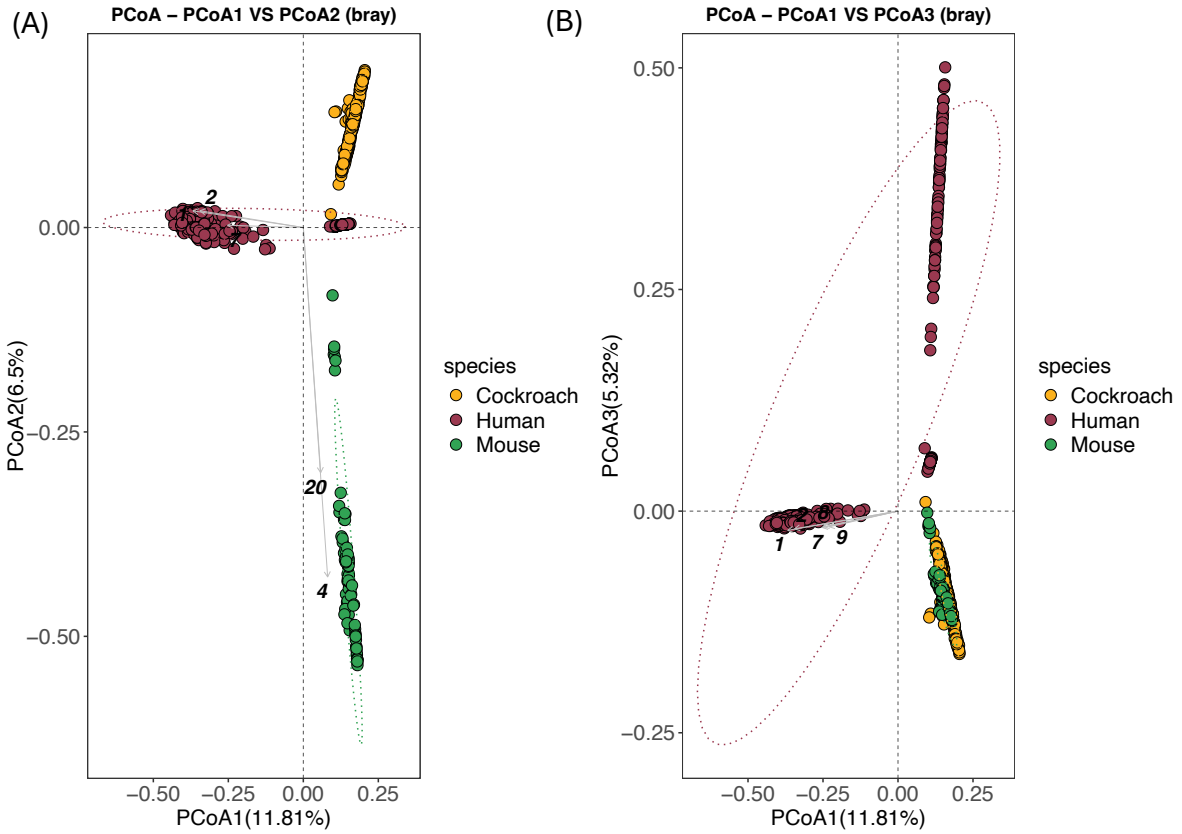


Figure 5 PCoA of bacterial communities across samples of cockroach, human, and mouse guts using Bray-Curtis dissimilarity matric. Plots of (A) PCoA1 and PCoA2 and (B) PCoA1 and PCoA3 explain the variance of the abundance of gut microbiome. The permutational multivariate analysis of variance (PERMANOVA) test was used ($P < 0.05$). Pairwise ADONIS ($P < 0.05$) indicates a substantial dissimilarity between the three samples.

2.3.2 The differences of bacterial communities across human, cockroach, and mouse samples

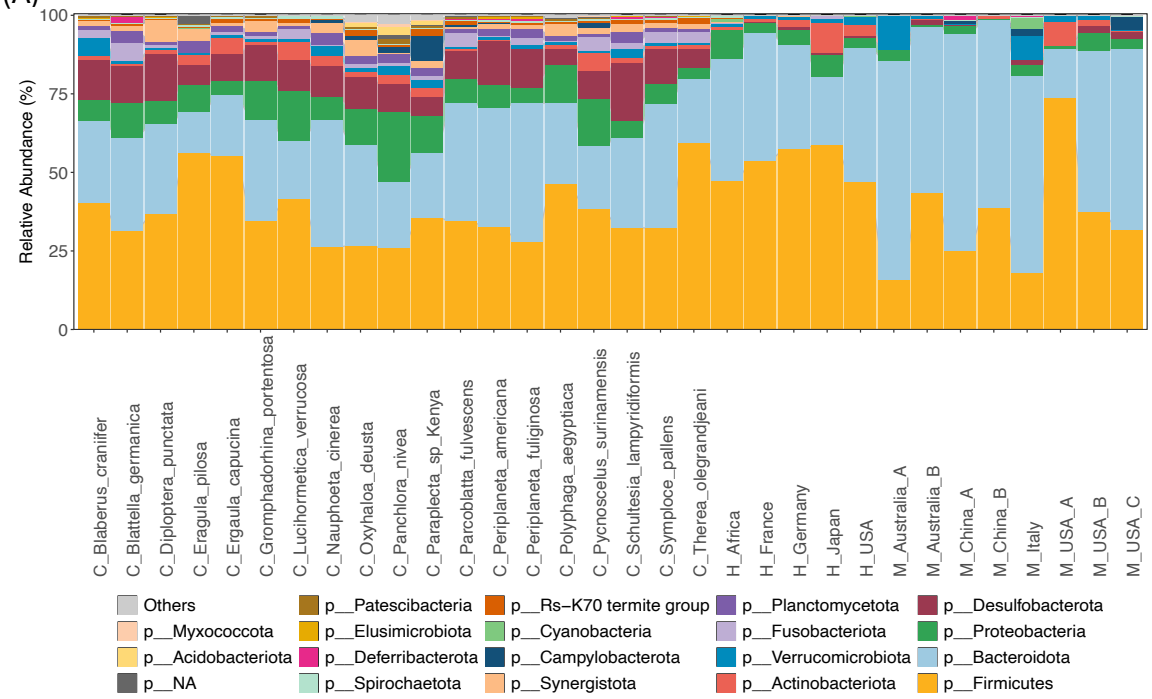
Firmicutes and *Bacteroidota* are the two most prevalent bacterial phyla in all samples (Figure 6A). More *Bacteroidota* than *Firmicutes*, accounting for 56.4% and 33.5% of the total bacterial communities in mice, respectively. In humans and cockroaches, on the other hand, the proportion of *Firmicutes* is higher than that of *Bacteroidota*, accounting for 52.3% and 34.2%, 36.3% and 30.3%, respectively. Apart from those two phyla, *Proteobacteria*, *Actinobacteriota*, and *Verrucomicrobiota* are the other bacterial phyla that predominate in humans. The other bacterial phyla that are most frequently found in cockroaches are *Fusobacteriota*, *Actinobacteriota*, *Synergistota*, *Planctomycetota*, *Desulfobacterota*, and *Proteobacteria*. Mice also contain members of the bacterial phyla *Verrucomicrobiota, Campylobacterota,* and *Proteobacteria.*

Some bacterial families that are prevalent in human and cockroach guts belong to *Lachnospiraceae*, *Bacteroidaceae*, *Ruminococcaceae*, *Rikenellaceae*, and *Christensenellaceae* (Figure 6B). However, there is less diversity of bacterial families in mouse guts, with approximately 40% of them being *Muribaculaceae*.

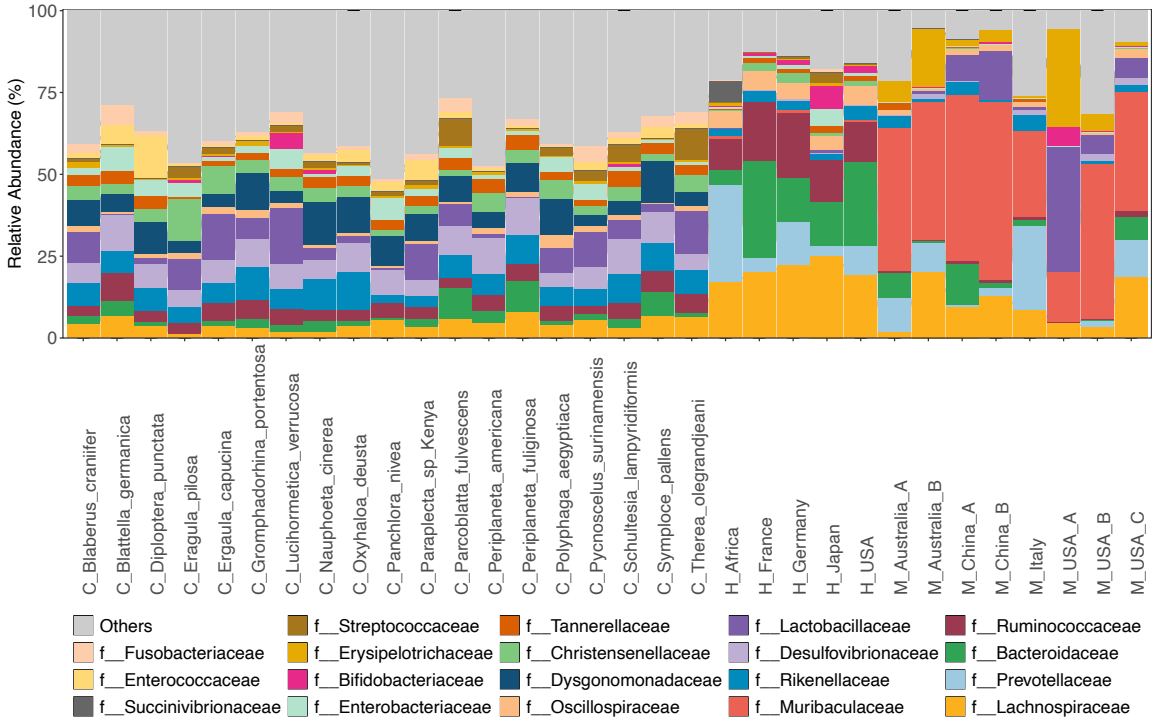
At the genus level, an unclassified genus of *Muribaculaceae* is the most predominant in mouse samples, with the occurrence of other genera, including *Bacteroides,* an unclassified genus of *Lachnospiraceae*, and *Lachnospiraceae* NK4A136 group (Figure 6C). More abundant genera of cockroaches are *Desulfovibrio, Alistipes, Bacteroides,* and *Christensenellaceae* R-7 group, while *Bacteroides, Prevotella_*9*,* and *Faecalibacterium* are predominant in human samples.

The heat map reveals the majority of 40 bacterial genera are present in the guts of cockroaches, humans, and mice (Figure 7). There are some bacterial communities that are found similarly in both human and cockroach guts. Specifically, *Bacteroides, Desulfovibrio, Alistipes, Christensenellaceae* R-7 group, and *Parabacteroides* are predominant among both human and cockroach samples.

(A)


Relative Abundance (%)
100
75
50
25
0
C_Blaberus_craniifer
C_Blattella_germanica
C_Diploptera_punctata
C_Eragula_pilosa
C_Ergaula_capucina
C_Gromphadorhina_portentosa
C_Lucihormetica_verrucosa
C_Nauphoeta_cinerea
C_Oxyhaloa_deusta
C_Panchlora_nivea
C_Paraplecta_sp_Kenya
C_Parcoblatta_fulvescens
C_Periplaneta_americana
C_Periplaneta_fuliginosa
C_Polyphaga_aegyptiaca
C_Pycnoscelus_surinamensis
C_Schultesia_lampyridiformis
C_Symploce_pallens
C_Therea_olegrandjeani
H_Africa
H_France
H_Germany
H_Japan
H_USA
M_Australia_A
M_Australia_B
M_China_A
M_China_B
M_Italy
M_USA_A
M_USA_B
M_USA_C
Others
p__Myxococcota
p__Acidobacteriota
p__NA
p__Patescibacteria
p__Elusimicrobiota
p__Deferribacterota
p__Spirochaetota
p__Rs−K70 termite group
p__Cyanobacteria
p__Campylobacterota
p__Synergistota
p__Planctomycetota
p__Fusobacteriota
p__Verrucomicrobiota
p__Actinobacteriota
p__Desulfobacterota
p__Proteobacteria
p__Bacteroidota
p__Firmicutes

(B)


Relative Abundance (%)
100
75
50
25
0
C_Blaberus_craniifer
C_Blattella_germanica
C_Diploptera_punctata
C_Eragula_pilosa
C_Ergaula_capucina
C_Gromphadorhina_portentosa
C_Lucihormetica_verrucosa
C_Nauphoeta_cinerea
C_Oxyhaloa_deusta
C_Panchlora_nivea
C_Paraplecta_sp_Kenya
C_Parcoblatta_fulvescens
C_Periplaneta_americana
C_Periplaneta_fuliginosa
C_Polyphaga_aegyptiaca
C_Pycnoscelus_surinamensis
C_Schultesia_lampyridiformis
C_Symploce_pallens
C_Therea_olegrandjeani
H_Africa
H_France
H_Germany
H_Japan
H_USA
M_Australia_A
M_Australia_B
M_China_A
M_China_B
M_Italy
M_USA_A
M_USA_B
M_USA_C
Others
f__Fusobacteriaceae
f__Enterococcaceae
f__Succinivibrionaceae
f__Streptococcaceae
f__Erysipelotrichaceae
f__Bifidobacteriaceae
f__Enterobacteriaceae
f__Tannerellaceae
f__Christensenellaceae
f__Dysgonomonadaceae
f__Oscillospiraceae
f__Lactobacillaceae
f__Desulfovibrionaceae
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f__Ruminococcaceae
f__Bacteroidaceae
f__Prevotellaceae
f__Lachnospiraceae

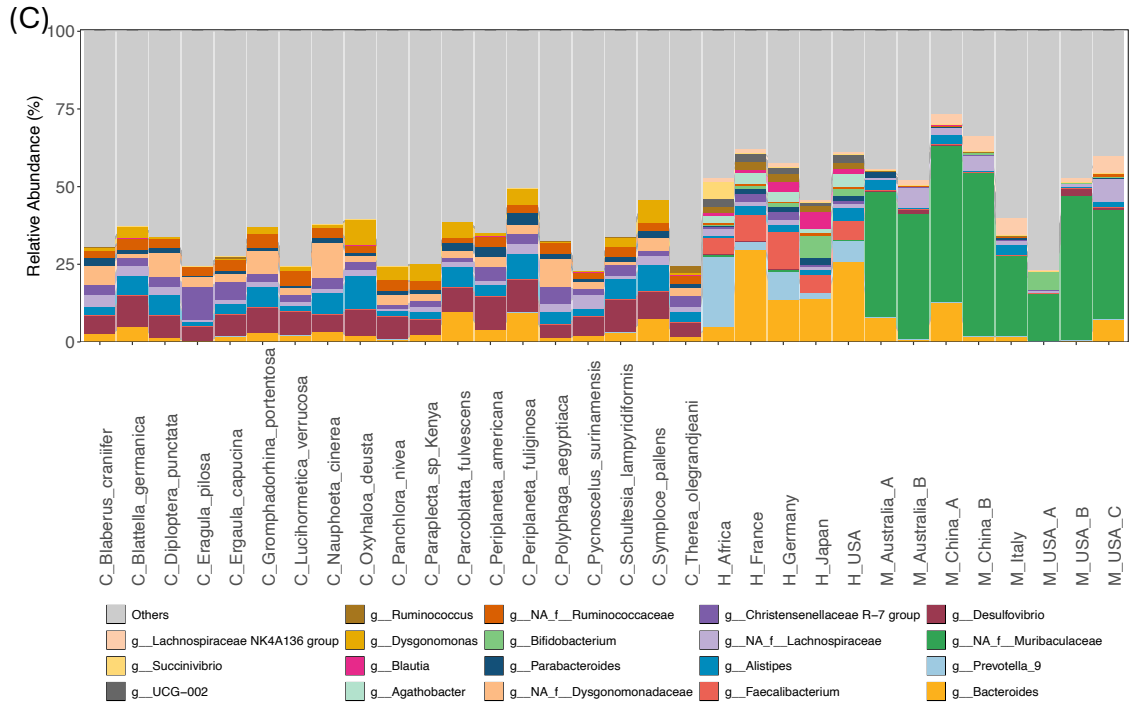


Figure 6 Relative abundance of bacterial communities associated with cockroach, human, and mouse guts. The most bacterial predominance with a total of 19 bacterial phyla (A), families (B), and genera (C) are shown. The initial letters of the sample names, C, H, and M, stand for cockroach, human, and mouse, respectively.

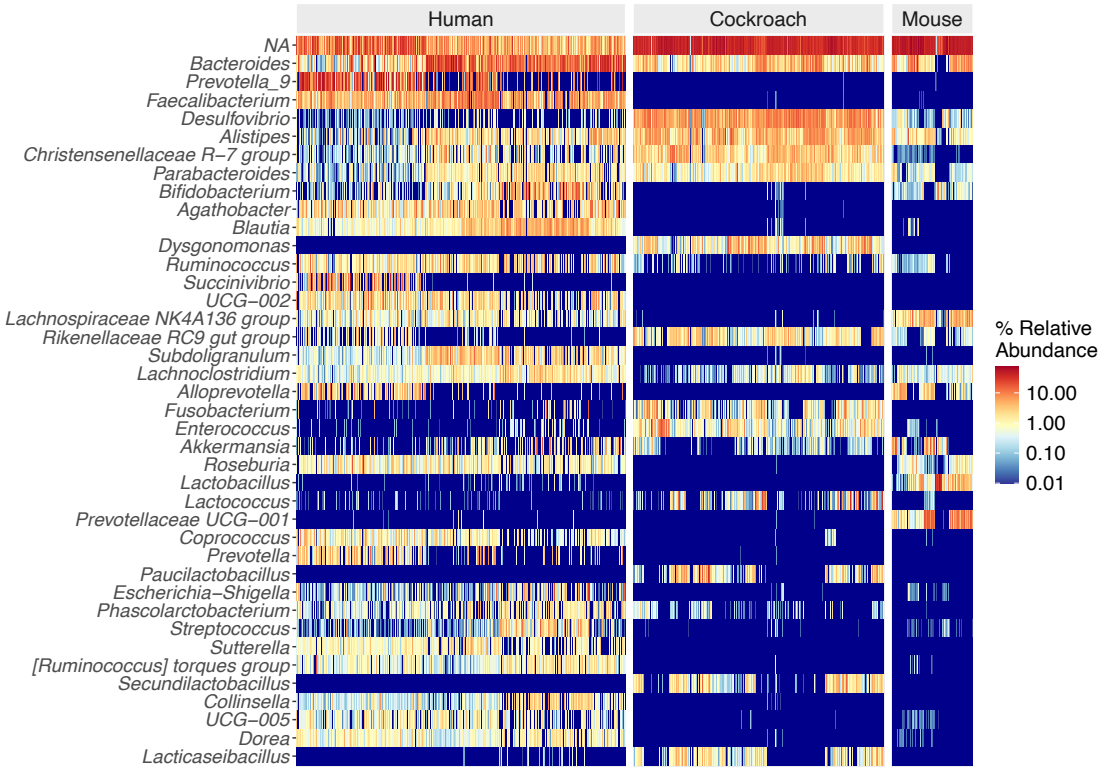


Figure 7 Heat map shows the most 40 bacterial genera are found in cockroach, human, and mouse guts. The most abundance shows in the red colour, while blue colour represents less abundance.

2.3.3 Functional prediction based on 16S rRNA gene sequencing using PICRUST2

The ASVs of bacterial communities from 16S rRNA gene sequencing were used to predict the bacterial functions from human, cockroach, and mouse samples. A heat map (Figure 8) showed the 30 most relative abundances of Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2012) Orthology (KO). There was some KO sharing in higher abundance compared to the rest KO across the three samples displayed in red. Several pathways are shared across all samples, for example, RNA polymerase sigma-70 factor ECF subfamily, ABC-2 type transport system ATP-binding protein, ATP-binding cassette subfamily B bacterial, ABC-2 type transport system permease protein, putative ABC transport system permease protein, and putative ABC transport system ATP-binding protein.

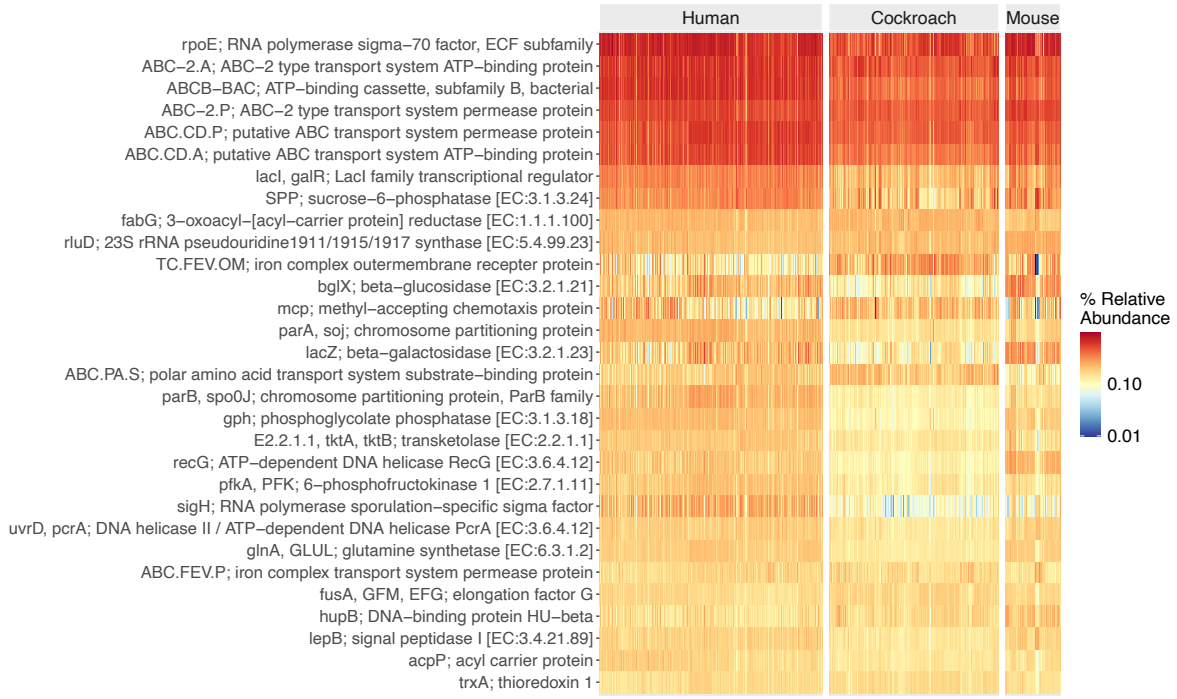


Figure 8 Heatmap of the 30 most predominant bacterial functions based on 16S rRNA gene sequencing using PICRUST2. The higher KO relative abundances are labelled in red and lower abundances are in yellow and blue, respectively.

2.4 Discussion

The 16S rRNA gene sequence data from the SRA in the GenBank database of 693 samples, which include 345 samples from human guts, 86 samples from mouse guts, and 262 samples from cockroach guts, were examined of a partial 16S rRNA gene. Overall, the intriguing findings indicate that cockroaches have a higher diversity of gut microbes than mice and humans.

The major phyla in both humans and cockroaches are *Firmicutes* and *Bacteroidota*, with similar trends indicating that *Firmicutes* is more numerous than *Bacteroidata* (Figure 6). On the other hand, although these two main phyla are the most common in mice, *Bacteroidata* is more prevalent than *Firmicutes* (Figure 6). These two bacterial phyla are found dominantly in humans and common laboratory mice (Gu et al., 2013; Kostic, Howitt and Garrett, 2013). Furthermore, compared to mice and humans, cockroaches' gut microbiomes appear to be more diverse in regard to bacterial families and genera (Figure 6). In humans, *Lachnospiraceae* and *Ruminococcaceae* from *Firmicutes*, *Bacteroidaceae*, and *Prevotellaceae* from *Bacteroidota* are predominant bacterial families. Moreover, *Lachnospiraceae*, *Bacteroidaceae*, and *Ruminococcaceae* are also found in the cockroach gut but in less abundance than in humans (Figure 6).

Dietary fibre is related to bacterial communities in *Firmicutes* (Sun et al., 2023). *Lachnospiraceae* and *Ruminococcaceae* were altered and were able to produce more short-chain fatty acids (SCFAs) of dietary fiber fermentation when consumed with whole-grain wheat in humans (Schutte et al., 2018). Additionally, consumption of a resistant starch diet enhanced the number of *Ruminococcus bromii* in humans (Walker et al., 2011). In addition, the human *Firmicutes*/*Bacteroidetes* ratio fluctuates with age, being higher in adults and lower in both infants and the elderly (Mariat et al., 2009). Moreover, both bacterial phyla may be useful in determining our health condition as they are altered by several factors, such as ageing (Vaiserman et al., 2020) and obesity (Ismail et al., 2011).

Several bacterial species share between cockroaches and humans, including *Bacteroides, Desulfovibrio, Alistipes, Christensenellaceae* R-7 group, and *Parabacteroides* (Figure 7). *Bacteroides* are found to be involved with polysaccharide metabolism (Comstock and Coyne, 2003). Specifically, exposure to non-digestible polysaccharides (inulin and pectin) enhanced the abundance of several of the most prevalent *Bacteroides* species in the human gut in *in vitro* experiment (Chung et al., 2016). In mice fed polysaccharide diets, intestinal *Bacteroides* species are shown to express higher levels of polysaccharide utilization loci (PULs) (Sonnenburg et al., 2010). *Desulfovibrio* is sulfate-reducing bacteria that harbour in the intestines of humans. Their function in human guts is disputable, though, as they are present in both healthy (Chen et al., 2021a) and humans with diseases such as Parkinson's disease (Murros et al., 2021; Nie et al., 2023), autism (Finegold, 2011), and inflammatory bowel

disease (Loubinoux et al., 2002a). *Desulfovibrio* is also a common bacterium found in cockroaches (Pérez-Cobas et al., 2015; Chen et al., 2020b) and termites (Sato et al., 2009a; Trinkerl et al., 1990). The *Christensenellaceae* was isolated from human faeces (Morotomi, Nagai and Watanabe, 2012) and it is positively associated with a normal body mass index and also a response to human diet and human diseases (Waters and Ley, 2019). The *Fusobacterium* was found and isolated from cockroach guts. One found that it was enhanced in German cockroaches fed diets high in protein (Pérez-Cobas et al., 2015). However, *Fusobacterium nucleatum* is found in colorectal human cancer patients (Bashir et al., 2016).

Bacteroides, Alistipes, and *Parabacteroides* from *Bacteroidota* phylum are common in human and cockroach guts (Figure 7). Within the same *Bacteroidota* phylum as all of the previously listed bacterial species, *Dysgonomonas* is also frequently found in the American cockroach guts (Chen et al., 2020b). Remarkably, *Bacteroides, Dysgonomonas, Paludibacter,* and *Parabacteroides* were isolated from the American cockroach guts which are closely related to these genera from other cockroach species, and they contain PULs and some carbohydrate-active enzymes (CAZymes) (Vera-Ponce De León et al., 2020).

The healthy human gut microbiota can be classified into three enterotypes: *Bacteroides, Prevotella,* and *Ruminococcaceae* (Costea et al., 2018). Two of these major indicator families, *Bacteroides* and *Ruminococcaceae*, are also present in the cockroach guts (Figure 6; Figure 7). It is important to note that, depending on the individuals, either *Prevotella*_9 or *Faecalibacterium* is the subsequent most common bacterial genera discovered in humans across geographic locations, following *Bacteroides*. With the exception that in African humans, *Prevotella*_9 is far more prevalent than *Bacteroides*. *Prevotella spp.* are more dominant in rural populations than in Westernization populations (De Filippis et al., 2019; Prasoodanan P. K. et al., 2021). In addition, Italian populations that consume high-fiber diets are more likely to have *Prevotella copri* (De Filippis et al., 2019). These lead to the conclusion that the bacterium may vary depending on the environment, diet, and lifestyle (De Filippis et al., 2019).

PICRUST2 was used to predict gut microbiome functions based on 16S rRNA gene sequencing. As shown on the heat map (Figure 7), there are some bacterial functions that are found to be similar across all samples. We could use this information to evaluate the bacterial communities associated with their functions in the cockroach gut model. Cockroaches are not only showing that they host a diverse bacterial community but also that they share some bacterial taxa with humans. Apart from focusing on bacterial interactions, metabolic pathways that are shared between human and cockroach gut microbiomes are also important to take into consideration.

In summary, we compared the bacterial communities associated with three different organisms: mice, cockroaches, and humans, allowing us to understand the similarities and differences among their microbiomes. These indicate that these organisms have very diverse gut microbiomes and that some of the bacteria are shared. It could lead us to study the benefits of microorganisms and how they interact with hosts. It is noteworthy that this is the first study to compare the bacterial communities of three distinct organisms, including mice, cockroaches, and humans. We confirmed that, in comparison to humans and mice, cockroaches harbour a greater diversity of bacteria. While genera in *Muribaculaceae* are approximately one-third of all bacterial genera in mice, several bacterial taxa were also shared by humans and cockroaches at the phylum and genus taxonomic levels. Furthermore, cockroaches appear to be superior in some respects to mice in an alternate human gut model, with a greater number of bacterial species and those that are shared by both humans and cockroaches. Therefore, we would like to recommend an alternative model for the cockroach to be considered.

Chapter 3: The effects of antibiotic treatments on the gut microbiome of American cockroach

3.1 Introduction

In the second chapter, we examined the differences in the gut microbiomes of cockroaches, humans, and mice. *Firmicutes*, *Bacteroidota*, and *Proteobacteria* are the most common bacterial phyla in the healthy human gut microbiome. Similar to the human gut microbiome, we found that the most predominant microorganisms in the cockroach gut also belong to *Firmicutes*, *Bacteroidota*, and *Proteobacteria*. Cockroach guts had an additional predominant phylum, the *Desulfobacterota*. Furthermore, compared to the guts of humans and mice, cockroaches have more diversified gut microbiota (Chapter 2).

Our gut microbiome is altered by numerous factors (David et al., 2014; Hagan et al., 2019). For instance, birth methods include vaginal and cesarean (Mancabelli et al., 2020), dietary consumption (David et al., 2014), human diseases (Cho and Blaser, 2012), and geographical environments (David et al., 2014). Due to their increased exposure to oxygen in the intestines, *Lactobacillaceae* and *Enterobacteriaceae*, two facultative anaerobes, can colonise newborns (Mancabelli et al., 2020). Neonates have more complicated guts, which facilitate the colonization of obligate anaerobes, including *Bifidobacteriaceae* and *Clostridiaceae* (Sanidad and Zeng, 2020). For most infants, human breast milk is the most necessary and their initial food. Additionally, *Bifidobacterium* and *Lactobacillus* were more prevalent in breast-feeding newborns (Bäckhed et al., 2015). Some *Bifidobacterium* strains are beneficial in helping in the digestion of oligosaccharides found in human milk (Hegar et al., 2019). Our immune system, digestion of food, and general health are all influenced by this gut flora (Clemente et al., 2012; Armour et al., 2019).

Antibiotics are another factor that can alter human gut microbiomes (Francino, 2016). Since the discovery of penicillin by Alexander Fleming in the early 20th century (Bear, Turck and Petersdorf, 1970), antibiotics have been widely used to treat infectious disorders (Francino, 2016) which can also disrupt the human gut microbiome. Antibiotics undeniably improve human and animal health (Francino, 2016). However, therapeutic use of antibiotics is not the only way that humans receive the effects of antibiotics. We can receive antibiotics through diet, as antibiotics have been widely used in livestock farming (Subirats, Domingues and Topp, 2019) and agriculture (Ben et al., 2022). Antibiotics may have both beneficial and detrimental effects on consumers. Additionally, inappropriate use may also have negative influences on human health, antimicrobial resistance, and other non-target human gut microbiota (Francino, 2016; Fishbein, Mahmud and Dantas, 2023).

Approximately 5,000 different cockroach species have been identified, indicating their diversity (Guzman and Vilcinskas, 2020). Cockroaches are easy to raise, require little space for experimentation, and can be raised in large batches. We examined the publicly available 16S rRNA gene sequencing data of mouse, cockroach, and human guts in the previous Chapter 2. The fact that cockroaches have diverse bacteria in their digestive tracts is further supported by our study. Additionally, they had several bacterial species in common with the intestinal organs of humans.

There are several methods used to eliminate gut microorganisms in cockroaches, such as antibiotics (Rosas et al., 2018; Domínguez-Santos et al., 2020, 2021) and germ-free conditions (Mikaelyan et al., 2016; Dukes, Dyer and Ottesen, 2021b). In order to generate germ-free insects, the eggs must first be surface sterilised. The insects are then kept in this sterilised environment for the duration of the experiment (Dukes, Dyer and Ottesen, 2021b). However, as American cockroaches typically take 1-2 years to reach adulthood, this method is laborious as well as costly (Lin et al., 2022a).

When pathogenic or foreign microorganisms invade the host's gut, diverse indigenous microorganisms in the host gut can inhibit those invading microorganisms from colonizing the host guts (Lawley and Walker, 2013). This phenomenon is called colonization resistance (Lawley and Walker, 2013). These can be exhibited in several ways, such as stimulating the immune system (Sassone-Corsi and Raffatellu, 2015) and producing short-chain fatty acids (Sun and O'Riordan, 2013). The term colonization resistance is essential for hosts to compete against invading pathogens or resident bacterial overgrowth (Ducarmon et al., 2019).

In addition to the effects of antibiotics on the cockroach gut microbiome, our primary aim was to use antibiotics to eliminate the gut microbiota in order to overcome the colonization resistance in the host gut. This could enable the study of colonization processes by commensal bacteria in the cockroach gut.

This phenomenon relates to human health and sickness as well as assisting in the microbiota's ability to maintain equilibrium (Lawley and Walker, 2013). Several antibiotics have been experimentally used on the German cockroaches, including kanamycin (Domínguez-Santos et al., 2021), rifampicin (Rosas et al., 2018; Muñoz-Benavent et al., 2021a), and ampicillin mixed with vancomycin (Domínguez-Santos et al., 2020). Apart from cockroach gut microbiota, the endosymbiotic bacterium (*Blattabacterium*) occurs in almost all cockroaches harbouring in the bacteriocytes located inside the fat body tissue of the cockroach (Sabree, Kambhampati and Moran, 2009). This bacterium plays an important role in nitrogen metabolism and helps with the nutritional diet of cockroach guts (Sabree, Kambhampati and Moran, 2009). This bacterium is transferred vertically via the ootheca to their offspring (Carrasco and Pérez-Cobas, 2014). The majority of the gut microbiome of cockroaches is

horizontally transferred, primarily receiving from their faeces (Carrasco and Pérez-Cobas, 2014; Chen et al., 2020b). The antibiotics that have no effect on the *Blattabacterium* endosymbiont would be selected. Fortunately, most types of antibiotics are unable to disrupt the *Blattabacterium* in cockroaches, although rifampicin has been shown to negatively impact both the gut microbiome and this bacterium (Rosas et al., 2018; Cazzaniga et al., 2023).

Kanamycin is an aminoglycoside that is considered a broad-range antibiotic and is effective against both Gram-negative and Gram-positive bacteria. It is water-soluble (Umezawa et al., 1957). Ampicillin is a beta-lactam antibiotic and is also a broad-spectrum antibiotic against both Gram-positive and Gram-negative bacteria (Bear, Turck and Petersdorf, 1970). Several studies found that both kanamycin (Domínguez-Santos et al., 2021; Wolfe and Scharf, 2021) and ampicillin (Domínguez-Santos et al., 2020; Lee et al., 2021) had no impact on *Blattabacterium*, so these two antibiotics were chosen in our experiment.

Our study aims to evaluate the use of the cockroach as a human gut microbiome model. In Chapter 2, we discovered that the human and cockroach gut microbiomes are similar. Antibiotic treatment in the cockroach was not only to overcome its colonization resistance situation, but antibiotic treatment might also provide us more about how antibiotics affect microbial communities. The different antibiotics, kanamycin and ampicillin, at a concentration of 0.3 $mg \cdot ml^{-1}$ were used to eradicate the cockroach gut microbiome.

This study aimed to:

1. To illustrate the effects of kanamycin and ampicillin on the gut microbiome of American cockroaches. The microbial communities in the cockroach guts were determined using the 16S rRNA gene of universal bacteria by Illumina sequencing.
2. The absolute number of bacterial copies was quantified by a quantitative PCR (qPCR), and both the relative abundance of bacterial communities from Illumina sequencing and the absolute number of bacteria from qPCR were compared.

3.2 Methods

3.2.1 Cockroach husbandry

Female American cockroaches (*Periplaneta americana*) were purchased from Blades Biological Ltd. in Cowden, Edenbridge, Kent, UK. All cockroaches were fed with dog food made by Pets at Home, composed of 22.0% protein, 4% crude fibre, 6.5% crude fat, 7% crude ash, 1.5% calcium, 1.6% omega 6, and 0.2% omega 3. The cockroach colony rearing is adapted from Dukes et al., 2021 (Dukes, Dyer and Ottesen, 2021b). All were raised under 12:12 hours of light: dark photoperiod at 28°C and 60-70% R.H. The fish tanks were stacked with cardboard tubes with hard cardboard inserted into their holes. Petroleum jelly was smeared on the top of the tank, about 2 inches deep, to prevent the insects from climbing out of the tanks. The tanks were changed and cleaned regularly with 2% bleach and rinsed several times with water. All insects were transferred into a new tank setting with the same conditions. Then, their frass, carcasses, and waste were autoclaved before disposal.

3.2.2 Cockroach dissection

The dissection was carried out in a sterilized environment. Cockroaches were knocked out by freezing and dissected on a Petri dish. To eliminate external contamination, their bodies were washed with 7% sodium hypochlorite and 70% ethyl alcohol. Then, samples were rinsed with deionized water several times. The insects were dissected under the stereo microscope, and their digestive organs were collected carefully. The digestive organs were rinsed again with sterile phosphate-buffered saline (PBS) three times before being preserved at -20°C until DNA extraction. Each gut represented an individual insect.

3.2.3 Pilot test: antibiotic susceptibility in cockroach guts

The female cockroaches were raised in a plastic container, and they were starved for three days before the antibiotic treatment. There were four treatments, including the control with sterilized drinking water and the antibiotic treatment with 0.1, 0.2, and 0.3 $mg \cdot ml^{-1}$ of Kanamycin, respectively. The cockroaches were fed with water containing antibiotics and sterile diets containing antibiotics during the experiment. The cockroaches were collected on day 0 as a control group. The cockroaches were collected on day 4 and day 5, which was the end of the antibiotic treatment. The cockroaches were kept at -20°C until DNA extraction.

3.2.4 Bacterial isolation and detection in the guts after the pilot antibiotic treatment

After antibiotic treatment, a quantitative PCR (qPCR) was used to quantify bacterial communities in the cockroach guts. The individuals from each antibiotic concentration from different time points were extracted genomic DNA. The universal 16S rRNA gene of bacteria was performed using qPCR (341F-CCTACGGGAGGCAGCAG and 534R-ATTACCGCGGCTGCTGGCA) (Watanabe, Kodama and Harayama, 2001). A total of 25 µl of qPCR master mix contains 0.5 µl of each primer, 12.5 µl SYBR Green dye, 0.5 µl H_2O, 5 µl template DNA (0.5 ng μl^{-1}), and water. The qPCR conditions were 3 min at 95°C, followed by 30 cycles of 95°C for 15 s, annealing at 65°C for 30 s, and 72°C for 1 min (Muyzer, de Waal and Uitterlinden, 1993) using a QuantStudio 3 (Thermo Fisher Scientific).

3.2.5 Amplicon analysis following antibiotic treatment

For the antibiotic treatment, kanamycin at a concentration of 0.3 $mg \cdot ml^{-1}$ and ampicillin of 0.3 $mg \cdot ml^{-1}$ were chosen. The control group received sterilised diets and water. The remaining three antibiotic groups were separated into two main groups, and four cockroaches from each group were collected on day 10 and the remaining four on day 20 of the continuing antibiotic treatment. The cockroaches were kept in Falcon tubes and stored at -20°C until DNA extraction. The genomic DNA of their guts was sequenced using the bacterial 16S rRNA gene (V3-V4 region: 515F-ill TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGANNNHNNNWNNNHGTGCCAGCMGCCGCGGTAA and 806R-ill GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011) using the Illumina sequencing.

3.2.6 Determination of bacterial communities in the cockroach guts after the antibiotic treatment using qPCR

The genomic DNA of the guts was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Each gut was represented individually. The gut was transferred into a 2 ml sterile Eppendorf tube, and a 0.5 mm stainless steel bead was added and then homogenized using a TissueLyser II (Qiagen) at 30 Hz for 1 min and the tube was flipped for another 1 min. The bacterial communities associated with their guts were determined using a quantitative Polymerase Chain Reaction (qPCR). Before doing the qPCR, the DNA samples were reassured that containing bacterial communities by using the PCR technique. The 16S rRNA gene of bacteria was performed using bacterial universal primers (341F and 534R). A total of 20 µl of PCR master mix contains 0.5 µM of each primer, 4.0 µl 5X Green buffer, 2 µl template DNA (25 ng μl^{-1}), 0.25 µl GoTaq G2 Polymerase, 0.2 mM dNTPs, and nuclease-free water. The PCR conditions were 5 min at 94°C, followed by 30 cycles of

94°C for 30 s, annealing at 64°C for 30 s and 72°C for 30 s, and 5 min at 72°C. A total of 20 µl of qPCR master mix contains 0.4 µM of each primer, 10.0 µl of Fast SYBR Green Mastermix, 2.0 µl of DNA template, and nuclease-free water. The conditions were 20 s at 95°C, 40 cycles of 1 s at 95°C, 30 s at 60°C, and a melting curve. The 16S rRNA cloned-plasmids of *Acinetobacter guillouiae* were used to form a standard curve at dilutions ranging from 10^{-1} to 10^{-5}.

3.2.7 Identification of *Blattabacterium* sp., the symbiont bacterium contaminating the genomic DNA of the cockroach guts

The bacterial symbiont in the fat body of cockroaches, *Blattabacterium* sp., resides in a bacteriocyte. However, it could become contaminated with bacterial communities in the cockroach guts during the dissection. A pair of primers was created to search for this bacterium in order to eliminate the interfering result.

The pair of primers of *Blattabacterium* sp. was designed using a partial 16S rRNA gene of *Blattabacterium* sp. from *Periplaneta americana* (American cockroach) (AM182513.1) from the GenBank database using Primer3web version 4.1.0, and the primers were then blasted in the RDP database. Using a new pair of primers, a 16S rRNA gene fragment of 173 bp was selected (qBlattaF-TCTAACAAGACTGCCGACGT and qBlattaR- TGCAGACTCCAATCCGAACT). By generating a single peak in qPCR, the primers' effectiveness was evaluated. Using the DNA gut samples described above, the primers were amplified, and the results were visualized on a 2% agarose gel electrophoresis. The PCR product was cloned and performed using One Shot TOP10 competent cells (ThermoFisher Scientific). The transformation colonies were selected by spreading on LB agar with 50 µg·ml^{-1} ampicillin and incubated overnight at 37°C. The performed colonies were randomly chosen and cultured in 5 ml of LB broth with 50 µg·ml^{-1} ampicillin overnight with a shaking incubator at 37°C and 225 rpm. The overnight culture was kept in 25% glycerol stock and kept at -80°C. The left culture was centrifuged, and the pellet was extracted using the QIAprep Spin Miniprep Kit (QIAGEN, Germany). Sanger sequencing was used to sequence the cloned fragment using M13 forward and M13 reverse primers. The sequence was confirmed against GenBank's publicly accessible data.

The 16S rRNA cloned plasmid was used as a standard curve in qPCR at dilutions from 10^{-1} to 10^{-7}. A total of 20 µl of qPCR master mix contains 0.7 µM of each primer, 10.0 µl of Fast SYBR Green Mastermix, 2.0 µl of DNA template, and nuclease-free water. The conditions were 20 s at 95°C, 40 cycles of 1 s at 95°C, 30 s at 60°C, and a melting curve.

3.2.8 Absolute abundance calculation based on qPCR and metagenome results

The relative abundances of bacterial communities from the Illumina sequencing were multiplied by the 16S rRNA gene copy number from qPCR. These were given the bacterial number in the unit of 16S rRNA gene copy number per gram of cockroach gut. These figures were performed using Phyloseq package (McMurdie and Holmes, 2013).

3.2.9 Illumina Data Analysis

A total of 22 samples were sequenced using the Illumina sequencing technique, which is an amplicon of the V3 and V4 regions of the 16S rRNA gene that were amplified. The dataset was analysed using the DADA2 (Callahan et al., 2016) in the RStudio programme (Allaire, 2012). The pipeline starts with paired-end fastq files from each sample that were deposited into DADA2 on RStudio programme and their primers were removed separately using Cutadapt (Martin, 2011). Those sequences that showed a quality value of less than 30 were eliminated. Filtering quality and denoising were applied. Chimeras were eliminated using BimeraDenovo after merging both paired ends, and the remaining sequences were classified taxonomically throughout the Silva reference database (Quast et al., 2012). Sequences belonging to unclassified phyla and identified as Archaea, Eukaryota, Chloroplasts, Mitochondria, and *Blattabacterium* (a cockroach endosymbiont) were removed from the study. The amplicon sequence variant (ASV) (Callahan, McMurdie and Holmes, 2017) abundance table was used to group the remaining samples. Finally, rarefy was used to normalise the samples without replacing any of them, based on 90 percent of the minimum sample depth.

3.2.10 Statistical analysis

All analyses were performed in RStudio on R Statistical Software version 4.4.0 (Allaire, 2012). Alpha diversity was measured using Shannon (Shannon, 1948) and Chao1 (Chao, 1984) indices. A statistical test of diversity indices was analysed using Kruskal-Wallis test between treatments and Dunn test for pairwise comparisons between time points in each treatment and visualized using microeco package (v1.7.1) (Liu et al., 2021b). The composition of microbial communities was processed using MicrobiotaProcess package (v1.16.0) (Xu et al., 2023) and visualized using ggplot2 (Wickham, 2016). Beta diversity analysis was performed using a distance method by Non-metric Multidimensional Scaling (NMDS) by phyloseq package (McMurdie and Holmes, 2013) and a bacterial composition through principal coordinate analysis (PCoA) based on Bray-Curtis distances by MicrobiotaProcess package. Permutational multivariate analysis of variance (PERMANOVA) measured the significant differences between antibiotic treatments and time points using adonis function in vegan package

(v2.6.6.1) (Oksanen et al., 2018). The differences of all bacterial taxonomic levels were analysed using linear discriminate analysis (LDA) effect size (LEfSe) methods with a cut-off log LDA score of 4 ($P < 0.05$) using the microeco package. The differences between antibiotic treatments were tested using the Kruskal-Wallis rank sum test ($P < 0.05$).

3.3 Results

3.3.1 Pilot test: Bacterial communities' determination after antibiotic treatment using qPCR in the cockroach guts

The bacterial communities associated with the cockroach guts were not significantly affected by 0.1, 0.2, and 0.3 mg·ml^{-1} of kanamycin by collecting samples on day 4 and 5. The bacterial abundances in antibiotic treatments were higher than in the control group. The conclusion drawn from the results is that longer-term antibiotic feeding should be taken into consideration. As shown on the graph (Figure 9), there were only two samples on day 4 and day 5 of 0.3 mg·ml^{-1} of Kanamycin. Although the results indicate that the bacterial populations were reduced, these samples were insufficient to draw firm conclusions. As a result, the subsequent experiment with extended feeding days would include kanamycin at a concentration of 0.3 mg·ml^{-1}.

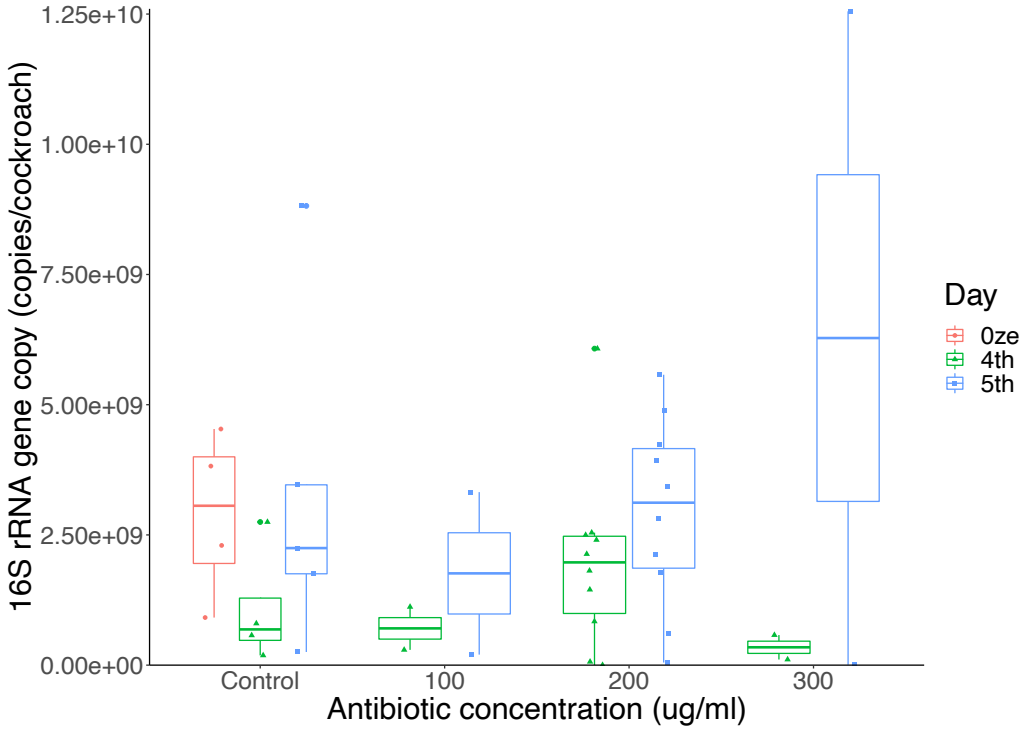


Figure 9 Quantitative PCR (qPCR) analysis shows bacterial communities' copies in cockroach guts. There are four treatments, including a control group with sterilized water and kanamycin-treated at concentrations of 0.1, 0.2, and 0.3 mg·ml^{-1} in drinking water.

3.3.2 Identification of *Blattabacterium* sp., the endosymbiont bacterium in a cockroach

To determine the *Blattabacterium* sp., the endosymbiont bacterium living in a bacteriocyte in a fat body of cockroaches. It is undeniable that the *Blattabacterium* sp. can be contaminated with gut tissue during the cockroaches' dissection (Kakumanu et al., 2018). However, the qPCR method would

be valuable for quantifying them in comparison to other bacteria. The *Blattabacterium* sp., however, was found in less than 1% of the total of other bacteria in the cockroach guts in our study (Figure 10). The endosymbiont contamination outside the intestines may be removed by repeatedly rinsing the whole cockroach guts in sterile PBS.

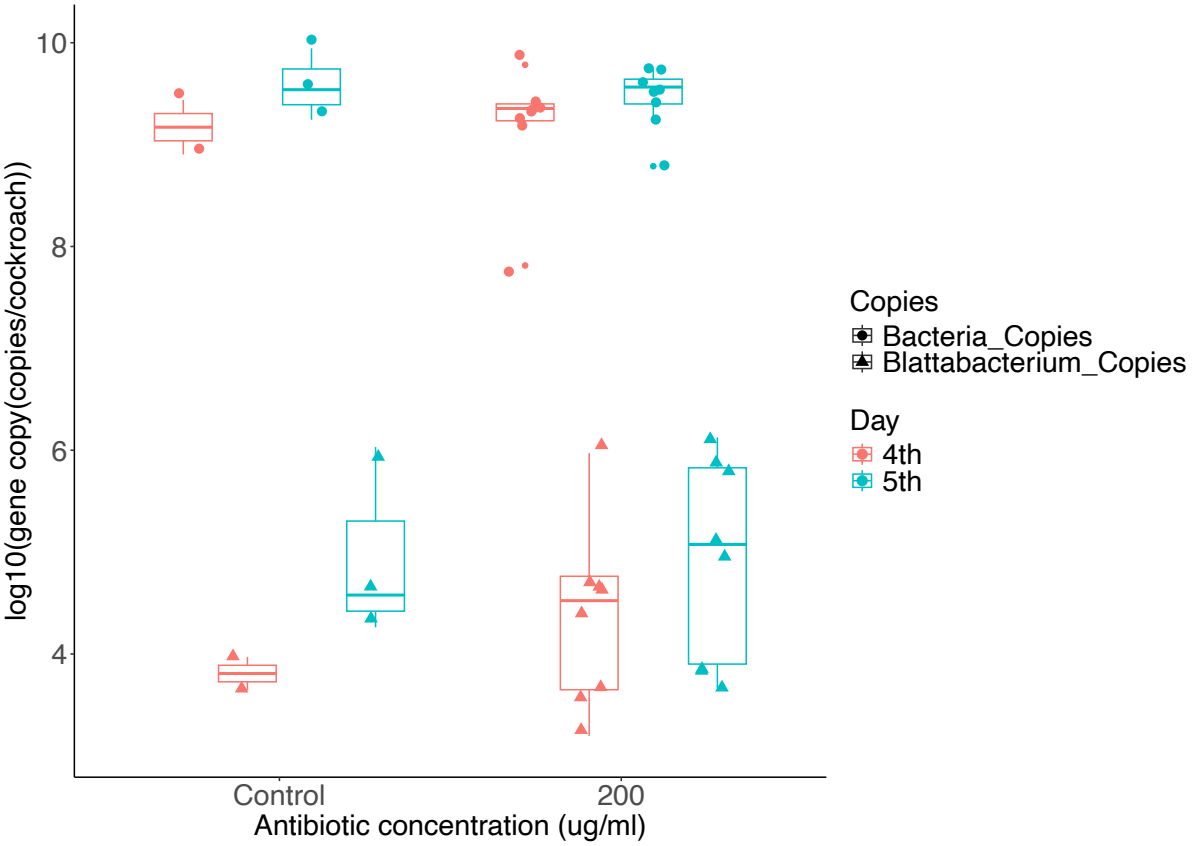


Figure 10 Quantitative PCR (qPCR) analysis indicates bacterial communities compared with Blattabacterium in cockroach guts. There are two treatments, including a control group with sterilized water and diet, and 0.2 mg·ml^{-1} of Kanamycin group.

3.3.3 Bacterial determination in the cockroach guts after the antibiotic treatment using qPCR

The bacterial communities associated with the cockroach guts were not significantly affected by 0.1, 0.2, and 0.3 mg·ml^{-1} of kanamycin by collecting samples on day 4 and 5 during the previous pilot experiment. The conclusion drawn from the results is that longer-term antibiotic feeding should be taken into consideration. The subsequent experiment with extended feeding days included kanamycin (0.3 mg·ml^{-1}) and ampicillin (0.2 and 0.3 mg·ml^{-1}). As shown on the graph (Figure 11), the results indicate that the bacterial populations were reduced, especially on day 20 in kanamycin groups. Inversely, there were higher bacterial copies on day 20 than on day 10 of ampicillin at a concentration of 0.3 mg·ml^{-1} with a variation within the treatment.

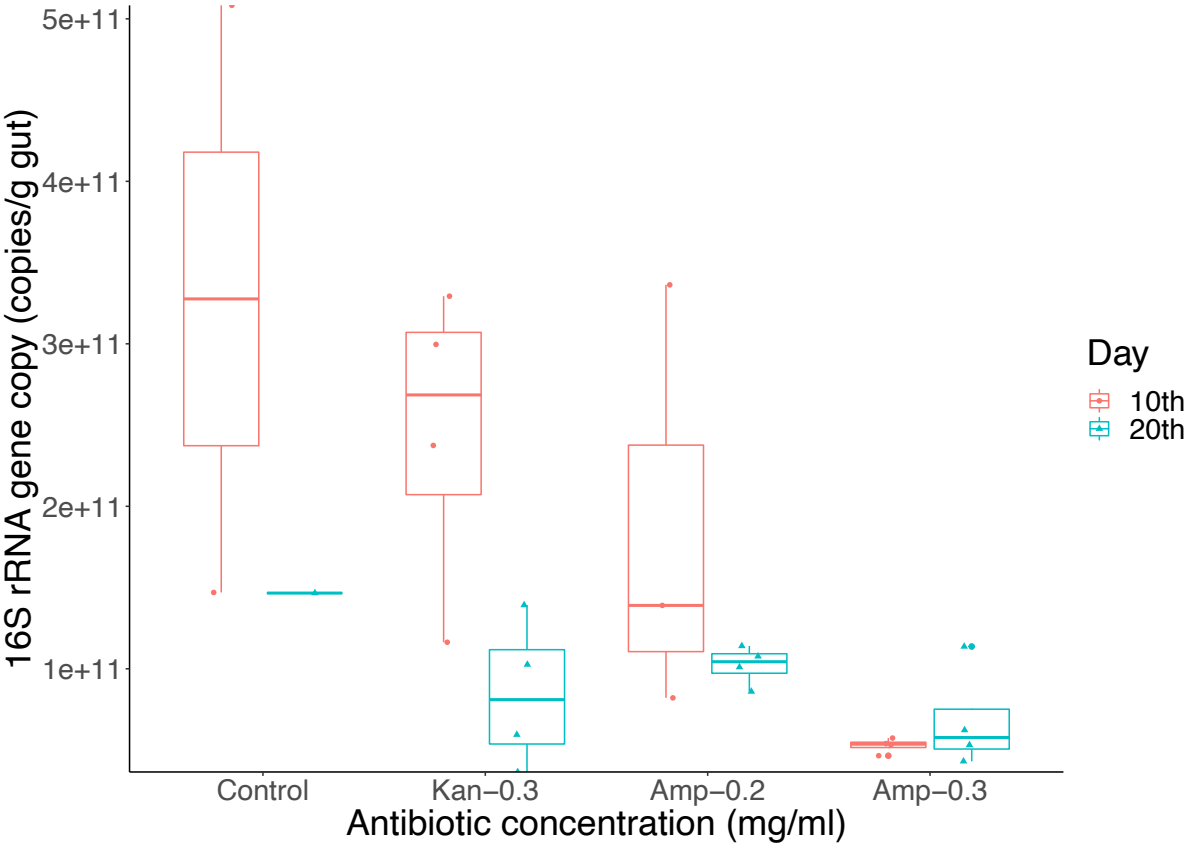


Figure 11 Quantitative PCR (qPCR) analysis shows bacterial communities in cockroach guts. There are four treatments, including a control group with sterilized water and 0.2 mg·ml^{-1} and 0.3 mg·ml^{-1} of ampicillin and 0.3 mg·ml^{-1} of kanamycin.

3.3.4 Bacterial determination after antibiotic treatment using Illumina sequencing

The antibiotic treatment effect on the reduction of bacterial diversity. Sequences ranged from 472,623 to 1,015,256 sequences, with a mean of 818,840.8 sequences. A total of 6,500 ASVs were grouped using DADA2 in the R programme to determine the bacterial communities associated with the gut of cockroaches treated with antibiotics. The bacterial diversities of the cockroach gut were determined using the Shannon and Chao1 indices (Figure 12). Both Shannon and Chao1 diversities showed no significant difference between time points (day 10 and day 20) within the same treatment. To consider between control and treated antibiotics, the bacterial diversity in the guts of cockroaches treated with antibiotics reduced; both indices showed that the antibiotics could eliminate the bacterial communities in the cockroach guts, particularly the ampicillin-treated group. Moreover, the bacterial diversity in the antibiotic-treated group was lower on day 20 than it was on day 10 (Figure

12). However, to identify the shift in the taxonomic levels of the bacterial populations, we compared the bacterial copies between the metagenomic and qPCR results.

However, according to the meta-analysis study in Chapter 2, there are approximately 62,000 ASVs among mice, cockroaches, and humans. About 34,000 ASVs of cockroaches are included in those numbers, which is considerably greater than this chapter. Approximately 6,500 ASVs were found in the bacterial communities in our recent study. There is only one species of cockroach (the American cockroach) in the recent study, while there are three main samples that include several publicly available data sets separated into 19 different cockroach species, as well as different data sets from mice and humans. More diversified species can produce more ASVs since each organism harbors distinct microorganisms.

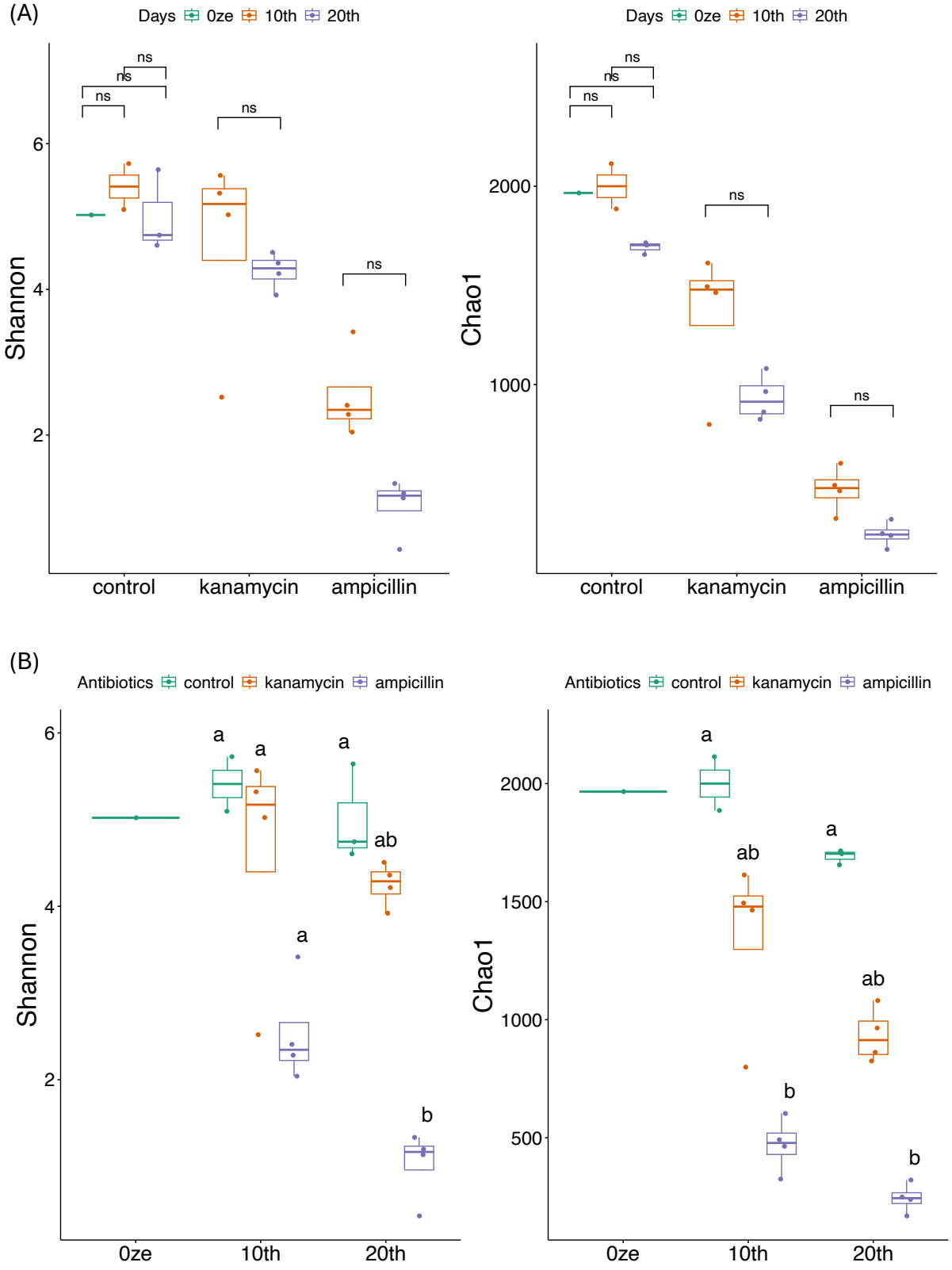

(A)
Days 0ze 10th 20th
Shannon
Chao1
ns
control
kanamycin
ampicillin
(B)
Antibiotics control kanamycin ampicillin
a
ab
b
0ze
10th
20th

Figure 12 Alpha diversity of bacterial communities associated with cockroach gut samples treated with or without antibiotics at day 0, 10, and 20. Shannon and Chao1 indices are shown in a box plot. The horizontal bars indicate the median for each box, while the hinges represent the lower and the higher quartiles. (A) The Wilcoxon test was tested between time-point within treatment (ns indicated a P value > 0.05). (B) The Kruskal Wallis test was tested across the treatments (control, kanamycin, and ampicillin) and the Dunn's test within the Kruskal Wallis was tested for paired test (a, P > 0.05; ab, P ≤ 0.05; c, P ≤ 0.01).

It is worth noting that the result from qPCR illustrated that kanamycin had an impact on the bacterial communities that it could eliminate some of them. However, the bacterial phyla prevalence in kanamycin-treated and control groups was similar (Figure 13). Their most frequent bacterial phyla were *Firmicutes* (control, ranging from 42.9-74.1%; kanamycin, ranging from 39.0-94.0%); *Bacteroidota* (control, ranging from 8.8-39.8%; kanamycin, ranging from 12.6-46.5%); and *Desulfobacteria* (control, ranging from 4.3-9.0%; kanamycin, ranging from 0.3-14.4%), respectively. Adversely, the microbiomes were different between ampicillin-treated and control groups together with kanamycin-treated groups. The ampicillin-treated group was dominated by phylum *Proteobacteria* (ranging from 23.1-97.7%) and *Firmicutes* (ranging from 8.4-19.7%). In addition, the phylum *Actinobacteria* was more frequently detected on day 10 (ranging from 1.2-7.0%) but they were almost unnoticeable on day 20 (ranging from 0.2-0.4%) of ampicillin treatment. However, *Desulfobacteria* were still detected in less abundance in some samples of kanamycin.

At the genus level, the bacterial genera have changed from day 10 to day 20, especially in the ampicillin group. The predominant genera were *Paralactobacillus* (control, ranging from 1.3-26.7%; kanamycin, ranging from 2.2-58.6%) and *Christensenellaceae R-7 group* (control, ranging from 4.9-13.0%; kanamycin, ranging from 5.6-20.4%) in the control and kanamycin microbiota. Those were not detected in the ampicillin-treated group in which *Klebsiella* (day 10, ranging from 4.2-49.8%; day 20, ranging from 69.1-93.3%) was found predominantly. Moreover, unclassified genera in *Neisseriaceae* showed high abundance across ampicillin-treated samples on day 10, but they were decreased on day 20. Additionally, *Termite planctomycete cluster* was also high (2/4 samples) on day 10.

The NMDS confirmed that the bacterial communities between control and kanamycin were clustered closely together (Figure 14A). The bacterial populations in ampicillin were distinct from the other two treatments. Similarly, the PCoA plot indicated the differences in bacterial communities were clustered separately across treatments (Figure 14B). PERMANOVA test confirmed that antibiotics had effects on bacterial communities (R = 0.517, *P* = 1e-04). Principal coordinate 1, which explains 40.79% of the dissimilarity, has the largest effect and is where ampicillin can be clearly distinguished from kanamycin and control. The impact sizes of coordinates 2 (12.99%) and 3 (8.87%) that separate kanamycin from control are much smaller.

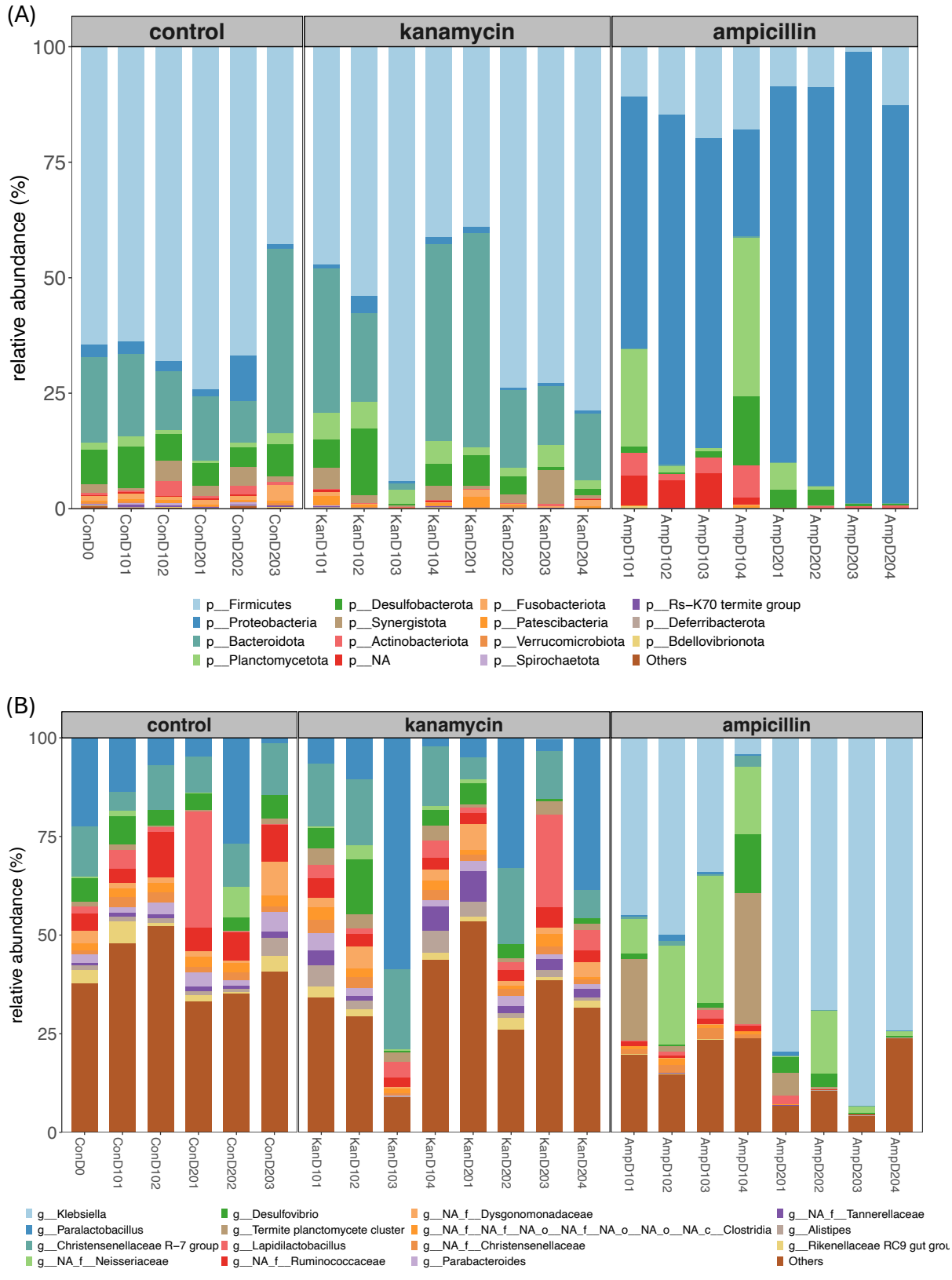


Figure 13 Relative abundance of bacterial communities associated with the cockroach guts treated with kanamycin, ampicillin, and control. (A) A total of 15 of the most bacterial phyla are shown and (B) the most abundant bacterial genera are shown. The numbers 10 and 20 were written after the names of the antibiotic-treated were indicated on day 10 and 20, respectively.

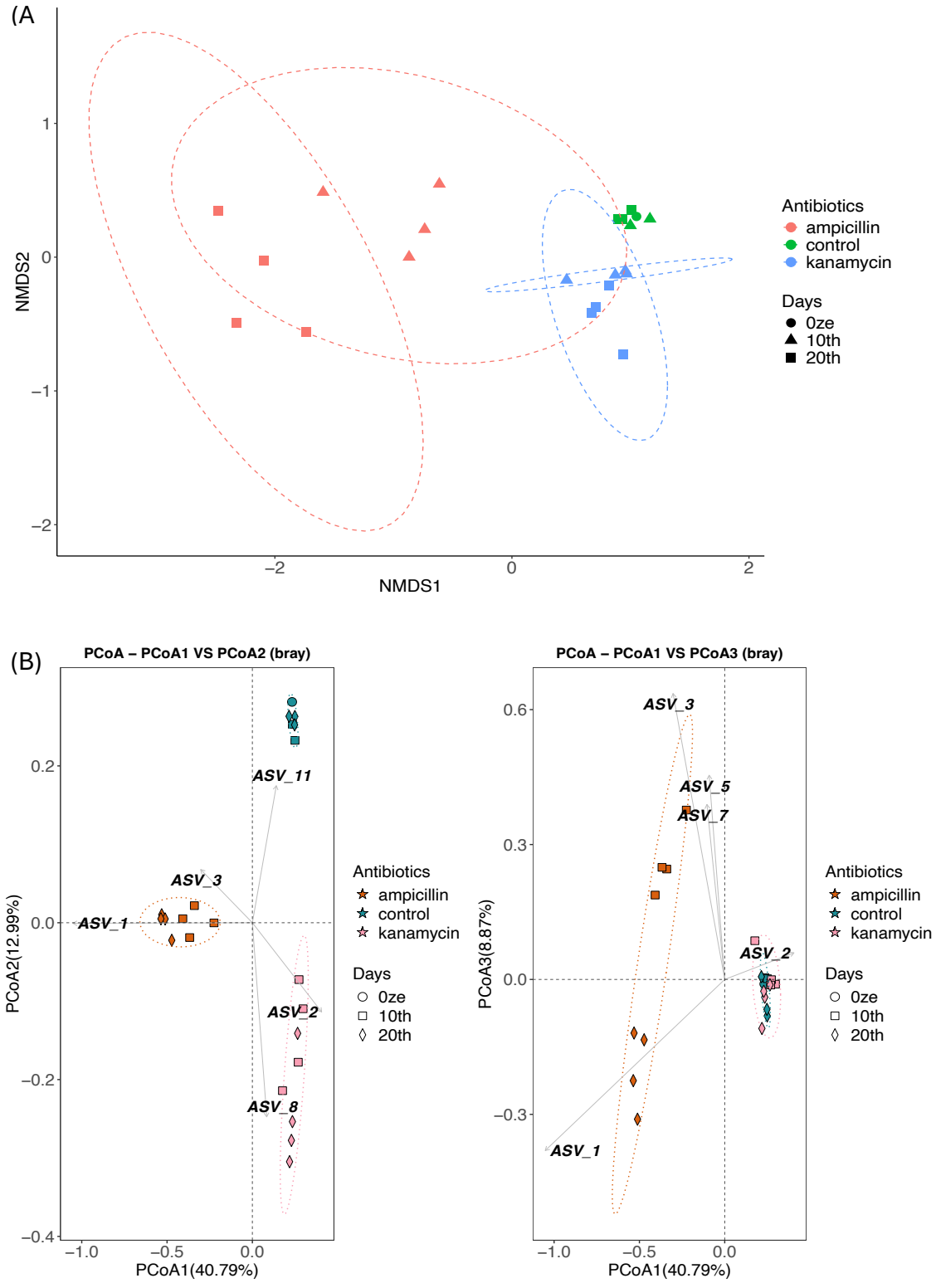
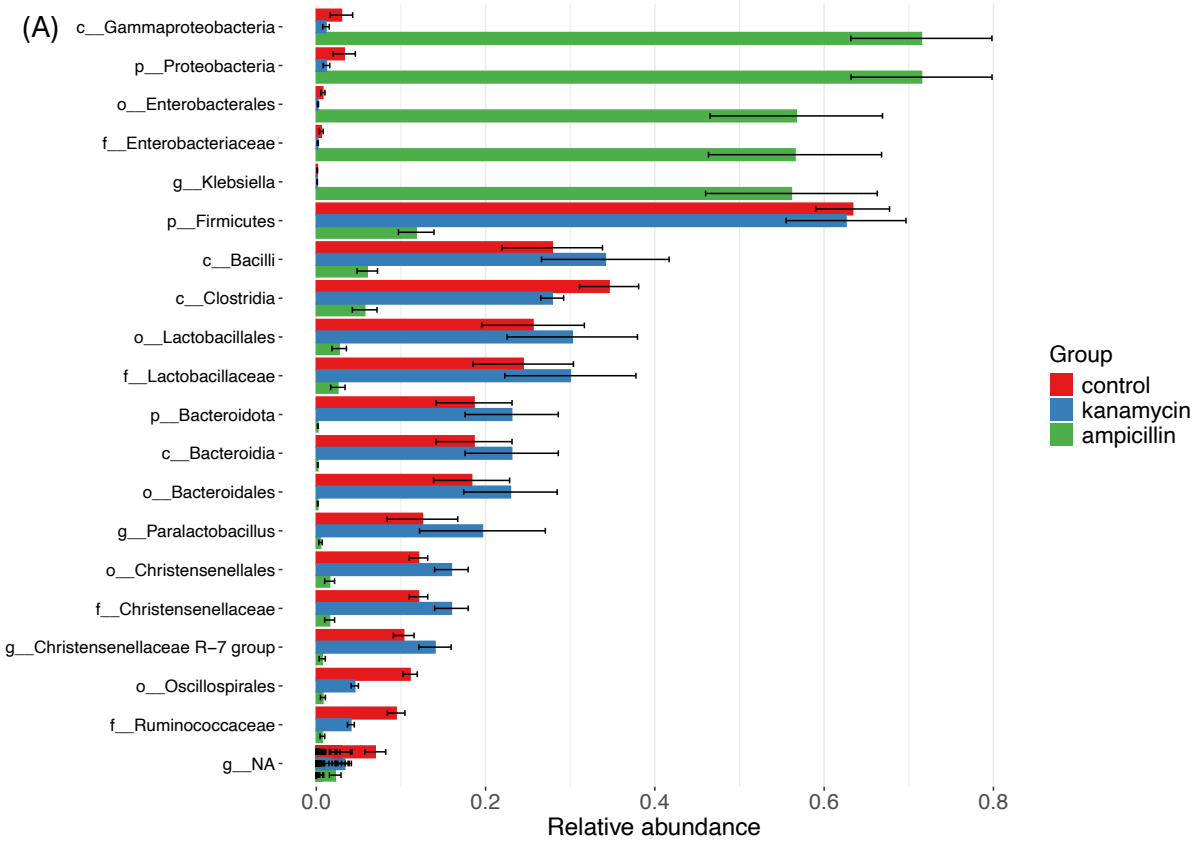


Figure 14 (A) Non-metric multidimensional scaling (NMDS) plot illustrates bacterial communities associated with the cockroaches (control, ampicillin, and kanamycin groups). (B) PCoA analysis based on Bray-Curtis distances that different treatments and days were coloured and shaped differently, respectively.

3.3.5 LDA effect size analysis (LEfSe)

The enriched bacterial taxa were analysed using linear discriminant analysis (LDA) effect size (LEfSe). The 10 most distinct bacterial taxa across antibiotic treatments are shown in bar charts (Figure 15). There were more bacteria in the ampicillin group belonging to the *Proteobacteria* (i.e. o_*Enterobacterales*, f_*Enterobacteriaceae*, and g_*Klebsiella*) were high in the ampicillin group. Although the bacterial taxa were more similar between the control and kanamycin groups, some distinct taxa were also present, such as p_*Firmicutes*, c_*Clostridia*, g_*paralactobacillus* were the three most enriched taxa in the control group. Several bacterial taxa were predominant in the kanamycin group, such as c_*Bacilli*, p_*Bacteroidota* (c_*Bacteroidia*, o_*Bacteroidales*, f_*Dysgonomonadaceae*), o_*Lactobacillales* (f_*Lactobacillaceae*), and some taxa of o_*Christensenellales*.



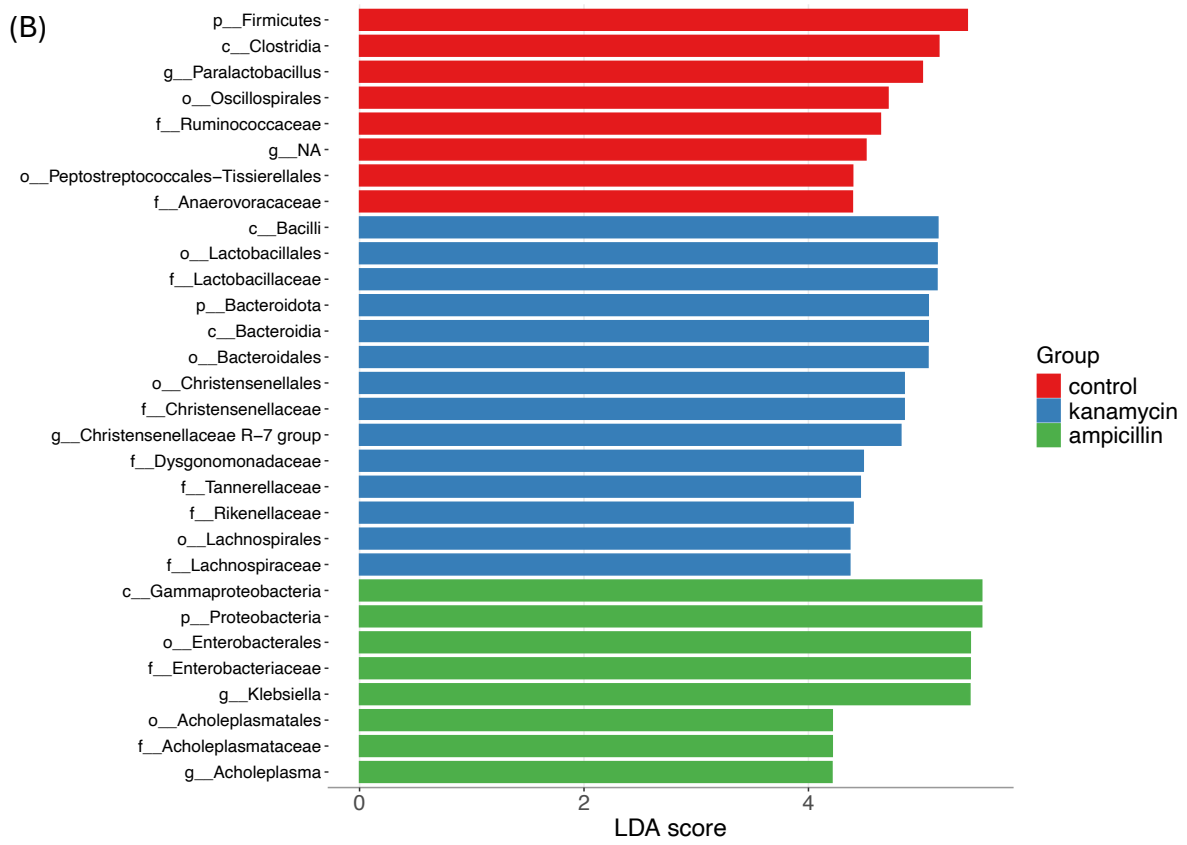
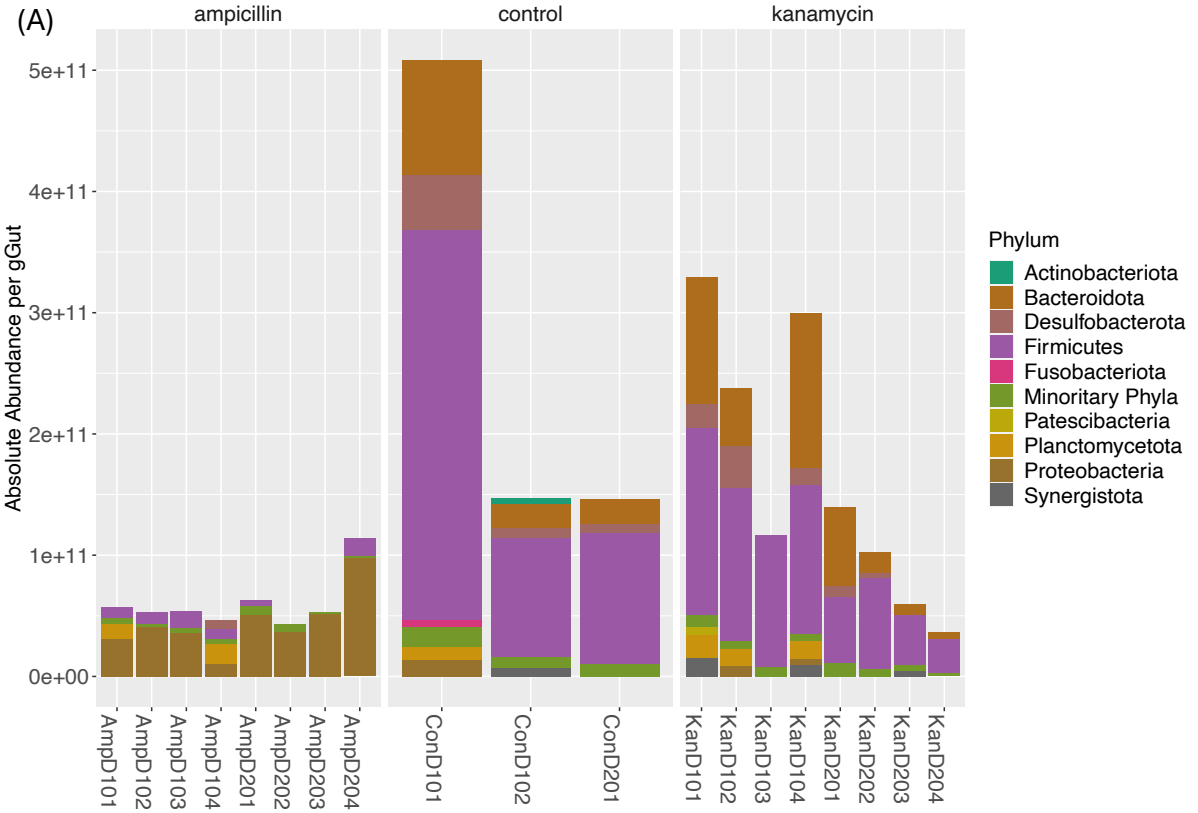


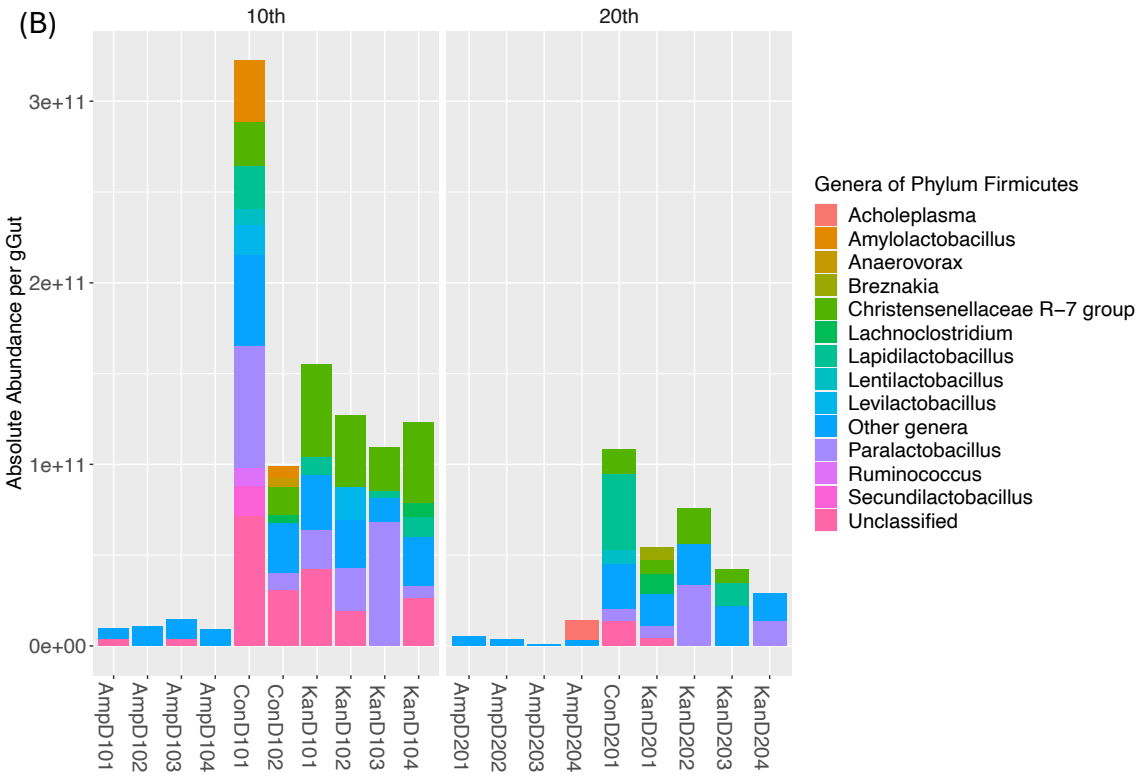
Figure 15 The differences of all bacterial taxonomic levels were analyzed using Linear Discriminant Analysis (LDA) effect size (LEfSe) methods. (A) The 20 most different taxa of bacterial populations show a relative abundance. (B) The enriched bacterial taxa show in LDA score with a greater value of 4 (Kruskal-Wallis test; P < 0.05).

3.3.6 Absolute abundance calculation based on qPCR and metagenome results

The absolute abundance of bacteria was estimated from the 16S rRNA gene copies using qPCR compared with metagenomic sequencing. This result indicated the actual number of bacteria that were eliminated by antibiotic treatment. Particularly, *Firmicutes* and *Bacteroidota* phyla are the predominant phyla in the control group, and this finding is obviously the same in the group that received kanamycin (Figure 16). The *Bacteroidota* were higher at the beginning of treatment with kanamycin (on day 10) than in the control group at the same period of time. Inversely, this phylum decreased on day 20 in the kanamycin-treated group. It is interesting to observe that *Proteobacteria* were more prevalent in the ampicillin-treated group on day 10 and 20 instead of a high abundance of *Firmicutes* as in the kanamycin and control groups. In the phylum *Firmicute* at the genus level, the genera of *Christensenellaceae R-7 group* and *Paralactobacillus* presented in similar abundance in both groups. However, there were fewer on day 20 of the kanamycin treatment group than on day 10.

Bacteria from the phylum *Bacteroidota* are present in one sample from the control group that has a high number of bacterial copies, around three times as many as the other two control samples. It had higher amounts of *Alistipes*, *Parabacteroides*, and *Rikenellaceae* than the other two control samples. These genera were also found in the kanamycin-treated group on day 10. However, these genera disappeared on day 20, except for only one sample that is still present but in a smaller number.

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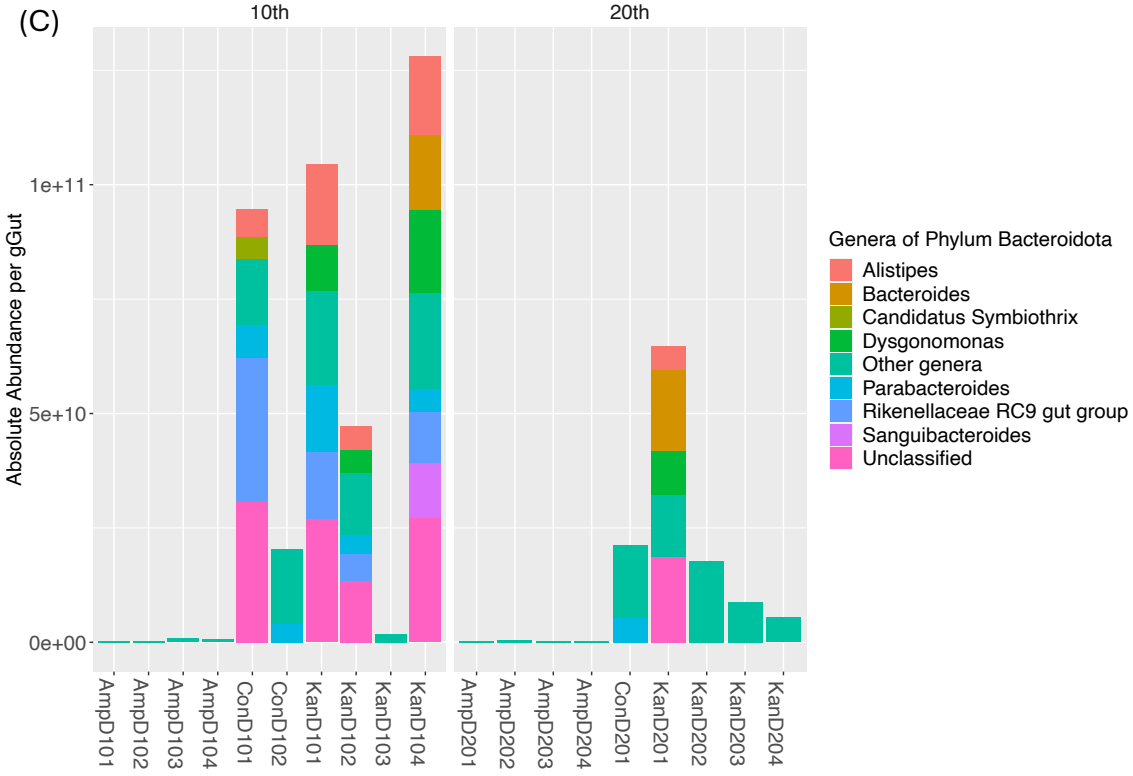
KanD204
Genera of Phylum Firmicutes
Acholeplasma
Amylolactobacillus
Anaerovorax
Breznakia
Christensenellaceae R−7 group
Lachnoclostridium
Lapidilactobacillus
Lentilactobacillus
Levilactobacillus
Other genera
Paralactobacillus
Ruminococcus
Secundilactobacillus
Unclassified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KanD204
Genera of Phylum Bacteroidota
Alistipes
Bacteroides
Candidatus Symbiothrix
Dysgonomonas
Other genera
Parabacteroides
Rikenellaceae RC9 gut group
Sanguibacteroides
Unclassified

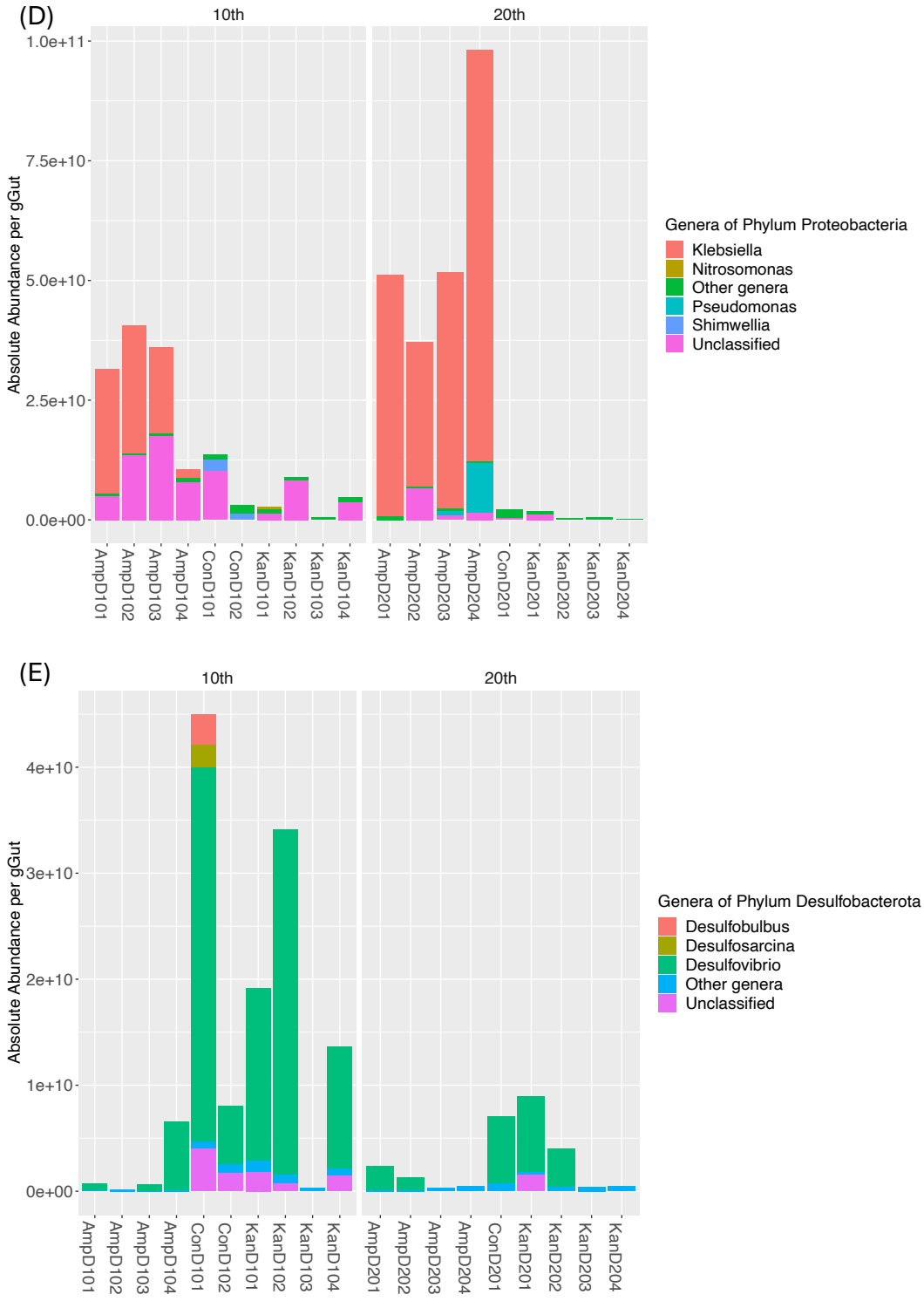


Figure 16 Absolute abundance of bacterial communities' effects from antibiotic treatments. The number of bacterial copies ((A): all phyla, (B): genera of Firmicutes, (C): genera of Bacteroidota, (D): genera of Proteobacteria, (E): genera of Desulfobacterota) was calculated based on the relative abundance of the 16S rRNA gene of the bacterial communities by Illumina sequencing.

3.4 Discussion

To overcome the colonisation resistance in the guts of the American cockroach, the cockroach gut microbiome was treated with two different antibiotics: kanamycin and ampicillin. The bacterial communities were determined using the 16S rRNA gene of bacteria by qPCR and Illumina sequencing. Our pilot study showed that, when administered for a short time (4-5 days), the antibiotic was unable to reduce the gut microbiome at concentrations of 0.1, 0.2, and 0.3 $mg \cdot ml^{-1}$ of kanamycin. However, we treated the cockroach with a concentration of 0.3 $mg \cdot ml^{-1}$ for a longer period of 10 and 20 days. The same antibiotic concentration over a longer length of time caused the disruption of the gut microbiota. The effects of antibiotics showed alteration of the gut microbiome in the cockroach.

The cockroach gut microbiome was altered by both antibiotics, particularly ampicillin. It is worth mentioning that after the cockroaches had been treated with antibiotics for both 10 and 20 days, their gut microbiome was obviously decreased in both antibiotic groups on day 20. On the other hand, the number of *Firmicutes*, *Bacteroidota*, and *Desulfobacterota* was increased on day 10 after treating with kanamycin. However, these bacteria decreased after treatment continued until day 20.

When comparing treatments with ampicillin and kanamycin, ampicillin was more effective than kanamycin in eliminating more diverse bacterial communities in the cockroach gut, as shown in bacterial copies and bacterial diversities from qPCR and Illumina sequencing, respectively. Interestingly, similar microbial communities between treated kanamycin and control were indicated. It showed that kanamycin could reduce the number of bacteria in the intestinal organs of the cockroach, but the bacterial communities were still similar albeit with less abundance than the control (*Firmicutes*, *Bacteroidota*, and *Desulfobacterota*, respectively).

Kanamycin had a significant effect on the *Desulfovibrio* in the German cockroach after continuing treatment for two generations that this bacterium was completely eradicated in the second generation (Domínguez-Santos et al., 2021). In contrast, 75% of cockroaches (3/4 samples) in our study showed proliferation of *Desulfovibrio* after being treated with kanamycin for 10 days but eventually decreased at day 20. Overall, kanamycin is more effective against *Desulfovibrio* when used over a longer period.

Desulfovibrio are found within the human gut microbiota both healthy and unhealthy (Chen et al., 2021a). According to the majority of research, this bacterium is a sulfate-reducing bacterium that frequently causes inflammatory gastrointestinal diseases in humans (Loubinoux et al., 2002a). Moreover, several studies discovered higher abundance in some diseases such as autism, Parkinson's

disease, and cancer (Murros et al., 2021; Singh, Carroll-Portillo and Lin, 2023). However, its actual roles in human diseases need further study.

Bacteroides increased after treating with kanamycin for two generations (Domínguez-Santos et al., 2021). According to our study in kanamycin treatment, some bacterial genera on *Bacteroidota* showed an increase at day 10, but most of them showed less abundance by day 20. *Bacteroides* is one of our commensal gut microbiomes; however, they can be opportunistic pathogens depending on their habitant organs (Zafar and Saier, 2021). *Firmicutes* was significantly altered by kanamycin in the German cockroach (Wolfe and Scharf, 2021). However, they were lower on day 20 than they were on day 10 in our study. The other study found that some bacteria, including *Bacteroides, Dysgonomonas, Alistipes, Lachnospiraceae,* and *Desulfovibrio*, contain antibiotic resistance genes (ARGs) to aminoglycosides, kanamycin studied in the German cockroach (Creus-Martí et al., 2023). This may be why, on day 10, we were able to detect some more bacteria from the kanamycin group.

Interestingly, ampicillin had more effectiveness in decreasing the gut microbiome after treatment continued for 10 days until day 20. Both Shannon and Chao1 indices confirmed the reduction of bacterial diversities after treatment continued for 10 days. As a broad-spectrum antibiotic, ampicillin was able to eliminate the majority of bacterial phyla in our study. Specifically, after day 10 of ampicillin treatment, *Firmicutes*, *Bacteroidota*, and *Desulfobacterota* almost vanished. On the other hand, we detected the resistance of *Proteobacteria* in ampicillin-treated with the longer period treatment. The genus *Klebsiella* in *Proteobacteria* was increased at day 10 and peaked at day 20 by comparing bacterial copies from the qPCR result with the relative abundance of total bacteria from Illumina sequencing. Antibiotic resistance has an impact on the host gut microbiome. Some bacteria that harbour antibiotic-resistant genes can outgrow after the elimination of those antibiotic-susceptible bacteria (Dey and Ray Chaudhuri, 2023).

Klebsiella is one of the indigenous microorganisms in the gastrointestinal tract of healthy humans (Lin et al., 2012). This bacterium is harmless in the human gut microbiome (Lin et al., 2012). However, *Klebsiella pneumoniae* is also one of the pathogenic bacteria in infectious diseases in humans (Paczosa and Mecsas, 2016). This bacterium frequently causes infection in the respiratory tract (Kaur, Vadivelu and Chandramathi, 2018). Moreover, it showed association with diseases of the lower gastrointestinal tract, including Crohn's disease, ulcerative colitis, colorectal cancer, and pyogenic liver abscess (Kaur, Vadivelu and Chandramathi, 2018).

Ampicillin is destroyed by the bacterial enzyme AmpC β-lactamase. This enzyme is encoded in several *Enterobacteriaceae* (Jacoby, 2009; Navon-Venezia, Kondratyeva and Carattoli, 2017). This leads to resistance to β-lactam antibiotics by some bacteria containing the enzyme. Numerous resistance

genes, including β-lactam, aminoglycoside, quinolone, polymyxin, tigecycline, and fosfomycin, are mostly present in *K. pneumoniae* (Li et al., 2023b). This has led this bacterium to become one of the most challenging infectious bacteria in the most common hospital outbreak diseases (Chung The et al., 2015; Navon-Venezia, Kondratyeva and Carattoli, 2017).

Moreover, Neisseriaceae and *Termite planctomycete cluster* in *Pirellulaceae* were high in some cockroaches on day 10 of ampicillin but decreased on day 20. Both commensal and pathogenic *Neisseria gonorrhoeae*, which cause sexually transmitted infections in humans, are resistant to several antibiotics, particularly penicillin-binding protein 2 (PBP2) (Goytia et al., 2021).

While *Paralactobacillus, Christensenellaceae R-7 group, Desulfovibrio, Lapidilactobacillus,* and *Rikenellaceae* RC9 gut group were predominant bacteria in both control and kanamycin treatments, these bacteria were almost undetected in ampicillin treatment. These bacteria are susceptible to ampicillin in our study. As a result, *Klebsiella* and some genera in *Neisseriaceae* increased in the cockroach guts.

A low concentration of antibiotic usages, including kanamycin (Domínguez-Santos et al., 2021) and ampicillin (Domínguez-Santos et al., 2020) are unable to affect the endosymbiont *Blattabacterium* within the bacteriocyte in the cockroach's fatty body. This suggests that any variation seen may not be the result of the endosymbiont being disrupted. However, insect physiology may suffer if their commensal bacteria are disrupted, which could lead to reduced fitness or even mortality. For example, the antibiotic treatment decreased the growth rate and reproduction capacity of German cockroaches (Liles, 1958; Pietri, Tiffany and Liang, 2018). These effects were worse when the cockroaches were continuously treated with antibiotics in the second generation, which showed higher mortality with only a low concentration of antibiotics than their first generation treated with the same concentration (Liles, 1958). This needs to be managed cautiously using antibiotics to reduce the number of bacteria in insects. It would be worthwhile to consider conducting a pilot study with varying antibiotic concentrations.

The efficiency of antibiotics depends on either the period of exposure or the concentrations of them. Our study discovered that antibiotics could successfully eradicate some gut microbiota in the cockroach. The different antibiotics illustrate their specific effects on altering different bacterial communities. Therefore, chosen antibiotics depend on bacterial taxa that they have impacts on. Both kanamycin and ampicillin would help us gain a better understanding of bacterial interactions involved in commensal bacterium inoculation for our next chapter.

Chapter 4: The inoculation of the commensal bacterium *Escherichia coli* into the cockroach guts

4.1 Introduction

The phrase "colonization resistance" refers to the ability of native microorganisms to cope with the introduction of new microorganisms into their natural environments (Van der Waaij and de Vries-Hospers, 1986). This competition phenomenon has benefits for the host. It can prevent disease and is beneficial for host health (Adlerberth, Marina Cerquetti, Isabe, 2000).

Antibiotic therapy is one effective strategy for dealing with colonization resistance. However, not all microbes can be eradicated by antibiotics (Ramirez et al., 2020). Some bacteria contain antibiotic resistance that can tolerate particular antibiotics. Antibiotics in particular have the potential to eradicate sensitive bacteria, leading to the overgrowth of resistant bacteria (Ebner, Kropec-Hübner and Daschner, 2000). However, using antibiotics also reduces the cost and duration of evaluation when compared to germ-free insect culture.

The human gastrointestinal (GI) tract contains the oral cavity, esophagus, stomach, small intestine, and colon (Ruan et al., 2020). Comparing different adult healthy human body habitats, it is seen that the gut exhibits a high density of bacterial populations (The Human Microbiome Project Consortium, 2012). The three main gut compartments of cockroaches are the foregut, midgut (anterior and posterior), and hindgut (colon and rectum) (Schauer, Thompson and Brune, 2012; Jahnes et al., 2021). There are high numbers and a more diverse microbial community in the hindgut in the cockroaches across several studies using different approaches, such as culture-dependent methods (Bignell, 1977), scanning and transmission electron microscopy (Bracke, Cruden and Markovetz, 1979), and 16S rRNA sequencing (Schauer, Thompson and Brune, 2012; Bauer et al., 2015). Diverse microbial communities are present in the American cockroach (*Periplaneta americana*) (Chapter 2). In particular, *Firmicutes*, *Bacteroidota*, *Desulfobacterota*, and *Proteobacterota* are the four most prevalent bacterial phyla. These bacterial phyla share similarities to the prevalent bacteria found in the human gut microbiome (*Firmicute*, *Bacteroidota*, and *Proteobacterota*) (Chapter 2 and 3).

Escherichia coli (*E. coli*) is a facultative anaerobe in commensal bacteria in humans (Conway and Cohen, 2015a) and in various vertebrates (Foster-Nyarko and Pallen, 2022) that has been known as a harmless bacterium, including in mammals such as cows (Massot et al., 2017) and pigs (Herrero-Fresno, Larsen and Olsen, 2015). *E. coli* is one of the ordinary bacterial genera that colonises the intestines of neonates, along with *Bifidobacterium, Clostridium, Bacteroides,* and *Staphylococcus*

(Mancabelli et al., 2020). Additionally, infants subsequently receive additional microbial communities through their surroundings and foods (Mackie, Sghir and Gaskins, 1999). However, when there is an imbalance in the intestinal flora, this might be harmful bacteria (Govindarajan et al., 2020). Moreover, some strains of *E. coli* are classified as harmful bacteria, which are typically found in an environment, including soil and water (Van Elsas et al., 2011b). Some *E. coli* strains are found to be obviously resilient in the human gut and have an impact on human health (Gao, Zhao and Huang, 2014). *E. coli* O157:H7 is a pathogenic bacterium that can produce Shiga toxins. This particular type of bacteria is commonly detected in cattle, and humans may contact it through the consumption of contaminated food (Lim, Yoon and Hovde, 2010). Diarrhea in humans can be caused by this; some patients can recover without treatment, but other patients may develop serious illnesses (Su and Brandt, 1995). Conversely, *E. coli* strain Nissle 1917 frequently produces antimicrobial peptides against pathogenic bacteria, such as *Salmonella* spp., and other *E. coli* strains (Trebichavsky et al., 2010). The study of the response of resident non-pathogenic *E. coli* to pathogens found that some residents could prevent the colonization of pathogens (Richter et al., 2018). *E. coli* MG1655 has a complete genome, and this strain has been commonly used as a laboratory strain (Blattner et al., 1997). Several studies have successfully colonized laboratory mice with this strain (Leatham et al., 2009; Leatham-Jensen et al., 2012).

E. coli is a Gram-negative bacterium that cell wall consists of a thin peptidoglycan layer, an inner plasma membrane, a periplasmic space, and lipopolysaccharides as the main component of their outer membrane (Silhavy, Kahne and Walker, 2010). Spheroplasts describe Gram-negative bacteria that still have an outer membrane but have lost their peptidoglycan layer (Smith, 1969). This happens when *E. coli* undergoes lysis following ampicillin treatment and some of them transform into spheroplasts (Greenwood and O'Grady, 1969). Kanamycin, an aminoglycoside antibiotic, inhibits translation by blocking protein elongation by binding to the 30S ribosomal subunit's 16S rRNA of *E. coli* (Semenkov et al., 1982). The impact of antibiotics on inoculated *E. coli*, however, should be cautiously considered.

From Chapter 3, we were successful in reducing bacterial communities from the intestinal organ of the American cockroach by using kanamycin and ampicillin. The results showed that after 10 or 20 days of treatment, both antibiotics could reduce the cockroach's gut microbiota. The purpose of this study was to establish a human gut microbiome model by inoculating the cockroach's gut with the commensal *E. coli* MG1655. To increase the possibility of success, antibiotic treatments were chosen to overcome the colonization-resistant phenomenon in the host. The female adult American cockroaches were treated with either kanamycin or ampicillin for 10 days before continuously

inoculating them with *E. coli* for 3 days. After the inoculation, the cockroaches were collected on days 1, 3, 7, and 14. The bacterial communities in the cockroach gut were determined using the 16S rRNA gene by Illumina sequencing.

4.2 Methods

4.2.1 Cockroach husbandry

Female American cockroaches (*Periplaneta americana*) were reared and fed with dog food (Pets at Home) as mentioned in Chapter 3: Methods.

4.2.2 *Escherichia coli* strain MG1655

To inoculate into adult female American cockroach guts, commensal *E. coli* strain MG1655 was chosen. The *E. coli* strain from glycerol stock was streaked on Lysogeny broth agars and incubated at 37°C overnight. A single colony was transferred to Lysogeny broth (LB) medium and shaken at 37°C overnight. The average bacterial concentration was determined using an optical density (OD_{600}) of about 0.7 with approximately 5.6×10^{8} bacterial cells·ml^{-1}. We sterilized the dog food using the autoclaving technique, which is adapted from the method used for maintaining insects in germ-free conditions (Wada-Katsumata et al., 2015; Dukes, Dyer and Ottesen, 2021a). The bacterial culture was resuspended in autoclaved dog food diets (Pets at home) at 121°C for 15 minutes and allowed to dry in a 60°C oven overnight. The control group of cockroaches was fed the autoclaved diet.

4.2.3 Pilot test: *Escherichia coli* strain MG1655 inoculation without the antibiotic treatment

To determine the ability of the commensal *E. coli* to inoculate into the cockroach guts, the pilot test contains two groups, an *E. coli*-fed group and a control group. Each group contained 3 cockroaches placed in a plastic box. The cockroaches were starved for two days before starting the experiment. The *E. coli*-fed group received the *E. coli* culture mixed with sterile diets and sterile LB for three days. Their faeces were collected daily for 8 days of the test. The faeces were weighed and ground by a sterile pestle in sterile phosphate-buffered saline (PBS). Then, the homogenized faeces were serially diluted, and 100 µl each was streaked on MacConkey agar plates and incubated at 44°C overnight. The number of culturable bacteria was calculated as colony forming units per gram of faeces.

4.2.4 Pilot test: Colony PCR from culturable bacteria from the faeces

A total of 5 bacterial colonies on MacConkey agar plates were chosen. The bacterial colonies were determined using universal bacterial primers on the 16S rRNA gene (27F-AGAGTTTGATCMTGGCTCAG and 1492R-GWTACCTTGTTACGACTT). The bacterial colonies were picked with sterile sticks and placed into a total of 25 µl of PCR master mix that contained 0.2 µM of each primer, 1x colourless Gotaq® Flexi buffer, 1.5 mM $MgCl_2$, 1.25 U GoTaq® Hot Start Polymerase (Promega), 0.2 mM dNTPs, and nuclease-free water. The PCR conditions were 5 min at 95°C, followed by 30 cycles of 95°C for 30 s,

annealing at 58°C for 30 s and 72°C for 1 min 30 s, and 10 min at 72°C. The PCR products were visualized on a 1% agarose gel electrophoresis and purified with a FastPure Gel DNA Extraction kit and then a total of five colonies were sequenced by Sanger sequencing. The selected culturable sequences were trimmed and merged using MEGAX (v10.1.8) (Kumar et al., 2018). They were then blasted in the GenBank database using BLASTN. A total of 51 nucleotide sequences were analyzed. Both selected sequences and the publicly accessible sequences of the *E. coli* strains were aligned and trimmed to the same length. The phylogenetic tree was constructed using the Neighbor-Joining method (Trees, 1987) to calculate the evolutionary distance with 1,000 bootstrap replicates (Felsenstein, 1985). Evolutionary analyses were conducted in MEGAX.

4.2.5 *Escherichia coli* inoculation

From the previous antibiotic treatment, kanamycin at a concentration of 300 $\mu g \cdot ml^{-1}$ and ampicillin of 300 $\mu g \cdot ml^{-1}$ were chosen. There are six groups in the experiment: the control, kanamycin-treated, and ampicillin-treated groups and with or without the *E. coli* inoculation. The cockroaches were treated with the antibiotic for ten days, and then *E. coli* was inoculated for three days. After inoculation, the sterilized dry food and sterilized water were provided as described above. The three cockroaches per group were collected on day 1, 3, 7, and 14 after stopping the *E. coli* inoculation. For each treatment and time point, three cockroaches were housed in a plastic container. At each time point, the three cockroaches from the same container were collected.

4.2.6 DNA extraction with a spike-DNA

The cockroaches were dissected under the stereo microscope as mentioned in Chapter 3: Methods. Each gut was represented individually. The gut was transferred into a 2 ml sterile Eppendorf tube and added 500 µl of sterile PBS and then ground using a sterile pestle. A 100 µl aliquot was spread on a MacConkey agar plate in triplicate and incubated overnight at 44°C. Another 200 µl left was used for the DNA extraction. A 0.5 mm stainless steel bead was added and then homogenized using a TissueLyser II (Qiagen) at 25 Hz for 1 min and flipped the tube for another 1 min. The bacterium *Thermus thermophilus* is chosen as an internal control. The DNA concentration from the genomic DNA of cockroach's guts from the previous experiment was calculated as an average. The *T. thermophilus* DNA was added into the gut's aliquot as approximately 4.26% of DNA concentration after homogenizing them. The *T. thermophilus* DNA concentration was diluted to 1 $ng \cdot \mu l^{-1}$ and 1.77 µl of DNA was used for each gut sample. The genomic DNA extraction followed the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as mentioned above (Chapter 3: Methods). The bacterial communities associated with the cockroach guts were determined using the bacterial 16S rRNA gene (515F and 806R primers) by Illumina sequencing (Chapter 3).

4.2.7 Data analysis and statistical tests

The sequence data from the Illumina sequencing were analyzed using the DADA2 in RStudio programme as mentioned in Chapter 3 (Methods: Illumina Data Analysis). All statistical analyses were performed in RStudio. Alpha diversity indices (Shannon and Chao1) were measured by Kruskal-Wallis test with a pairwise Dunn test between treatments with or without *E. coli* inoculation. Wilcoxon tests were used to compare differences between time points within the same treatment. The statistical tests were performed using microeco package (v1.7.1) (Liu et al., 2021b). A two-dimensional principal coordinate analysis (PCoA) was performed based on Bray-Curtis distances using MicrobiotaProcess package (v1.16.0) (Xu et al., 2023). The statistical test of bacterial communities across treatments was analyzed using permutational multivariate analysis of variance (PERMANOVA) by vegan package (v2.6.6.1) (Oksanen et al., 2018).

4.3 Results

4.3.1 Pilot test: Culturable bacteria from *E. coli* inoculating into cockroaches

To check the probability of *E. coli* inoculation in the cockroach guts, the *E. coli* culture mixed with sterile diets and sterile LB was introduced to the cockroaches for three days. Their faeces were collected before the experiment and daily until the end of the experiment on day 8. Bacteria were isolated from their faeces on MacConkey agar. The number of culturable bacteria peaked on day 2 and then gradually declined until day 6 when none were found (Figure 17). However, we cannot be assured that there were no contaminated faeces present from day 1 to day 3 of the experiment. Nevertheless, the bacteria were also detected on day 4 and day 5, which would indicate that they derived from the cockroach's gut. A total of five bacterial isolates and a colony of *E. coli* strain MG1655 were selected for colony PCR and sequenced using universal primers (27F and 1492R) on the 16S rRNA gene. However, the only samples with high-quality nucleotides that could be joined between forward and reverse sequences were B1, B2, B5, and *E. coli* MG1655 (B6). The phylogenetic tree of the chosen bacteria with publicly available *E. coli* strains from the GenBank database was reconstructed (Figure 18). The bacterial sequences were blasted and were discovered in a high match with *E. coli* on the GenBank database. Even though all of them are classified as *E. coli*, they are uncertain about strains.

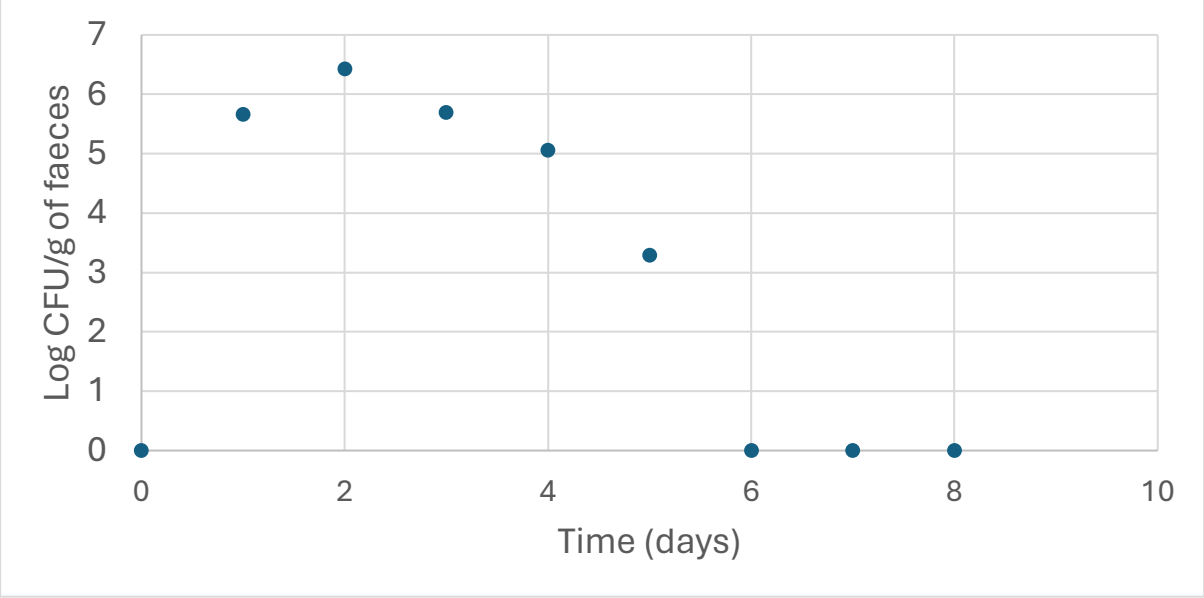


Figure 17 Culturable bacteria were isolated from the cockroach faeces inoculated E. coli strain MG1655 using MacConkey agar. Log CFU per gram of faeces was determined for eight days of the experiment.

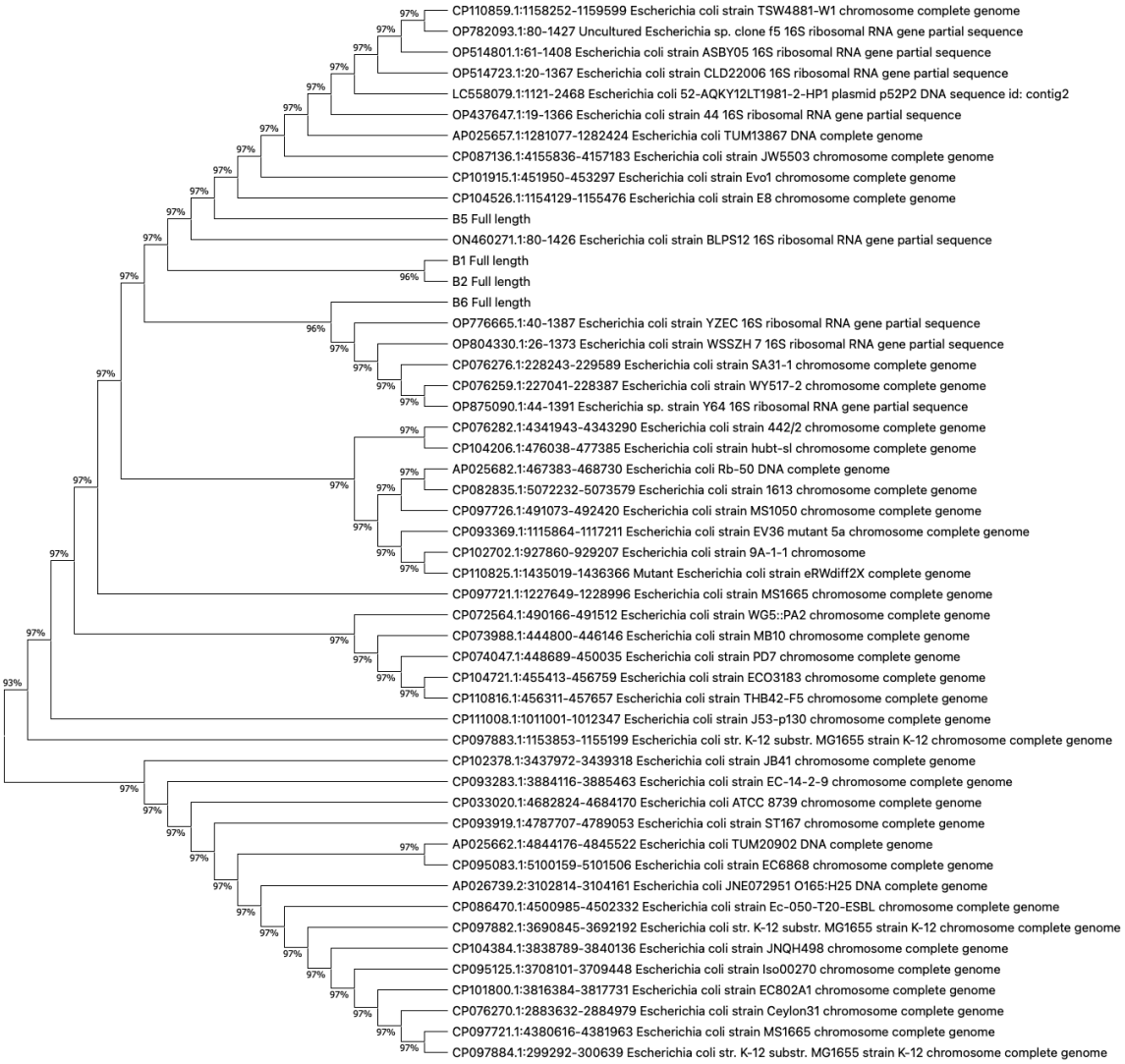


Figure 18 The phylogenetic tree of culturable bacteria from the pilot test against some E. coli strains from the GenBank database was constructed. The optimal tree is displayed with a branch length sum of 1.74. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. A total of 1392 nucleotide sequences were analysed. E. coli MG1655 (B6) was used in our study.

4.3.2 Absolute abundance of bacterial communities using spike-in DNA

A total of 10,871 amplicon sequence variants (ASVs) were left after sequence analysis. *Thermus thermophilus* DNA was used as a spike-in DNA in order to quantify the absolute abundance of the 16S rRNA gene using Illumina sequencing. *T. thermophilus* DNA was undetected in several samples. In order to eliminate the unclear result, we decided to exclude the copies of *T. thermophilus* DNA from the data.

4.3.3 Alpha diversity of bacterial communities after treatment with antibiotics and inoculated *E. coli*

The sequences ranging from 56,409 to 394,225 reads were obtained after data analysis and a total of 10,817 ASVs were classified after eliminating *T. thermophilus* data. The Shannon and Chao1 indices among the antibiotic treatment groups (kanamycin and ampicillin groups) and the control group were significantly different among the three groups, indicating that antibiotics had an impact on bacterial diversity (Kruskal-Wallis test with a pairwise Dunn test; $P < 0.05$) (Figure 19A). It appears that ampicillin could eliminate some bacteria more effectively than kanamycin since both species richness and diversity were reduced more in the ampicillin group than in the kanamycin group. However, neither of the antibiotic treatment groups showed any significant change during the period of the study (Kruskal-Wallis test; $P > 0.05$) within the same treatment (Figure 19B).

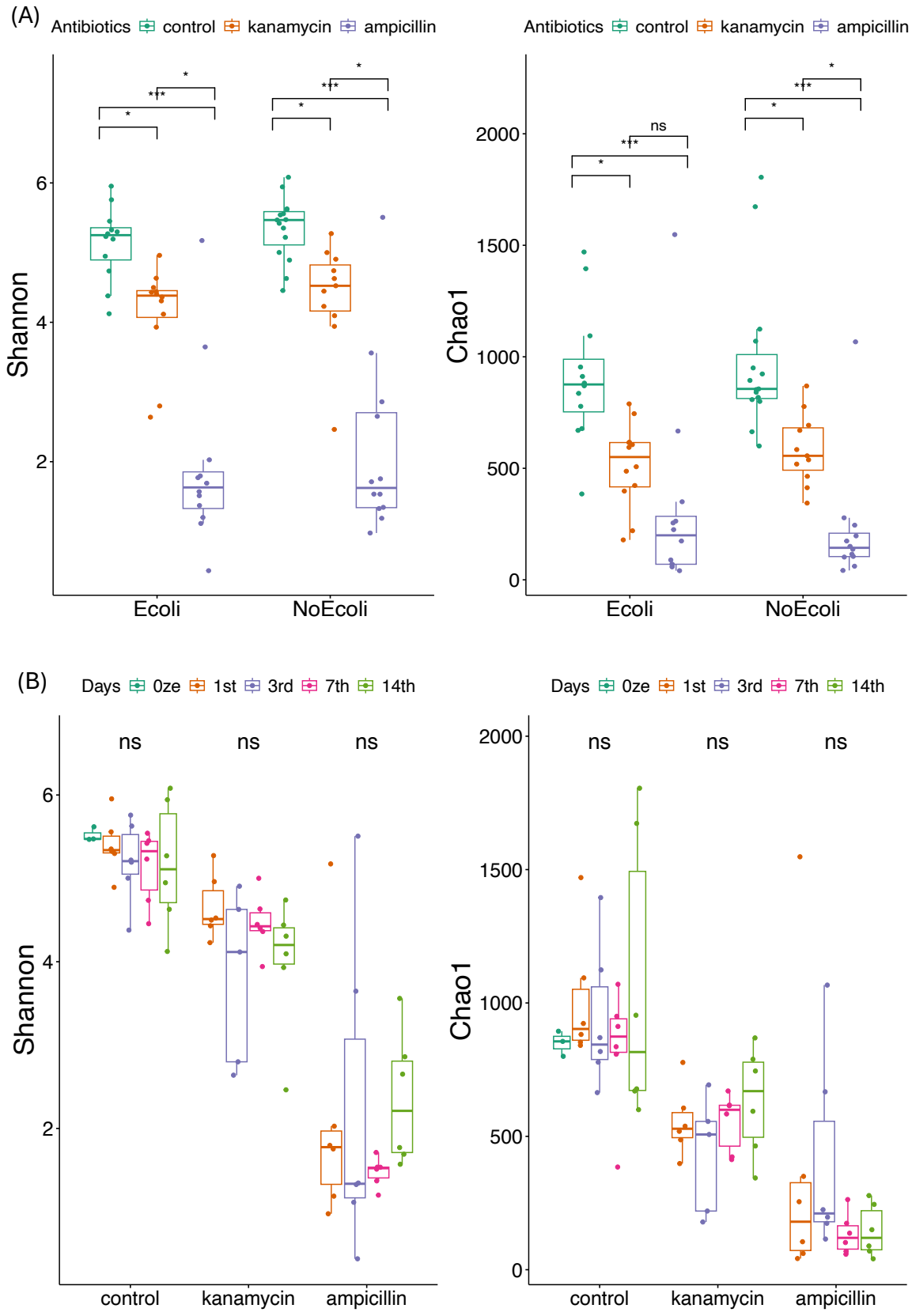


Figure 19 (A) Shannon and Chao1 alpha diversity indices indicate bacterial communities with or without E. coli inoculation from different antibiotic treatments (control, kanamycin, ampicillin). The Kruskal-Wallis test with a pairwise Dunn test was analysed. (B) The alpha diversity indices show across periods of time (day 0, 1, 3, 7, and 14) after E. coli inoculation by different antibiotic treatments. The Kruskal-Wallis test was analysed within the same treatment. The median is depicted by the middle lines in the box plot.

4.3.4 Antibiotics affect the bacterial communities associated with the cockroaches' gut

Antibiotics had an impact on the diversity and species richness of the microbial communities in the cockroaches' gut. At the phylum level, the bacterial communities associated with control and kanamycin showed similarity (Figure 20A). *Firmicutes* were the most prevalent phylum when comparing the bacterial communities in the control and kanamycin groups, followed by *Bacteroidota*, *Desulfobacterota*, and *Proteobacteria*. However, the bacterial populations of the ampicillin group were distinct from both the control and kanamycin groups at the phylum level (Figure 20A). *Bacteroidota*, *Firmicutes*, and *Proteobacteria* were the three predominant bacterial phyla in ampicillin without E. *coli* inoculation treatment.

4.3.5 *E. coli* inoculation affects the bacterial communities associated with the cockroaches' gut

There was little difference in the bacterial communities with or without *E. coli* inoculation in the kanamycin and control groups. It appeared that *E. coli* inoculation had a mild influence on the bacterial communities in these treatments. While the microbial communities in the control and kanamycin groups were similar at the phylum level, the composition of the bacterial genera showed little difference. *Desulfovibrio* and *Christensenellaceae* R-7 group were less prevalent in the kanamycin group compared to the control group, while *Paralactobacillus* and *Rikenellaceae* were detected more often in some samples from the kanamycin group (Figure 20C). Interestingly, *Lapidilactobacillus* increased in kanamycin-inoculated *E. coli* on day 3. However, they were hardly detected on day 7 and 14.

Comparing between with or without *E. coli* inoculation in the control group, while there were no differences between bacterial phyla (Figure 20A), there was little difference between bacteria at the genus level (Figure 20C). While we could detect more abundance of *Lapidilactobacillus* in *E. coli*-treated group, *Desulfovibrio* decreased in the same treatment.

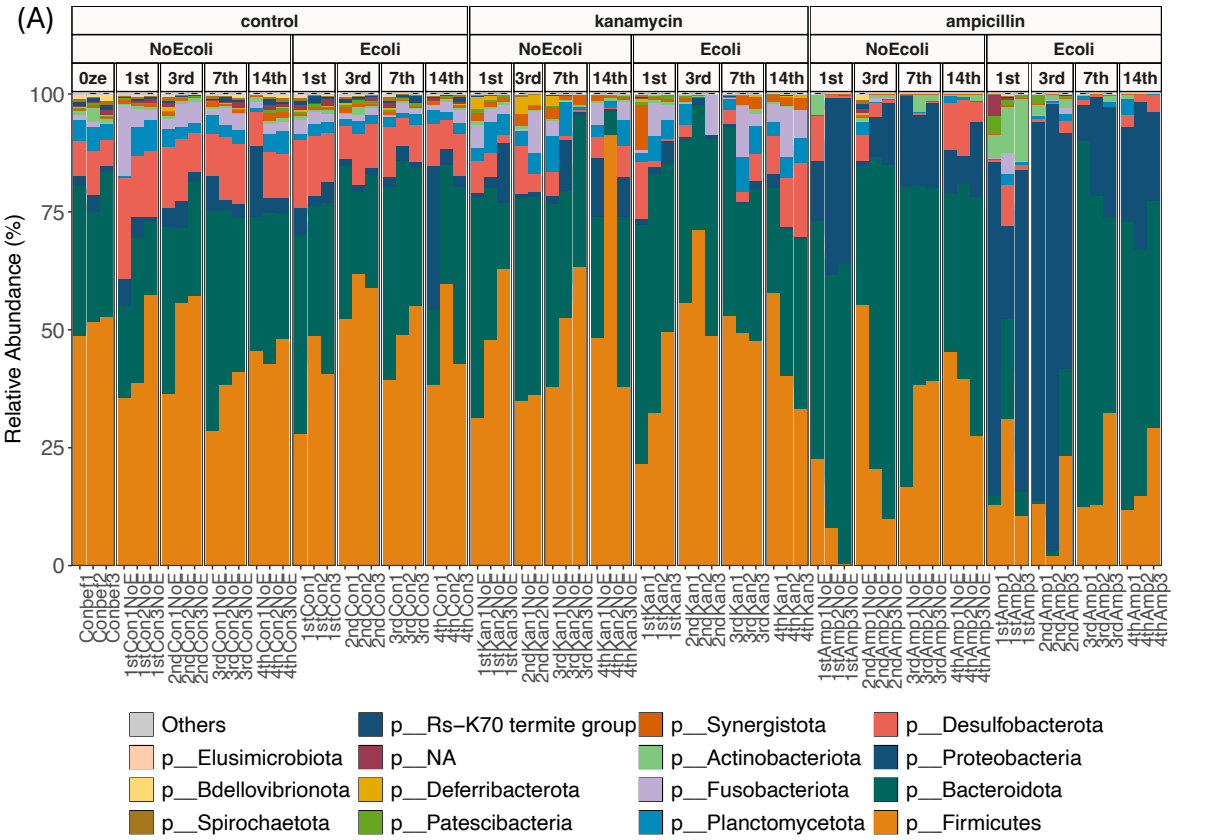
There was also no difference between bacterial phyla in kanamycin treatment with or without *E. coli*. However, there were more obvious differences in bacterial populations at the genus level. Comparing between with or without *E. coli* inoculation on day 1 of the kanamycin group, there was more bacterial diversity than in the ampicillin group, and we detected more *Paralactobacillus* in *E. coli*-untreated group. Inversely, *Bacteroides* was more abundant in *E. coli*-treated group. Moreover, there was more diversity of bacteria after day 7 and we could find more *Lapidilactobacillus* in *E. coli*-treated group.

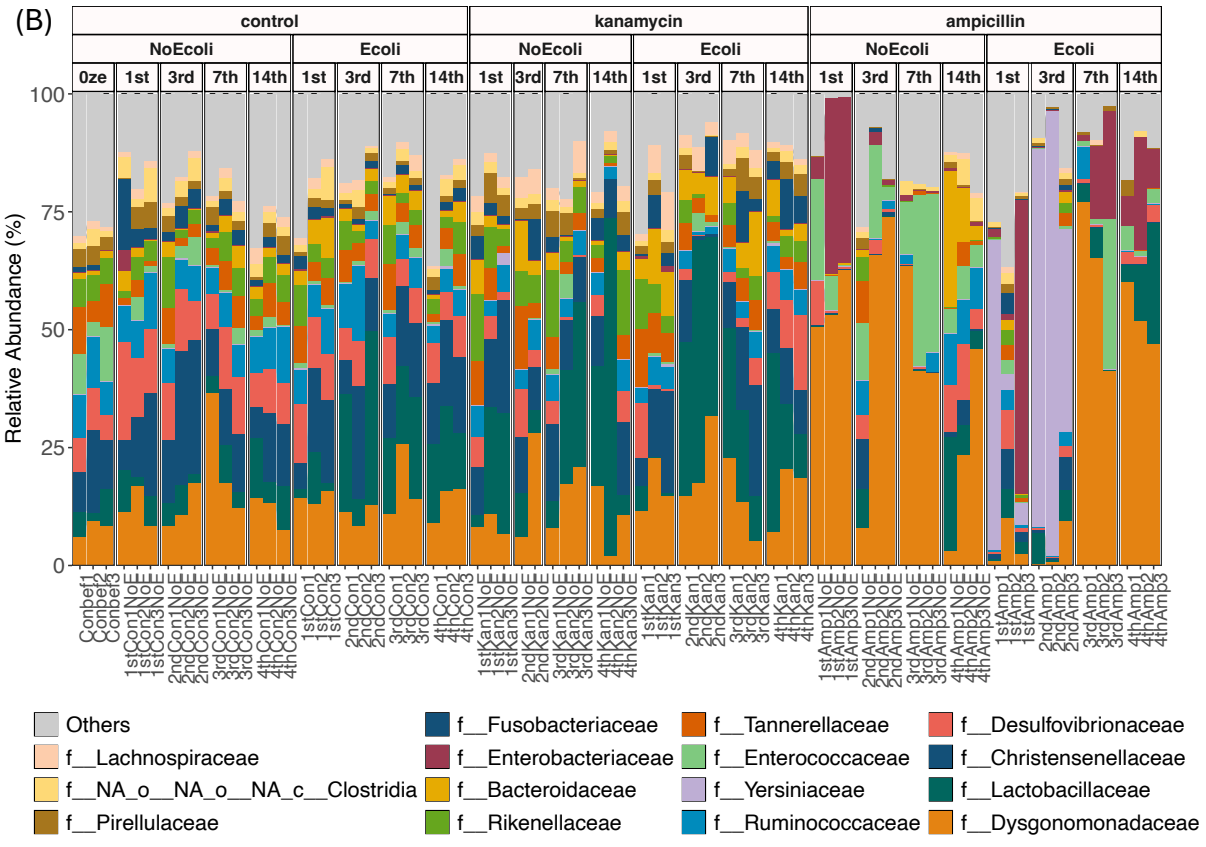
On day 1 of the ampicillin group without *E. coli* inoculation (Figure 20C), unclassified genera of *Dysgonomonadaceae* were discovered in all samples in this treatment. Additionally, we could detect *Klebsiella* and *Enterococcus* in some samples from the same treatment group. Interestingly, their bacterial populations were similar to *E. coli*-treated of the ampicillin group on day 7. However, there was a huge difference starting from day 7 as *Dysgonomonas* and *Enterococcus* increased predominantly. After that, there was a slight alteration in the bacterial populations, and on day 14, the bacterial communities were diverse and recovered. *Bacteroides, Desulfovibrio, Dysgonomonas*, and *Enterococcus* increased by day 14.

In ampicillin treatment, the proportion of *Proteobacteria* increased in both groups with and without the *E. coli* inoculation; however, this phylum was more prevalent in the group with the *E. coli* inoculation than in the untreated group. *Proteobacteria* did, however, decline in both groups after 7 days. *Bacteroidota* and *Firmicutes* recovered after day 7 in *E. coli* group, while it started early on day 3 in the absence of the *E. coli* group, *Desulfobacterota* was still found in some samples from the ampicillin-treated group whether with or without *E. coli.*

However, in the ampicillin-fed *E. coli* group, bacterial genera appeared different across all three samples from the first day after stopping *E. coli* inoculation in which *Serratia* was detected in one sample. There was more diversity in the other sample. The last sample was predominant with *Escherichia-Shigella* in *Enterobacteriaceae* (Figure 20). On day 3, *Serratia* became the dominant bacterial genus in all three samples from the *E. coli*-treated group. However, we hardly detected them after day 7 in the same treatment. It turned out that unclassified genera of *Dysgonomonadaceae* were predominant after day 7. Additionally, *Klebsiella* increased on day 7 (2/3 samples) and *Enterococcus* was also detected in one sample from the same treatment. At day 14, while unclassified genera of *Dysgonomonadaceae* were predominant, we could detect more abundance of *Lapidilactobacillus,* unclassified genera of *Neisseriaceae*, *Enterococcus,* and *Desulfovibrio* in this treatment.

Overall, it seemed like inoculation of *E. coli* had impacts on bacterial communities in the cockroach gut, even though the *E. coli* itself appeared not to survive inside the intestinal organ of the cockroach. Overall, *E. coli* could be detected in large numbers in only one sample on the first day after their inoculation from the ampicillin-treated group. However, they were hardly found after day 3. However, these patterns are inferred from the relative abundance of the amplicon 16S rRNA sequence without statistical support (Figure 20).


(A)
control
kanamycin
ampicillin
NoEcoli
Ecoli
0ze
1st
3rd
7th
14th
Relative Abundance (%)
100
75
50
25
0
Others
p__Elusimicrobiota
p__Bdellovibrionota
p__Spirochaetota
p__Rs−K70 termite group
p__NA
p__Deferribacterota
p__Patescibacteria
p__Synergistota
p__Actinobacteriota
p__Fusobacteriota
p__Planctomycetota
p__Desulfobacterota
p__Proteobacteria
p__Bacteroidota
p__Firmicutes


(B)
control
kanamycin
ampicillin
NoEcoli
Ecoli
0ze
1st
3rd
7th
14th
Relative Abundance (%)
100
75
50
25
0
Others
f__Lachnospiraceae
f__NA_o__NA_o__NA_c__Clostridia
f__Pirellulaceae
f__Fusobacteriaceae
f__Enterobacteriaceae
f__Bacteroidaceae
f__Rikenellaceae
f__Tannerellaceae
f__Enterococcaceae
f__Yersiniaceae
f__Ruminococcaceae
f__Desulfovibrionaceae
f__Christensenellaceae
f__Lactobacillaceae
f__Dysgonomonadaceae

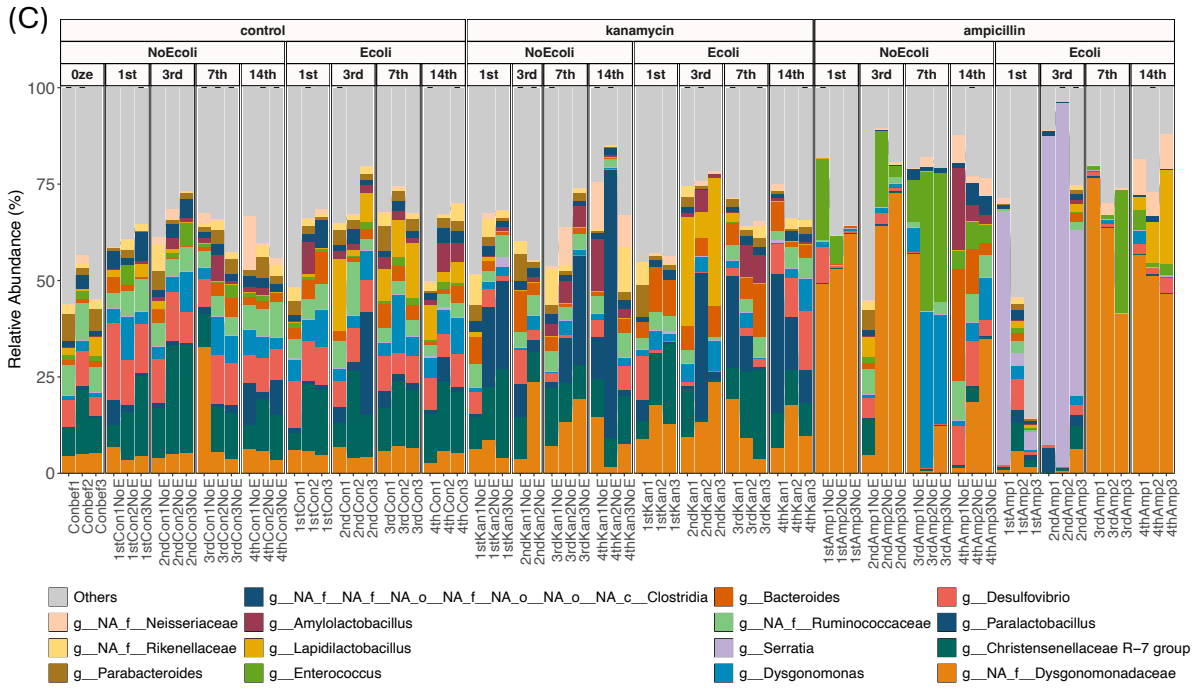
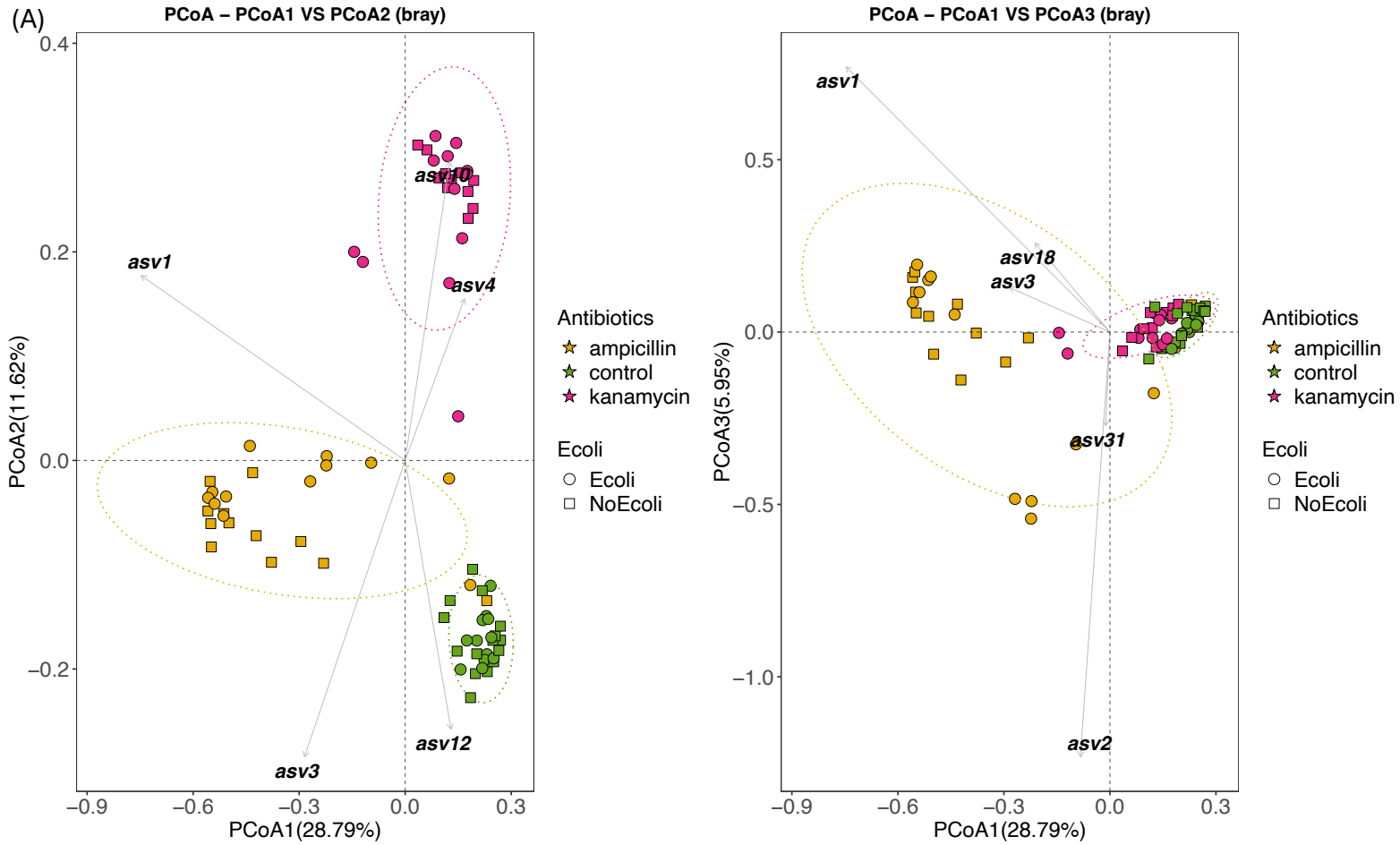


Figure 20 Relative abundance of bacterial communities associated with Escherichia coli inoculation after fed antibiotic treatments (kanamycin and ampicillin groups). Relative abundance at the phylum level (A), the family level (B), and the genus level (C).

PCoA indicates the dissimilarity of bacterial communities across antibiotic treatment with or without *E. coli* inoculation (Figure 21A). Figure 21A shows the first three axes, PCoA1, PCoA2, and PCoA3 which explain 28.79%, 11.62%, and 5.95%, respectively. The PERMANOVA test confirmed that bacterial communities were significantly different across antibiotic treatments (R^2 = 0.3283, P = 0.0001) with or without *E. coli* inoculation (R^2 = 0.0190, P = 0.0335). While there was no difference between antibiotics and *E. coli* inoculation (R^2 = 0.0285, P = 0.0629), the interactions between antibiotics and days were significantly different (R^2 = 0.0809, P = 0.0131; Figure 21B).

The bacterial communities were clustered distantly against antibiotic treatments (PCoA1 and PCoA2). The bacterial composition of the ampicillin group, in contrast, is clustered separately from the other two groups, showing that its communities differed greatly from those of the control and kanamycin groups (PCoA1 and PCoA3) (Figure 21A:B).

(A)


PCoA – PCoA1 VS PCoA2 (bray)
PCoA2(11.62%)
PCoA1(28.79%)
asv1
asv10
asv4
asv12
asv3
Antibiotics
ampicillin
control
kanamycin
Ecoli
Ecoli
NoEcoli
PCoA – PCoA1 VS PCoA3 (bray)
PCoA3(5.95%)
PCoA1(28.79%)
asv1
asv18
asv3
asv31
asv2
Antibiotics
ampicillin
control
kanamycin
Ecoli
Ecoli
NoEcoli

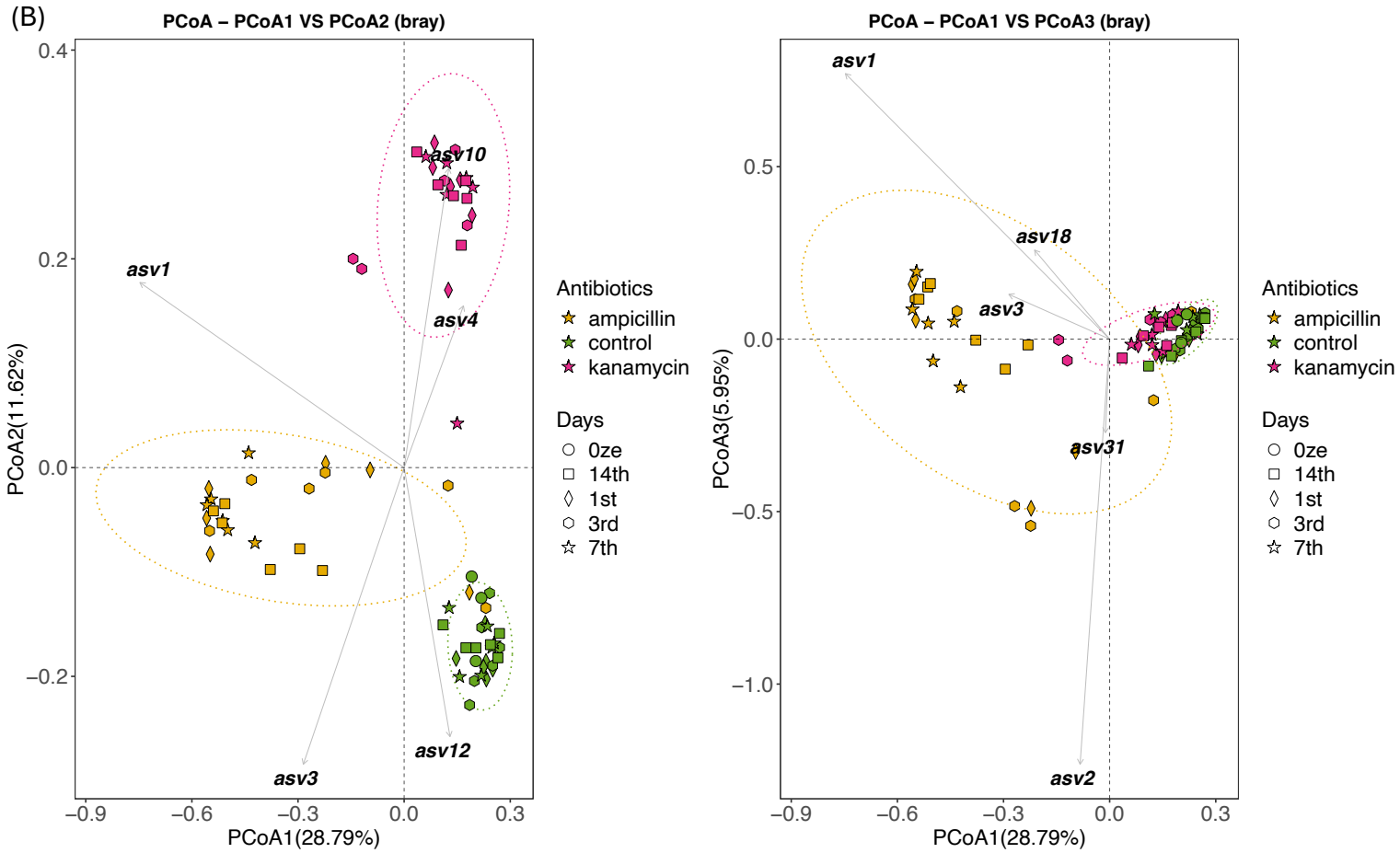


Figure 21 Beta diversity analysis using PCoA plots based on Bray-curtis dissimilarity of bacterial genera in the cockroaches' gut with or without E. coli inoculation after antibiotic treatment (control, kanamycin, and ampicillin). The first three axes (PCoA1, PCoA2, and PCoA3) are represented 28.79%, 11.62%, and 5.95%, respectively. (A) The PCoA shows the similarity of bacterial communities across antibiotic treatment with or without E. coli inoculation. (B) The PCoA shows the similarity of bacterial communities across antibiotics treatment across time-points (day 0, 1, 3, 7, and 14).

4.4 Discussion

Antibiotic treatment was chosen to overcome the colonization resistance in the American cockroach in our study. As they obviously show, reducing some bacterial communities associated with the intestinal organ of the American cockroach (Chapter 3). According to the previous study, kanamycin and ampicillin, both at a concentration of 0.3 $mg \cdot ml^{-1}$, were effective against the gut microbiome following continual administration for 10 or 20 days. In this study, the American cockroaches were continuously treated with antibiotics for 10 days at the same concentration of both ampicillin and kanamycin. Subsequently, *E. coli* MG1655 was inoculated for 3 days. The bacterial communities associated with their intestinal organs were observed on day 1, 3, 7, and 14 after the bacterium inoculation by the 16S rRNA gene using Illumina sequencing.

On the first day following the inoculation, we discovered that *E. coli* could only colonize in one sample from the ampicillin treatment. The other two samples from the same treatment differed; one was predominant with *Serratia* while the other had a diversified bacterial population on day 1. However, *E. coli* could not survive in the intestinal organ of the cockroach and had completely vanished after day 3. Antibiotic treatment could obviously eradicate bacterial communities in the cockroach guts. It showed us that antibiotics may potentially be able to overcome the colonization resistance; their effectiveness may vary depending on the individual. Specifically, as only one cockroach contained *E. coli* in its guts, for the other cockroaches from the same treatment, they might harbour different microorganisms in their intestinal organs. Because no two hosts carry the exact same microorganisms (Yatsunenko et al., 2012; Suzuki and Worobey, 2014). The amount or variety of microorganisms could cause them to respond differently when they encounter invading microbes.

We found that *E. coli* could colonize in one sample from ampicillin treatment on day 1. It is presumed that this was due to ampicillin being able to eradicate more of the bacterial community than could kanamycin. Moreover, the bacterial populations from the ampicillin group were also changed and less diverse than both the control and kanamycin groups. In the kanamycin group, this could be as a result of the native bacterial communities in the cockroach's intestinal organs recovering from the antibiotic and we could notice a greater prevalence of the colonization resistance, which prevents the invading bacterium from colonizing. One explanation for why *E. coli* failed to survive inside the cockroaches after being treated with kanamycin could be that the native bacteria were similar to conventional cockroaches, even in smaller numbers, but could perform their colonization resistance against the invading *E. coli*.

Even though we were unable to successfully inoculate the cockroach guts with *E. coli*, the inoculation of this bacterium did alter bacterial communities in the cockroach gut. When comparing the control

and antibiotic treatments with or without *E. coli* inoculation, we discovered that the *E. coli* inoculation groups had greater levels of *Lapidilactobacillus*. *Lapidilactobacillus* is Gram-positive *Lactobacillus* bacteria (Zheng et al., 2020). Several species within this genus can be isolated from various environments, including fermented Chinese cabbage, beer, silage, and traditional Chinese pickles (Zheng et al., 2020; Zhang and Gu, 2022). The genus *Lactobacillus* is Gram-positive and also one of the indigenous intestinal microbiomes (Tannock, 2002). It is also commonly recognized as one of the probiotic bacteria that could improve intestinal health in humans (Kechagia et al., 2013). These bacteria are members of the lactic acid bacteria, which ferment lactose to lactic acid. They are normally used in the process of fermented foods and dairy products (Giraffa, Chanishvili and Widyastuti, 2010).

Some studies have discovered that *Lactobacillus* was able to lessen diarrhea caused by pathogenic bacteria or rotaviruses (Van Niel et al., 2002; Kale-Pradhan, Jassaly and Wilhelm, 2010). For example, *L. plantarum* 299v, when given as probiotics, was able to reduce *E. coli*, which was causing intestinal permeability in a rat model (Mangell et al., 2002). Additionally, *L. reuteri* HCM2 infected mice could suppress enterotoxigenic *E. coli* that causes infectious diarrhea in children (Wang et al., 2018a). These would suggest that there is competition in the interactions between *Lactobacillus* and *Escherichia*. We could detect more abundance of *Lapidilactobacillus* in cockroaches inoculated with *E. coli* from the control group on day 3 following the inoculation. Furthermore, samples collected on days 3, 7, and 14 of the study were all positive for this bacterium in the control treatment. In the kanamycin group, it peaked only on day 3, whereas in the ampicillin group, it peaked on day 14. Since day 14, the bacterial communities in the cockroach gut treated with ampicillin recovered and became more diverse faster than when *E. coli*-inoculated was present. However, the majority of unclassified genera from *Dysgonomonadaceae* predominated instead.

Serratia species are commonly one of the indigenous gut microbiomes in some insects (Grimont and Grimont, 2006). β-lactam antibiotic-resistant genes have been found in several bacteria belonging to the *Serratia* genus (Sandner-Miranda et al., 2018). This might be responsible for their predominance in the ampicillin-treated *E. coli* group on days 1 and 3 of our study. On the other hand, *Dysgonomonas, Klebsiella*, and *Enterococcus* were the most common bacteria found in cockroach guts of no-*E. coli* treatment from the ampicillin group. The β-lactam resistance genes found in *Dysgonomonas* (Gao et al., 2022), *Klebsiella*, and *Serratia* (Jacoby, 2009; Sandner-Miranda et al., 2018) cause them to proliferate when treated with ampicillin. Nevertheless, only one sample from the first day after stopping *E. coli* inoculation in the ampicillin group was able to be colonized by *E. coli* along with a lower number of *Serratia*. Overall, *Serratia* appeared to outcompete *Dysgonomonas*, *Klebsiella,* and

Enterococcus when *E. coli* was inoculated into the cockroach guts. However, in the case that *E. coli* colonized the intestinal organ, the remaining bacteria were decreased.

Serratia and *Dysgonomonas* are essential in lignin degradation and are present in many insects, including bamboo snout beetles (Tang et al., 2023). *Dysgonomonas* is a Gram-negative and an anaerobic bacterium (Olsen, 2015). This bacterium can be found commonly in insects, such as black soldier fly (Khamis et al., 2020; Li et al., 2023a) and termites (Pramono et al., 2015; Su et al., 2016), which it assists in food digestion and enhances host development (Li et al., 2023a). Moreover, it can be found in high abundance in human clinical samples such as the nasal cavity of hospital staff members (Chen et al., 2019a), blood of patients (Gao et al., 2022), and human gall bladders (Hofstad et al., 2000). However, its pathogenic process in humans is uncertain and needs more discovery.

It is worth noting that the results of ampicillin treatments between Chapter 3 and this study were different. Specifically, at the genus level, *Klebsiella,* unclassified genera in Neisseriaceae, and *Termite planctomycete cluster* were predominant after 10 days of treatment from the previous chapter. However, the most prevalent species of non-*E. coli* from this study were unclassified *Dysgonomonadaceae*, *Klebsiella,* and *Enterococcus.* During the three days that the cockroaches from ampicillin in this chapter that had not had an *E. coli* inoculation were given a control diet and water, the others received an *E. coli* inoculation. This could have resulted in the recovery of bacterial communities once the antibiotic treatment was discontinued.

One possible reason that *E. coli* could not survive inside the cockroach gut is that it is not the optimal temperature for them to grow. In our experiment, we raised the American cockroaches at 30°C, which is the optimum temperature for their growth. It should not go above 33°C (Gunn and Notley, 1936). The American cockroach cannot survive at temperatures as high as 37–39°C, particularly in dry air rather than moist air (Gunn and Notley, 1936). However, *E. coli* K12 can grow at 37°C, which is their optimal temperature. (Akkermans, Logist and Van Impe, 2018). Because the body temperatures of mice and most animals, including humans, are between 36°C and 37°C, they are able to survive and proliferate in their digestive tracts (Refinetti, 2020). This may be one of the explanations for *E. coli* MG1655's failure to survive in the guts of American cockroaches.

In addition, some studies found that commensal *E. coli* could be isolated from the American cockroach (Pai, Chen and Peng, 2005; Us, Uma and Ram, 2013; Akbari et al., 2015). Therefore, *E. coli* MG1655 might encounter competition from other bacteria or from nutritional competition. *E. coli* was unable to grow and colonize in the guts of cockroaches, despite the fact that we used antibiotics to reduce colonization resistance by either decreasing the number of gut microbiomes (in both kanamycin and ampicillin) or altering the microorganism populations (in ampicillin). It is proven that

there could be a number of reasons why the cockroach gut inoculation with *E. coli* was unsuccessful. Even though *E. coli* was unable to thrive and propagate throughout the cockroach's digestive organ, we were still able to determine interactions between the bacteria. Eventually, despite the fact that the antibiotics were used to prevent the cockroach guts' colonization resistance, they were unable to lessen this phenomenon since the inoculated *E. coli* were totally removed from the guts. Future studies on gnotobiotic or germ-free cockroaches will be worthwhile. Our study included six treatments in all, with twelve replicates of each treatment. This might limit the power of statistical analysis (Muller and Benignus, 1992). For every treatment, there are only three replicates for every time point. Increasing the sample size at each time point could result in more reliable data. Additionally, for each time point, we used three replicate cockroaches from the same box. Cohabitation may have an impact on the gut microbiota, which requires further awareness (Song et al., 2013).

The phylogenetic tree demonstrates that *E. coli* MG1655 (B6) and isolated *E. coli* (B1, B2, and B5) were unable to differentiate from other publicly available sequences of *E. coli* strains (Figure 18). We cannot be certain that the isolated *E. coli* is from the strain that was inoculated because American cockroaches also harbour commensal *E. coli* (Pai, Chen and Peng, 2005; Us, Uma and Ram, 2013; Akbari et al., 2015). There is insufficient information to differentiate among closely related bacterial strains using the 16S rRNA sequencing in our study.

The commonly used technique for defining *E. coli* strains is MLST, with the sequence type representing each clone (Pitout and DeVinney, 2017). Multiple housekeeping genes are sequenced using multi-locus sequence typing (MLST); typically, seven to eight housekeeping genes are sequenced, with each gene's nucleotide sequences consisting of about 500 bp fragments (Maiden et al., 1998). According to recent research, the species-level taxonomic distinction may require more than just a full-length 16S region. The use of entire genome sequences along with multiple coding ribosomal gene sequences is recommended for the classification of species and phylogenetics (Hassler et al., 2022). Additionally, it would be more accurate to identify closely related bacteria that are different by whole-genome MLST (Maiden et al., 2013).

In this study, we applied both culture-dependent and culture-independent approaches. The pilot test was conducted using a culture-based approach. Gram-negative enteric bacteria, including *E. coli*, are specific to the selective media MacConkey agar (Jung and Hoilat, 2024). However, not all bacterial communities can be isolated using the culturable method because some are not culturable and require particular nutrients and specific environments (Stewart, 2012). For example, facultative anaerobes, which prefer oxygen but can survive on anaerobic respiration if necessary, or obligate anaerobic bacteria, which can survive and grow in an oxygen-free environment (Kaspar and Tiedje,

1982). However, the whole-genome sequencing of the isolates, which enables deep classification into the species or strain level, phylogenetic analysis, and understanding of novel genes and their functions from pure sequence data, are all benefits of this culture method (Stewart, 2012).

Next-generation sequencing, Illumina sequencing, is used in our study. This method provides us with both culturable and unculturable gut microbiomes. Compared to the culture-dependent approach, this could provide us with more data and greater gut microbial diversity. However, because of its short-read sequence, approximately 400 bp on the 16S rRNA gene. It is not enough to distinguish bacterial species. A whole-genome sequencing or a full-length 16S rRNA sequencing would be more suitable.

The Illumina sequencing usually determines bacterial communities in relative abundance. While relative abundance provides the dominating species in communities, it is less effective when explaining population dynamics within a community (Vandeputte et al., 2017). The absolute abundances of microbial communities are more suitable and have been determined using a variety of techniques, including quantitative PCR (qPCR) and spike-in DNA (Tkacz, Hortala and Poole, 2018b; Smets et al., 2016).

We used *T. thermophilus* DNA as the internal control for absolute quantification of the gut microbiome, as it does not exist in the gut microbiome. However, *T. thermophilus* DNA was undetected in several samples. Therefore, to get rid of the unclear result, we decided to remove DNA copies of *T. thermophilus* from our data. This is not quite clear why the copies of spike-DNA were not found in all samples. We adapted this approach from the environmental soil samples (Smets et al., 2016) and the DNA copies of spike-DNA were added into the dissected cockroach gut prior to the DNA extraction. The spike-DNA might be lost during the DNA extraction steps. Also, the commercial Blood and Tissue DNA extraction kit is used in our experiment, which is different from the environmental soil samples that usually use commercial DNA extraction kits such as the PowerSoil DNA Isolation Kit (MoBio) (Smets et al., 2016).

It is worth varying the concentration of spike-in DNA. In this case, we recommend considering the spike-in bacteria prior to DNA extraction, as they have been successful with the gut microbiome research (Stämmler et al., 2016). This could decrease the chance of losing spike-in DNA copies during DNA extraction. Moreover, testing with a spike-in DNA dilution would help us determine the ideal concentration for our samples. Additionally, it will be worthwhile to explore performing multiple types of spike-in DNA (Zaramela et al., 2022).

The ampicillin and kanamycin reduced the cockroach's gut microbiome significantly. The gut microbiota was comparable to the control after kanamycin treatment, while ampicillin treatment significantly altered the bacterial populations. Both outcomes could be beneficial in different ways, depending on the intended objectives of the study. Both results may help us understand how commensal microbes interact with invasive bacteria in our study. One study demonstrated how the invading bacterium interacted with the same bacterial communities (kanamycin treatment), whereas the other study showed how the invading bacterium interacted with other commensal microbe groups (ampicillin treatment). However, antibiotics that solely decrease bacterial abundance while maintaining the same bacterial groups may be more advantageous than those that significantly change the gut microbiome in terms of the faster recovery of the gut microbiota after stopping medication. Additionally, the primary goal of administering antibiotics is to eradicate harmful microorganisms. Antibiotics that are highly specific to their pathogenic bacteria and are not effective against other commensal microbes may be worth taking into consideration.

Chapter 5: Sulfate supplement influences the sulfate reduction in *Desulfovibrio* bacteria in the cockroach guts

5.1 Introduction

When the body suffers an injury or microbial infection, inflammation is one of its defense mechanisms (Ahmed, 2011). Redness, pain, swelling, and heat are common symptoms of inflammation (Hannoodee and Nasuruddin, 2024). Numerous illnesses are linked to inflammation, including inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC) (MacDonald and Monteleone, 2005), and neurological diseases, such as Parkinson's and Alzheimer's (Voet et al., 2019). Chronic inflammation of CD occurs in any part of the gastrointestinal tract, while UC is restricted to the colon (MacDonald and Monteleone, 2005). Although the exact causes of both CD and UC are unknown, several factors have been identified, such as host genetic, environmental, and intestinal dysbiosis factors (Miyoshi and Chang, 2017).

The gut microbiome plays an important role in gut inflammation (Al Bander et al., 2020). Both CD and UC are associated with gut microbiome dysbiosis (Miyoshi and Chang, 2017). Change in the composition of the gut microbiota, particularly reduction in species diversity, is associated with IBD (Sha et al., 2013; Alam et al., 2020). The gut microbiomes of IBD patients fluctuated more than those of individuals in good health (Halfvarson et al., 2017b). There are numbers of microorganisms that have been found associated with these diseases, including an increase in *Proteobacteria* species and a decrease in *Firmicutes* and *Bacteroidetes* species (Frank et al., 2011; Glassner, Abraham and Quigley, 2020). Sulfate-reducing bacteria (SRB) have also been found associated with IBD patients (Loubinoux et al., 2002a; Dordević et al., 2021).

SRB are anaerobic microbes that use sulfate in their respiration as a final electron acceptor (Postgate, 1965). There are two distinct pathways for sulfate reduction, which are assimilatory sulfate reduction (ASR) and dissimilatory sulfate reduction (DSR). ASR is more complex with a need of seven different enzymes necessary in the pathway for the biosynthesis of the amino acids cysteine and methionine (Kushkevych et al., 2020b). ASR is widely distributed in plants and some microorganisms (Schiff and Fankhauser, 1981), such as *Escherichia coli* and yeast (Peck Jr, 1961). DSR, on the other hand, reduces sulfate by using hydrogen or organic compounds as reductants, with hydrogen sulfide (H_2S) as a final product. There are three essential genes associated with the DSR pathway: *sat*: ATP sulfurylase, *dsrAB*: dissimilatory sulfite reductase, and *aprAB*: dissimilatory adenosine-5'-phosphosulphate

reductase (Figure 22) (Muyzer and Stams, 2008). H_2S is mainly produced through cysteine catabolism and by sulfate-reducing bacteria in the gut microbiome (Stummer et al., 2023). The risk of gut inflammation may rise as a result of the gut's elevated H_2S concentration (Blachier et al., 2021; Stummer et al., 2023).

There are five known genera of sulfate-reducing bacteria, including *Desulfobacter, Desulfomonas, Desulfobulbus, Desulfotomaculum,* and *Desulfovibrio* (DSV) that use DSR, leading to the production of H_2S in the human gut microbiome (Dordević et al., 2021). In addition, there are several microorganisms defined as SRB, which can be divided into four groups based on rRNA sequence analysis: Gram-negative mesophilic SRB, Gram-positive spore-forming SRB, thermophilic bacterial SRB, and thermophilic archaeal SRB (Castro, Williams and Ogram, 2000). These organisms are mainly distributed in natural environments and are major contributors to several biotechnological processes, causing a serious problem in various industries, such as in oil production (Tiburcio et al., 2021; Cord-Ruwisch, Kleinitz and Widdel, 1987), rice field soil (Wind, Stubner and Conrad, 1999), and freshwater sediments (Bak, Scheff and Jansen, 1991). However, they are also beneficial in several industries, especially in industrial wastewater treatment (Muyzer and Stams, 2008; Zhang et al., 2022).

Desulfovibrio is one of the Gram-negative mesophilic SRB (Castro, Williams and Ogram, 2000). These bacteria generally form a minor microbiome group in the human gastrointestinal tract. SRB has been reported to be associated with inflammatory conditions in humans' guts (Loubinoux et al., 2002a) with the abundance of *Desulfovibrio* being greater in the inflammatory bowel disease patients than in the healthy ones (Loubinoux et al., 2002a). Apart from the inflammatory intestinal diseases, several studies found that *Desulfovibrio* is also associated with other diseases, including liver cirrhosis (Lu et al., 2023), autism (Finegold, 2011), and bacteremia (Hagiya et al., 2018). Interestingly, the same isolated *Desulfovibrio* species from the patients showed more virulence activities, such as producing H_2S levels and antibiotic resistance, than the same bacterial species from the healthy ones (Lu et al., 2023). In addition, in comparison to healthy individuals, patients with Parkinson's disease had greater amounts of *Desulfovibrio*. Moreover, the greater the quantity of *Desulfovibrio* the more severe the illness is correlated (Lu et al., 2023). Conversely, the study of the DSV had a positive correlation with the healthy population in Guangdong, China (Chen et al., 2021b). Moreover, they were positively associated with some beneficial bacteria but negatively correlated with several harmful bacteria in humans (Chen et al., 2021b). The *Desulfovibrio* were correlated with age in healthy humans, where the mucosal *Desulfovibrio* were more abundant in infants and elderly people than in young adults (Fite, 2004). However, the way that these bacterial genera can be found in both healthy (Fite, 2004) and unhealthy humans (Chen et al., 2021b) leads us to reconsider their uncertain roles in human intestinal organs which require more research and clarification.

Sulfate can be found in drinking tap water, beverages, processed foods, dried fruits, food additives, breads, *Allium* vegetables, and cruciferous vegetables (Doleman et al., 2017; Florin et al., 1993). Additionally, it appears that inorganic sulfate and protein-derived sulfate are common in a westernized diet. Overconsumption of sulfate might lead to detrimental effects on human health. Considering that diets are one of the main factors influencing the gut microbiota (Zhang et al., 2015).

Studies of sulfate reduction pathways and the *Desulfovibrio* activities would be challenging in human guts. The alternative models would have more possibilities and be less expensive, for instance, the use of a cockroach model. *Desulfovibrio* spp. is one of the significant bacterial genera inhabiting cockroach guts (Lee et al., 2020; Tinker and Ottesen, 2021; Chen et al., 2023). Examining how sulfate content in the diet affects the *Desulfovibrio* and other bacterial communities in the cockroach model is therefore worthwhile.

The purpose of this chapter is to examine the interaction between sulfate-supplemented drinking water and the *Desulfovibrio* spp. bacteria in the cockroach guts, with a view to exploring whether the cockroach could be a model for the inflamed human/mammalian gut microbiome. The number of *Desulfovibrio* spp. was investigated compared with the total number of bacteria using qPCR and Illumina sequencing of the 16S rRNA gene. Additionally, we evaluated the expression of two SRB genes, *dsrA* and *aprA*, using quantitative reverse transcription-PCR (qRT-PCR) to confirm the activity of sulfate reduction.

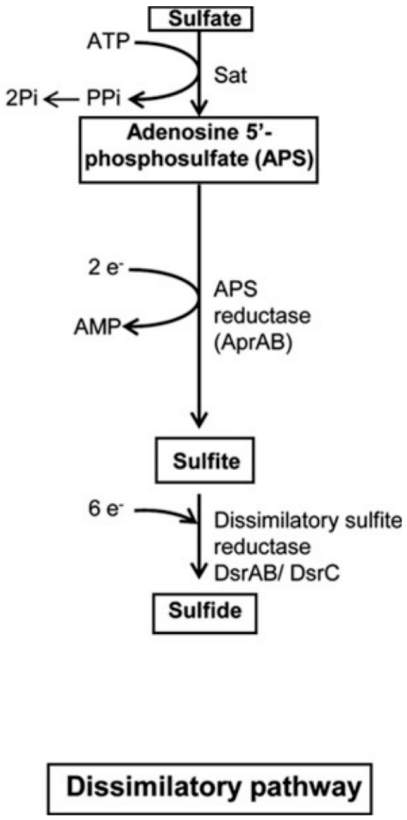


Figure 22 The dissimilatory sulfate reduction pathway (Chandra et al., 2020).

5.2 Methods

5.2.1 Sulfate consumption

A total of 51 cockroaches were starved for two days before starting the experiment. Three cockroaches were collected as a control group before starting the experiment and kept at -80°C for DNA and RNA extraction. Three cockroaches were raised in each box and three cockroaches from the same boxes were collected by the end of each week for a total of four weeks. Potassium sulfate (K_2SO_4) was chosen as a sulfate source. There are three separate treatments for the first two weeks including the control group fed with normal diet and sterile water, 0.1% sulfate-supplement water, and 0.2% sulfate-supplement water. Subsequently, half of the sulfate-supplement group was treated with the sterile, unsupplemented diet and sterile water for a further two weeks. However, the other half of the sulfate-supplement groups remained ongoing with the same treatment until the end of the experiment at week 4.

Table 3 Experimental plan of different sulfate concentration supplements through the experiment.

Samples	Week 1	Week 2	Week 3	Week 4
control	sterile diet/water ★	sterile diet/water ★	sterile diet/water ★	sterile diet/water ★
0.1% sulfate	0.1% sulfate diet + sterile water ★	0.1% sulfate diet + sterile water ★	0.1% sulfate diet + sterile water ★	0.1% sulfate diet + sterile water ★
0.2% sulfate	0.2% sulfate water + sterile diet ★	0.2% sulfate water + sterile diet ★	0.2% sulfate water + sterile diet ★	0.2% sulfate water + sterile diet ★
0.1% sulfate-con	0.1% sulfate diet + sterile water	0.1% sulfate diet + sterile water	sterile diet/water ★	sterile diet/water ★
0.2% sulfate-con	0.2% sulfate water + sterile diet	0.2% sulfate water + sterile diet	sterile diet/water ★	sterile diet/water ★

★ Collecting day

5.2.2 Gut dissection

The cockroach gut was dissected as mentioned in 3.2.2 Cockroach dissection (Chapter 3). The dissection equipment was autoclaved for 15 minutes and oven at 80°C overnight. All the equipment and surface dissection area were cleaned with 70% ethanol and RNase AWAY (Thermo Fisher

Scientific) before dissecting. The whole gut was weighed and kept in RNAlater solution (Thermo Fisher Scientific) and left in a fridge overnight before transferring to -80°C until a molecular method.

5.2.3 DNA extraction and the Illumina next-generation sequencing

The DNA extraction was performed using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany. The 16S rRNA gene of bacteria was amplified using 515F and 806R primers (Caporaso et al., 2011). A total of 20 µl of PCR master mix that contains 0.2 µM of each primer, 1x colourless buffer, 1.25 U GoTaq Hot Start Polymerase, 0.2 mM dNTPs, and nuclease-free water. The PCR conditions were 2 min at 95°C, followed by 30 cycles of 95°C for 30 s, annealing at 60°C for 30 s and 72°C for 30 s, and final extension for 10 min at 72°C. The PCR products were visualized on a 2% agarose gel electrophoresis to confirm the expected band. The PCR product was purified using 0.8x AMPure XP beads and eluted in 20 µl nuclease-free water. The concentration of purified PCR product was quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and sequenced using 250 bp paired-end Illumina Novaseq sequencing.

5.2.4 RNA extraction and cDNA synthesis

Following extraction, whole cockroach guts were washed in sterile PBS twice and then rinsed with sterile PBS. The samples were homogenized using a Tissue Lyser II (Qiagen) at 30 Hz for 1 minute, with the tube flipped and homogenization continued for another 1 minute. The individual homogenized tissue was divided equally for DNA and RNA extractions. The RNA was extracted using a RNeasy Plus kit (Qiagen) and finally eluted with 30 µl of RNase-free water. To get rid of the DNA, the RNA samples were treated with a Turbo DNA-free kit (Invitrogen) as followed the manufacturer's instructions with the RNA concentration of no more than 200 $\mu g \cdot ml^{-1}$ in a total of 14 µl reaction and 12 µl RNA was eluted and kept at -80°C. The quality of RNA was examined with a Nanodrop and eight representative samples were chosen for quality analysis using an Agilent 2100 Bioanalyzer.

Expression of the dissimilatory sulfate reduction of *Desulfovibrio* was investigated in the sulfate-supplement experiment using the *dsrA* and *aprA* genes. cDNA was synthesised using SuperScript IV Reverse Transcriptase Kit (Invitrogen) following the company's instructions. Briefly, 20 µl reaction, including 1 µg total RNA were used, 1 µl of 50 µM random primers (Promega), 1 µl of 10 mM dNTP, 4 µl of 5x SSIV buffer, 1 µl of 100 mM DTT, 1 µl of RNaseOUT Recombinant RNase inhibitor, 1 µl of SuperScript IV Reverse Transcriptase, and nuclease-free water. The cDNA was diluted 1/20 and used 3 µl for qPCR. The 16S rRNA universal bacterial primers (1114F and 1275R) were used to normalize the gene abundances. The negative (no nucleic acid) control and a no reverse transcriptase control were also included.

5.2.5 PCR primer efficiency by qPCR

The 16S rRNA gene (1114F and 1275R) of total bacteria, 16S rRNA genes of *Desulfovibrio* bacterium, *dsrA*, and *aprA* genes (Table 4) were validated using qPCR in a total of 10 µl reactions, performed using a QuantStudio 7 Pro Real-Time PCR system (Thermofisher).

The primer efficiency was tested using a standard curve from the PCR products of each pair of primers. A total of 20 µl of PCR master mix contained 0.2 µM of each primer, 4.0 µl 5X Colorless buffer, 1.5 mM of $MgCl_2$, 2 µl template DNA (10 ng μl^{-1}), 0.25 µl GoTaq Hot Start Polymerase, 0.2 mM dNTPs and nuclease-free water. The PCR conditions were 2 min at 95°C, followed by 30 cycles of 95°C for 30 s, annealing at 60°C for 30 s and 72°C for 30 s, and a final extension of 5 min at 72°C. The PCR products were run on a 2% agarose gel and the band was cut out of the gel. The gel was purified using a QIAquick PCR and Gel Cleanup kit (Qiagen). The purified products were quantified using Qubit dsDNA Quantification Assay Kit and then dilutions were made from 10^{-1} to 10^{5}.

The cDNA was diluted for 10-fold dilutions (10^{-1} to 10^{-5}) after the reverse transcription, starting with 10 ng·μl^{-1} of cDNA, and 3 µl of each sample was used in a 10 µl qPCR reaction to determine the PCR efficiency of *dsrA* and *aprA* genes. However, the genomic DNA was used as a template in the 16S rRNA gene of total bacteria (1114F and 1275R) and *Desulfovibrio* (DSV691-F and DSV826-R).

A total of 10 µl of qPCR master mix contains 0.2 µM of each primer, 5.0 µl of Fast SYBR Green Mastermix, 2.0 µl of DNA template or 3.0 µl of cDNA, and nuclease-free water. The conditions were 20 s at 95°C, 40 cycles of 1 s at 95°C, 30 s at 60°C, and a melting curve. The qPCR reaction was run on a QuantStudio 7 (Thermo Fisher Scientific). The standard curve was quantified using a qPCRtools package (Li et al., 2022) in RStudio.

5.2.6 Quantification of bacterial and *Desulfovibrio* communities using qPCR

All cDNA samples were performed in triplicate. A total of 10 µl of qPCR master mix contained 0.2 µM of each primer, 5.0 µl of Fast SYBR Green Mastermix, 3.0 µl of cDNA template, and nuclease-free water. The conditions were 20 s at 95°C, 40 cycles of 1 s at 95°C, 35 s at 60°C, and a melting curve. The qPCR reaction ran on a QuantStudio 7 Pro (Thermo Fisher Scientific). The relative expression levels of the *dsrA* and *aprA* genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and performed in a bar chart using ggplot2 package (Wickham, 2016).

Table 4 The primers used for qPCR for both DNA and cDNA.

Gene	Primer	Sequence	Reference
16S rRNA of *Desulfovibrio*	DSV691-F DSV826-R	CCGTAGATATCTGGAGGAACATCAG ACATCTAGCATCCATCGTTTACAGC	(Fite, 2004)
dsrA	dsrA-F dsrA-R	GCCGTTACTGTGACCAGCC GGTGGAGCCGTGCATGTT	(Ben-Dov, Brenner and Kushmaro, 2007)
aprA	aprA-F aprA-R	CGCGAAGACCTKATCTTCGAC ATCATGATCTGCCAGCGGCCGGA	(Ben-Dov, Brenner and Kushmaro, 2007)
16S rRNA of universal bacteria	1114F 1275R	CGGCAACGAGCGCAACCC CCATTGTAGCACGTGTGTAGCC	(Denman and McSweeney, 2006)

5.2.7 Diversity analysis and statistical analyses

The sequencing data was analysed using DADA2 running on RStudio as described in 3.2.9 Illumina data analysis. Microbiota analysis from Illumina sequencing was performed using MicrobiotaProcess package (Xu et al., 2023). Bacterial diversity analyses using Chao1 and Shannon indices were analysed in RStudio. The statistical significance of alpha diversity was tested using Wilcoxon test in MicrobiotaProcess package. The principal coordinate analysis (PCoA) was measured using the Bray-Curtis distance.

The different relative abundances of bacterial communities were determined using linear discriminate analysis (LDA) effect size (LEfSe) (Segata et al., 2011) with LDA score greater than 2 at a *P* value more than 0.05. The different bacterial taxa were shown in LDA log score as a bar plot and a cladogram using a microbiomeMarker R package (v1.10.0) (Cao et al., 2022). Metabolic pathways were predicted based on the representative ASVs and bacterial abundance of 16S rRNA Illumina sequencing using PICRUST2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; v2.5.2) (Douglas et al., 2020). The pathways were visualized using STAMP (Statistical Analysis of Metagenomic Profiles; v2.1.3)

The ggplot2 (Wickham, 2016) package was used to illustrate the total number of bacteria and DSV copies as well as to visualise the expressions of *aprA* and *dsrA.* The Kruskal-Wallis test was used to

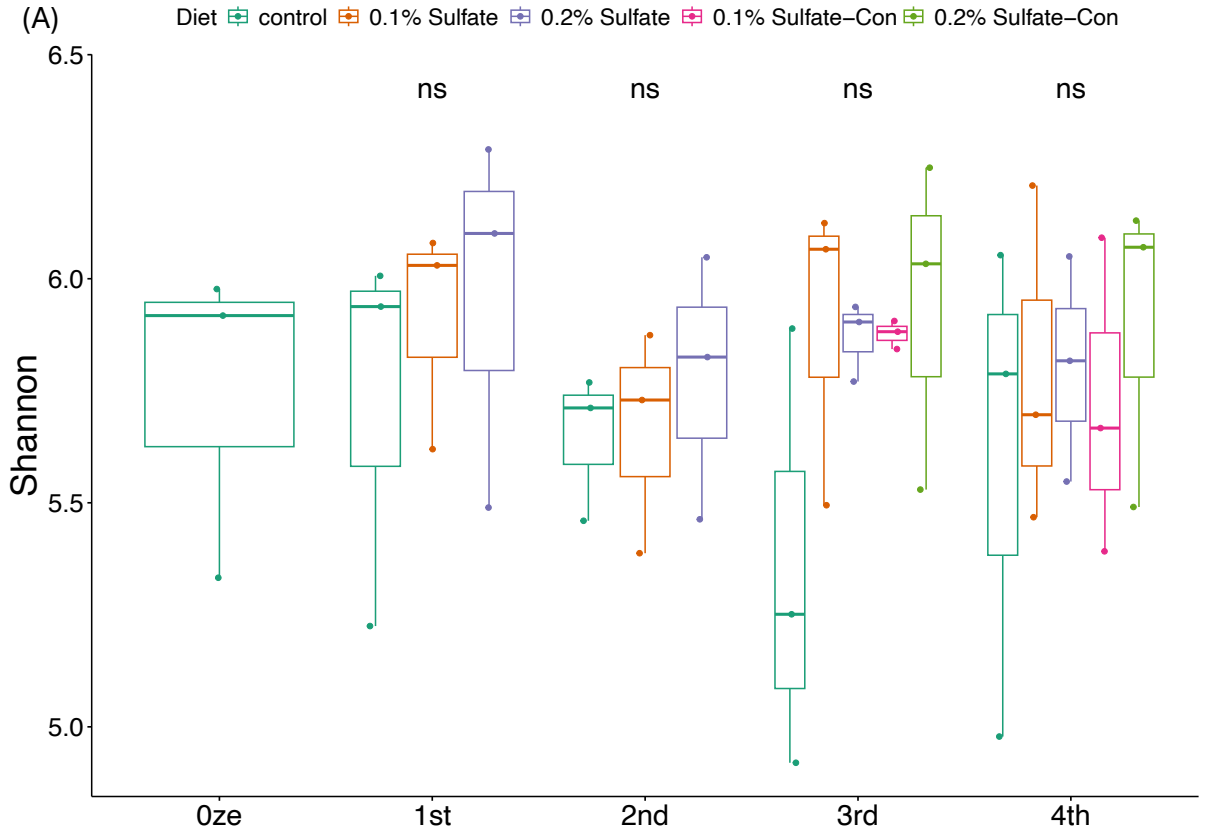
compare the variations in total bacterium copies, DSV copies, *aprA* expression, and *dsrA* expression between time points within the same treatment. Pairwise comparisons using Dunn's test and the Kruskal-Wallis test were used to examine the copies of total bacteria and DSV across treatments.

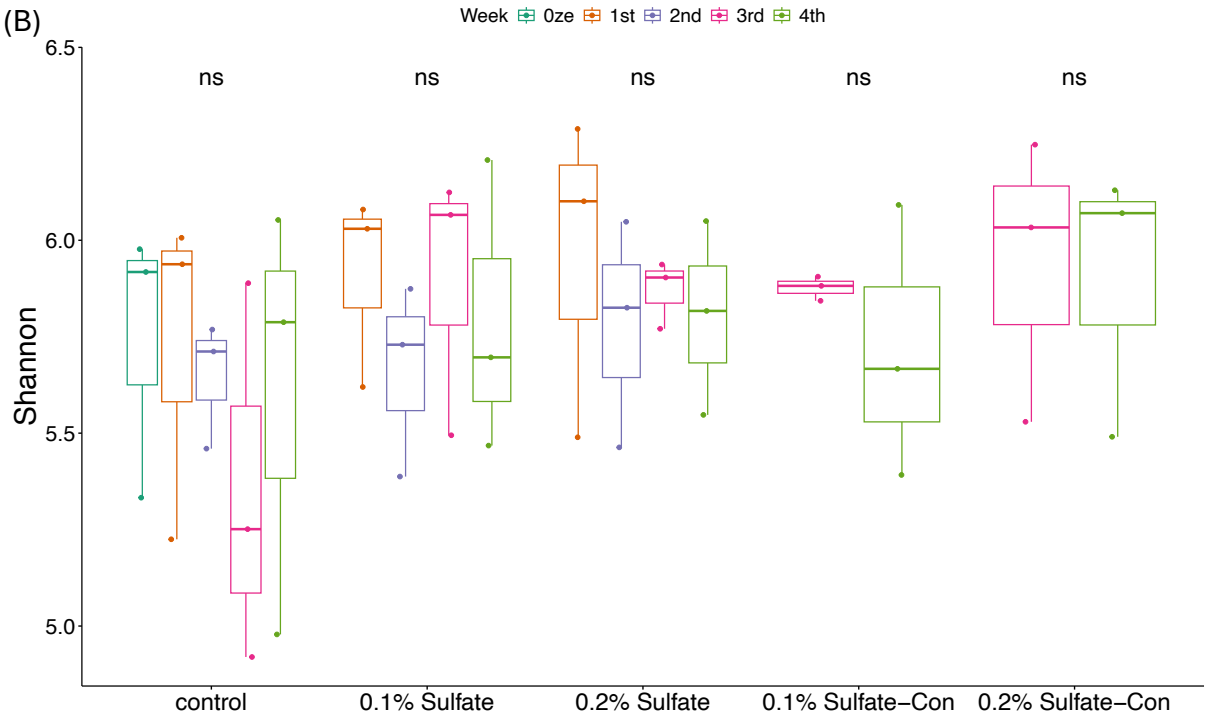
5.3 Results

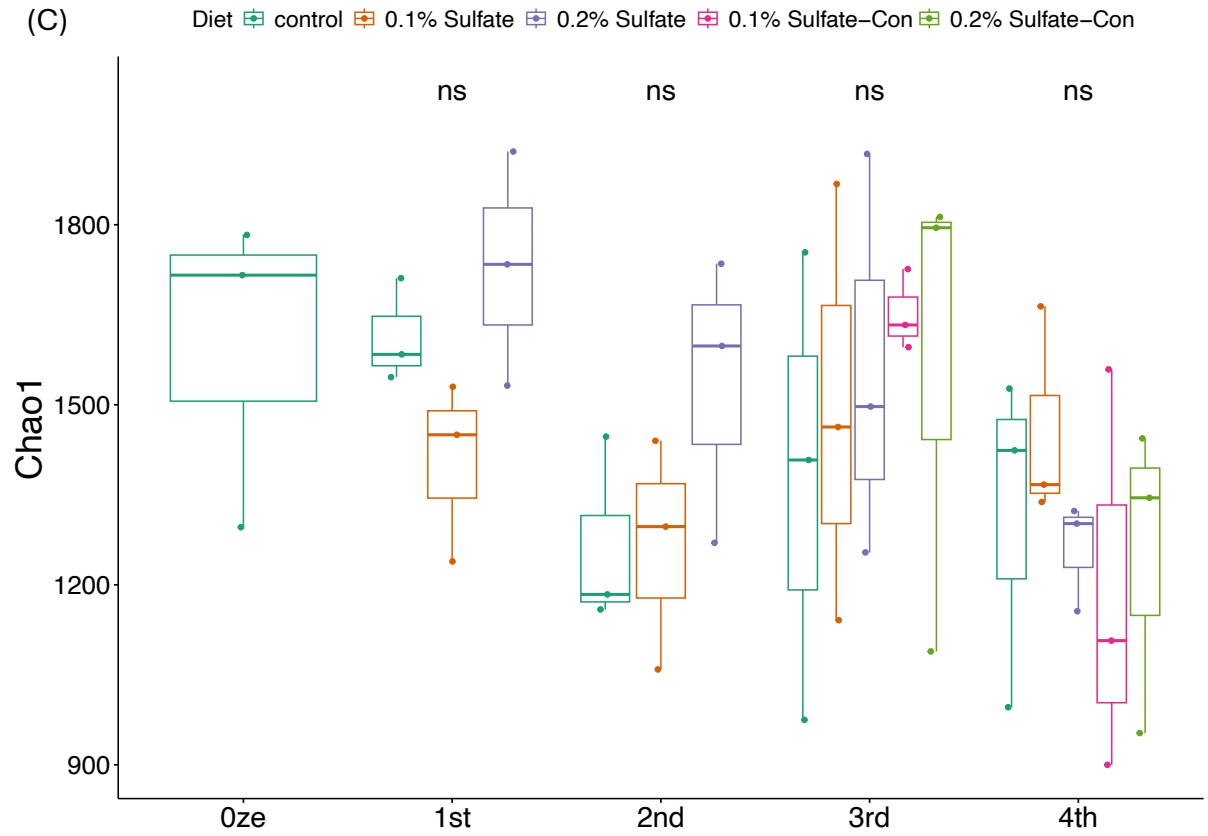
5.3.1 Bacterial communities associated with the cockroaches treated with sulfate diet based on 16S rRNA Illumina sequencing

The microbial diversity from different time points was combined for each diet group. The alpha diversity (Chao1 and Shannon) of bacterial communities indicates that there is no significant difference between the diet groups (Kruskal-Wallis test). Additionally, the diversities for each treatment and each week were also analyzed. The Chao1 and Shannon indices were not significantly different from either all time points or the diet treatment (Figure 23).

The most abundant four phyla were *Firmicutes*, followed by *Bacteroidota*, *Desulfobacterota*, and *Proteobacteria*, respectively (Figure 24A). At the genus level, the relative abundances of *Desulfovibrio* were dominant across all samples (ranging from 4.2 to 14.5%) but showed no difference between the time points and the diet groups. Apart from *Desulfovibrio*, the other dominant genera across all samples were *Paralactobacillus*, *Parabacteroides*, *Christensenellaceae R-7 group*, and *Bacteroides* with similar relative amounts (Figure 24B). Both at the phylum and genus levels, bacterial communities showed no apparent differences across the samples.




(B)
Week 0ze 1st 2nd 3rd 4th
Shannon
6.5
6.0
5.5
5.0
ns
ns
ns
ns
ns
control
0.1% Sulfate
0.2% Sulfate
0.1% Sulfate−Con
0.2% Sulfate−Con


(C)
Diet control 0.1% Sulfate 0.2% Sulfate 0.1% Sulfate−Con 0.2% Sulfate−Con
Chao1
1800
1500
1200
900
ns
ns
ns
ns
0ze
1st
2nd
3rd
4th

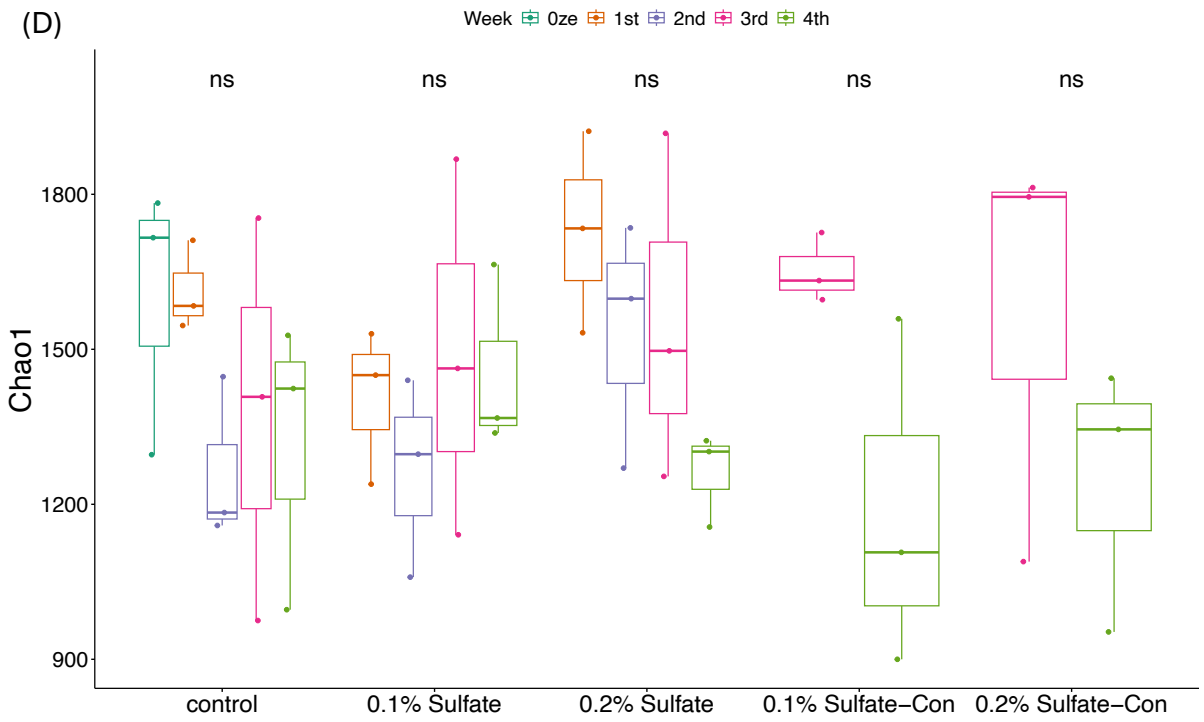
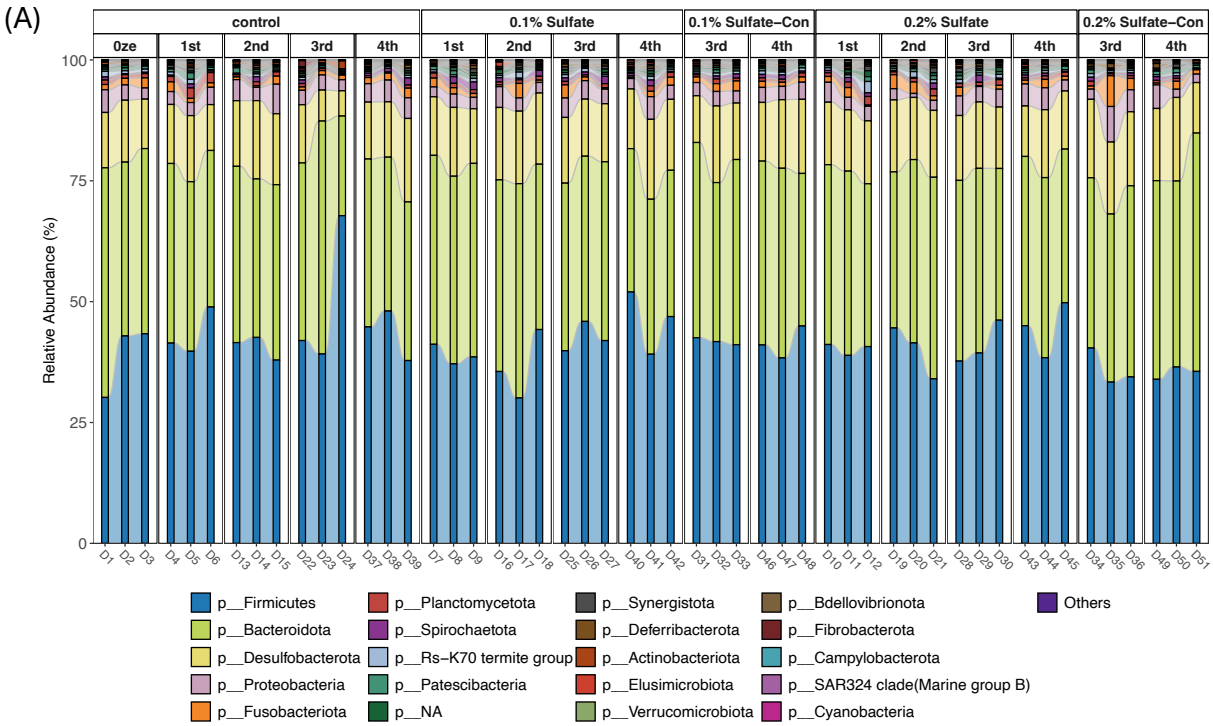


Figure 23 The box plots of alpha diversity (Shannon and Chao1 indices) of bacterial communities from the Illumina sequencing were performed using Kruskal-Wallis test across treatment in different weeks (Shannon; A, Chao1; C), across time points in each treatment (Shannon; B, Chao1; D).



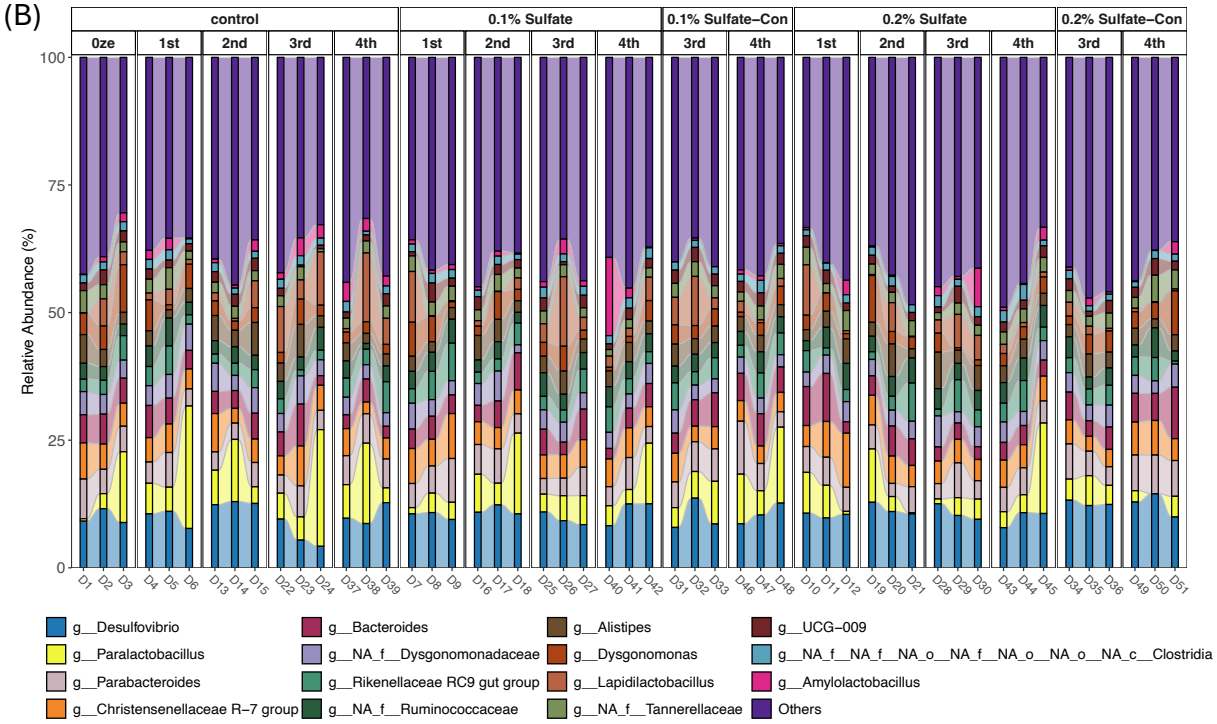


Figure 24 The relative abundance of bacterial communities associated with the sulfate supplement groups at the phylum (A) and genus (B) levels.

The beta diversity of bacterial communities between different sulfate treatments and the control group was calculated and visualized using PCoA (Figure 25). The PCoA confirms that there is no distinction of bacterial compositions across the cockroaches' groups feeding with or without the supplement of sulfate in drinking water with the PERMANOVA test (R^2 = 0.0908, P = 0.1202). However, the distribution of bacteria in the 0.2% sulfate-con caused its ellipse to be a bit further away from the other groups, but still no significant differences.

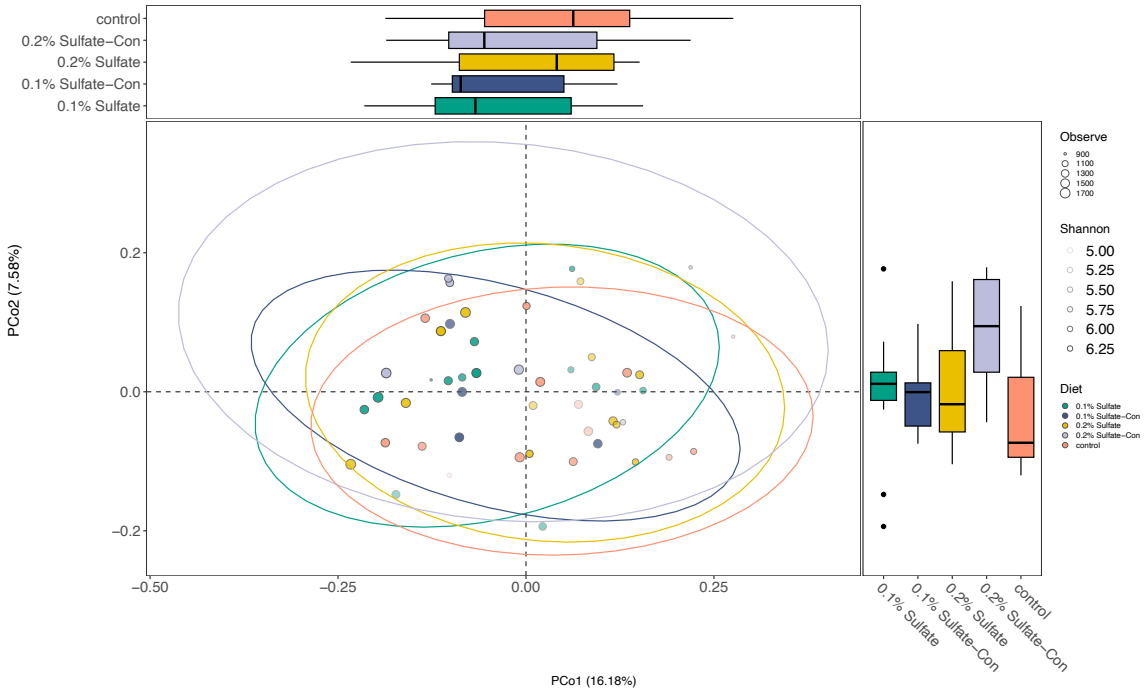
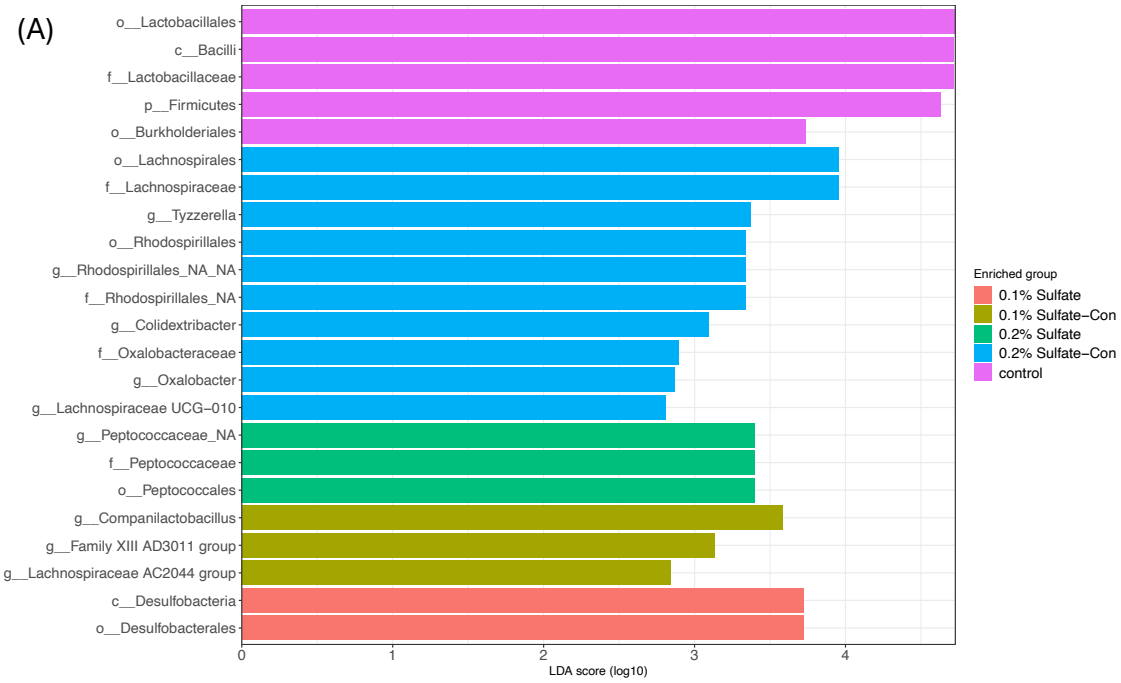


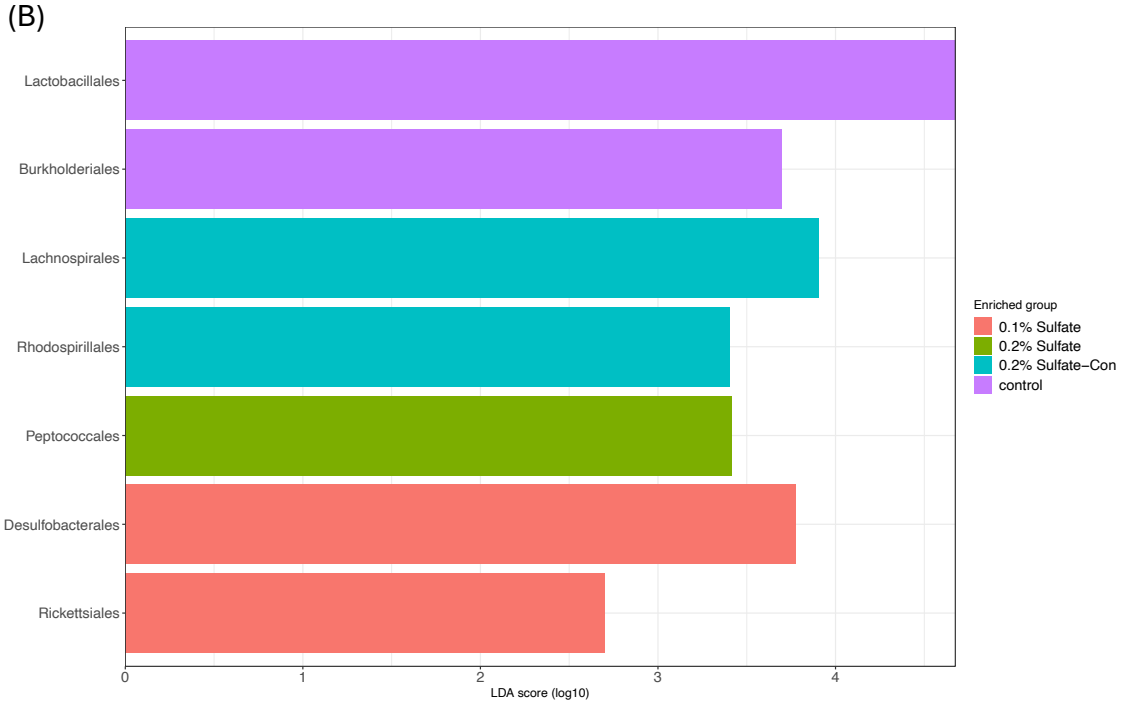
Figure 25 PCoA of bacterial communities associated with the cockroaches' gut feeding with or without sulfate-supplement diets. The different colors and sizes of ellipses represent different diet groups and the number of bacteria, respectively. PERMANOVA test based on bacterial dissimilarities across treatments was performed. The ellipses represent each treatment (control, 0.1% sulfate, 0.2% sulfate, 0.1% sulfate-con, and 0.2% sulfate-con, respectively).

We could not see the distinct bacterial communities from their relative abundance and the cluster analysis. The enriched taxa amongst the bacterial communities were determined using LDA score set at 2. There are 23 different bacterial taxa with LDA log scores greater than 2 across all treatments (Figure 26A). The cladogram shows the enriched bacterial taxa across treatments by labelling with different colours (Figure 26C). *Firmicutes* phylum was predominant in the control group, and it was the main bacterial phylum that appeared different across all treatments. Additionally, bacterial taxa, including *c_Bacilli, o_Lactobacillales,* and *f_Lactobacillaceae* belonging to the *Firmicutes* phyla, were more abundant in the control group (LDA >2; $P < 0.05$). Moreover, *o_ Burkholderiales* was also predominantly found in the control group. In the 0.1% sulfate group, however, *c_Desulfobacteria* and *o_Desulfobacterales* were enriched.

(A)


o__Lactobacillales
c__Bacilli
f__Lactobacillaceae
p__Firmicutes
o__Burkholderiales
o__Lachnospirales
f__Lachnospiraceae
g__Tyzzerella
o__Rhodospirillales
g__Rhodospirillales_NA_NA
f__Rhodospirillales_NA
g__Colidextribacter
f__Oxalobacteraceae
g__Oxalobacter
g__Lachnospiraceae UCG−010
g__Peptococcaceae_NA
f__Peptococcaceae
o__Peptococcales
g__Companilactobacillus
g__Family XIII AD3011 group
g__Lachnospiraceae AC2044 group
c__Desulfobacteria
o__Desulfobacterales
0
1
2
3
4
LDA score (log10)
Enriched group
0.1% Sulfate
0.1% Sulfate−Con
0.2% Sulfate
0.2% Sulfate−Con
control

(B)


Lactobacillales
Burkholderiales
Lachnospirales
Rhodospirillales
Peptococcales
Desulfobacterales
Rickettsiales
0
1
2
3
4
LDA score (log10)
Enriched group
0.1% Sulfate
0.2% Sulfate
0.2% Sulfate−Con
control

(C)

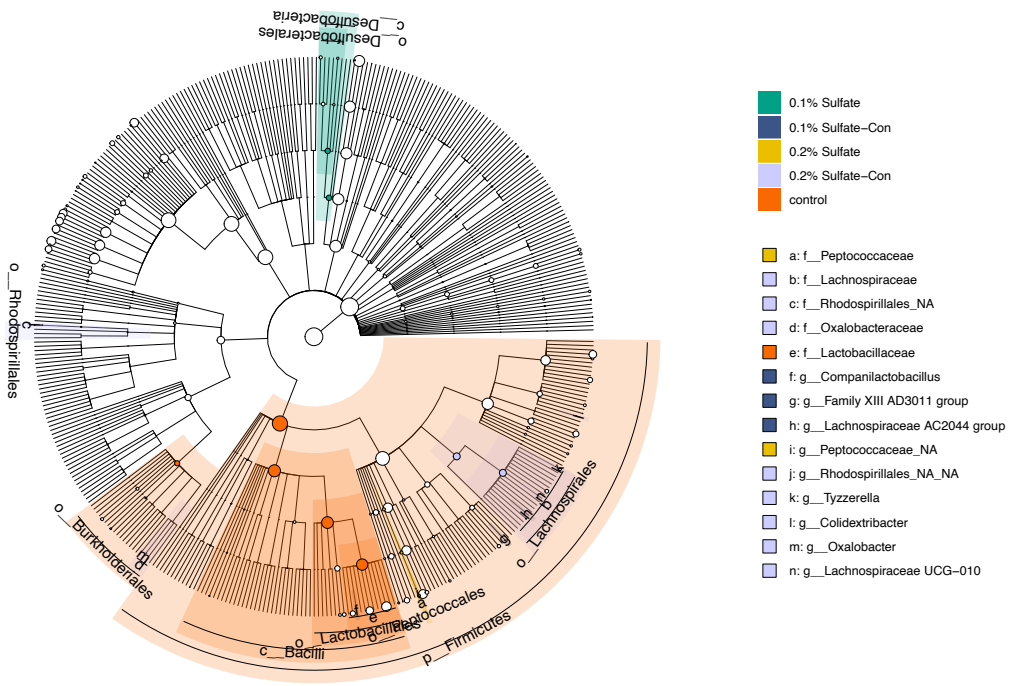


Figure 26 The enriched taxa of bacterial communities of the cockroaches from with or without sulfate-supplement groups were determined using LEfSe. The different abundant bacteria are shown in all taxa (A) and order (B) with the LDA score greater than 2 with a P value less than 0.05 of the factorial Kruskal-Wallis test. (C) The cladogram shows different taxa enriched from the treatment groups with different colours.

5.3.2 Amplification efficiency

All four pairs of primers were tested for their amplification efficiency using qPCR. All primers show a proper amplification efficiency (81.30% to 101.78%) and all the R^2 are about 0.98-0.99 (Table 5). The results confirm that all four pairs of primers could be used for amplification of the 16S rRNA gene of total bacteria, *dsrA*, aprA, and the 16S rRNA gene of *Desulfovibrio* using qPCR.

Table 5 The table shows amplification efficiency of four pairs of primers using qPCR.

Primers	Amplification (%)	R^2	Formula
Total bacteria (1114F and 1275R)	93.43	0.9987	y = -3.49*x + 48.22
Desulfovibrio	86.64	0.9959	y = -3.69*x + 42.48
aprA	81.30	0.9883	y = -3.87*x + 66.36
dsrA	101.78	0.9912	y = -3.28*x + 61.35

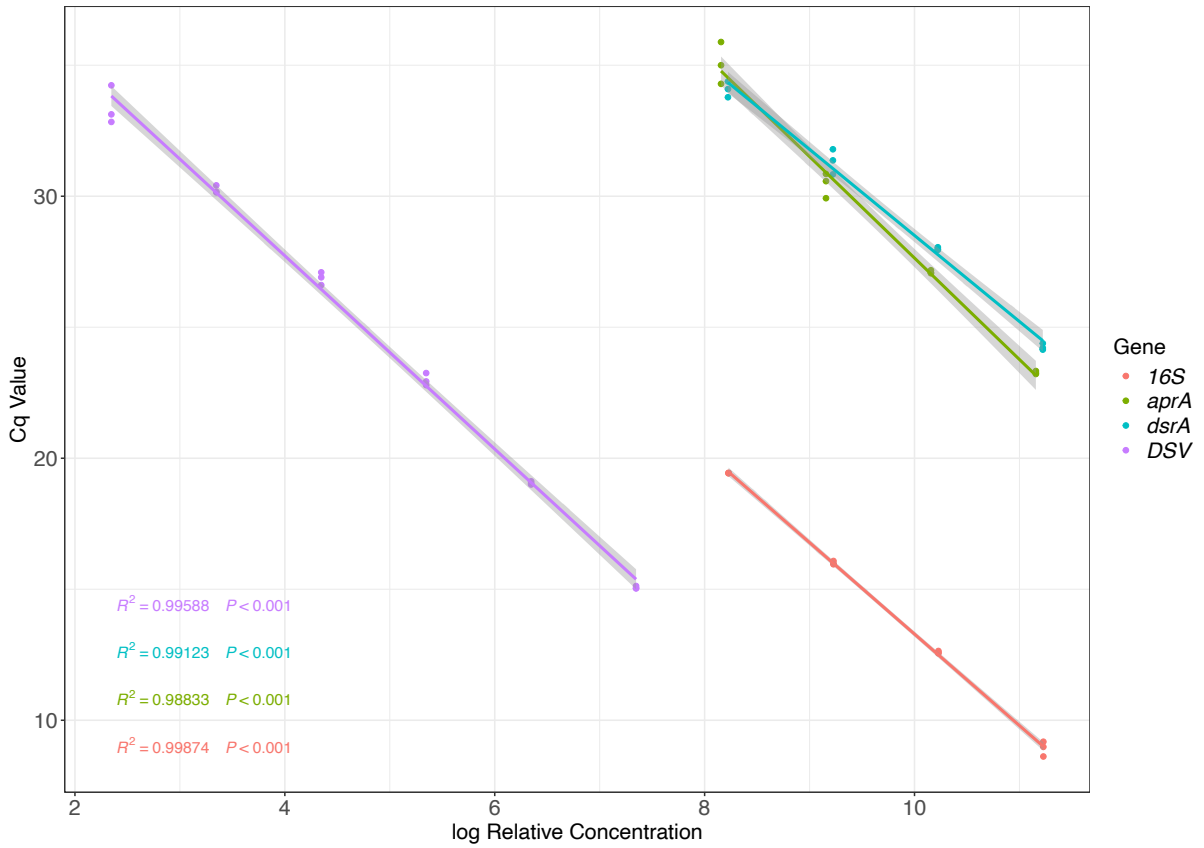


Figure 27 The amplification efficiency of the four primers used in qPCR.

5.3.3 Quantification of bacterial and *Desulfovibrio* communities using qPCR

Having shown no overall effect of sulfate availability in the cockroach diet on the relative abundance of the main taxa in the microbiome, we analysed the impact of sulfate on the absolute abundance of bacteria in general and DSV in particular. The abundance of gene copies of universal 16S rRNA of bacteria and *Desulfovibrio* (DSV) is presented in Figure 28. The sulfate supplement had no significant effect on the total bacterial communities in the cockroach guts, with an average of 1.98×10^4, 2.25×10^4, 1.92×10^4, 2.18×10^4, and 2.44×10^4 copies of the 16S rRNA gene per ng DNA for control, 0.1% sulfate, 0.2% sulfate, 0.1% sulfate-con, and 0.2% sulfate-con, respectively (Figure 28A).

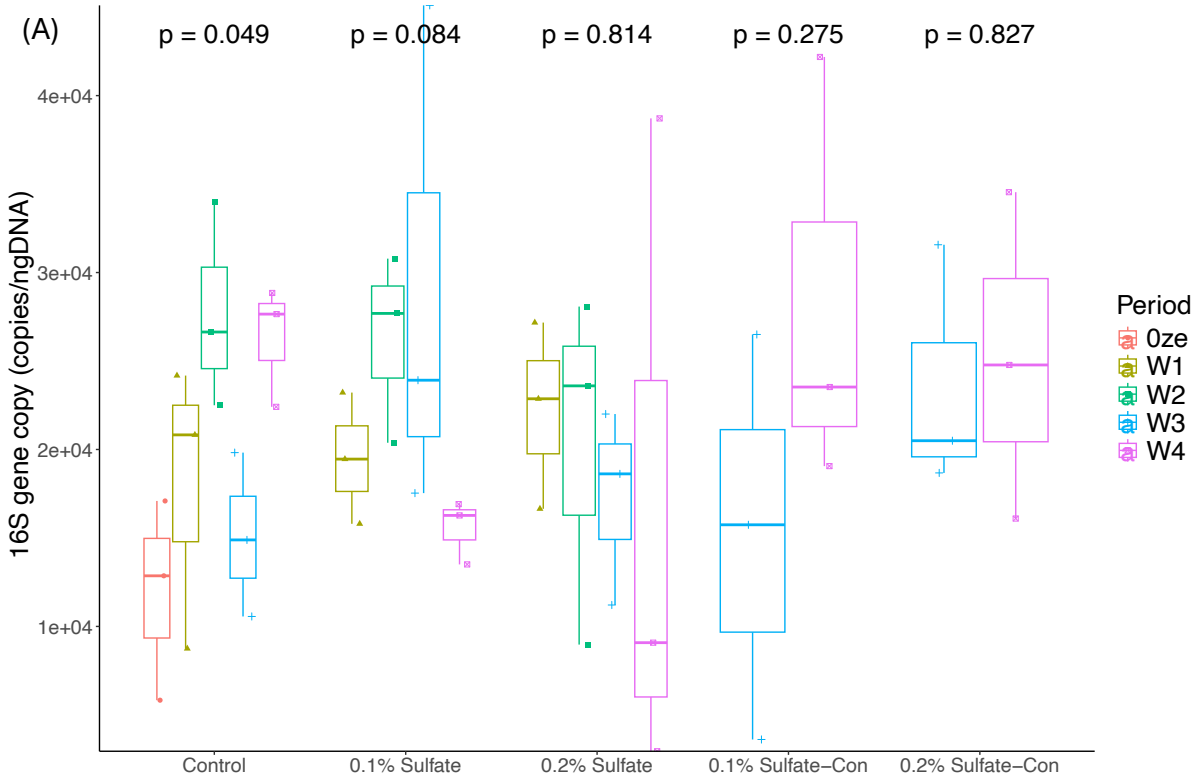
There were no significant differences in the abundance of DSV across treatments (Figure 28C). Interestingly, some treatment groups showed wide variation in the proportion of DSV compared to the total bacteria within the same group. For example, the individual percentages of DSV per total bacteria in the control group before starting the experiment were 13.97%, 43.71%, and 47.69%, respectively. In addition, the DSV percentages ranged from 11.87 to 56.38% in the other control groups.

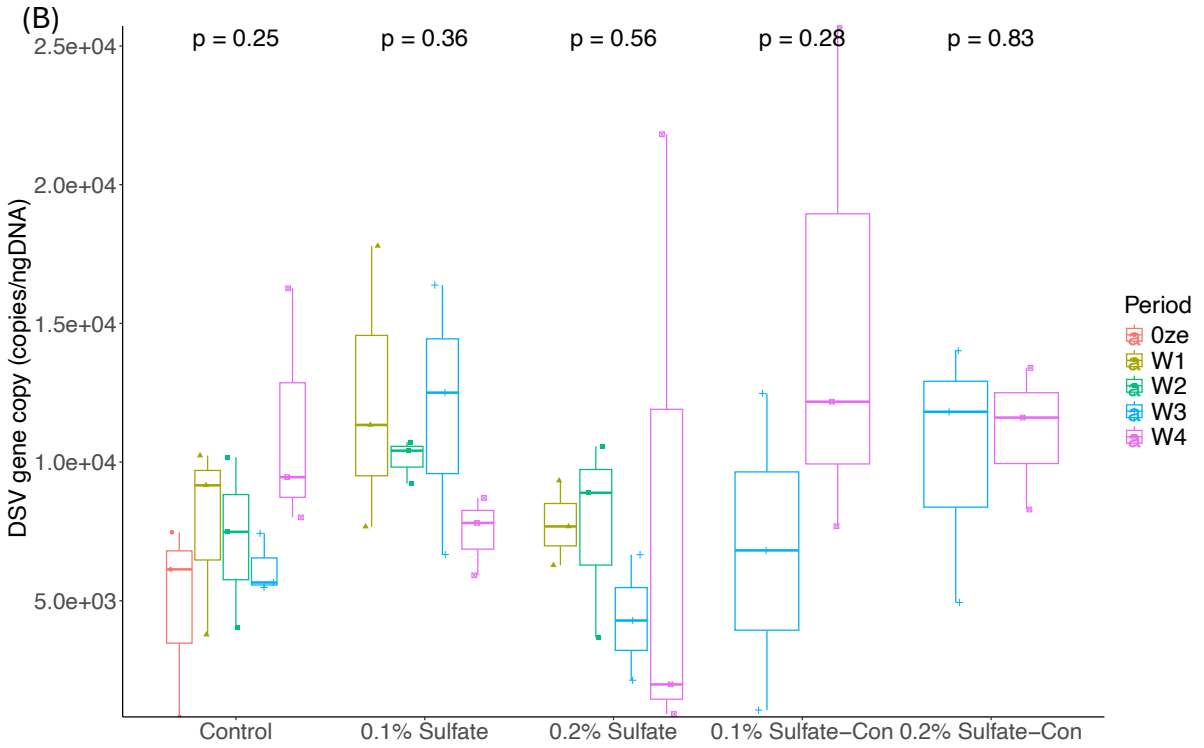
There was the abundant of DSV, ranging from 5.92×10^3 to 1.78×10^4 copies of DSV per ng DNA, with an average of 1.04×10^4 copies of DSV per ng DNA in the 0.1% sulfate group. The DSV abundance of

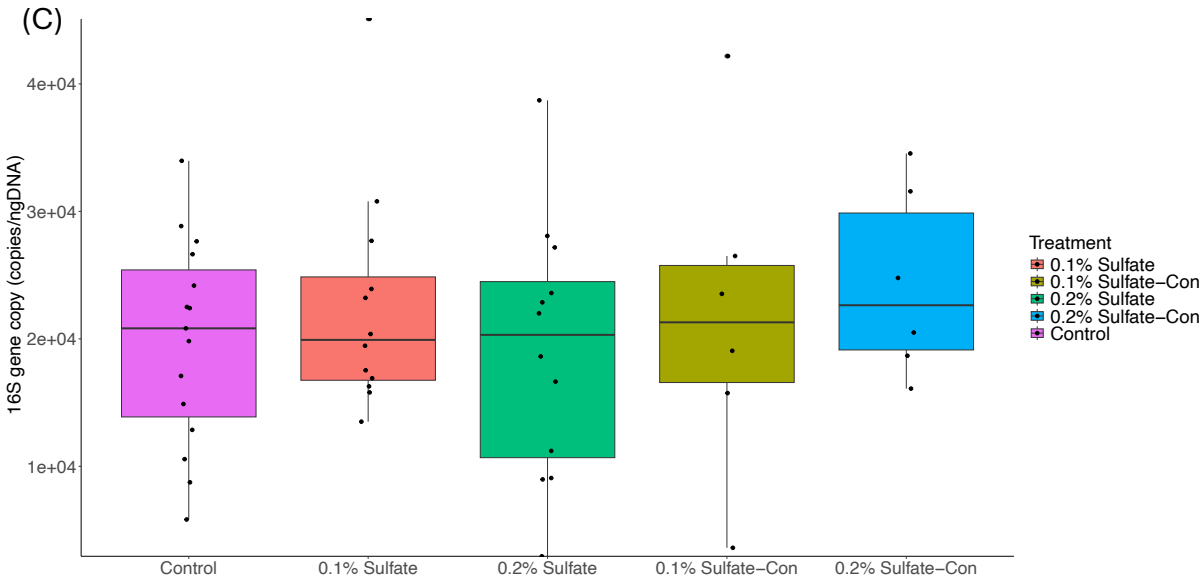
0.1% sulfate-con showed no significant difference at week 3, ranging from 1.06×10^3 to 1.25×10^4 copies of DSV per ng DNA, with an average of 6.78×10^3 copies of DSV per ng DNA. Adversely, the number of DSV was little higher at week 4 after going back to the control diet with an average of 1.52×10^4 copies of DSV per ng DNA.

The DSV abundance of 0.2% sulfate showed no difference from the control group, with an average of 7.02×10^3 copies of DSV per ng DNA. However, after going back to the control diet at week 3 and 4 from starting treated 0.2% sulfate, the number of DSV was a little higher ranging from 4.94×10^3 to 1.63×10^4 copies of DSV per ng DNA with an average of 1.07×10^4 copies of DSV per ng DNA.

When all time points in the same treatment were combined, there were no significant differences between treatments for both total bacteria and DSV (Figure 28C:D).




(B)
p = 0.25
p = 0.36
p = 0.56
p = 0.28
p = 0.83
DSV gene copy (copies/ngDNA)
2.5e+04
2.0e+04
1.5e+04
1.0e+04
5.0e+03
Control
0.1% Sulfate
0.2% Sulfate
0.1% Sulfate−Con
0.2% Sulfate−Con
Period
0ze
W1
W2
W3
W4


(C)
16S gene copy (copies/ngDNA)
4e+04
3e+04
2e+04
1e+04
Control
0.1% Sulfate
0.2% Sulfate
0.1% Sulfate−Con
0.2% Sulfate−Con
Treatment
0.1% Sulfate
0.1% Sulfate−Con
0.2% Sulfate
0.2% Sulfate−Con
Control

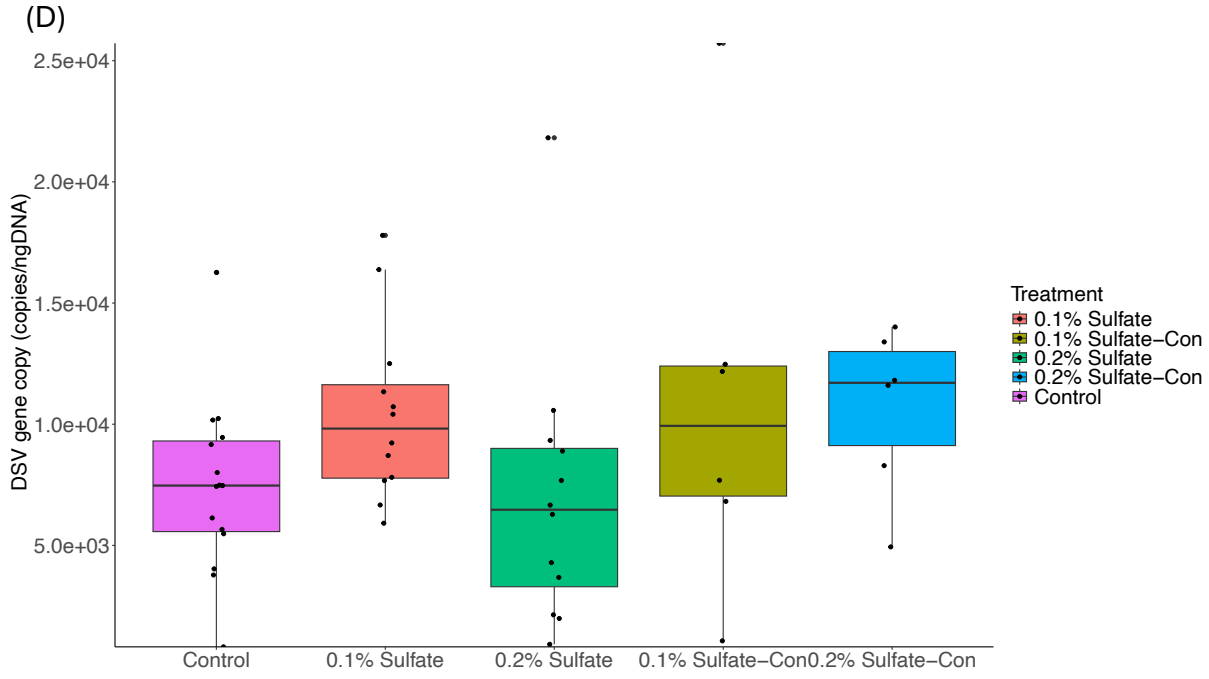


Figure 28 The number of gene copies of the 16S rRNA gene of total bacteria (A) and DSV (Desulfovibrio) (B). Kruskal-Wallis test was used to test the differences between time points in each treatment. Samples from the same treatment were grouped and shown in total bacteria (C) and DSV (D). Kruskal-Wallis test and pairwise comparisons using Dunn's test were used.

5.3.4 *dsrA* and *aprA* expressions compared with the 16S rRNA gene of bacterial communities

The expressions of *dsrA* and *aprA* genes in cockroaches' guts were estimated using qRT-PCR by normalizing with the expression of 16S rRNA genes of total bacteria from the control diet before starting the experiment. The expressions of *aprA* and *dsrA* were expressed consistently across almost all samples from different treatments and time periods. There were no significant differences in both *aprA* and *dsrA* expressions between time points within the same treatment ($P > 0.05$; Kruskal-Wallis test) (Figure 29). Moreover, the expression of *dsrA* was higher than *aprA* in all samples (Figure 29). There were no significant differences between the treatments in a pairwise test performed the same week between the control and the other treatments ($P > 0.05$; Wilcoxon test, Appendix 1).

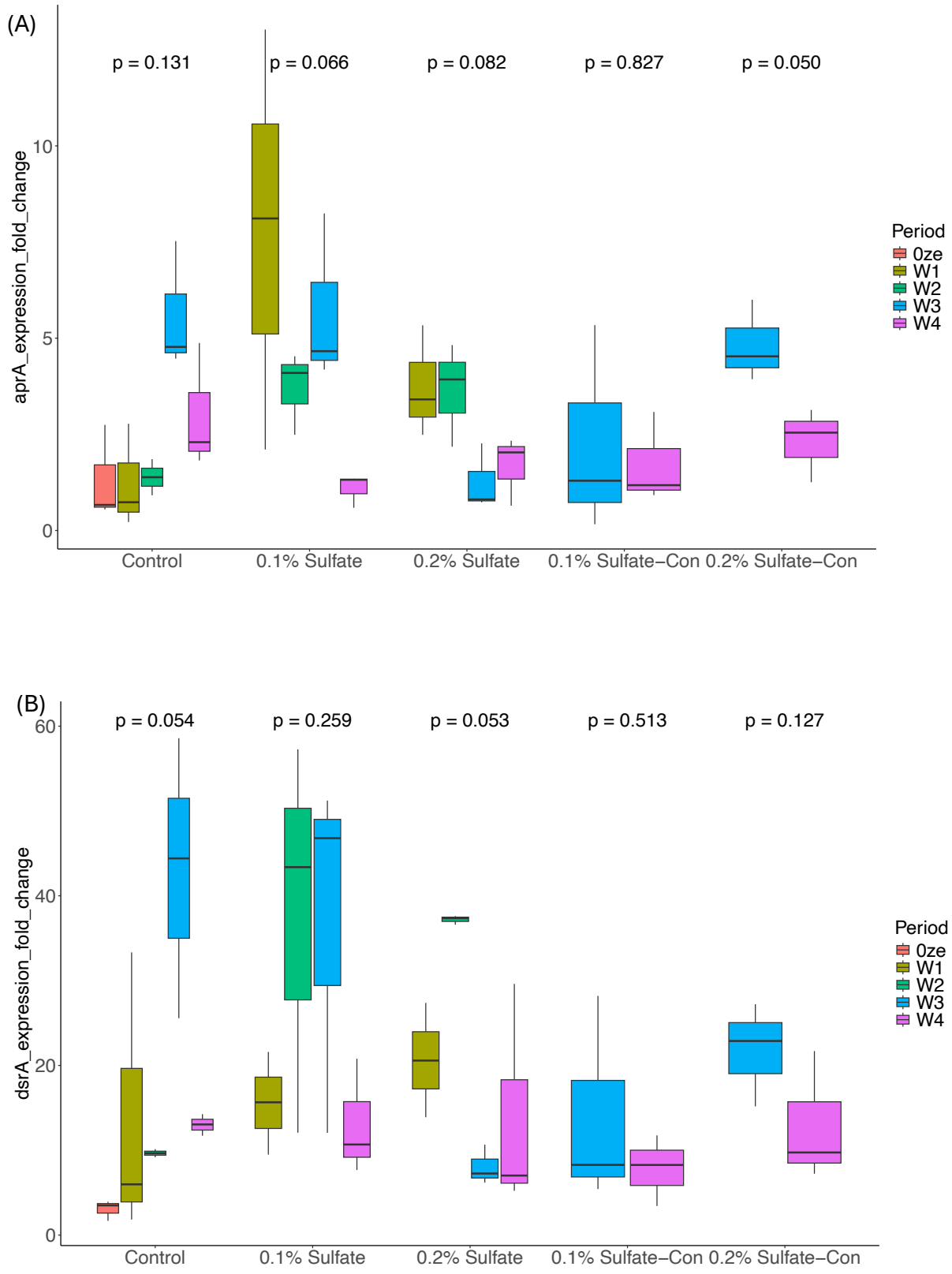


Figure 29 The expression levels of the genes dsrA (A) and aprA (B), which are involved in the dissimilatory sulfate reduction of Desulfovibrio spp. in the cockroaches' guts, are represented in the bar charts. The qRT-PCR was used to measure the gene expressions in triplicate. The Kruskal-Wallis test revealed no significant change in the aprA and dsrA genes between time points in each treatment (P > 0.05).

5.4 Discussion

It is as yet unknown how *Desulfovibrio* and human gut inflammatory diseases interact (Chen et al., 2021b; Singh, Carroll-Portillo and Lin, 2023). This bacterium is not just involved in gut inflammation; it can be detected in other human diseases (Singh, Carroll-Portillo and Lin, 2023). In our study, the sulfate supplement in drinking water and together with their normal sterile diets treated in the adult female American cockroaches were investigated. Based on the Illumina sequencing data, the results showed that the sulfate supplement had effects on neither the cockroach gut microbiome nor *Desulfovibrio*. Although the cockroaches received a sulfate supplement for as long as one month, the DSV populations were not affected by these extended feeding durations. However, the relative abundance could not completely illustrate the gut microbiome as much as the absolute abundance does. The absolute abundances of bacterial communities and *Desulfovibrio* were examined using qPCR. Nevertheless, total bacterial copies and *Desulfovibrio* copies did not significantly differ across treatments, according to the qPCR data.

The influence of the sulfate was minor, as evidenced by the 0.1% sulfate diet slightly increasing DSV, even though there was still no statistical difference between treatments. Remarkably, raising the sulfate to 0.2% had no effect on the DSV abundance, with the exception of week 4, when there was a variation across three replicates. It is interesting to note that, in contrast to 0.1% sulfate, which slightly influenced the expression of genes and DSV abundance, a higher level of sulfate (0.2%) had no effect on even bacterial communities or the abundance of *Desulfovibrio*. Overall, neither the DSV nor the other bacterial populations were affected by the rising sulfate concentrations.

Desulfovibrio is capable of using sulfate in dissimilatory sulfate reduction, which produces hydrogen sulfide as the end product. The *aprA* and *dsrA* genes are two of the most important genes in this pathway (Grein et al., 2013). The qPCR results showed that the sulfate supplement had a minor effect on the number of DSV, but it is interesting to note that the expression of *aprA* and *dsrA*, which are involved in the DSR, was little elevated in the sulfate supplement group, notably *dsrA* expression, which was much higher than *aprA* expression. However, these subunits are not restricted to *Desulfovibrio*; they can be found in other bacteria as well. These genes spread among certain bacteria for a number of reasons, such as lateral gene transfer and vertical transmission from their parents (Loy, Duller and Wagner, 2008). These genes are also detectable in other sulfate-reducing microorganisms such as *Desulfotomaculum* spp., *Desulfobacter* spp., and *Desulfobacula* spp. (Zverlov et al., 2005). *Desulfovibrio* was also altered by the ages of the cockroach as they were the predominant genus in the nymphs and adults in the American cockroach (Chen et al., 2020b). However, different protein contents in diets influenced the gut microbiome in German cockroaches, as *Desulfovibrionaceae* abundance

was lower in cockroaches on a high-protein diet (Pérez-Cobas et al., 2015). Moreover, other sulfate-reducing bacteria inhabit the intestinal organs of the cockroaches in our studies, including *Desulfosarcina* and *Desulfobotulus*. Apart from sulfate-reducing bacteria, our study found *Methanimicrococcus* of Archaea from the *Euryarchaeota* phylum, which some methanogens possess enzymes in sulfate and sulfite respiration (Wagner et al., 1998). Methanogens are normally present in cockroaches (Kane and Breznak, 1991; Domínguez-Santos et al., 2021). These results support that we detected the high expression level of *dsrA* and *aprA* genes, which was not quite according to the number of *Desulfovibrio.*

Although unlike the dominant Archaea found in cockroaches, *Methanobrevibacter smithii* is the predominant methanogen in the human gut (Eckburg et al., 2005). Moreover, methanogens also inhabit termites (Brune, 2018). Several termite species, including flagellate termites, have been shown to harbour *Desulfovibrio* in their guts (Sato et al., 2009b).

Apart from dissimilatory sulfate reduction, assimilatory sulfate reduction is a metabolic pathway in prokaryotes such as *Escherichia coli* that uses sulfate to synthesize the amino acid cysteine (Kushkevych et al., 2020b). It is interesting to note that both *E. coli* (Pai, Chen and Peng, 2005; Akbari et al., 2015) and *Desulfovibrio* are found in the intestinal organs of the American cockroaches (Lee et al., 2020; Tinker and Ottesen, 2021). This is also one of the explanations for supporting the possibility that different bacteria might utilize sulfate in both assimilatory and dissimilarity sulfate reduction.

In volunteers ingesting sulfate supplements, Lewis and Cochrane observed enhanced faecal sulfate reduction rate and faecal H_2S excretion (Lewis and Cochrane, 2007). Humans were fed diets containing low and high concentrations of sulfur-containing amino acids for 10–14 days. The findings indicated that diets had no effect on microbial populations or *Desulfovibrio* (Dostal Webster et al., 2019). Interestingly, individual variations existed in *Desulfovibrio* abundances. Small sample sizes were a constraint of this study, however (Dostal Webster et al., 2019).

However, many studies discovered the associations between dietary consumption and the human gut microbiome over a short-term or long period of consumption (David et al., 2014; Leeming et al., 2019). On the other hand, after treating the American cockroaches for a month with a 0.1% or 0.2% sulfate supplement, we discovered that the bacterial populations in them did not dramatically change. Tinker and Ottesen (Tinker and Ottesen, 2016b) investigated several diets, such as butter, honey, white flour, whole wheat flour, or mixed diets, for two weeks. The study showed that the gut microbiota of American cockroaches remained consistent after all treatments (Tinker and Ottesen, 2016b). Inversely, some bacterial genera were altered in the American cockroaches fed with purified synthetic diets for 14 days (Dockman and Ottesen, 2024b). Interestingly, the American cockroaches

on high-fiber diets produced less acetate and lactate than those fed a normal dog chow diet. Furthermore, scanning electron microscopy revealed that cockroaches fed high-fiber diets have fewer lactic acid bacteria in their foreguts (Kane and Breznak, 1991).

Interestingly, the bacterial populations in the cockroaches' guts did not significantly alter between the control and sulfate-supplemented groups. Sulfate reducers, which are found in members of the *Desulfobacteria* family in the cockroach (Dukes, Tinker, and Ottesen, 2023). In our investigation, these microbes may be able to use the sulfate in sufficient amounts resulting in their bacterial communities remaining unchanged.

As in humans, our gut microbiome is significantly influenced by what we consume (De Angelis et al., 2019). Nonetheless, a number of investigations into how diets affect the intestinal microbiota of cockroaches have produced varying findings. The gut microbiota diversity of cockroaches can be influenced by their foods, according to some research (Pérez-Cobas et al., 2015; Zhu et al., 2023; Dockman and Ottesen, 2024a), while other studies show that the gut microbiome remains rather stable after dietary changes (Tinker and Ottesen, 2016b; Lampert, Mikaelyan and Brune, 2019). Remarkably, the complex or whole food diet experiments showed no effect on the gut microbiota, whereas the experiments utilizing synthetic diets were related to a shift in the gut microbiota. Dockman and Ottesen's study suggested that using whole food diets may require several microbes to work on food digestion, resulting in a stable gut microbiome, while synthetic polysaccharide diets that contain specific nutrients may narrow to a smaller number of bacteria specific to each diet type, causing a drastic shift in the gut microbiome (Dockman and Ottesen, 2024a). Even though they were supplemented with either 0.1% or 0.2% sulfate, the dog diet offers complex and sufficient nutrients, which may be an explanation for our experiment's consistent gut microbiota.

Understanding the distinctions and similarities between the cockroach and human immune systems is crucial for developing the cockroach as a model of intestinal inflammation in humans. The two major innate immune responses in cockroaches are the Toll and immune deficiency (IMD) signaling pathways (Li et al., 2018). The IMD pathway is activated by Gram-positive bacteria and fungi, whereas the Toll pathway is essential in resisting Gram-negative bacteria (Tzou, 2002). These processes result in the production and release of AMPs into the hemolymph, where they can travel throughout the body and destroy invasive microbes (Igboin, Griffen and Leys, 2012). These innate immune responses are also comparable to tumor necrosis factor-α (TNF-α) and Toll-like pathways in humans (Sheehan et al., 2018b). Insects mainly have an innate immune system, whereas humans have both an innate and an adaptive immune system (Jiminez et al., 2015). The absence of an adaptive immune system, which is crucial for humans, is one of the cockroach's main limitations. Furthermore, there are notable distinctions between the mechanisms and components of each response in mammals and insects,

including humoral receptors, antimicrobial peptides, cascades, and enzymes between mammals and insects (Sheehan et al., 2018b). For example, human Toll-like genes are important in the synthesis of cytokines upon pathogen recognition (Akira and Hemmi, 2003), but Toll signaling is crucial in insects' synthesis of AMPs in response to invading microbes (Sheehan et al., 2018b).

According to Martínez-Porchas et al. (Martínez-Porchas, Villalpando-Canchola and Vargas-Albores, 2016), the metagenomic of 16S rRNA gene sequencing may not be sensitive enough to identify minor changes in the gut microbiome. In addition, compared to an absolute abundance, the Illumina sequence data's representation of bacterial communities' relative abundance may be deceptive (Gloor et al., 2017). More importantly, the small sample size is a limitation in our study, as there are only three samples in each treatment, even though there are two or four time points for each treatment. Even though DSV abundances in the 0.1% sulfate group were slightly higher than in the control group, there was still no significant difference across treatments. One potential restriction of the statistical test could be the small sample size. Finally, we could conclude that neither *Desulfovibrio* nor the microbial communities in our investigation were impacted by the sulfate supplement. It will be crucial to increase the sample size in order to obtain more precise results.

Chapter 6: Summary and General Discussion

6.1 Summary

The human gut microbiome has been extensively studied for several years after the human microbiome project. In order to handle ethical issues in research on humans, the majority of studies use animal models, particularly mouse models. Even though mouse studies are extensively used, there are certain drawbacks, such as limited replication, and the 3Rs (replacement, reduction, and refinement) are widely encouraged in the use of vertebrate animals in scientific research (MacArthur Clark, 2018). This is worth considering an alternative human gut microbiome model as the cockroach. Our objective is to investigate the cockroach as a model of the human gut microbiome. Firstly, we utilized publicly available data to compare the bacterial communities of cockroaches, humans, and mice (Chapter 2). In the following chapter (Chapter 3), two distinct antibiotics, kanamycin, and ampicillin, were used to eradicate some bacteria and reduce the colonization resistance of the gut microbial populations. Additionally, the same two distinct antibiotics were used before studying the commensal *E. coli* colonization in the cockroach (Chapter 4). Finally, we observed the effects of sulfate on the cockroach's gut microbiome, specifically the sulfate-reducing bacterium, *Desulfovibrio*.

6.1.1 Meta-analysis

In our first chapter, we compare the microbial communities in the guts of mice, cockroaches, and humans using partial 16S rRNA gene analysis from publicly available data from the GenBank database. The results illustrated that all mouse, cockroach, and human gastrointestinal tracts are dominated by the phyla *Firmicutes* and *Bacteroidota*. It is intriguing that cockroaches exhibit higher species richness and variety in bacterial communities than do humans and mice. More importantly, our meta-analysis confirmed that not only their shared bacterial phyla between cockroaches and humans, but also their bacterial genera are also shared between them, namely, *Bacteroides, Alistipes, Christensenellaceae* R-7 group, *Parabacteroides,* and *Desulfovibrio*. These bacterial genera are predominant microorganisms and play an important role in human intestinal health and human fitness. We suggest the American cockroach might be a worthwhile choice to use as a human gut microbiome model. In addition to sharing some bacterial taxa with humans, the study can yield lower costs and more effective results by increasing the number of repetitions, shortening the life span, and requiring less space and consumption. For this investigation, American cockroaches are recommended.

6.1.2 Antibiotic treatment

The primary objective of our study is to suggest commensal bacteria from the human gut into the cockroach stomach to develop the cockroach as a model of the human gut. To accomplish this, we had to deal with the concept of colonization resistance, in which the host microorganisms attempt to outcompete the invading ones (Lawley and Walker, 2013). To overcome this phenomenon, a germ-free condition and antibiotics have been utilized frequently in investigations on animals. Our first goal was to get rid of some bacteria that were present in the cockroach guts. Kanamycin and ampicillin were chosen in our study. We tried the varied concentrations at 0.1, 0.2, and 0.3 $mg \cdot ml^{-1}$. The findings indicated that certain antibiotic concentrations had an effect on microbial communities on days 10 and 20 while not being toxic to cockroaches. These results led to our next experiment, in which cockroaches were fed kanamycin and ampicillin at a concentration of 0.3 $mg \cdot ml^{-1}$ for 10 days before their changes in the gut microbiome were determined. Overall, both kanamycin and ampicillin could eliminate some of the cockroach gut microbiome. More importantly, different antibiotics, kanamycin and ampicillin, alter distinct bacterial communities. The cockroach gut microbiome given kanamycin exhibits similarities to the control group, but with reduced community abundance. Conversely, the bacterial communities fed ampicillin experienced significant alterations and had a lower abundance than those fed either kanamycin or control.

Using Illumina sequencing, we were unable to determine the exact effect of antibiotics on quantitative bacterial populations. Therefore, the absolute abundance of the bacterial populations was measured by qPCR. Kanamycin is an aminoglycoside antibiotic with broad-spectrum activity that inhibits protein synthesis in both Gram-negative and some Gram-positive bacteria by binding to the bacterial ribosome (Fosso, Li and Garneau-Tsodikova, 2014). Interestingly, kanamycin significantly affected *Proteobacteria* and this effect could be seen on day 10 and particularly on day 20, applying the absolute abundance by comparing Illumina sequencing to qPCR data. Kanamycin also caused a slight decrease in the amount of *Desulfobacterota* on day 20. Kanamycin 0.2 $mg \cdot ml^{-1}$ was studied on two generations of *Blattella germanica* (German cockroaches), and it was discovered that the antibiotic's effects were low on the first generation but greater on the second. Due to this, *Fusobacterium* and *Desulfovibrio* were largely eradicated in the second generation (Domínguez-Santos et al., 2021). *Bacteroidota* phylum increased after 10 days of kanamycin treatment, and some bacterial genera, including *Alilipes, Bacteroides, Dysgonomonas, Parabacteroides,* and *Rikenellaceae*, increased. However, they were eradicated after day 20. This is because *Bacteroides* are resistant to aminoglycoside antibiotics and they can proliferate after those sensitive microbes are eliminated

(Garrett and Onderdonk, 2015). However, when treated for an extended length of time (20 days) in our study, it was able to eradicate the majority of *Bacteroidota* genera.

Ampicillin is a beta-lactam antibiotic that is effective against both Gram-positive and some Gram-negative bacteria (Bear, Turck and Petersdorf, 1970). In our study, ampicillin considerably affected the bacterial populations in the cockroach guts, in contrast to the control and kanamycin groups. Ampicillin treatment decreased *Bacteroidota* and *Firmicutes* since it was continuously fed for 10 days. In contrast, this antibiotic offered *Proteobacteria* the opportunity to increase since day 10 and to increase even more by day 20. This phenomenon could be caused by a number of factors, including ampicillin resistance or proliferating after the other bacteria, have been eradicated. It is possible that the absence of competitors for their resources in the gut environment was caused by the decline of *Firmicutes* and *Bacteroidota*. In contrast, *B. germanica* was treated with ampicillin at 0.02% (w/v) for 10 and 30 days (Domínguez-Santos et al., 2020). They noticed a slight decline in two bacterial families belonging to the phylum *Bacteroidetes*. It was likely that the impact on the microbial communities was stronger because we applied a higher concentration at 0.3 $mg \cdot ml^{-1}$ than they had at 0.2 $mg \cdot ml^{-1}$. However, ampicillin 0.025% for 21 days in adult German cockroaches had an impact as it was found that the microbial communities were almost entirely disappearing. Beta-lactam antibiotics have been proven to be ineffective against *Proteobacteria*.

This chapter provided confirmation that some bacterial populations in the cockroach guts could be eliminated by the antibiotics, kanamycin and ampicillin, particularly in the ampicillin group, which had different and less abundant bacterial communities. Given that this treatment may lessen the cockroach gut's colonisation resistance. It may pave the way for our upcoming experiment, which involves introducing commensal *Escherichia coli* into the cockroach gut.

6.1.3 *Escherichia coli* inoculation

Escherichia coli (*E. coli*) is introduced as a commensal bacterium in humans (Conway and Cohen, 2015b). This bacterium was inoculated into the intestinal cockroach in our study. Antibiotic treatment was used to overcome the colonization resistance in the cockroach gut. Following ten days of antibiotic treatment, the cockroaches were given *E. coli* strain MG1655 for three consecutive days. In the control and kanamycin groups, we discovered that the bacterial populations did not significantly alter regardless of whether *E. coli* was inoculated or not. It was because inoculated *E. coli* was eliminated when they attempted to invade the cockroach guts.

It is interesting to note that the microbial population differed between the ampicillin-treated groups with and without *E. coli*. We found that *Firmicutes* and *Bacteroidota* were reduced when E. *coli* was

inoculated, whereas *Proteobacteria* were increased. However, we detected *Escherichia-Shigella* in one out of three samples from the ampicillin group on day 1. Despite the fact that they were eradicated after three days, it appeared that *E. coli* could not survive inside the cockroach intestines. *Serratia* and *Acinetobacter*, however, were also discovered in high numbers in samples 2 and 3 from the *E. coli*-treated group, respectively. Then, *Serratia* was dominant across all three samples on day 3. On the other hand, it disappeared after day 7.

Originally, *Klebsiella* predominated in the cockroach gut following treatment with ampicillin from our earlier study. Only one out of three samples showed positive for *E. coli* after an inoculation of a commensal *E. coli* into the cockroaches. The study interaction between *K. pneumoniae* and *E. coli* found that *E. coli* were shown to be outcompeted at the beginning, but they could coexist for a longer period (Juarez and Galván, 2018). It might be possible if we could plan the experiment so that they could coexist and support one another inside the cockroach's stomach.

In conclusion, the results showed that invading *E. coli* could not survive in the intestinal organ of the cockroach. This could be because the invading *E. coli* could not overcome the colonization resistance of the indigenous microorganisms in the cockroach guts. However, we could detect the bacterial interaction between *E. coli* and other bacteria such as *Serratia* that increased their abundance after *E. coli* inoculation in ampicillin treatment.

6.1.4 Sulfate supplement diets and *Desulfovibrio*

However, because cockroaches have a diverse microbiome in their intestinal organs, it is intriguing to research the microbial population in the cockroaches. It is worth studying the interaction between microorganisms and their interaction within hosts, and this fundamental knowledge could be linked with our human gut microbiome. In a recent experiment, *Desulfovibrio* was discovered as commensal bacteria in cockroaches, and this bacterium has also been found in the human gut microbiome. This bacterium is known as sulfate-reducing bacteria (SRB), and they produce hydrogen sulfide gas and are involved in human gastrointestinal diseases, such as inflammatory bowel disease, colorectal cancer, and gut inflammation (Singh, Carroll-Portillo and Lin, 2023). However, we are still uncertain about their relationship with humans. By treating this bacterium with various types of diets, whether containing high or low levels of sulfate components, the goal is to try and manipulate it and learn how it interacts with the host.

Increase the sulfate to 0.1% and 0.2% sulfate diets, and for up to a month, the cockroaches were given a sulfate supplement. The results demonstrated that the sulfate supplement had no influence on *Desulfovibrio* or the cockroach gut microbiome. The gut microbiome cannot be fully illustrated by

relative abundance in the same way that absolute abundance can. The qPCR was used to determine the absolute abundances of *Desulfovibrio* and total bacteria. The qPCR results confirmed that neither total bacterial copies nor *Desulfovibrio* copies varied significantly between treatments.

Quantitative reverse transcription-PCR (qRT-PCR) was used to assess the expression of two SRB genes, *aprA* and *dsrA*. *Desulfovibrio* is able to use sulfate in dissimilatory sulfate reduction, which results in the production of hydrogen sulfide. The *sat, aprAB*, and *dsrAB* genes are among the most crucial genes in this pathway (Grein et al., 2013). In general, in all samples from different treatments and times, the expressions of *aprA* and *dsrA* were consistent. Within the same treatment, there were no significant differences in the expressions of either *aprA* or *dsrA* between time points. However, *dsrA* expression was greater than *aprA* expression in all samples. This is intriguing because, according to the DSR pathway, we expected that both genes would appear in a comparable amount. The *aprA* and *dsrA* expressions in the 0.1% sulfate group were slightly greater than in the other treatments, but they were not significantly different. The fact that we only have three replicates for each time point and treatment is one of the study's weaknesses. Additional replication will provide additional insight into the actual impact of the sulfate on the expression of both genes.

However, the *Desulfovibrio* that was expected to interact with the sulfate remained in the same abundance relative to the control as we raised the sulfate concentration in the diet. There could be other explanations for it. Other microbes that are capable of using sulfate may exist. However, *Desulfovibrio* is not only the sulfate-reducing bacteria harbor in the cockroach intestinal organs. *Methanimicrococcus* of the phylum *Euryarchaeota*, which are methanogens having enzymes involved in the respiration of sulfate and sulfite (Wagner et al., 1998). Methanogens are usually found in cockroaches (Kane and Breznak, 1991; Domínguez-Santos et al., 2021).

6.2 General discussion

We proposed using the cockroach as a model of the human gut microbiome. In order to develop this model, we first compared the bacterial communities of humans, cockroaches, and well-known lab mice (Chapter 2). It is interesting to note that cockroaches have a more diversified gut flora than mice and humans. The findings revealed the bacterial communities that humans and cockroaches share. Compared to previous well-known animal models, differences in the dominant gut microbial taxa between humans and other animals were observed. While humans (MetaHIT Consortium (additional members) et al., 2011; The Human Microbiome Project Consortium, 2012; Frioux et al., 2023b), mice (Yang et al., 2019; Yang and Chun, 2021), and cockroaches (Tinker and Ottesen, 2016a, 2021; Chen et al., 2023) are mainly dominated by *Bacteroidetes* and *Firmicutes*, the microbiome in *Drosophila*

(Broderick and Lemaitre, 2012) and honeybees (Moran et al., 2012; Moran, 2015) are mainly composed of *Firmicutes* and *Proteobacteria*. Additionally, our meta-analysis indicated that humans and cockroaches share not just bacterial phyla but also bacterial genera. Compared to mammals, the bacterial communities of the *Drosophila* are far less diverse (Chandler et al., 2011). Moreover, certain anaerobic bacteria are most prevalent in the digestive organs of humans and cockroaches. Inversely, they are rarely detected in commonly used animal models like *Drosophila* and mice. In conclusion, cockroaches would be a more interesting model for gut microbiome research due to their extremely diversified gut microbiota, which has some common bacteria with humans.

In the following chapter (Chapter 3), the study's objective was to use antibiotics to eradicate some bacteria and weaken the microbial communities' colonisation resistance. Our research confirmed that each antibiotic influenced the gut microbiota of cockroaches. In comparison to the control, the cockroaches' bacterial communities were reduced after receiving antibiotic treatments. The bacterial communities were affected differently by each medication. Specifically, the bacterial communities in the ampicillin treatment were quite distinct from those in the kanamycin treatment, which showed bacterial taxa that were comparable to the control. Next chapter, we investigated the commensal *E. coli* colonization in the cockroach (Chapter 4). Two different antibiotics, kanamycin and ampicillin, were used. The results revealed that *E. coli* was eliminated from the cockroaches' digestive tracts after the colonization. Nevertheless, we were able to identify the variations in the bacterial populations between the treatments that fed and did not feed *E. coli.* This indicates that the presence of invasive bacteria in the host's gut may change the host's commensal bacterial communities. Our study showed that the cockroach's diversified gut microbiome is resistant to invasive *E. coli*. Our study is the first investigation on commensal *E. coli* colonization in the intestines of cockroaches. Studying bacterial colonization models is challenging, as the hosts' gut microbiomes are resistant to invading microbes colonizing, especially a highly diverse gut microbiome in the cockroach. It would be intriguing to consider the studies on how these beneficial bacteria fight invaders.

It is still unclear how *E. coli* colonizes, causes disease, or interacts with other microbes in humans. The majority of studies focus on the colonization of pathogenic *E. coli*, while commensal *E. coli* has not been extensively studied. However, mice have been extensively used in colonization models by administration of streptomycin sulfate prior to the colonization (Wadolkowski, Laux and Cohen, 1988; Gauger et al., 2007). Furthermore, it is suggested that commensal colonizer succession could be facilitated by providing colonized *E. coli* with appropriate nutrients (Doranga et al., 2024). The development of a cockroach colonization model will require greater attention to a number of factors that support colonization, such as reducing the host's colonization resistance, the use of antibiotics or

a germ-free model, and providing the invaders with adequate nutrients. Cockroaches and mice have different gut microbiomes (Chapter 2). The fact that cockroaches have a higher gut microbiota than mice is one thing that could be useful. These will help us comprehend how colonized *E. coli* interacts with the other gut microbiota that is shared by humans but different from that of mice.

Desulfovibrio was discovered as the predominant commensal bacteria in the cockroach guts (Chapter 2-5). Hydrogen sulfide, which is produced by sulfate-reducing bacteria (SRB) like *Desulfovibrio*, has been associated with gastrointestinal disorders in humans, including inflammatory bowel disease (Singh, Carroll-Portillo and Lin, 2023; Loubinoux et al., 2002; Singh, Carroll-Portillo and Lin, 2023). In the last chapter, we examined how sulfate affected the cockroach's gut microbiome, including the sulfate-reducing bacteria, *Desulfovibrio* (Chapter 5). The results showed that sulfate supplementation had no effect on the gut microbiota or *Desulfovibrio*. Additionally, there was no significant difference in the expression of two genes (*aprA* and *dsrA*) involved in dissimilatory sulfate reduction across all treatments.

Why sulfate has no noticeable effect on *Desulfovibrio* or other gut microbiomes is unclear. 0.1% and 0.2% were given with magnesium sulfate. Dextran sulfate sodium (DSS), a polymer of sulfated polysaccharide substance, is extensively being used in mouse models to induce inflammation in the gut. In mice models, intestinal damage is induced by administration of 1-5% DSS in drinking water (Mizoguchi, 2012). However, the lack of a pilot test of an appropriate sulfate for the cockroach is one of the study's weaknesses. These concentrations may be appropriate for the commensal gut microbiota and not excessive for them. Alternatively, sulfate could be efficiently used by the cockroach's gut microbiota. Second, in order to obtain more reliable results, the sample sizes for each time point should be increased. Lastly, the force-feeding approach may be worthwhile to take into account as it may provide a reliable and effective method for evaluating infections or bacterial colonization.

This study is the first to use a cockroach as a model of inflammation in the gut. Nevertheless, we were unable to alter the gut microbiota and *Desulfovibrio* using sulfate supplementation. This could open the door to developing a suitable approach for cockroach research. Studies that solely focus on gut microbiome changes are not sufficient to assess gut inflammation, which may be measured using a variety of indicators, such as clinical observations, intestinal microbiota, inflammation markers, immunological responses, metabolic pathways, and so on (Wen et al., 2023). Additionally, we currently have limited knowledge of the molecular pathways by which gut microbes cause IBD, but several microorganisms are thought to be implicated (Kushkevych et al., 2024). Nevertheless, the relatively low diversity of gut microbiota in *Drosophila* and mice has limitations. It would be more

appropriate for other insects, such as cockroaches, that have a highly diverse gut microbiota. If invertebrate models could offer more replicates, be less expensive, and be representative in some studies, it would be advisable to utilize insects rather than vertebrates. The lack of the adaptive immune system, an essential part of the human immune response, makes studying inflammation in insects challenging. We discussed the differences between insects' and humans' immunities in 1.1.3.4 (Chapter 1).

Overall, the diet and living conditions of mice, *Drosophila*, and zebrafish are very different from those of humans. Humans are omnivores, and their diets differ greatly depending on their ethnicity and geographical location, whereas mice, rats, and *Drosophila* are primarily herbivores. Cockroaches may not have as much information as other laboratory animals like fruit flies (*Drosophila melanogaster*), nematodes (*Caenorhabditis elegans*), zebrafish (*Danio rerio*), and honeybees (*Apis mellifera*). However, the increased research and attention on the cockroaches' genetic history and gut flora in recent years (Dukes, Tinker and Ottesen, 2023) would be very beneficial to the investigations. Given their diversified gut microbiome, the fact that they share some anaerobic microorganisms with humans, and their lengthy history with the earth spanning several million years, as well as the fact that they can be raised in an axenic condition, the study can yield lower costs, and more effective results by increasing the number of repetitions, being omnivorous like humans, shortening the life span, and requiring less space and consumption. Cockroaches may be an interesting insect model with their benefits, although they are not a complete representation of human models.

6.3 Future work

It is noteworthy to emphasize that, in comparison to humans and mice, cockroaches have a higher bacterial richness. In addition to harboring several bacterial taxa that are shared with humans, they also contain some pathogenic microbes. Thus, it might be worthwhile to use the cockroach as a model when examining bacterial interactions, bacterial functions, and infectious microbes. Two antibiotics, ampicillin and kanamycin, were used in our investigation. Both medicines change different bacterial populations, but unfortunately, they are unable to reduce colonization resistance and allow commensal *E. coli* to survive within the cockroach's internal organs. It will be encouraged to apply a combination of antibiotics that can eradicate various bacteria. This might make it more likely for the invading bacteria to survive and proliferate throughout the cockroach intestines. Increasing the number of replicates in the experiment will also be worthwhile.

In general, the 16S rRNA gene's variation region offers insufficient details for classifying bacteria at the species level. Furthermore, 16S rRNA amplicon sequencing does not reveal the gut microbiome's

functional capacities. Despite the fact that the metabolic pathway can be predicted by PICRUST2 using the 16S rRNA sequence. Metagenomic data does not provide enough information to predict transcript abundance (Heintz-Buschart et al., 2016). The comparison of the cockroaches' and humans' metaproteome, metatranscriptome, or whole metagenome sequencing, will assist us to better understand their similarities and differences.

A diverse cockroach gut microbiome that shares certain microorganisms with humans (Chapter 2). This will be important to take into account how harmful microbes interact with their hosts or other bacteria. Even though *E. coli* were eradicated from the cockroach's gut shortly after the inoculation (Chapter 4). It illustrated how the presence of invasive bacteria in the cockroach's gut changed the bacterial communities. The gut microbiota of cockroaches has an effective immune system and resistance to colonization. This suggests that the cockroach could be an effective model for learning about invasive microbes in the intestines. It will also be used for studying how insects' natural immune systems respond to invasive microbes. Drawing and comprehending how the innate immune system reacts to invasive microorganisms may benefit from this. Furthermore, cockroaches can live and thrive in a variety of environments, including proximity to human households. They come into contact with human habitats; thus pathogenic microorganisms studies in the cockroach should also be taken into account.

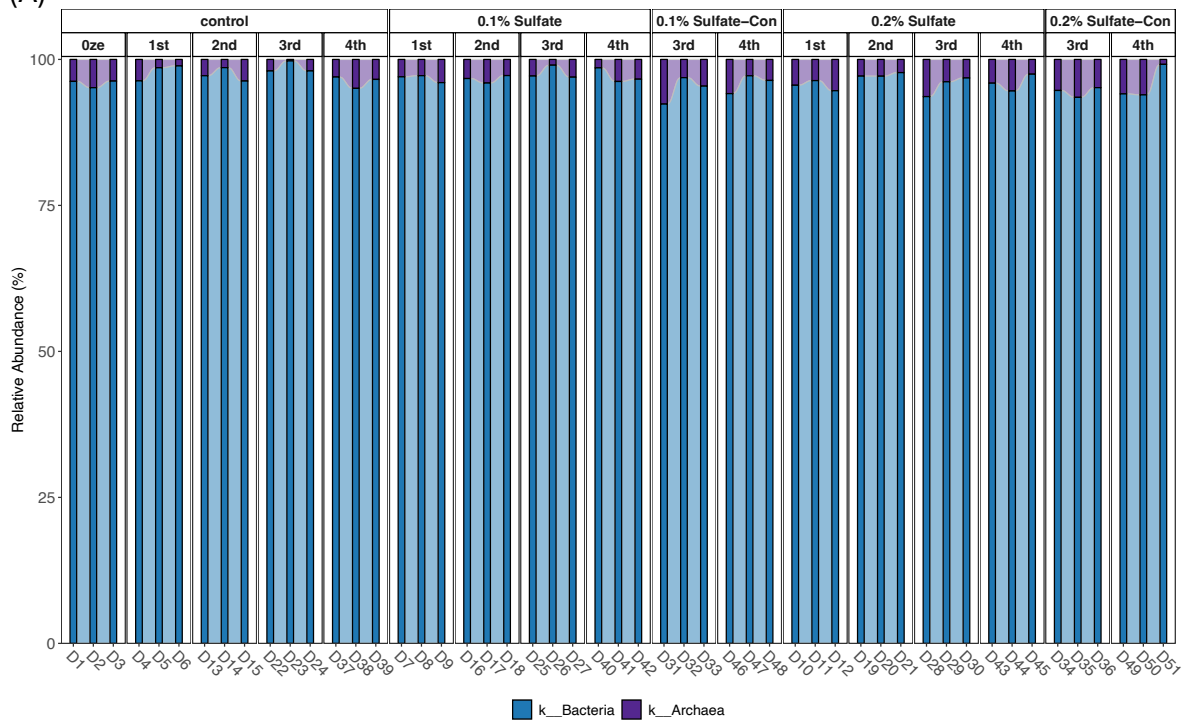
We used two different antibiotics to eliminate some gut microbes (Chapters 3 and 4). Both ampicillin and kanamycin reduced the gut microbiome while affecting distinct bacterial groups. The bacterial populations under kanamycin treatment were still the same bacterial group, although they differed greatly from those under ampicillin treatment. Because the cockroach's gut microbiome contains distinct bacterial communities following treatment with distinct antibiotics, investigations of the effects of dietary intake on the gut microbiome would be beneficial. However, several conflicting findings regarding how diets affect the gut microbiota of cockroaches, though, with some demonstrating that diets had an impact on the microbiota while others found no significant changes (Pérez-Cobas et al., 2015; Zhu et al., 2023; Dockman and Ottesen, 2024a; Tinker and Ottesen, 2016b; Lampert, Mikaelyan and Brune, 2019). This may be because the amplicon next-generation sequencing of the 16S rRNA gene, which shows the relative abundance of the microbial communities, was used in the majority of those investigations. According to studies, when there is a slight alteration in the gut microbiome, the relative abundance might not be sufficient (Vandeputte et al., 2017). Therefore, several methods, including flow cytometry, digital PCR, and qPCR, would be recommended to determine the absolute abundance of the microbial communities. Moreover, studies on transcriptome information and metabolic pathways will be valuable.

Appendix 1: Wilcoxon test was used to compare the expression of *aprA* and *dsrA* across treatments at each time point.

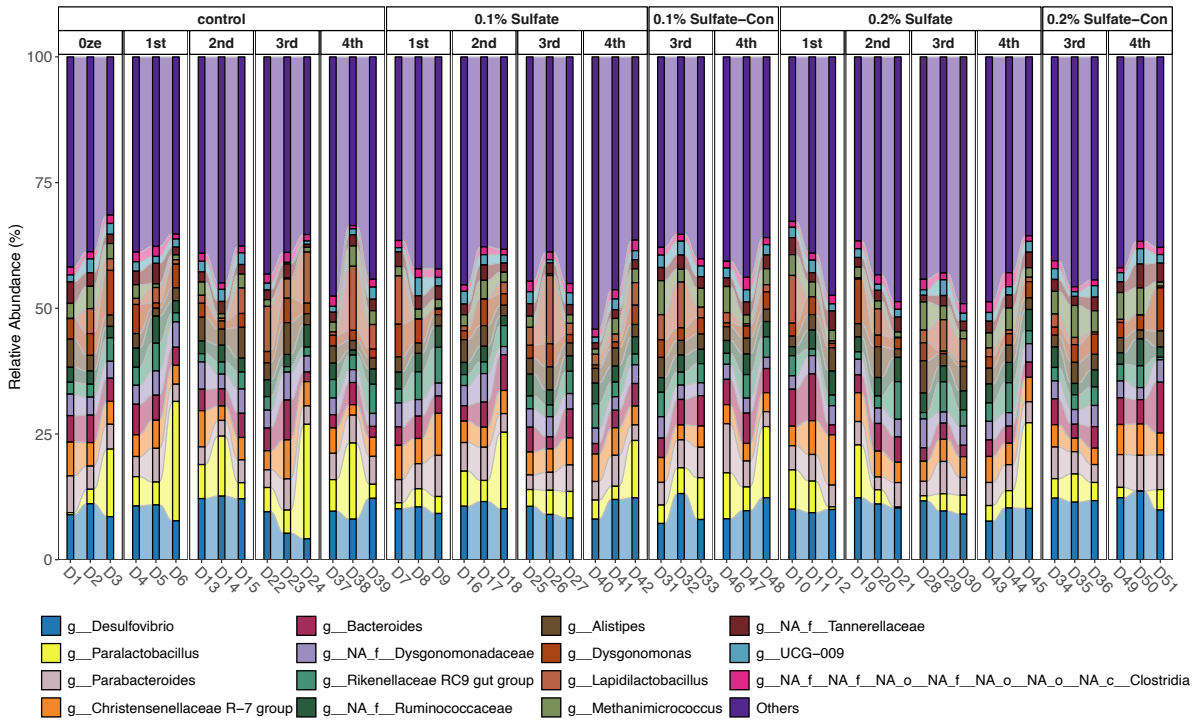
	aprA	***dsrA***
Treatment Comparison	(Wilcoxon test; p value)	(Wilcoxon test; p value)
Week 1		
Control and 0.1% Sulfate	0.2	0.7
Control and 0.2% Sulfate	0.2	0.7
Week 2		
Control and 0.1% Sulfate	0.2	0.2
Control and 0.2% Sulfate	0.2	0.2
Week 3		
Control and 0.1% Sulfate	1.0	1.0
Control and 0.2% Sulfate	0.1	0.1
Control and 0.1% Sulfate-Con	0.4	0.2
Control and 0.2% Sulfate-Con	0.7	0.2
Week 4		
Control and 0.1% Sulfate	0.1	0.7
Control and 0.2% Sulfate	0.7	0.7
Control and 0.1% Sulfate-Con	0.4	0.2
Control and 0.2% Sulfate-Con	1.0	0.7

Appendix 2: The relative abundance of microbes' communities (Archaea and Bacteria) associated with the sulfate supplement groups at the Kingdom (A) and Genus (B) levels.

(A)



(B)


control
0.1% Sulfate
0.1% Sulfate−Con
0.2% Sulfate
0.2% Sulfate−Con
0ze
1st
2nd
3rd
4th
Relative Abundance (%)
100
75
50
25
0
g__Desulfovibrio
g__Paralactobacillus
g__Parabacteroides
g__Christensenellaceae R−7 group
g__Bacteroides
g__NA_f__Dysgonomonadaceae
g__Rikenellaceae RC9 gut group
g__NA_f__Ruminococcaceae
g__Alistipes
g__Dysgonomonas
g__Lapidilactobacillus
g__Methanimicrococcus
g__NA_f__Tannerellaceae
g__UCG−009
g__NA_f__NA_f__NA_o__NA_f__NA_o__NA_o__NA_c__Clostridia
Others

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