A longitudinal study of perturbations on gut microbiota of *Blattella germanica* due to periodic antibiotic treatment

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Jesús Marín Miret

Doctoral Program in Biodiversity and Evolutionary Biology

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Directors:
Prof. Andrés Moya Simarro
Prof. Amparo Latorre Castillo

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Andrés Moya Simarro y Amparo Latorre Castillo, Catedráticos del Departamento de Genética de la Universidad de Valencia,

Hacemos constar que somos los directores de la tesis doctoral de Jesús Marín Miret, estudiante de doctorado del programa de Biodiversidad y Biología Evolutiva de la Universitat de València, titulada **A longitudinal study of perturbations on gut microbiota of Blattella germanica due to periodic antibiotic treatment.**

Asímismo damos nuestro visto bueno al documento de tesis que ha presentado para optar al grado de doctor por la Universitat de València. Lo que hacemos constar a los efectos oportunos en Valencia a 21 de Octubre de 2022.

Andrés Moya Simarro

Amparo Latorre Castillo

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1. Introduction

1.1. Symbiosis

Symbiosis is a widespread phenomenon in nature. The term was coined by Anton de Bary in 1879 in his work "*Erscheinung der Symbiose*" (Bary, 1879). He defined symbiosis as "the living together of unlike organisms". He was the first to propose "that microbes and insects show an amazing biodiversity in themselves, but they often come together and take evolutionary paths to persistent physical association" Symbiosis is the close, long-term biological interaction between two or more species. This phenomenon can be classified depending on many factors. Considering the effects on the biological fitness of the participants, we distinguish between a) **commensalism,** when one of the partners' benefits of the relationship and the other is not negatively affected; b) **mutualism** when both parts of the equation are making a profit (i.e., increasing their fitness); and c) **parasitism,** when one of the members is taking advantage of the other, leading to a decrease of the fitness of the later. According to the location of the symbiont, there are two types of relations: a) **ectosymbiosis,** when the symbiont is located outside the cells of the host, which includes the intestinal tract from mouth to anus, and b) **endosymbiosis,** when the symbiont is confined inside the cells of the host, called bacteriocytes. Lastly, the partnership is **obligate** when the associates cannot survive unattached from each other or **facultative** if the association is not vital for the members (Martin and Schwab, 2012).

Lynn Margulis introduced symbiosis in modern science, postulating a theory about the origin of the eukaryotic cell (Sagan, 1966). Despite the long controversy of her theory, it is now widely accepted that mitochondria and chloroplast originated from free-living bacteria.

Thus, a free-living Alphaproteobacteria and a proto-eukaryotic cell started the endosymbiotic process that led to mitochondria, providing the new cell a mechanism for respiration. The process that led to plant cells was similar. Free-living cyanobacteria and the proto-eukaryotic cell initiated an endosymbiosis, which gave the host the capability of photosynthesizing.

Symbiosis associations have occurred multiple times since the origin of life between groups through the tree of life (McKenna *et al*., 2021; Joy, 2013)). These relationships are mainly based on utilizing biochemical compounds from one of the parts by directly using them or by recycling waste compounds (normally, they are derivatives from the nitrogen metabolism). Most frequently, bacteria are the ones that utilize specific prokaryotic-unique metabolic pathways to take profit from compounds that eukaryotes are unable to use (Moya *et al*., 2008; Feng and Li, 2019; Kneip *et al*., 2007; Schink, 1997; Stewart *et al*., 2005; Minic and Hervé, 2004). Although most iconic symbiosis relationships are between eukaryotes (i.e., the clownfish or hermit crab with the anemones, or fungi with algae, giving rise to lichens), there are also many associations between prokaryotes and eukaryotes (Holman and Gzyl, 2019; Ochman *et al*., 2010; Sundset *et al*., 2007McFall-Ngai, 1994; Mujer *et al*., 1996). Bacteria and archaea have formed associations with a wide range of eukaryotes (Figure 1).

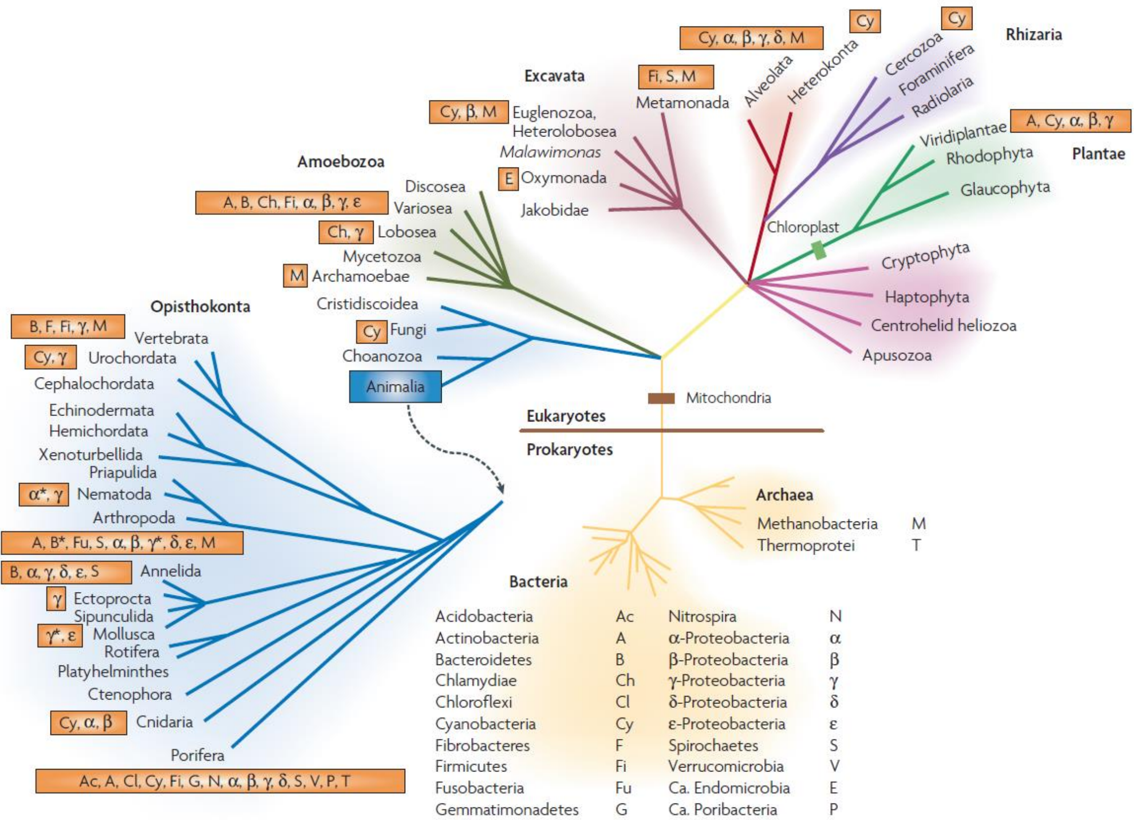

Figure 1. Phylogenetic distribution of symbioses. The class of bacteria and archaea associated with eukaryotes are indicated with one or two letters. Figure extracted from Moya et al. (2008).

Although symbiosis is a diverse and widespread phenomenon through the tree of life, this thesis focuses mostly on the interactions between insects and bacteria. It has been estimated that about 15% of insects have established a mutualistic relationship with bacteria (Buchner, 1965). Interactions between these two groups are widespread and have significantly impacted insect evolution and insects' capacity to colonize different environments (Douglas, 2011; Schmidt and Engel, 2021). The most typical benefit for insects is the capability of feeding on poor-nutrient diets, which are complemented thanks to bacterial metabolism. Thus, the role of the endosymbionts is mainly nutritional (Brune, 2014; Hu *et al.*, 2018; Consuegra *et al.*, 2020) These complete and complement the host's diet (Baumann, 2005). It is, for example, the case of aphids with *Buchnera aphidicola*, which

provides the insect with essential aminaocids; the tsetse flies that feed on blood with *Wigglesworthia glossinidia,* which provides vitamins to the poor diet of the flies; or the carpenter ants with *Blochmannia floridanus* which grants the host with nitrogen and sulfur compounds (Shingenobu *et al*., 2000; Akman *et al*., 2002; Gil *et al*., 2003). Meanwhile, endosymbionts receive a very protective and controlled environment with nutritional resources and the host metabolic machinery. These kinds of associations are the narrowest of the symbiosis. The endosymbiont goes through a process of genome reduction by non-essential gene loss. Due to the intracellular environment, these genes are no longer needed either because of redundancy with host genes or because the new protective environment does not require them. It exists a mutation accumulation process without harm for the endosymbiont. Furthermore, there is also a high genetic drift because the vertical transmission of endosymbionts prompts bottlenecks since only a few endosymbionts are transmitted (Pérez-Brocal *et al*., 2006; Moya *et al*., 2008; Latorre and Manzano-Marin, 2017).

Insects are a very diverse group and maintain not only endosymbiosis with prokaryotes, but also gut ectosymbiosis (Jang and Kikuchi, 2020; Adair *et al*., 2018; Berasategui *et al*., 2016; Coon *et al*., 2014; Romero *et al*., 2019). This phenomenon is widespread in insects and animals in general, including humans (Frank and Pace, 2008; Clemente *et al*., 2012; Thursby and Juge, 2017; Piquer-Esteban *et al*., 2022). The gut has one of the most remarkable microbial communities associated with animals. Usually, insects have a low diverse gut microbiota when compared to mammals (Engel and Moran, 2013; Colman *et al*., 2012; Dillon and Dillon, 2004). But some of them, contrarily, harbor large communities of specialized bacteria (Engel and Moran, 2013). It is the case of social insects like termites or cockroaches (Brune and Dietrich, 2015).

1.2. Insects

Insects (phylum Arthropoda, class Insecta) are the most successful animal group. In the Early Ordovician, they were among the first animals conquering Earth's terrestrial surface around 479 million years ago (mya). Since then, they evolved in almost endless forms that allowed them to live in a wide range of different habitats establishing relationships with other living groups, such as plants and bacteria, which shaped the biota of this planet. Insects were the first animals to develop the flying capacity 406 mya in Early Devonian (Misof *et al.*, 2014). It is undeniable that their early colonization of the surface of Earth and their incredible capability to adapt to different environments has played an essential role in the great diversity of this animal group.

The class Insecta has around one million described species (Zhang, 2013), and the number increases each year with new species assignments, but this does not represent the actual number of species of insects. Recent estimations indicate around 5.5 million species of this group, meaning that up to 80% of them are still undescribed. The most diverse clades at the order level are Coleoptera, Lepidoptera, Diptera, and Hymenoptera, representing more than 90% of the diversity of insects (Stork, 2018) (Figure 2).

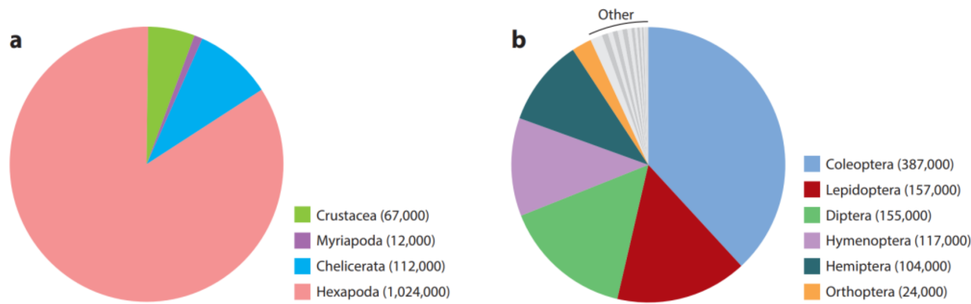

Figure 2. Relative proportions of named species in a) the four subphyla constituting the Arthropoda and b) the orders in the Insecta, with numbers in parentheses. Data from the Catalogue of Life summarized by Zhang Z, 2011. Figure extracted from Stork (2018).

1.2.1. Cockroaches

Cockroaches are hemimetabolous insects with approximately 4,500 species described today. Furthermore, the earliest fossil records of cockroaches are as ancient as 400 mya. These insects of the order Blattodea are one of the first winged insects (Hashemi-Aghdam *et al*., 2014; Kambhampati, 1995). Morphological traits (i.e., forming ootheca with discrete outer case) and phylogenetic studies suggest that the superorder Dictyoptera is formed by cockroaches (Blattodea), termites (Isoptera), and mantids (Mantodea) (Kristensen 1981; Lo, 2003). After revision of the superorder Dictyoptera, the order Isoptera has been demoted to family level (Termitidae), inside de order Blattodea (Inward *et al*., 2007). Mantids and termites form a monophyletic group while cockroaches form a paraphyletic group, since termites are the most recent branch inside Blattodea (Legendre. *et al*. 2015; Thorne *et al*. 1992).

Cockroaches can colonize a broad spectrum of habitats. Usually, these habitats share some common traits, such as dark, humid, poorly ventilated, and often narrow spaces. Their range of habitats includes five subdivisions: i) loose substrates as uncompacted soil or plant litter; ii) under logs, stones, and other crevices; iii) burrows in solid soil excavate by themselves; iv) other insect or small vertebrate

burrows and v) extensive burrows like caves or sewers (Bell *et al.*, 2007).

Cockroaches are pest insects due to their capability of prospering in rural and urban areas, such as homes, restaurants, hotels, hospitals, etc. They can be a source of diseases mainly because of their habit of feeding in the organic matter regardless of its procedence, including wastes and then roaming through human-shared spaces, like hospitals. In this environment, cockroaches (mostly *Periplaneta americana,* and *Blattella germanica*) may be acting as vectors of pathogenic bacteria such as *Klebsiella sp.* However, the direct involvement of cockroaches in the transmission of pathogenic bacteria is difficult to prove even when different studies have found that cockroaches in hospitals carry pathogenic bacteria that are typically responsible of nosocomial infections (Fakoorziba *et al.*, 2010; Fotedar *et al.*, 1991; Donkor, 2019). Furthermore, they can promote allergies and asthma in humans, especially given constant exposure. Despite all of this and considering the whole clade, less than 1% of the cockroaches act as pest insects. *B. germanica* is one of these (Cochran, 2009).

1.2.2. *Blattella germanica*

The German cockroach *B. germanica* has a worldwide distribution, and it is associated with humans. It lives in buildings, hospitals, wells, sewers, steam tunnels, caves, and mines. The limiting factors that determine whether human structures will provide suitable habitats for cockroaches are temperature and availability of water and food. Ideal temperatures range between 20-29 ºC, and the upper limit is around 33 ºC. Drinking water is available for these cockroaches, such as traps of sinks, toilet bowls, condensation on cold pipes, flush tanks, etc. *B. germanica* is omnivorous, and food is usually very accessible in human structures since they can feed on human or pets' food and

glues and pastes on cartons, boxes, or envelops, among others. They can also feed on other dead insects or living plants (Roth and Willis, 1960). In short, the habitat of this cockroach is dark, humid, poorly ventilated, and often in cramped spaces (Bell, 2007). *B. germanica* is a heavily gregarious species mediated by body secretions (Pettit, 1940; Ishii and Kuwahara, 1967) that attract individuals, causing migrations from less populated nests to more populated refuges (Denzer *et al*. 1988). The study carried out by Berthold and Wilson in 1967 showed that 82% of cockroaches prefer already colonized hideouts. Moreover, social interactions such as coprophagy (Nalepa *et al*. 2001), acoustic communication related to choosing shelter (Wijenberg *et al.* 2008), and reproduction (Bret *et al*. 1983) take place within the shelters. These cockroaches usually hide in their shelters during light hours and forage at night. When the source of water and food is far from the nest, they tend to exit less from their nest than when the nest is near the resources. Gravid females are fewer active feeders than non-gravid females (Silverman, 1986).

Regarding their development from nymph to adult, *B. germanica*, as any other cockroach, is hemimetabolous, which means that the insect goes through a series of molts after hatching from an egg, to finally become an adult (Figure 3). During the nymphal stages, the nymphs resemble to adults, but they lack wings and reproductive organs and

are smaller. This species undergoes 5 or 6 molts. The number of molts can vary if there are bad conditions for insects, such as low temperatures or a shortage of food. Furthermore, females tend to have more molts than males (Tanaka, 1981).

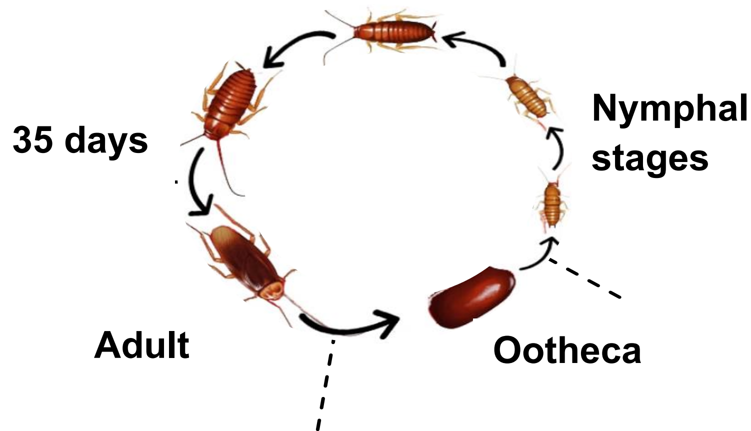

Figure 3: Life cycle of the cockroach Blattella germanica

B. germanica, despite being omnivorous and feed on complex diet possesses an endosymbiont, *Blattabacterium cuenoti* (hereafter *Blattabacterium*) which lives inside specialized cells called bacteriocytes. The genome analysis of *Blattabacterium* from *B. germanica* revealed the role of the endosymbiont in the synthesis of essential amino acids (López-Sánchez *et al*, 2008). However, the most striking result was that it had retained the complete urea cycle, plus the two genes that encode the catalytic core of the urease (ureAB and ureC; EC 3.5.1.5). Apart from the bacteriocytes, there are two different cellular types in forming the fat body: the adipocytes, which are the main cellular type of this tissue, storing energy as fat, and the uricocytes, that are specialized in storing the excess of nitrogen as uric acid. Urea produced by the host in the urea cycle is degraded into CO_2 and ammonium by the endosymbiont urease enzyme. This ammonium can be used by the host to produce organic compounds with the synthesis of glutamine or can be used by the endosymbiont to produce glutamate (Latorre *et al*., 2022). After the ootheca hatch, on

the 5th day of nymph life, some *Blattabacterium* travels from the fat body to the nymph's ovarioles. Then, between the 11th and 17th day, endosymbionts migrate as free cells and reach the space between the follicular epithelium and the oocyte. They stay in this space until the end of the vitellogenic phase when they are phagocytosed by the oocyte before the chorion formation, just before ovulation takes place in the adult (Sacchi *et al*. 1988). It is also known that *Blattabacterium* is the only bacterium transmitted vertically from the mothers to their offspring (Carrasco *et al*., 2014).

B. germanica and cockroaches in general are paradigmatic model of symbioses because they share the endosymbiont *Blattabacterium* in the fat body and a rich and complex gut microbiota (López-Sánchez *et al*., 2008). Gut microbes aid their host in different ways, such as helping in digestion and processing nutrients, in the immune system development, or producing key metabolites for the host, among others. In humans, when uncontrolled or altered, the microbiota can cause different diseases and medical conditions such as obesity and can affect the normal development of insect larvae (Rojo *et al*., 2017). Gut microbiota is key for the physiological homeostasis of individuals. This work focused on this feature.

The German cockroach is an excellent animal model due to some biological properties. It is omnivorous, which favors studies with different diets, and diet is one of the main shapers of the gut microbiota composition; it is easy to maintain in laboratory conditions; it has a relatively short life cycle, high reproducibility rate and possess a rich and diverse microbiota.

1.3. Microbiota

First, we need to define the terms microbiota and microbiome, since both are related concepts and sometimes are interchanged in the literature. We define microbiota according to the Berg description

(Berg *et al*., 2020). Following this definition, microbiota is formed by the living components of the community (bacteria, archaea, fungi, protist, and algae) and virus. Microbiome is a broader concept; all microorganisms in the symbiotic relationship are englobed in this concept, this includes bacteria, archaea, algae, fungi and viruses (Lederberg and Mccray, 2001). A more simplistic and human-centered vision of the microbiome defines it as the genomes of our affiliated microbial partners (Bäckhed *et al*., 2005; Turnbaugh *et al*., 2007). These two definitions lack one important aspect of what the microbiome is. The microbiome is formed not only by the microorganisms in a symbiosis, but also the theatre of activity which is formed by the environment, including biochemical compounds and conditions (Whipps *et al*., 1988; Rosenberg, 2021).

Microbiome studies started at the beginning of this millennium, with the commercialization of the 454-pyrosequencing platform in 2005. The Next Generation Sequencing (NGS) allowed investigators to perform approximations to different biological problems through whole genome sequencing, such as identification of genomic variation across the whole tree of life much more accessible than ever (Hillier *et al*., 2008; Von Bubnoff, 2008; Srivatsan *et al*., 2008; Cao *et al*., 2011). Furthermore, it also favored the ecological studies of microbial communities in different habitats such as soil, marine water, or different animal surfaces and organs (Gill *et al*., 2006; Roesch *et al*., 2007; Sogin *et al*., 2006; Grice *et al*., 2009). We can now study communities that can prosper under conditions such as hypersalinity, extreme temperature, pressure, and alkalinity (Price, 2000; Ollivier *et al*., 1994; Brazylinski, 2013). Two general techniques allowed the study of these communities: metagenomics (the study of genetic material recovered directly from samples) and metatranscriptomics (the study of the gene expression of microbes within natural environments). As

stated, they are now a key tool to unravel many unanswered questions about these communities (Vezzulli *et al*., 2022).

Regarding bacterial symbionts, they can live in a wide range of body locations in animals like the skin or cuticle, gut, urogenital and respiratory tract. The two most accessible locations in insects are the cuticle and the gut. In some cases, the first can be modified in different cuticular structures that favor the process of colonization and maintaining of bacteria. It is the case of the fungus-growing attine ants and ambrosia beetles (Mueller *et al*., 2008). Ants rear the antibiotic-producing bacteria in specialized crypts mediated by exocrine glands (Currie *et al*., 2006). Ambrosia beetles harbor bacteria and fungi in specialized membranous invaginations that are equipped with secretory glands, called mycangia (Hulcr *et al*., 2012; Ibarra-Juarez *et al*., 2020). As a curiosity, the name of these invaginations, suggesting fungi housing, is anterior to the knowledge of bacteria inhabiting it too. On one hand, these specialized structures offer different advantages for the microbes that inhabit them, such as protection from UV light exposure, abrasion, and even nutrients (Happ *et al*., 1971). On the other hand, bacterial symbionts provide the host with defense mechanisms by forming biofilms or competing with pathogens for the niche and producing antimicrobial products to eliminate them and other mechanisms by which they protect the host from parasitic symbionts and predators (Cogen *et al*., 2008; Brownlie and Johnson, 2009).

1.3.1. Gut microbiota

Gut is the most bacteria-populated location in animals. It evolved from a simple tube of the ancient cyclostomatida to the complex mammal gastrointestinal tract. During this process, the complexity of the composition of bacteria inhabiting it has also increased (Figure 4) (Kostic *et al*., 2013).

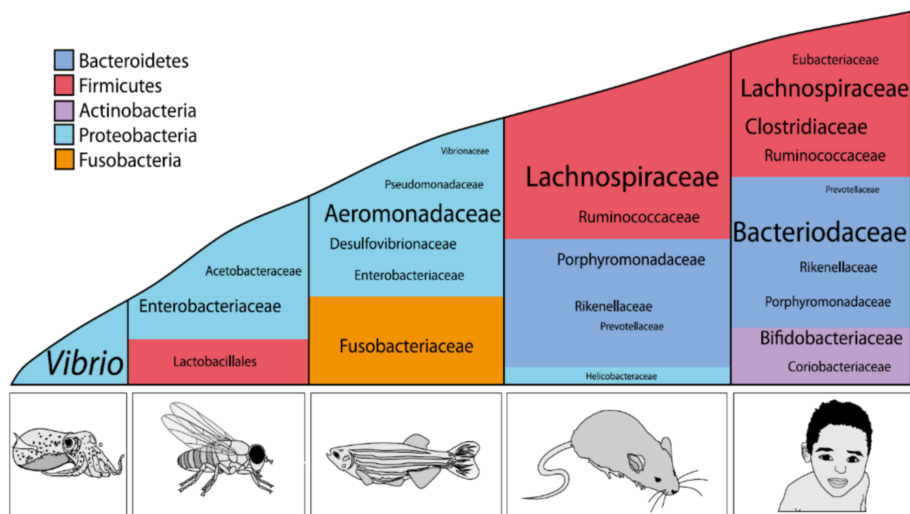

Figure 4. Structure of gut microbiota across species. Although there can be significant interindividual variation in the composition of the microbiota, broad trends exist within a given species, particularly at the phylum level. Phyla are represented by color, and the relative abundance of the lower taxonomic levels is indicated by font size. This figure was produced with data adapted from Arumugam et al. (2011), Brinkman et al. (2011), Chandler et al. (2011), and Roeselers et al. (2011). Figure extracted from Kostic et al. (2013).

The gut is especially attractive for microorganisms for different reasons. The most evident is the easy food access, but it is not the only one. In the gut, bacteria find protection to outer damaging sources like UV irradiation or desiccation. There are also negative factors that make the colonization of the gut more difficult for some bacteria attached to the different regions of the gut.

In particular, the insect gut is divided into three main regions (Figure 5). From the more distal part of the gut, the hindgut harbors the most quantity of bacteria in many insects. The hindgut is not an aggressive environment in general, but desiccation stress is present in the more distal parts of this region due to the active reabsorption of water. Malpighian tubules providing ions and metabolites may be a positive factor for the bacterial diversity of this region (Douglas, 2015). Less distally, there is the midgut in which many different enzymes such as

lysozymes and immunological products like antimicrobial peptides (AMP) are excreted by its epithelium. These components make the midgut a more challenging to colonize place for microbes. Most commonly, the pH of this region is between 6 and 7, which supposes no challenge for most bacteria. Still, in some cases, like in lepidopterans the pH is more alkaline, between 8 and 12, which presents a lower surviving range of bacteria (Harrison, 2001). Finally, the most proximal region is the foregut. This region specializes in some insects such as *Bactrocera oleae*, which houses a symbiont that aids in the detoxification of secondary plant metabolites and will be explained further in this section (Capuzzo *et al.*, 2005).

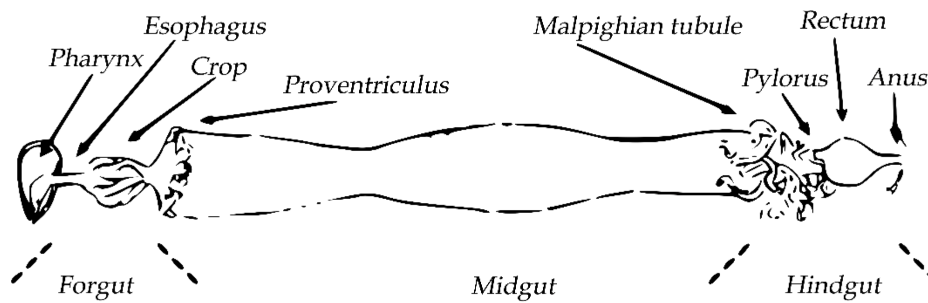

Figure 5. General scheme of the gut of insects, divided in three parts.

Although there is variability among the gut microbiota of insects, four are the most abundant and typical bacterial phyla: Actinomycecota, Bacteroidota, Bacillota , and Pseudomonadota. Previously known as Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria respectively (Oren & Garrity, 2021). It is also inhabited to a lower extent by archaea (related to methanogenesis metabolic pathways), fungi (related to fermentation and organic matter decomposition), and other eukaryotes such as protists. These represent a major source of cellulolytic and xylanolytic activity in the hindgut of lower termites (Santana, 2015; Brune, 2009). There is the

possibility that even helminths may have a positive impact in the stability of the gut microbiome and on the host's healthy status (Chabé *et al.*, 2017).

It has been demonstrated that the gut microbiome is a key component of health in humans and other animals (Jani *et al.*, 2017; Abrahamsson *et al.*, 2015; Cornejo-Pareja *et al.*, 2019; Morais *et al.*, 2021; Nicholson *et al.*, 2012; Shang *et al.*, 2018). It is related to modulating the diversity and quantity of a wide range of different metabolites that are highly important in the homeostasis and normal functioning of more complex animals. We show here a few examples. One of them is the case of bile acids, which are cholesterol derivatives synthesized in the liver and then conjugated with glycine or taurine before secretion into the bile and small intestine. Once there, microbes perform a series of transformations to bile acids (Midtvedt and Norman, 1967; Sinha *et al.*, 2020). Deconjugated bile acids have been reported in control animal feces but in germ-free animals (animals that does not harbor microorganisms because of a treatment) the bile acids found are conjugated (Midtvedt, 1974). Reduction in the abundance of certain genera such as Ruminococcaceae are related to a reduction in the deconjugated bile acids levels in feces (Sinha *et al.*, 2020). This reaction is carried out by bacteria such as *Bacteroides melaninogenicus* (now *Prevotela melaninogenica*) or *Proteus mirabilis* (Shimada, 1969; Midtvedt and Norman, 1967). Mammal enzymes are not capable of cleavage the carbon-nitrogen bond formed in the bile acid conjugates. Oxidation-reduction of hydroxyl groups in bile acids in different carbon positions is also carried out by bacterial enzymes, mostly by dehydrogenases. A broad spectrum of bacteria is capable of synthesizing these enzymes, including all strains of the genus *Eubacterium*, belonging to the phylum Bacillota, a predominant phylum in mammals and insects (Midtvedt, 1974). Other reactions performed by microbiota on bile acids are hydroxylation,

dehydroxylation, and the formation of β-OH derivatives (Midtvedt, 1974).

In addition to their traditional assigned role as maintainers of cholesterol homeostasis in liver and dietary fats absorption components, they are now recognized as important signaling molecules with systematic endocrine functions like regulation of lipid, glucose, and energy homeostasis (Swann *et al.*, 2010). In their study, they tested the impact of the microbiota on the bile acid profile in different tissues (liver, kidney heart, and plasma) of rats under different conditions: conventional, germ-free, antibiotic-treated, and control. They found that gut microbiota significantly affects the composition and abundance of bile acids in all studied organs and tissues, but also the global transcript profiles of the liver (Figure 6). In this organ, the majority of bile acids are non-conjugated in control and conventional rats but not in germ-free rats. It is due to the gut microbial-mediated deconjugation and later distribution to other organs. These gut microbiota-mediated metabolism changes suggest that bile acids may perform as signaling molecules outside the enterohepatic circulation which may be another mechanism of host

metabolism regulation mediated by gut microbiota (Swann *et al.*, 2010).

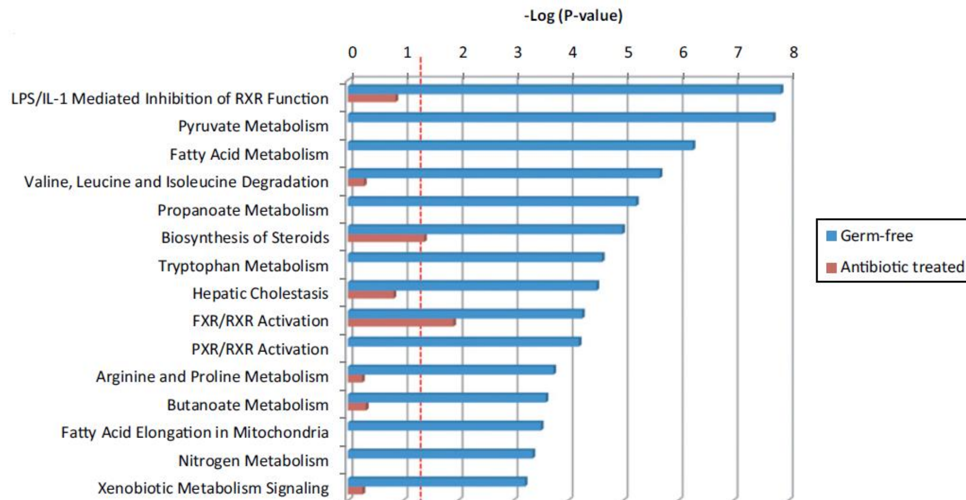

Figure 6. Hepatic metabolic and signaling pathways modulated by the gut microbiota. Significantly regulated canonical gene expression pathways in GF rats (blue bars) and pathways common to AB rats (red bars). Scale is a log-transformed P value calculated by Fisher's exact test that a pathway is overrepresented within the altered genes. Dotted red lines indicate threshold value (P < 0.05) for pathway change to be considered statistically significant. Figure extracted *from Swann et al. (2010).*

Another key metabolite related to gut microbiota is choline. Choline is needed in the body for many functions, such as acetylcholine synthesis as a neurotransmitter, cell-membrane signaling through phospholipids, transport of lipoproteins, and the metabolism (reduction) of methyl-groups like homocysteine. It is also essential for the synthesis of phospholipids of cell membrane components such as phosphatidylcholine, lysophosphatidylcholine, choline plasmalogen, and sphingomyelin. Besides, it is related in brain and memory development in the fetus and seems to be associated with a decrease of risk in the development of neural tube defects (Penry and Manore, 2008; Zeisel *et al.*, 1991; Shaw *et al.*, 2004). Low levels of choline are

linked to an increase of the risk of health problems like muscle damage, hepatic steatosis, also known as fatty liver and inflammation, cancer, and heart diseases, among others (Fischer *et al*., 2008; Zeisel and da Costa, 2009; Strilakou *et al*., 2013). When altered, gut microbiota may reduce the bioavailability of choline to the host. In normal conditions, some bacteria from the gut use choline in their metabolism, transforming it into trimethylamine (TMA), which later is converted in the liver into trimethylamine-N-oxide (TMAO). TMAO exacerbates atherosclerosis in mice. This route is favored by some bacterial groups that are not that abundant in normal conditions, but when enriched may deplete the amount of choline accessible for the host. In their study, Romano *et al*. (2015) supplied three different mixtures of microbiotas to germ-free mice. One of the mixtures was similar to control microbiota, other had an additional TMA producer (*Clostridium sporogenes)* and the last one had eight additional TMA producers (*Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *C. sporogenes*, *Edwardsiella tarda*, *Escherichia fergusonii*, *Proteus penneri*, and *Providencia rettgeri*). The last-mentioned mixture leads to a significant level decrease of choline in serum in comparison to the only core mixture and core plus *C. sporogenes.*

Short-chain fatty acids (SFCA) are the result of bacterial fermentation of complex carbohydrates, but also of polysaccharides, oligosaccharides, proteins, peptides, and glycoproteins, being the first one, the most important in quantity (Cummings and Macfarlane, 1991). Acetate, propionate, and butyrate are the main SCFAs produced by the microbiota. In their work Smith *et al*. (2013) tested the effect of SCFAs in the immune system by controlling the levels of regulatory T Cells (T_{reg} cells). T_{reg} cells regulate intestinal homeostasis and control inflammation by limiting the proliferation of effector CD4+ T cells (T_{eff}). They conducted their experiment on specific pathogen-free

(SPF), gnotobiotic Schaedler flora (ASF)-colonized, and germ-free (GF) mice. The ASF is a model community of eight microorganisms derived from mice that have been in use since 1970s. The bacteria in this mix are two different *Clostridium spp.*, *Lactobacillus intestinalis*, *Lactobacillus murinus*, *Eubacterium plexicaudatum*, *Pseudoflavonifactor spp.*, and *Parabacteroides goldsteinii* (Wymore Brand *et al.*, 2015). First, they checked SCFAs levels in these three mice groups and found that GF mice had the least quantity of SCFA (acetic acid, propionic acid, and butyric acid), but also presented a lack of T_{reg} cells. This state was reversed when they supplied SCFAs in water to GF mice. Moreover, they treated SPF mice with the antibiotic vancomycin and observed that the number of T_{reg} cells decreased to GF mice levels. This decrease was everted to SPF mice levels again by supplementing the diet with SCFAs.

Other studies have associated the gut microbiota with different aspects of the immune system, such as the development of gut-associated lymphoid tissue (GALT), Peyer's patches, mesenteric lymph nodes, and cellular lamina propria (Falk *et al.*, 1998; Pollard and Sharon, 1970; Hoshi *et al.*, 1992; Gleister, 1973; Lee and Mazmanian, 2010). But also, the expression of pathogen sensing receptors as Toll-like receptors (TLRs) and antigen presentation molecules like the major histocompatibility complex class II (Semin *et al.*, 2021; Lundin *et al.*, 2008; Roland *et al.*, 2021; Matsumoto *et al.*, 1992). There is a reduction in the number of different components of the immune system when microbiota is eradicated of the gut, as done with germ-free mice, such as intraepithelial lymphocytes (IELs) and $CD4^+$ T cells in lamina propria (Umesaki *et al.*, 1993; Niess *et al.*, 2008). These results make clearer that gut microbiota is a beneficial factor in the immune system by producing metabolites that regulate intestinal adaptive immune responses and promote health, helping in the immune system

development and stimulating the production of a wide range of immunity-related molecules.

Gut microbiota is also related to pathologies like obesity, autoimmune and neurological diseases (Surana and Kasper, 2017; Sampson *et al*., 2016; Turnbaugh *et al*., 2006). In their study, Surana and Kasper (2017) find taxa related to the severity of colitis, an autoimmune disease, by a triangulation method. This method consists of using different gnotobiotic mice colonized by different precedence microbiotas and inducing colitis to all of them. Then, comparing the bacterial taxa of the more severe and less severely affected mice groups they obtain a shortlist of bacterial candidates that are potentially related to the affection of the disease. Using this method, they found a new species, *Clostridium immunis*, which protects mice from colitis. Regarding neurological diseases, Parkinson's disease (PD) is the second most common neurodegenerative disease in the United States (Nalls *et al*., 2014). Sampson *et al*. (2016) used mice that overexpress α-synuclein, linking this fact to the increasing difficulties in the movement capabilities, similar to PD of the rodents when impaired with GF conditions. It suggests that there are gene-microbiome interactions. They also induced PD-like motor deficiencies by colonizing the gut of mice with the microbiota of PD patients, suggesting that the patients' altered microbiota may contribute to the disease. They found that the microglia activation and maturation are reduced when gut microbiota is removed (Sampson *et al*., 2016). Finally, the study of Turnbaugh *et al*. (2006) tested the relationship between obesity and the energy metabolism of the gut microbiome in obese and lean mice and humans. They found that the relative abundance of Bacteroidota and Bacillota is associated with obesity by increasing the ability of the microbiota of harvesting energy from food. All of this supports that gut microbiota is an important factor that, when uncontrolled, favors obesity.

1.3.2. Gut microbiota in insects

Not only humans and other mammals are highly favored by gut microbiota. As stated above, the gut has been colonized by microbes, and the insect gut is no exception. It has been demonstrated that different insects are in close symbiosis with their microbiota and that their microbes play a key role in their way of life for them. Let's see some examples.

The olive fly, *Bactrocera oleae*, is associated with the bacteria *Candidatus Erwinia dadicola* (*Ca. E. dadicola*). It is considered an obligate association consequence of the co-evolution between the two organisms (Capuzzo *et al*., 2005). The bacterium is transmitted vertically, not being able to survive *in vitro* or in the fruit by its own. Regarding its location, *Ca. E. dadicola* is hosted intracellularly in the gastric caeca of the midgut, during the larval stages, while it is located in the foregut lumen and ovipositor diverticulum during adult stages of *B. oleae* (Estes *et al*., 2009). *B. oleae* is monophagous and feeds only on unripe olives. The olive tree, *Olea europaea,* as other plants in the race of evolution, has developed a defense mechanism, involving the production of secondary metabolites, while the fruits are in the process of ripening. Unripe olives carry high levels of oleuropein, a bitter phenolic glycoside with antimicrobial and protein alkylator properties which maintaining unripe olives out of the scope of most of herbivores and pathogens (Amiot, 1989; Bennet and Wallsgrove, 1994; Fleming *et al*., 1973). It has been tested whether larvae of olive flies are capable of developing in unripe olives without *Ca E. dadicola*. These experiments demonstrated that this symbiont is key for the correct development of the larvae, which are affected negatively in weight and length when devoid of the symbiont (Ben-Yosef *et al*. 2015). In this case, microbiota is helping the colonization of an

unoccupied niche through the detoxification of secondary plant metabolites.

Termites are another notable example of the interaction between gut microbiota's role and the host's success in occupying complex and less occupied niches. These insects can survive on a strict wood diet. In this case, microbiota housed in specialized regions aids their host by degrading cellulose and hemicelluloses by hydrolysis, which is not a common feature in animals (Brune, 2009). Structurally, these wood-feeding insects have a dilated hindgut, which increases digestion time in this region, favoring the action of the microbiota. The hindgut is a bioreactor where the symbionts ferment lignocellulose to acetate and methane, which are the host's major carbon and energy sources. It has been demonstrated that gut microbiota is also responsible for the nitrogen fixation and recycling of nitrogenous waste components that occur in the gut (Brune and Ohkuma, 2010). In the present Thesis, we use B. germanica as animal model to understand how the microbiota works in different conditions. The gut microbiota of this insect has been studied under different situations. In our group, we mainly exposed the cockroach to different antibiotics such as Rifampicin (Rosas *et al.*, 2018), Kanamycin (Domínguez-Santos *et al.*, 2021), vancomycin and ampicillin (Domínguez-Santos *et al.*, 2020) to assess their effect on the microbiota, compare to control conditions. With those studies, we defined the core of gut microbiota, mainly formed by the phylums Bacteroidota, Bacillota, and Pseudomonadota, and checked for the effect of the different antibiotics. We found that each antibiotic affects the microbiota differently, which is restored once the treatment disappears. Domínguez-Santos *et al*. (2020) also demonstrated that bacteria found in the food were similar to that of the gut microbiota, but with different proportions, but also that bacterial species present in feces contribute to the establishment of gut microbiota. Interestingly, the most abundant bacteria in food

(Enterobacter, Lactobacillus, and Pantoea) were scarce in feces and gut microbiota. This may indicate that gut, in some manner, selects which bacteria are included in the acquisition process of the microbiota. We also studied the effect of different protein content diets in the gut microbiota composition (Pérez-Cobas *et al*., 2015). In this study, the protein content was demonstrated to be an important factor in the gut microbiota composition. When the cockroaches were fed a protein-free diet, their gut bacterial composition were significantly different from control and diets with varying proportions of proteins. The differences between lab-reared cockroaches and wild cockroaches were also assessed. Bacteria like Fusobacteriaceae or Bacteroidota were more abundant in lab-reared cockroaches while wild cockroaches presented a higher abundance in Deulfovibrionaceae or Enterococcaceae.

So far, here lie some examples about how the gut microbiota is vital to the host's health, nutrition, detoxification of diet components, immune and nervous system development, and correct function independently of the host position in the tree of life. However, a crucial question is: does the host control the bacteria that form the gut microbiota? This question has been tackled by investigating the relationship between the immune system of insects, mainly *Drosophila melanogaster,* and gut microbes. The mechanisms of regulation and production of AMPs are well understood in *Drosophila*. These are immune effectors that shape microbiota profiles by different mechanisms (Douglas, 2014). Two different signaling pathways regulate the balance of gene expression of AMPs. These are the immune deficiency (IMD), activated mainly by Gram-negative bacteria and the Toll signaling pathway, activated by fungi and Gram-positive bacteria (Lemaitre and Hoffmann, 2007). The activation of these pathways does not eliminate bacteria in the gut but controls the total amount of them. The alteration of other genes such as *caudal*,

which is implicated in the negative regulation of the IMD signaling pathway through RNA interference, produces a significant increase in AMP production, which leads to an alteration of the gut microbiota composition but not to a fully depleted microbiota. These changes can be restored when *Caudal* is reintroduced (Ryu *et al*., 2008). In normal conditions, *caudal* is repressing the nuclear factor kappa B, which is responsible for the induction of epithelial AMP expression. These studies suggest that coevolution has led to, at least in the fruit fly, the development of a master control gene (*caudal*) to avoid the eradication of commensal bacteria in the gut, and when disturbed the whole community is completely altered.

1.4. Longitudinal studies in gut microbiota

The gut microbiota composition can vary depending on factors like diet, age, site, host genetics, or treatment (Bokulich *et al*., 2016; Lozupone *et al*., 2012; Pérez-Cobas *et al*., 2015; Goodrich *et al*., 2014; Odamaki *et al*., 2016; Yatsunenko *et al*., 2012; Parata *et al*., 2020). The relevance of all these factors on the gut microbiota could be better understood by introducing the element of time. The number of longitudinal studies increased in the last years but are still not very common (Faust *et al*., 2015; Dinleyici *et al*., 2018; Martí *et al*., 2017; Wang *et al*., 2017; Dimitriu *et al*., 2013). They usually lack a sufficient number of time points or individuals

Longitudinal studies allow us to take pictures of the state of microbiota in different moments and then use that data to make more solid affirmations about what a normal or altered microbiota is in response to a given factor and even the time that gut microbiota needs to recover after a perturbation, that is, its resilience. Resilience refers to microbial communities that absorb biotic or abiotic perturbations without losing their structure and functionality and then recovering their normal state. After the perturbation, for instance, of antibiotic

intake, gut microbiota composition is altered, and two main things can occur, as shown in Figure 7. Either the microbial population returns to its original state thanks to the population's resilience, or it enters into a long-stable state called dysbiosis. During this state, microbiota loses important taxa and diversity, suffers in metabolic capacity, and presents less resistance against invading pathogens (Lange *et al*., 2016; Perez-Cobas *et al*., 2013). Furthermore, pathogenicity is favored due to an increase in the gene transfer from pathogens to local microbiota of, for example, antibiotic resistance genes and virulence related genes (Stecher *et al*., 2013.)

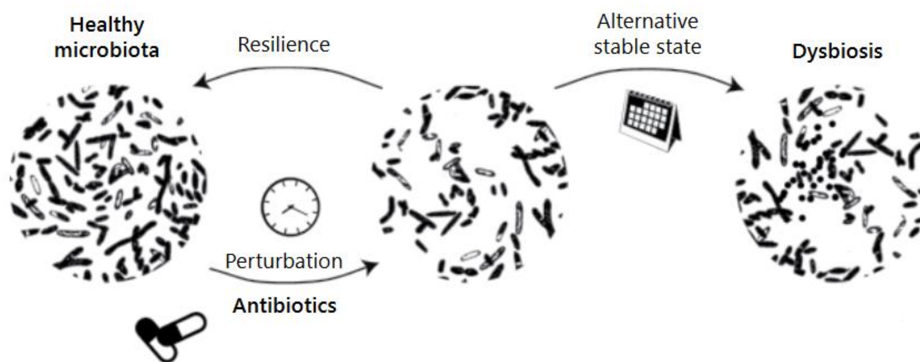

Figure 7. Effects of antibiotics in healthy microbiota, which can lead to dysbiosis. Figure is taken and simplified from Lange et al., 2016.

1.4.1. Time series analyses and compositional data

Studying the microbiome across time has been a challenge during the last few years. Different groups have approached the analysis of time series of microbiome data with different tools and techniques (Faust *et al*., 2015). Here we show a summary of it. The time-decay method is one technique that uses a log-linear model comparing the similarities (calculated extracting one from Bray-Curtys, Unifrac, and unweighted Unifrac dissimilarities) and time changes among samples (Shade *et al*., 2013). Augmented Dickey Fuller (Said and Dickey, 1984) test is also used in time series analysis. The null hypothesis of this test is that the

community is stable and tests it by fitting an autoregressive model. Thus, this test is used to determine when the microbial populations reached stability (David *et al*., 2014).

Local similarity analysis (LSA) is a technique that is able to identify dependence associations between species or between species and other factors such as environmental factors (Ruan *et al*., 2006; Chow *et al*., 2014). This technique has been improved to reveal statically significant associations in time series with replicates. Their authors termed it extended LSA or eLSA (Xia *et al*., 2011). There is also at least one web application, which performs analysis of longitudinal microbiome data. It is TIME (Temporal Insights into Microbial Ecology) and is prepared to use different kinds of formats and can perform different analyses classified in workflows such as clustering of taxa based on temporal behavior or identification time point similarities, among others (Baksi *et al*., 2018).

Some of these techniques require experimental design considerations and previous pre-processing such as having equidistant time points data, and filtering the least abundant bacteria in order to obtain robust results. Nevertheless, others like log transformations are also required. Otherwise, due to the nature of the microbiome data, spurious correlations can be made during the statistical analysis (Pearson, 1897). Thus, compositionality is a trait of microbiome datasets that has to be taken into account during analysis.

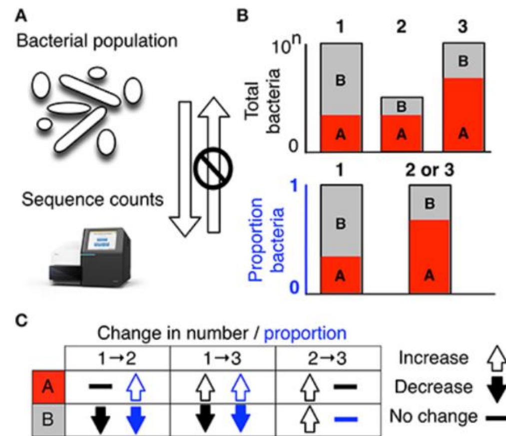

Figure 8. A) Nucleic acids from a bacterial population can only inform of proportions of the real composition. B) Difference between the count of molecules and their proportion for two features in three samples. When proportional, samples 2 and 3 seem identical, but in absolute terms, they are not. C) Real and perceived changes for each sample if we transition from one sample to another. Figure extracted from Gloor et al. (2017).

In their work, Gloor *et al*. (2017) presented a very useful example to compare classic and new microbial ecology studies that involve high-throughput sequencing, and hence, compositional data. In their example, an ecosystem is inhabited by tigers and ladybugs. Then, another population of ladybugs arrives in the same area. In a classic study, we could expect no changes in the number of tigers because of the newcomers. The problem starts when we bring this to the high-throughput sequencing. As of today, sequencing instruments can only deliver a certain number of reads, according to their capacity. Suppose these migrating ladybugs arrive at a full system (the instrument reached its maximum capacity). In that case, they have to displace either tigers or other ladybugs that were already counted by the machine. Thus, the counts of sequences yielded by the machine are, in reality, proportions, as seen in Figure 8, and cannot be related to the absolute real number of molecules that were used as input.

1.4.2 Time series in humans

As in many other fields of biological research, most of the longitudinal studies of gut microbiota are focused on human health and therefore, use humans for their projects with the problems it may suppose. Since the moment that theoretical and technical advancements allowed investigators to start working on this issue, different studies have raised, some with more impact than others (Yassour *et al*., 2016; Fisher Mehta, 2014; Gibbons *et al*., 2017; Trosvik *et al*., 2010; Wang *et al*., 2017).

One of the most impactful of these works is the one carried out by Caporaso *et al*. (2011). It was the largest longitudinal study at the time it came out, covering four body sites (right palm, left palm, tongue, and gut) over 396-time points, but only two individuals (Caporaso *et al*., 2011). In that study, they classified the taxa as transient and persistent, depending on the consecutive time points of observed taxa. They found that the human microbiota is very variable over time but that there were stable patterns along with body habitats and individuals. Due to the high number of time points, other researchers have used the data produced in this work to perform other analyses (Fisher and Mehta, 2014; Bashan *et al*., 2016).

La Rosa *et al*. (2014) did an extensive analysis with a good number of subjects (58) and samples (922) from premature infants. They found that the sequence progression of the bacterial colonizers was the same for all infants and that other factors such as antibiotics, diet, age, or vaginal vs. cesarean birth influenced only the rate of the progression, but not the bacterial order of colonization. As with the previous works, others have used this one to do further analysis. For example, Dynamic Bayesian Networks has been constructed from this dataset, which identified relationships between taxa and predicted the evolution in the bacterial composition in the samples from initial conditions (McGeachie *et al*., 2016).

Finally, the most complete longitudinal study to date was performed by Lloyd-Price *et al.* (2019) in which they followed 132 subjects with and without inflammatory bowel disease (IBD) for a year. They studied three different sample types: colon biopsy, blood, and stool, and carried our metagenomics, metaproteomics, metatranscriptomics, metabolomics, viromics, and the taxonomy through 16S rRNA gene. They also generated molecular profiles of the host. The analyses, identifies samples with a microbiota in a dysbiotic composition by defining a score based on Bray-Curtis dissimilarities comparing IBD samples with non-IBD samples. They found that in concordance with other studies, periods of disease have increased variability in gut microbial composition and function, but also an increase in gene expression by Clostridia during disease.

1.4.3 Time series in other animals

A fast search through PubMed (https://www.ncbi.nlm.nih.gov/) shows less available gut microbiota longitudinal studies in animals than in humans. Moreover, the studies in wildlife animals are even rarer. Sampling is more difficult in the wildlife; thus, these studies are usually less extensive, with fewer individuals and fewer time points. Even with these limitations, they are valuable since they describe how microbiota is shaped in nature, without human intervention.

One of these studies was performed with primates, specifically the threatened species of sifakas *Propithecus verreauxi*, which inhabits the coast of southwest Madagascar (Springer *et al.*, 2017). A total of 32 individuals were studied during dry and wet seasons. Stool sampling was taken once a month per individual, and the patterns of seasonality and group membership through their gut microbiota composition were studied. They found a pattern that could be driven by changes in diet during the different seasons. Gut microbial diversity increases with the high fiber content on the leaf diet taken by the primates

during the dry season, while it decreases during the wet season when the animals consume mostly fruit. Thus, they concluded that diet is one of the main diversity conditioners. More specifically, the non-structural carbohydrates in the diet present in the leaves that these primates eat during the dry season. Similar studies have been conducted with gorillas (*Gorilla gorilla gorilla* and *Gorilla beringei beringei;* Gomez *et al*., 2016). Differences in fruit availability during seasons had an impact on the composition of gut microbiota. For example, the abundance of Spirochaetes was lower during drier periods (November and December) for *G. g. gorilla,* and this change caused convergence in the composition of both species during low fruit season. Seasonality also has an impact on the metabolism of these animals. An increase of metabolites related to the fiber degradations was observed in *G. gorilla*, which as with the microbiome composition, caused convergence with its cousin subspecies, *G. b. beringei*.

There are not many longitudinal studies comprising different stages of life of the same individuals. Wang *et al*. (2019) carried out a study where they followed the gut microbiota of 18 swine from birth to market age (from day 0 to day 178). Thus, this does not include aged swines. They found that the diversity increases over time and that shifts in microbiome structure characterize the different stages of life. As a longitudinal study, it brings the opportunity of finding the order of colonizing bacteria. They identified the early and later colonizers but also those bacteria that are present in all stages (residents), those that are present in specific stages (stage-associated) and those that appear sporadically and disappear (passengers). Then, in a new set of experiments, they performed Fecal Microbiota Transplantation (FMT) in weanlings from growing stage pigs to assess the effect of microbiota in the body mass growth in the pigs. This assay found that FMT swines experienced a growth increase in almost every stage and that the animals were heavier at the final stage. However, the final gut

microbiome composition was not much affected, which reinforces the idea that the gut microbiota is somewhat controlled by the host, and thus, is not the result of just the colonization of the fittest bacteria.

Finally, there are also few longitudinal studies on insects. In their work, Rothman *et al*., (2018) followed the microbiota of the honey bee (*Apis mellifera)* over time. They studied 40 bee colonies over four months. Their objective was to assess the effects of adding supplemental floral forage to see how the gut microbiota of bees changed over time. They also moved the colonies long distances to different environments to check if migration was an important factor in the bee's gut microbiota diversity. They found that the gut microbiota of *A. melifera* is very stable over time. They also found that the gut microbiota of sister honey bees varies as much as those from different colonies. Regarding the supplemental floral forage, it was only affecting the microbiota significantly during March. Some specific bacteria from the strains *Lactobacillus, Bartonella, or Gilliamella* were less abundant to winter forage exposed bees. Finally, they did not find that moving honey bee colonies long distances affected the microbiota. Only the combination of migration and forage resulted in minor changes. Another study in bees followed the midgut/pyloric bacteria microbiota composition over a month. They used 16S rRNA gene sequencing combined with qPCR analysis to give a clearer idea of the compositions and the bacterial load present in samples (Ludvigsen *et al*., 2015). There are also longitudinal studies in *Drosophila melanogaster* that focus on the compositions of populations exposed to different temperatures for nearly ten years, translating to 180 host generations (Mazzucco and Schlötterer, 2021).

2. Objectives

The present work mainly focuses on the dynamics of the gut microbiota of insects, more specifically *B. germanica*. The best way to assess dynamics is to study the system over time, which is the central point of this thesis. The study was carried out during adulthood and under the effect of periodically supplied antibiotics.

The general objective of this work is to obtain a picture of the dynamics of the gut microbiota under antibiotic pressure and the capability of the microbiota to restore itself. Furthermore, this work has five specific objectives that are the following:

The first objective is to determine the diversity of the gut microbiota for the set of time points in normal conditions. It will allow us to observe variations in diversity across time and the appearance and disappearance of some specific taxonomic groups that may be important.

The second objective is to evaluate the microbiota's response to the antibiotic kanamycin periodically supplied to the cockroaches. We expect to find high variability values of the microbiota during the antibiotic periods followed by more stable periods and less cohesive networks for the antibiotic-treated populations.

The third objective is to determine whether the microbiota can return to the previous state after the antibiotic treatment by comparing initial states with the ones after antibiotic treatment. Also, to identify possible resilient taxa. This way, we can ascertain if there is resilience and, if there is, how fast it is achieved by the microbiota.

The fourth objective is to check the functional profile of the samples in both conditions to see the changes that kanamycin generates in treated samples. This objective also includes to study the robustness

of the cockroach gut microbiota, in other words, to check if the taxonomical changes are big enough in order to make significant functional changes.

Finally, the fifth objective is to explore if antibiotic kanamycin affects the cockroach populations' fitness.

3. Material and methods

3.1. Cockroach rearing and population maintenance

A population of *B. germanica* originating from a laboratory population housed by Dr. X. Bellés' group at the Institute of Evolutionary Biology (CSIC-UPF, Barcelona) was reared at the Institute for Integrative Systems Biology (I2SysBio) in climatic chambers at 25 ºC temperature; 65% of humidity and a photoperiod of 12L:12D, from 8 a.m. until 8 p.m. They were fed dog pellets and water "at libitum" from small flasks covered with cotton. Individuals are kept in plastic bottles and are closed with clean gauze to allow airflow. Furthermore, to provide shade and increase the habitat's total surface, each bottle has one paper with undulations (Figure 9). Around 200-300 individuals inhabit these bottles, which cleaned and renewed every week.

Figure 9: Cockroaches laboratory habitat. The plastic bottles are covered with clean gauze to allow air exchange. In the case of the antibiotic treatment experiment, bottles had two gauzes, one with a hole for the bug vacuum, and the other, on top of the other, without any hole. They contain dog pellets food and folded paper in order to increase the total surface and provide shadow for the insects. Water is provided in glass bottles capped with white cotton.

3.2. Experimental design

In this thesis, two main experiments were carried out. First, to study the dynamics of the microbiota under periodic antibiotic treatment, and second to measure the fitness of the cockroaches under the same kind of treatment. Each experiment required at least 600 adult cockroaches (300 males and 300 females).

It is worth mentioning that cockroaches for all the populations were synchronized. For that, late state nymphs were kept together, and after 48 h only those that molted into adults were selected. We were sure that all cockroaches were the same age with a 48h difference window.

3.2.1. Antibiotic treatment experiment

To study the microbiota dynamics, we designed a longitudinal experiment with equidistant distributed time points of sampling. We introduced high time sampling points (105) to avoid the background noise associated with stochasticity of the microbiota composition. We also applied a periodic pulse of an antibiotic (AB) to the treated populations to disturb the microbiota dynamics. The AB pulse was 10 days long, and was followed by 25 days without AB treatment. The cockroaches were exposed to AB three times during the experiment in what we called periods A, B and C, which makes a total of 105 days of experiment. Control populations were untreated.

We did not want to disturb *B. cuenoti*, the primary endosymbiont of *B. germanica*, with the treatment, since, as already stated, it is an important component in the metabolism of nitrogen for the cockroach. For this reason, we needed to choose an AB that only affects the gut microbiota. Otherwise, we could not ensure that the effects on the fitness of the cockroaches or even on the gut microbiota were a consequence of the changes in the gut microbiota. Additionally, since the gut microbiota of the German cockroach is very diverse, we

needed that the AB affected a broad spectrum of taxa. In conclusion, we required an AB that cannot enter the bacteriocytes, with low absorption and a broad-spectrum effect. Only kanamycin met the requirements of the four antibiotics we usually use in the laboratory for disturbing gut microbiota. Then, we chose a dose of 0.2 mg/mL of kanamycin taking into account previous results in our laboratory (Dominguez-Santos *et al*. 2021).

With these prerequisites, we designed an experiment with a duration of 105 days. The experiment had five populations of cockroaches, each one with around 120 individuals. To guarantee the required number of synchronized individuals, each population was divided into two subpopulations, a and b, (see Figure 10) formed by around 60 individuals. To avoid sex bias, we usually use only females for the experiments in our group. However, in this case, and due to the great number of individuals needed, we decided to include male cockroaches in the experiments. Therefore, each population consisted of half males and half females. Three out of the five populations were AB-treated populations (K1, K2, and K3), and the other two were control populations (C1 and C2) (Figure 10). Since each population was divided in two, there were ten subpopulations (C1a, C1b, C2a, C2b, K1a, K1b, K2a, K2b, K3a, and K3b).

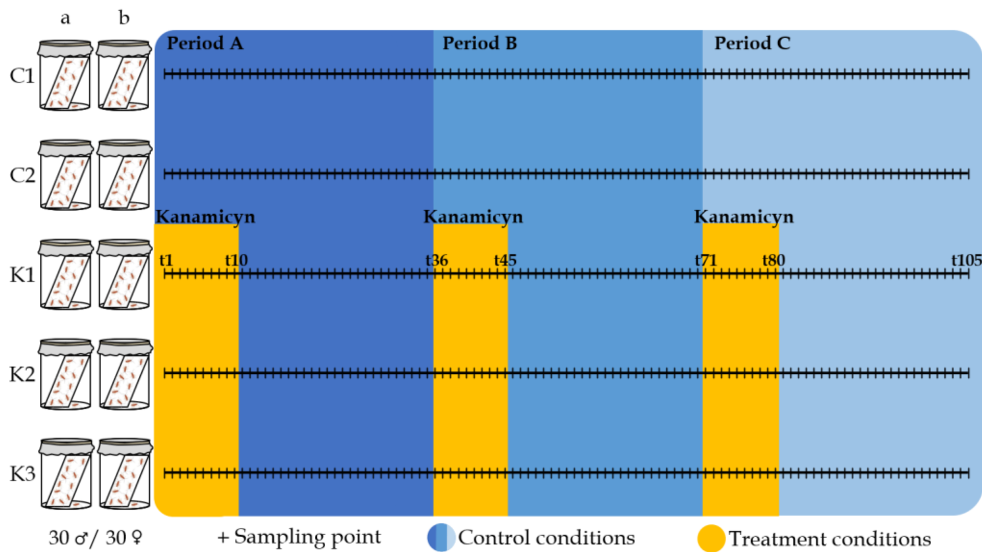

Figure 10: Design of the antibiotic treatment experiment. Each vertical line is one measurement day. Blue colors represent periods A, B and C and control conditions and yellow squares are periods of time with treatment conditions. The total time of the experiment was 105 days.

For treated populations, as already indicated, we supplied kanamycin in the water at a concentration of 0.2 mg/mL for 10 days. Treatment periods were the first ten days of the experiment, the days 36 to 45, and days 71 to 80. The rest of the days, treated populations were in control conditions. We decided to separate the AB periods with 25 days of control conditions to give time to the gut microbiota of individuals to recover from initial conditions, in case they could.

We sampled one cockroach from each population every day during 105 days. To reduce the impact of the gut microbiota being not equal between sexes, we decided to do the sampling alternating between females and males. Females were sampled in odd days and males in even days. Thus, if the microbiota were significantly different between sexes, we would still have two longitudinal studies, with samples every two days.

We used a bug vacuum with a thin cylinder for the individual abduction. Cockroach habitat bottles were prepared with two gauzes,

one with a small hole for the bug vacuum and the other on top of the previous one, without a hole to avoid that cockroaches could scape (Figure 9). This method allowed us to avoid the use of CO_2 daily, which might be harmful to the cockroaches. After the abduction, we anesthetized the individual cockroach with CO_2 and placed it on a thin plastic container in ice. Once we did this for the five cockroaches of the day, we started the dissections.

3.2.2. Fitness experiment

As stated in the previous section, two main experiments were done in the present thesis. The second one consisted of an analysis of several fitness parameters. To make comparable the fitness results with the previous experiment, the conditions for the populations were the same in both cases, and the experiment was equally long: 105 days divided in three equally long periods (A, B and C). The main difference with the antibiotic treatment experiment was that instead of setting two control and three treated populations, this time, we followed the evolution of three control and three treated ones (Figure 11). We performed a trial experiment and then a modified, improved version.

3.2.2.1. Trial fitness experiment

We first designed an experiment to follow the effect of the AB on the biological fitness of cockroaches. In this trial experiment, a total of 15 males and 15 females formed each of the six populations (Figure 11).

We measured four different parameters, twice a week, every Monday, Wednesday, and Friday for 105 days. The first fitness parameter was the weight of male and female individuals. We weighted all individuals of each of the six populations. To do so, we anesthetized all the populations with CO_2 simultaneously and then kept them in plastic containers while they were weighted. All cockroaches were anesthetized with ice for at least 30-45 minutes in

each measurement session. The other fitness parameters were taken while cockroaches were anesthetized with CO_2. The second was the number of oothecae in each population, attached to a female or not, at each sampling point. The third fitness component was the number of nymphs present in the population at each sampling point. Every nymph was removed from the population after we counted them. Finally, the last parameter was the number of dead cockroaches found in the bottles during the sampling.

During this experiment, almost all cockroaches died before 105 days, most probably because of the ice exposure, so we decided to repeat the experiment with some significant changes to avoid ice exposure as much as possible.

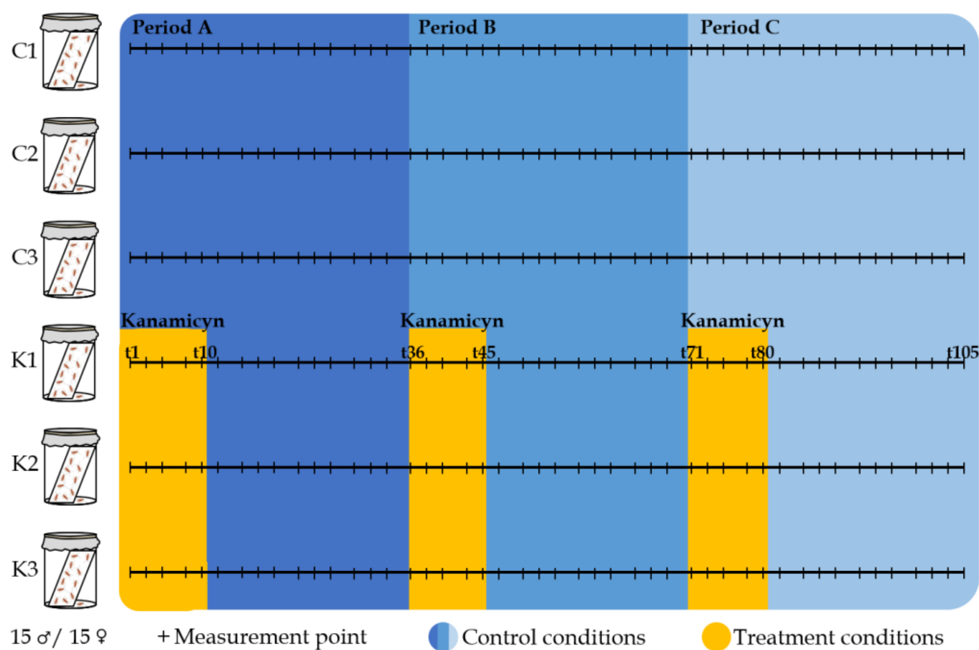

Figure 11: Design of the trial fitness experiment. Each vertical line is one measurement day. Blue colors represent periods A, B and C and control conditions and yellow squares are periods of time with treatment conditions. The total time of the experiment was 105 days.

3.2.2.2. New fitness experiment

In this new experiment, we increased the number of total cockroaches. We used 30 males and 30 females for each population. All the

parameters were measured twice a week, every Monday and Thursday, to maximize the distance between samplings. Regarding weight (first fitness parameter), five individuals of each sex were randomly selected after anesthetizing them with CO_2 and then weighted. To avoid individuals to wake up, we kept them on thin plastic containers surrounded by ice. This process was done separately for each population to minimize the time they were in contact with ice. After the measurement, they were returned to their corresponding habitat. The other three fitness parameters (number of ootheca, nymphs, and death cockroaches) were measured while cockroaches were anesthetized with CO_2, in the same way as we did in the trial experiment.

In both fitness experiments, plastic bottles were cleaned once a week, and during the AB treatment periods, water was renewed at least once (see Figure 9).

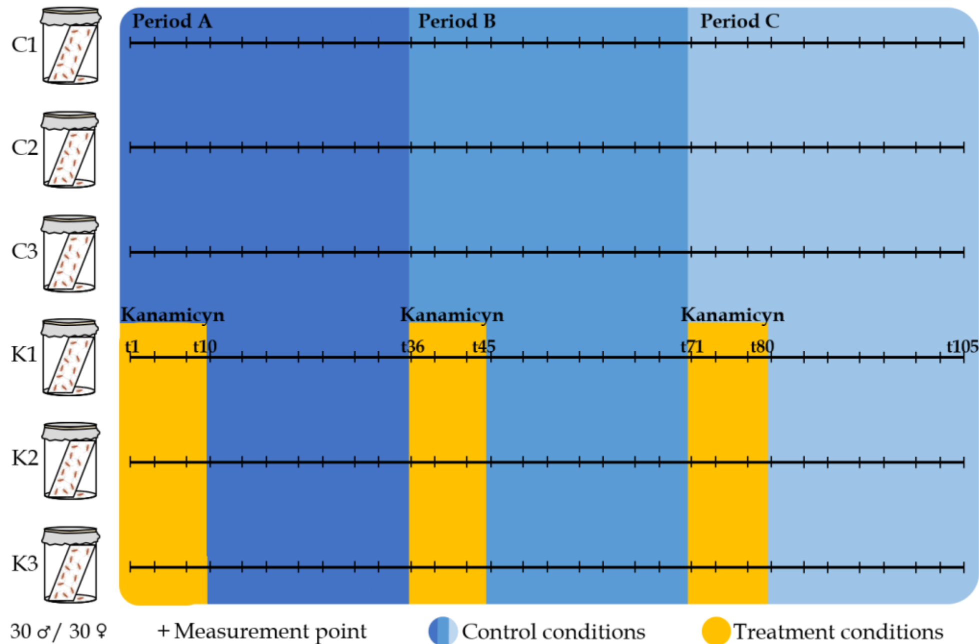

Figure 12: Design of the new fitness experiment. Each vertical line is one measurement day. Blue colors represent periods A, B and C and control conditions and yellow squares are periods of time with treatment conditions. The total time of the experiment was 105 days.

3.3. Hindgut dissection and tissue storage

Hindgut is the distal part of the gut, and it harbors the most bacteria in the gut for many insects (Douglas, 2015). It is relatively accessible by dissection of the insect (see Figure 5). Dissection was carried out in sterile conditions; thus, dissection material was autoclaved after every session of dissections and systematically sterilized during dissection through different methods. Cockroaches were pinned to a dish filled with silicone while anesthetized with ice. Then, the abdomen was opened with dissection scissors and fine forceps that were then sterilized with bleach and a temperature of 250 ºC inside a sterilizer. This sterilization step was done to avoid contamination by cuticle microbiota in the samples. Once opened, the fat body was moistened in Ringer solution (NaCl 0.5 g/L, $CaCl_2$ 0.30 g/L, KCl 0.25 g/L, $NaHCO_3$ 0.20 g/L, in H_2O Milli-Q) and absorbed with a pipette. This process was essential since the fat body harbors the primary bacterial symbiont, *B. cuenoti*, which may contaminate the samples. When the abdominal cavity was clear of fat body, eggs, and ovaries (in the case of females) two cuts were performed, first at the end of the gut, the anus, and the other at the end of the midgut. The hindgut was then placed in a sterile petri dish, opened longitudinally, and cleaned with Ringer solution.

Once the dissection process was completed, and the hindgut was clean of feces and parasites, it was placed in an Eppendorf tube of 1.5 ml and frozen with liquid nitrogen to avoid cell damage. Finally, each sample was stored individually at -80 ºC until DNA extraction.

3.4. DNA extraction and quantification

3.4.1. DNA extraction process

Every gut sample was processed to obtain total DNA that was then used for sequencing and further analysis. All the samples were DNA

extracted following the same protocol to ensure the minimum variability. We used the JetFlex ™ Genomic DNA Purification Kit.

In every round of extractions, we followed the same steps. First, we prepared a mix of 300µL of cell lysis buffer and 20 µL of proteinase K (20 mg/mL) for every sample plus one sample to account for the error while pipetting. When prepared, 320 µL of the mix is supplied to each sample. Then, we added 1 µL of lysozime (20 mg/mL) to facilitate the degradation of the Gram-positive cell wall. Samples are then homogenized with plastic pestles using a pestle motor. To improve the efficiency of the process, samples were vortexed and then placed at 58 ºC for 4 h in a thermoblock. Then, samples were shortly centrifuged to avoid losing material and contaminations since droplets were formed in the top of tubes during the previous step. When the temperature of the samples reached room temperature (rt), we added 10 µL of RNase (4 mg/mL), vortexed them, and placed 10 minutes in the thermoblock at 37 ºC. The next step requires the samples to be at room temperature, so we let them cool for at least 20 minutes and then added 150 µL of protein precipitation buffer. After this, we vortexed each sample for 20 s and centrifuged them for 5 min at 13,000 g. Pellets of proteins should be visible after this process. If they do not appear, an additional step was done. We added 50 µL of pellet compactor and repeated the vortex and centrifuge steps. Recovery of the supernatant, which was usually 450 µL, was performed, and the volume was placed in new 1.5 mL tubes. The tubes with the pellets were discarded. We then added 1 volume of isopropanol to each sample to favor the precipitation of the DNA, and after five minutes of incubation, they were centrifuged for ten minutes at 13,000 g. Then the supernatant was removed by inversion to avoid removing DNA that should be forming a quasi-invisible pellet. We added 1 mL of ethanol 70 %, mixed it by inversion, and centrifuged for 5 min at 13,000 g, removed the supernatant by inversion, short the

samples with a centrifuge. With the help of a pipette, we removed the remaining supernatant, avoiding contact with the pellet. To ensure no remains of ethanol, we put the samples in the thermoblock at 55 ºC until they were dried. Finally, they were resuspended in 20 µL of distilled water and placed at 4 ºC for 24 h for their quantification.

3.4.2. Quantification

After the 24 h, samples were placed in a thermoblock for 30 min at 65 ºC to facilitate resuspension prior the quantification process. We used the Qubit fluorometer for the quantification, with the Invitrogen's Qubit dsDNA HS assay Kit, and performed the following protocol. First, we prepared a mix containing 199 µL of dsDNA HS Buffer and 1 µL of reagent for each sample, plus three more samples, two for standards and an extra one to account for the error while pipetting. Two standard dilutions were prepared with 190 µL of the mix plus 10 µL of the corresponding standard to calibrate the fluorometer. One of the standards contained no DNA, and the other contained 10 mg/mL. For every sample, 198 µL of the mix plus 2 µL of the sample were prepared. Standards and samples were mixed by 2-3 s of vortexing and then incubated in darkness for 2 min. Finally, we calibrated the device with the standards and quantified the DNA concentration of each sample. As last step, samples were stored at -20 ºC until the moment of sending them for sequencing.

3.5. 16S rDNA gene sequencing

After DNA extraction, a total of 512 samples were sent for sequencing the region V3-V4 of the 16S rRNA gene by Illumina Myseq technology to FISABIO (Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comundidad Valenciana). Five different runs were needed to sequence all the samples. One of the most interesting advantages of this method is the cost per sequence, which allows

studies with a great number of samples, which is ideal for this kind of temporal studies.

3.6. Bioinformatic analysis

For the analysis of the data obtained from the sequencer, we used QIIME 2 (Bolyen *et al.*, 2019). QIIME 2 is a software that allows the analysis of 16S rRNA gene sequences, and it is extensible, free, open-source, and community developed. It means that new plugins and functionalities are being added from time to time. Some examples are metaplhan2 (Truong *et al.*, 2015), for the analysis of metagenomic shotgun data, or picrust2 (Langille *et al.*, 2013), for the prediction of functional microbial profiles from amplicons, that were not in the first version of the software, but have been added later as plugins. One advantage of this software is that the parameters of almost every plugin can be changed, which allows for getting better results.

3.6.1. Taxonomic assignment and processing

One of the most important steps for the correct analysis of WGS and amplicon data is the quality control of the sequences. Low-quality reads can lead to erroneous taxonomic assignments, distorting the results, and false conclusions. To avoid this, we used the implemented plugin DADA2 (Callahan *et al.*, 2016). We set the parameters as follows. Trimming 22 nucleotides in the 5′ end to remove primers that can interfere with the DADA2 error model. Taking into account the quality decrease when sequencing the last nucleotides of every read, we truncated at 270 and 210 nucleotides for forward and reverse reads, respectively. These differences in length are due to the faster decrease of quality in reverse reads during the sequencing process. The command line used inside de QIIME2 environment for this step was: "qiime dada2 denoise-paired", with the mentioned parameters. The

same parameters were used for the five runs since they all got similar quality after sequencing.

After this step, the five runs were merged into one construct, with all the experiment samples. We checked if there were batch effects due to different sequencing runs since this is a common problem in these kinds of studies (Goh *et al.*, 2017). We also checked the number of reads after the quality control step and removed any sample with less than 5,000 reads. A total of 9 samples were removed using this criterion. This step was performed outside the QIIME2 environment with R (R Core Team, 2019).

The next procedure was to classify the reads into bacterial taxa. For this process we used the SILVA release 132 (Quast *et al.*, 2013). This release was published in July of 2017 and contains a total of 6,087,080 small subunit rRNA sequences. This database was at 99% identity since the before canonical 97% seems too low to classify at species level reliably (Edgar, 2018; Stackebrandt and Goebel, 1994). The command line used inside de QIIME2 environment for this step was: "qiime feature-classifier classify-sklearn". After the classification process, we collapsed the bacterial taxa to genus level, which means that every read classified at species level would be collapsed to genus level.

To check that there were no artifacts or problems with the bioinformatic process of the reads, we plotted the taxa in a preliminary stacked barplot and found no inconsistencies. This step is of great importance since if, for example, we had found that most of the samples were dominated by a single taxon, we could infer that there was an error or a bad parameter at some point of the processing, typically in the quality control step, with DADA2.

We used the samples from the two control populations to establish the bacterial taxa forming the core. We performed the mean abundance of each time point to construct a new matrix We then

selected only those bacterial groups whose abundances are greater than 0 for all time points. We defined these taxa as the core of *B. germanica* gut.

3.6.2. Diversity

Diversity is regarded as the number and proportion of different species in a given location. It has been a very used metric in ecology since its definition in the middle of the past century by the American plant ecologist Robert Harding Whittaker who, as curiosity, was also the first to propose a five-kingdom classification for the life of the planet. In his work, he defines three different types of diversity (Whittaker, 1960). Alpha diversity, which comes from the alpha index from Fisher's work (Fisher *et al.*, 1943), is the quantity of each species found at a specific location or sample. In this way, alpha diversity is greater when more species and high numbers are in the sample. On the other hand, beta diversity studies the changes in species among different locations or samples. It means that if two samples are very similar in the number and quantity of species, their beta diversity will be low; otherwise, beta diversity will be high, as shown in the Figure 13. Finally, gamma diversity is the product of alpha and beta diversity values and represents the diversity of a whole ecosystem or landscape.

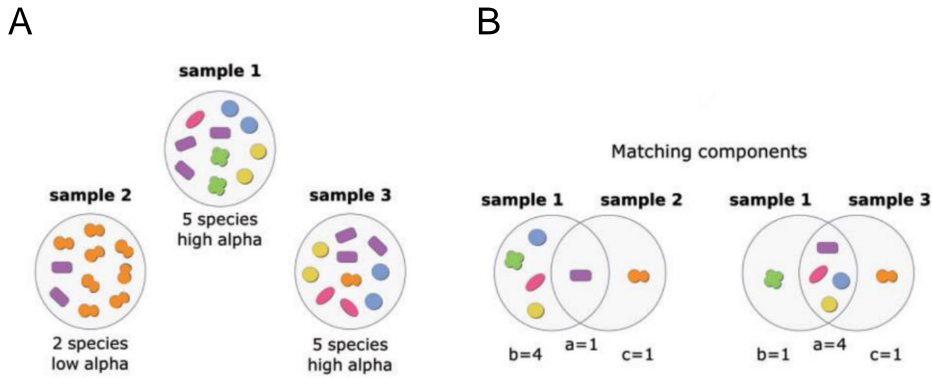

Figure 13. Graphic example of alpha and beta diversity. A) Samples 1 and 3 have a high alpha diversity when compared to sample 2 since they harbor a greater number of different species. B) When comparing them in terms of matching components, we can observe that samples 1 and 2 comparison results in a high beta diversity since they have only 1 species in common, while when comparing samples 1 and 3 the beta diversity is low, since they share almost all species. Figure modified from Finotello et al. (2018).

3.6.2.1 Alpha diversity

There are different ways of calculating alpha diversity. Different indexes have been proposed since the Whittaker work was published in 1960. In this work, we used the Shannon entropy index. This index was first developed to quantify the entropy in a string of letters (Shannon, 1948), in other words, to measure the probability of correctly assigning the next letter in the string, taking into account the known letters and their abundance. The following equation calculates it:

$$H = -\sum_{i=1}^{R} p_i \; ln \, p_i \, ,$$

where p_i represents the proportion of a certain (i) species and R the total of species.

If diversity accounts for the number and proportion of species, richness only accounts for the number of different species. Thus, richness is a simpler approach to understand the most basic properties

of a system. However, it is a very popular measure in ecological studies. As with diversity, the richness can be measured with different indexes. In this thesis, we used the Chao1 index to assess the richness of the samples (Chao, 1987). This index corrects the fact that some rare species are lost during the sequencing process. For this, the observed richness is corrected taking into account the distribution of the less abundant species. These are the singletons (f_1) and doubletons (f_2). Singletons are those species that appear only once in the sample, and doubletons, those that appear twice. The equation used in this index is the following:

$$S^{Chao1} = S^{obs} + \frac{f_1(f_1 - 1)}{2(f_2 + 1)}$$

where S^{obs} represent the number of observed species in the sample.

These two indices were used to compare the diversity in the gut of treated and untreated cockroaches and males and females, and also if periods A (from day 1 to day 35), B (from day 36 to day 70) and C (from day 71 to day 105). To do so, we used a combination of QIIME2 with the command line: "qiime diversity alpha" inside de QIIME2 environment, and R with the vegan package (Oksanen *et al.*, 2019) for the Shannon index and the fossil (Vavrek, 2011) for the Chao1 index.

3.6.2.2 Beta diversity

As previously said, beta-diversity explains how similar or different are the problem samples regarding their alpha diversity. To mathematically assess this problem, different indexes have been developed. In this thesis, we have used the Jaccard index of similarity (Jaccard, 1908). This index calculates how similar are the samples by dividing the size of the intersection by the size of the union of the samples using the following equation:

$$J(A,B) = \frac{|A \cap B|}{|A| + |B| - |A \cap B|},$$

The Jaccard index is always between 0 and 1, 0 meaning that two samples do not share any species, and 1 meaning that two samples are identical in their composition.

There are other indexes that are also very used in microbiome studies. Bray-Curtis dissimilarity index (Bray and Curtis, 1957) is one of them, and it calculates the opposite that Jaccard index. Instead of calculating how similar the samples are, it calculates how different they are. Doing so adds the minor value of the common species of the comparison. Then divide it by the sum of the total number of specimens found in all the samples. Finally, it subtracts this value to 1 as shown in the following equation:

$$BC_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j},$$

As the Jaccard index, the Bray-Curtis index is always between 0 and 1. When Bray-Curtis's index equals 0, the samples are identical, while if the value is 1, the samples are not sharing any species.

To simplify the interpretation and representation, this kind of data is usually represented in ordination plots in 2 or 3 dimensions, such a canonical correspondence analysis (CCA), Principal components/coordinates analysis (PCA/PCoA), or non-metric multidimensional scaling (NMDS). A distance matrix is required for this analysis, and it is calculated with the previous indexes. In this work, we used PCoAs to test if treated/untreated and male/female samples were clustering together. In these plots, when two samples are very similar in their composition, they are represented close to each other and far if they are different. I performed these plots inside the QIIME2 environment, using the command line: "qiime emperor plot" inside de QIIME2 environment.

These plots are useful for an exploratory data analysis, but statistical tests are needed to confirm what the ordination plot suggests. We used the test to assess if treated and untreated, and male and female samples were compositionally different. We used the multivariate, nonparametric Adonis test, based on permutations of the distance matrices. The function is included in the vegan R package.

3.6.3. Linear discriminant analysis effect size

In order to better understand which taxa were the ones with the most effect in the differences between studied groups, we performed a linear discriminant analysis effect size (LEfSe) (Segata *et al.*, 2011). We used the online Galaxy framework, implemented by the Huttenhower lab "https://huttenhower.sph.harvard.edu/galaxy/"). This method finds biomarkers, such as bacteria, metabolites, genes, functions, etc., between groups of samples using robust statistical tools and orders them in importance regarding their effect on the changes using a linear discriminant analysis (LDA) (Fisher, 1936).

One of the steps to prepare the data for this framework is to add a row with the study variables to the data matrix. We divided our data in two variables: control and treatment.

3.6.4. Co-occurrence microbial networks

One of the things we wanted to clarify with this experiment was how strong was the effect of antibiotics in the community equilibrium and how it may shift the interactions between bacterial members. For this, we constructed the co-occurrence networks of our samples. These networks are based on the assumption that, if two species are in similar abundances in enough samples, they are most probably positively correlated. The problem comes when trying to compare the abundance of different samples. This cannot be done straightforwardly due to the compositionality nature of the

metagenomic data (Gloor *et al.*, 2017). When data is normalized by dividing each value by the sum of all the values in the sample, it "enters" the simplex space. This is an arbitrary, small area of the Euclidean space. Most of the statistical approaches and tools we use are developed to work in the Euclidean space. This means that we have to "move out" our compositional data from the simplex to the Euclidean space. Fortunately, there are different ways of handling this correctly, like using log-ratio transformation for the data matrix. These are the additive log-ratio (alr), the centered log-ratio (clr), that were first introduced by Aitchison (Aitchison, 1982) and the isometric log-ratio (ilr) transformations (Egozcue *et al.*, 2003). The principal operation of these transformations is the logarithm, which means that any sum of the data equals 0, will be an undefined number. This is why zeros must be treated before applying any of these transformations. There are different approaches to the problem, but one of the most used is to substitute zeros with numbers close to zero.

We choose the free software SparCC (Friedman and Alm, 2012), programmed in Python (Rossum and Drake, 1995). Log-ratio transformations and pseudo count addition are implemented in this tool, making the results robust, evading more spurious correlations than software that does not implement these measures.

We ran this module for the control and treated samples separately to compare the effect of kanamycin treatment on the structure of the interactions of the populations with the normal conditions. To avoid spurious correlations due to the nature of the data, we used strict parameters. We counted only those *p*-values that were 0.01 or lower and used only the correlations with an index equal to or greater than 0.5. The resulting matrix of this procedure with the pseudo-*p*-values of the correlations is the input we used to construct the network plots

for more straightforward interpretation. The software used was the igraph R package v.1.2.4 (Csardi and Nepusz, 2006).

3.6.5. Temporal analysis

Time is a crucial variable in this thesis, which is why we used different longitudinal approaches in this work. Classically, analysis has been performed by grouping samples and statistically comparing them depending on the experimental conditions. That is what is done in this section, but the day-to-day analysis is shown later in this work.

First, we wanted to assess the usual dynamics of the diversity in control populations and then compare it to the treated populations.

For this analysis, we divided the experiment into three different periods of time: A, B and C for treated and control populations. As already mentioned, since the experiment is 105 days long, each of the periods is 35 days long and they are consecutive. In treated populations, the first 10 days of each period cockroaches are exposed to kanamycin (0.2 mg/mL) through the provided water. The other 25 days are equal to the control population, with normal conditions of diet and without antibiotic treatment (Figure 10).

Then, we analyzed the alpha diversity of each of the mentioned periods for control and treated populations. We used the Shannon index for diversity. Finally, we statistically compared the three periods of control and treatment using Kruskal-Wallis, implemented in the QIIME2 pipeline.

3.6.5.1. Taxon dynamics

In order to achieve a better understanding of the AB effect on each bacterial group during the 105 days of the experiment, we used the metagenomeseq R package (Paulson *et al.*, 2017). This package includes a function called fitTimeSeries which calculates the time intervals in which there are statically significant differences in

abundance. To find them, it performs the Smoothing Spline Anova method (SSANOVA). Smoothing splines are functions that minimize the variation of the sampled data; this means that these functions do not necessarily pass through the sampled data points. This method simulates approximately where the real points, abundances in this case, may be. This function also adds an interval confidence level.

We compared the control samples with the treatment samples to assess the differences in abundance during the key time points. These are the three 10-day periods when cockroaches were supplied with AB through the water. But we were also interested in the capability of the taxa to recover the abundance previous to the treatment, this is, their resilience. This method allows us to find and name resilient taxa in the treated populations. We can also find sensible, resistant and opportunistic taxa. The last group is formed by taxa whose abundance grows when the AB affects the sensible taxa due to the free niches that the AB is creating.

3.6.5.2. Rank stability index

In order to study temporal variation in the diversity of control and treated samples, we used a specialized software developed specifically for this purpose (Martí *et al.*, 2017). This software, complexCruncher, requires other packages such as pandas, numpy, and matplotlib to work. It takes into account the relative abundance and the rank of each of the 50 most abundant taxons for every time point. Then, it calculates the rank stability index (RSI), which represents the probability of each taxon staying at the same rank at any time point. This index ranges from 0, which means that one specific taxon changes its rank at every time point, to 1, representing a taxon that stayed at the same rank during all the time points. The software calculates this index using the following equation:

$$RSI = \left(1 - \frac{D}{(N-1)(t-1)}\right)^{p},$$

where D represent the total of the ranks changed by one specific taxon, N represents the total of taxa in study, and t represents the number of time points in the experiment. Finally, p is the power index and is selected arbitrarily, in this case, it equals 4 to increase the resolution in the stable regions.

3.6.5.3. *V* and β parameters

The Taylor's law (Taylor, 1961), can be found in a wide range of biological and non-biological phenomena such as cancer metastasis (Kendal and Frost, 1987), evolution of demographics (Cohen *et al.*, 2013), spatial distribution of urban facilities (Wu *et al.*, 2014) or regional monthly crime reports (Hanley *et al.*, 2014). But it also applies to the temporal variation in the gut microbiota (Martí *et al.*, 2017). In ecology, this law relates the variances of the number of individuals of a species per unit with the population's mean by a power law relationship. In this work the, Taylor's power law is applied through a software that uses the following equation:

$$\sigma_i = V \cdot x_i^{\beta} \ ,$$

where σ_i represents the dispersion of the population, x_i represents the mean of the abundance, V is the standard deviation of the abundances and β is the slope of the fit.

In this work, we wanted to assess the changes in variability for the treated samples compared to the control samples. To check if our samples followed Taylor's law, we used the software complexCruncher to check the fits. As we checked that our data fitted the model, we studied the parameters V and β of our samples. We compared the values of said parameters for each population (C1, C2, K1, K2, and K3) and then compared two groups, one formed by the control populations (C1 and C2) and other by the treated populations (K1, K2, and K3).

The β parameter is normally between 0 and 1, depending on the behavior of the system. When the β parameter is lower than 0.5, that is, β tends to 0, the system follows a Poisson distribution. When the β parameter is 1, the system operates as an exponential distribution. Finally, when this parameter is in between, we say that it follows Taylor's Law. As seen in Taylor's definition of σ, this parameter will be equal to the V constant, since $x_i^{\beta} \rightarrow 1$. As assumed by Poisson distribution definition, the expected value and the variance are equal to the parameter of the distribution and then constant.

3.7. Functional analysis with PICRUSt2

To check the functional effect of the AB on the gut microbiota of the cockroaches in the experiment, we used the open-source software PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). This software is designed to infer the functional abundances of the input samples using the abundance of 16S rRNA genes (Douglas et al., 2020), although other markers can also be used. PICRUSt2 uses its own database, which contains 20,000 different 16S rRNA genes from bacteria and archaea from the Integrated Microbial Genomes (IMG) database (Markowitz et al., 2012). The outputs of this program are two tables, one containing the pathway abundances, and the other containing the gene family profiles.

We used PICRUSt2 to find if there are differences in the abundance of the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways between treatment and control populations for any of the three periods studied. But also, to find if any of these pathways were markers of the studied conditions. For this step we performed a LEfSe analysis using the Galaxy framework, implemented by the Huttenhower lab (https://huttenhower.sph.harvard.edu/galaxy/). We also checked if the kanamycin kinase enzyme (EC: 2.7.1.95), the

enzyme responsible for the inactivation of Kanamycin A, was part of the metabolism of the taxa in our samples. Finally, we listed the bacterial taxa that harbored that enzyme.

3.8. Functional robustness

In order to check the effect of the changes in the taxa abundance due to the AB treatment, we studied the community functional robustness of our samples. As defined by Eng and Borenstein (2018), robustness represents the impact on the functional capacities of a community after suffering compositional changes. This parameter is calculated using the following equation:

$$f = \frac{1}{e^{a}} t^{b}$$

where *t* represents the weight of taxonomical changes, *f* represents the expected changes in functions, and *a* and *b* are two specific factors that give a quantitative result to make comparisons. The first one is "attenuation", which explains how quickly functional changes occur in response to increasing taxonomical compositional shifts. The higher the attenuation value, the higher the robustness of the samples. The other factor is "buffering", which shows the magnitude that the taxonomical alterations must have so that the changes in the functions are appreciable.

We have compared these parameters for control and treatment populations, for each of the three periods (A, B, and C) of the experiment. Using the results provided by PICRUSt2, we tested attenuation and buffering for different pathways such as xenobiotics biodegradation and metabolism, transport and catabolism, membrane transport, and others. For the statistical analysis, we tested the comparisons using the Wilcoxon test for attenuation and buffering.

4. Results

4.1. Sample description and sequencing data

A total of 512 individuals were analyzed. Half of the hindguts were obtained from males, and the other half from females. The individuals were divided in five different populations with more than 100 individuals each. Two populations were under control conditions (C1 and C2) and the other three were treated with the antibiotic kanamycin in water at a concentration of 0.2 mg/mL. The kanamycin was supplied in three periods of 10 days separated by 25 days of untreated water supply. The experiment lasted 105 days. Experimental design is shown in Figure 9.

We sequence the V3-V4 region of the 16S rDNA gene, and obtained an average of 116,564 reads per sample. The range of the reads were from 9 to 1,696,169 reads. We then performed the quality control step using DADA2 implemented in QIIME2. We also removed all samples with less than 5,000 reads. After this process, the average of reads per sample was 82,657 ranging from 5,344 to 1,283,730 reads which means that 64% of reads passed the filtering.

4.2. Bacterial core of the microbiota of *B. germanica*

In order to set the bacterial core of *B. germanic*a we used only the control samples from the two populations. We calculated the mean abundance of every bacterium at genus level for each time point and then selected only those taxa that were present, independently of their frequency in all time points.

We found that the bacterial core of the cockroaches is formed by 18 different genera or the closest found taxon level, grouped in 4 phyla: *Dysgonomonas* (15.7%), *Alistipes* (9.2%), *Bacteroides* (5.3%),

Rikenellaceae (4.4%), Tannerellaceae (3.8%), Bacteroidia (2.6%), Dysgonomonadaceae (1.3%) and *Parabacteroides* (1.1%) (Phylum Bacteroidota); *Candidatus Soleaferrea* (5.1%), Ruminococcaceae (4.4%), *Christensenellaceae R-7 group* (3.3%), Lachnospiraceae (1.9%) and *Tyzzerella 3* (1.4%) (Phylum Bacillota); *Desulfovibrio* (13.1%), Rs-K70_termite_group (2.0%) *Desulfatiferula* (0.7%) (Phylum Pseudomonadota); *Fusobacterium* (11.0%) (Fusobacteriota, previously known as Fusobacteria) and finally *Mucispirillum* (0.6%) (Phylum Deferribacterota, previously known as Deferribacter). These results are in agreement with the previous kanamycin study performed in our laboratory (Domínguez-Santos *et al*., 2021).

4.3. Gut microbiota comparison between sexes

Differences between males and females regarding gut microbiota composition have not been assessed in cockroaches in previous experiments. We wanted to know if the sex factor affects the composition of the microbiota and if males and females respond equally to the AB treatment. We estimated alpha and beta diversity of males and females, and applied the Adonis to the abundance matrix to check if there were statistical differences between sexes. We used all the populations and time points for these tests.

We used the Shannon and Chao1 indexes for the alpha diversity and richness, respectively (Figure 14). Using the Wilcoxon test, we found no differences for these indexes between males and females. The *p*-value for the Shannon index comparison was of 0.2270 while the Chao1 was 0.9949. These results demonstrate that the microbiota of males and females are equally diverse and rich, independently of the treatment. This means that male and female individuals could be used together for experiments where the microbiota is the focus of the investigation.

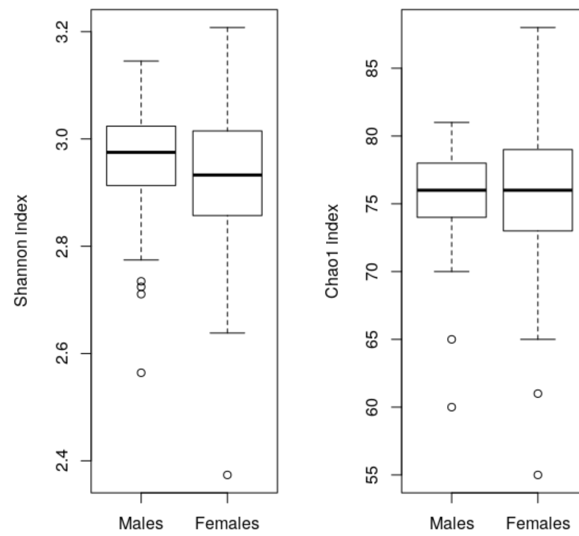

Figure 14: Alpha diversity boxplot showing Shannon (left) and Chao1 (right) indexes for males and females.

Regarding beta-diversity, we performed a PCoA using the Jaccard similarities distance. It can be seen in Figure 15 that female and male samples scattered in a cloud, all mixed together. This means that the composition of the samples is very similar. The three most explaining

components of this PCoA represents the 20.66% of the total variability in the samples.

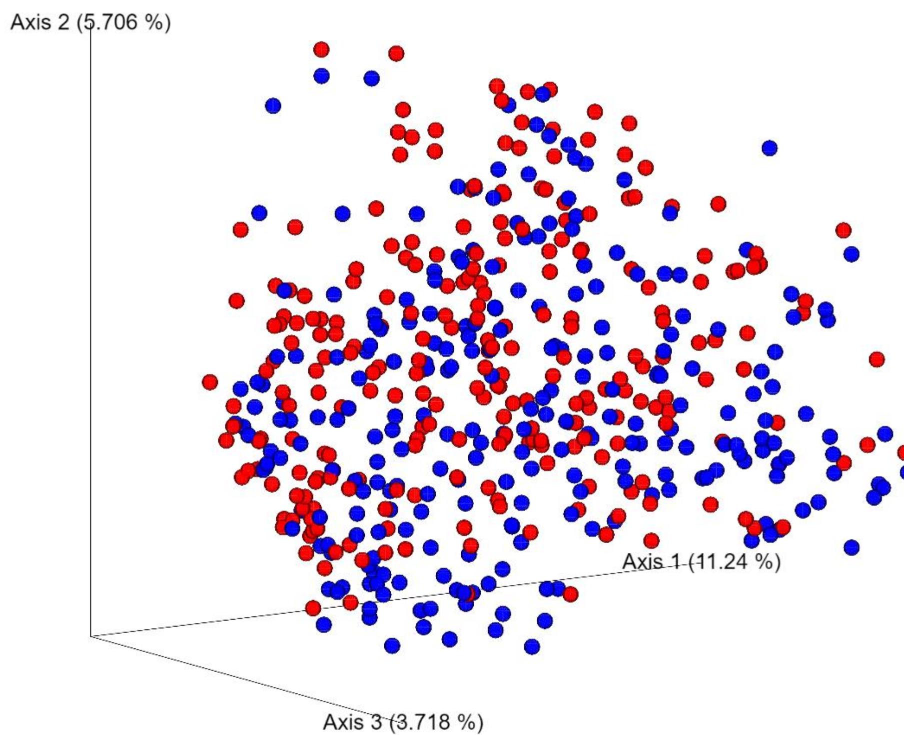

Figure 15: Three-dimensional Principal Coordinates Analysis (PCoA) of all samples. Female samples in red and male samples in blue.

Finally, we statically tested if there were differences between male and female composition using the Adonis function in the R package vegan. We found no differences between both groups (*p*-value = 0.1180). Thus, we can conclude that there are no differences in alpha and beta diversity between males and females and that they seem to react in the same way when exposed to AB treatment. With this result in mind, we performed the rest of the analysis not taking into account the sex of the samples.

4.4. Gut microbiota comparison between treated and control population

We analyzed the abundance of bacteria at phylum and genus levels in all the samples. We found that, on average, the most abundant phyla in the populations are Bacteroidota (C: 45.6%, K: 48.0%, *p*-value = 2.6803e+04), followed by Bacillota (C: 21.9%, K: 20.4%, *p*-value = 0.0203), Pseudomonadota (C: 18.4%, K: 17.7%, *p*-value = 0.0441) and Fusobacteriota (C: 10.7%, K: 10.5%, *p*-value = 0.5565). Bacteroidota, Bacillota and Pseudomonadota significantly differ between control (C) and treated (K) populations under the Wilcoxon test, while Fusobacteriota do not.

Regarding the genus level, we found that those bacterial groups which abundances greater than 1% in at least one of the conditions are: *Dysgonomonas* (C: 15.7%, K: 22.0%, *p*-value = 5.7092e-18), *Alistipes* (C: 9.2%, K: 7.2%, *p*-value = 9.8005e-10), *Bacteroides* (C: 5.3%, K: 5.2%, *p*-value = 0.0617) and *Parabacteroides* (C: 1.1%, K: 0.8%, *p*-value = 1.1688e-07) from the phylum Bacteroidota; *Candidatus Soleaferrea* (C: 5.8%, K: 4.9%, *p*-value = 0.1945), *Christensenellaceae_R-7_group* (C: 3.3%, K: 1.9%, *p*-value = 2.6470e-20), *Tyzzerella_3* (C: 1.4%, K: 1.2%, *p*-value = 6.3589e-17) and *Ruminiclostridium_5* (C: 1.3%, K: 0.9%, *p*-value = 0.4029) from the phylum Bacillota; *Fusobacterium* (C: 11.0%, K: 10.7%, *p*-value = 0.4307); *Desulfovibrio* (C: 13.1%, K: 13.7%, *p*-value = 0.2741) from the phylum Pseudomonadota. At this level, *Dysgonomonas, Alistipes, Parabacteroides, Christensenellaceae_R-7_group* and, *Tyzzerella_3* differ significantly in abundance from control to treated populations. A table ordered by average abundance for control and treatment populations at genus level with the *p*-value associated is shown in the appendix, as Supplementary Table 1.

We also compared the alpha-diversity of control and treated samples at genus level using the Wilcoxon test. We found that control

populations had a greater diversity for Shannon and Chao1 indexes as shown in figure 16 (p-value = 1.1768e-18, and p-value = 5.5790e-12 for Shannon and Chao1, respectively).

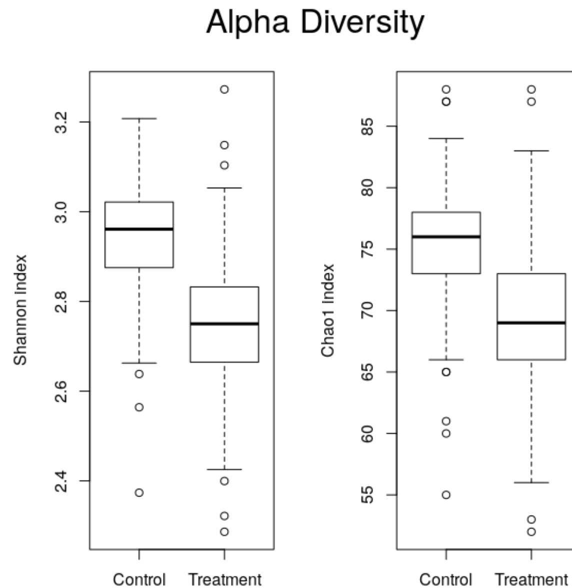

Figure 16: Alpha diversity boxplot showing Shannon (left) and Chao1 (right) indexes for control and treatment populations.

As we did for comparing males and females, we performed a PCoA using the Jaccard similarities distances, this time to check if the AB was impactful on the bacterial composition of the microbiota. The PCoA (Figure 17) showed a clear separation between control and treatment populations, indicating that kanamycin is an important factor affecting the composition of microbiota. The Adonis test (p-value < 0.001) supported this result. Thus, we conclude that kanamycin had the effect of decreasing the diversity and altering the composition of the cockroach's gut microbiota.

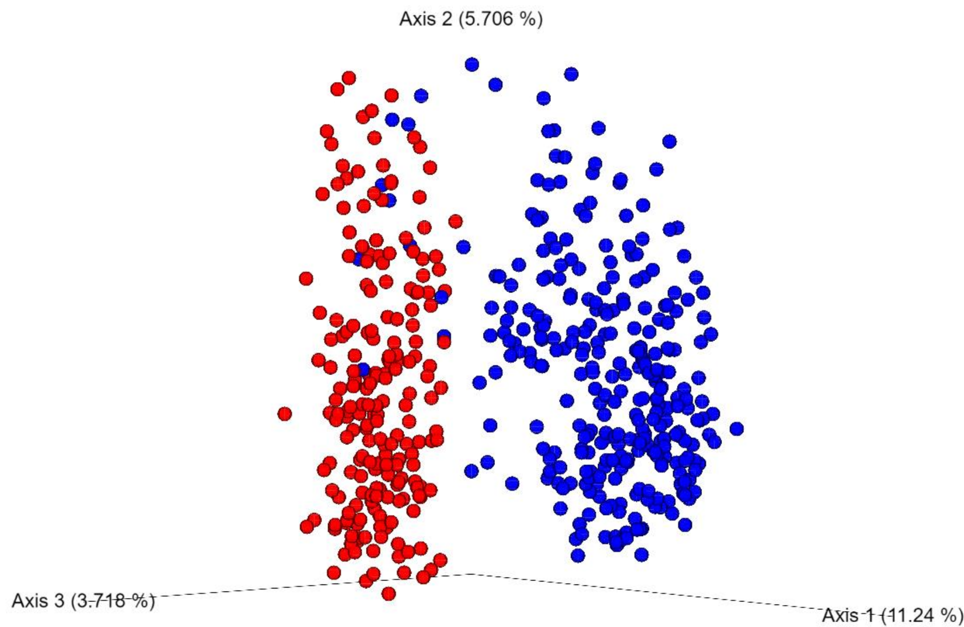

Figure 17: Three-dimensional Principal Coordinates Analysis (PCoA) of all samples. Control samples in red and treatment samples in blue.

Once we checked that there were significant differences between control and treatment samples, we performed a Linear discriminant analysis Effect Size (LEfSe) to determine the specific taxa that are, most likely, causing these differences. We found 24 taxa that were significantly more abundant in control than treatment samples. From these, 18 belong to the phylum Bacillota (Acidaminococcaceae, *Anaerovorax*, Christensenellaceae R-7 group, Clostridiales vadinBB60 group, *Enterococcus*, *Erysipelatoclostridium*, *Eubacterium coprostanoligenes group*, *Fournierella*, GCA-900066225 [Ruminococcaceae], *Hydrogenoanaerobacterium*, Incertae Sedis [Lachnospiraceae], *Papillibacter*, *Robinsoniella*, Ruminococcaceae NK4A214 group, Ruminococcaceae_UCG_009, Ruminococcaceae_UCG_013, Syntrophomonadaceae and *Tyzzerella* 3), 1 to the phylum Actinomycecota (*Raoultibacter*) and 3 to the phylum Pseudomonadota (*Pseudomonas*, Rhodospirillales and Rs-K70 termite group), 1 to the phylum Bacteroidota (Tannerellaceae) and 1 to the

phylum Synergistota, previously known as Synergistetes (Synergistaceae).

On the other hand, 4 bacterial groups were in higher abundance in treated populations than in control ones, 1 belongs to Bacteroidota phylum (*Dysgonomonas*) and 3 to Bacillota one (*Anaerotruncus, Breznakia* and Lachnospiraceae). The described LEfSe results are shown in Figure 18.

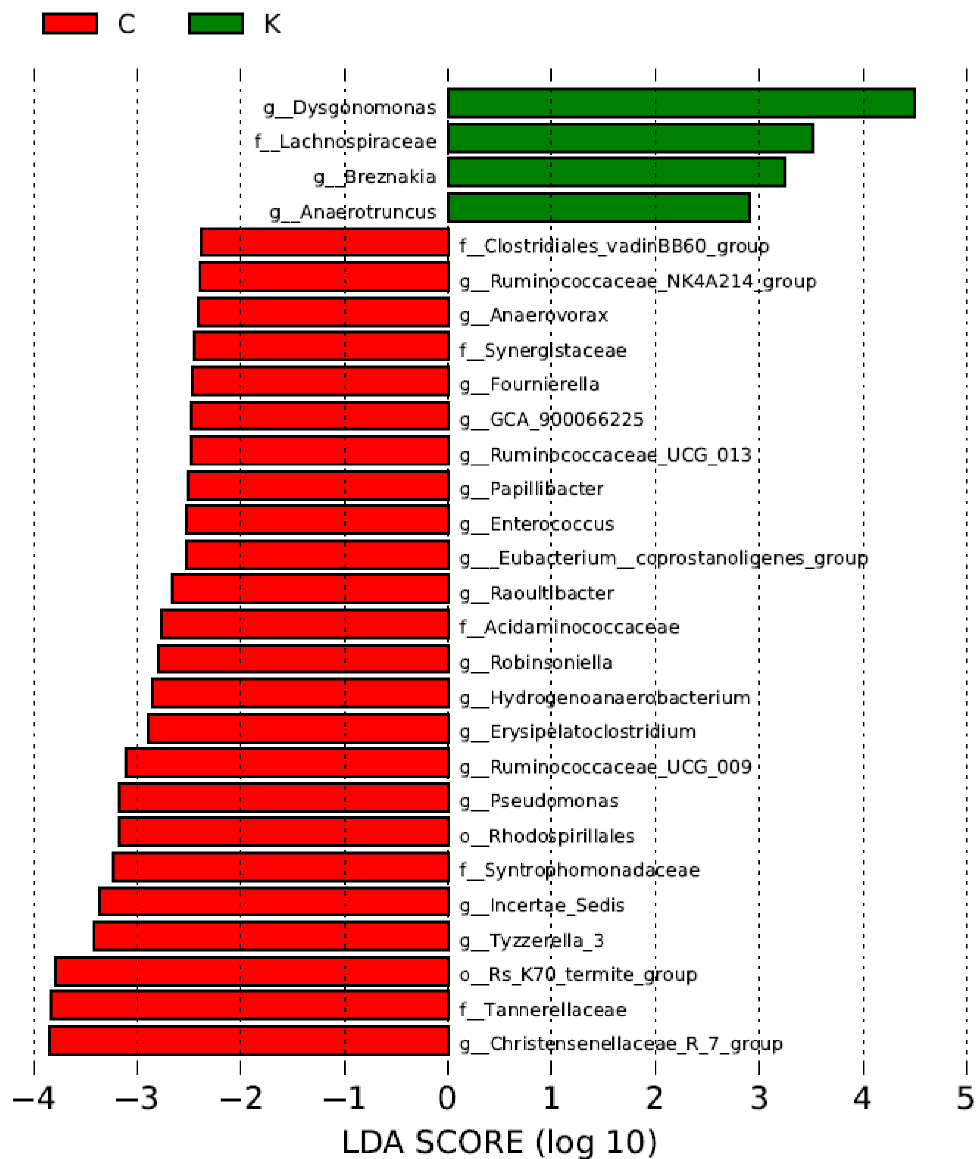

Figure 18: Linear discriminant analysis Effect Size graph showing taxa that significantly differ from control (red) to treatment (green) samples.

4.5. Co-occurrence network of the gut microbiota

To obtain a deeper characterization of the gut microbiota, and once we know that there were significant differences in some taxa between control and treated populations, we decided to run an analysis that shows groups of taxa that increase or decrease their abundance coordinately. We used the software SparCC implemented for R and Shell for the analysis, which includes methodology to treat compositional data, and the igraph R package to make the plots. All the co-occurrences shown have a *p*-value lower than 0.01. The analysis was run separately for control and treatment populations and, thus the results are shown separately

4.5.1. Control population

We found that, for control populations, different groups co-occurred and some of them are connected. More specifically, the analysis detected 6 groups, each one formed of different bacterial taxa (Figure 19).

The first group, in dark blue, is formed by 7 different taxons grouped in 3 phyla: *Ruminococcaceae UCG 013*, *Eubacterium coprostanoligenes group*, *Papillibacter* and *Ruminococcaceae UCG 009* from the Bacillota phylum; *Akkermansia* from Verrucomicrobiota, previously known as Verrucomicrobia; Rhodospirillales from Pseudomonadota; and *Parabacteroides* from Bacteroidota. Taxa with most connections in this group are *Eubacterium coprostanoligenes group* and *Ruminococcaceae UCG 009*, with 4 links, and they are connected to each other, which makes them the most important taxa of this group. The total number of links is 8. Only one bacterium is part of the core, *Parabacteroides.*

The following three groups are connected. Yellow and pink are connected through vadinHA49-Rs K70 termite group link and *Desulfovibrio-Bacteroides* link. Yellow and green are connected through

the Odoribacter-Dysgonomonadaceae link. There are no connections between the pink and green group.

Yellow group is formed by 10 different taxa grouped in 5 phyla: Desulfovibrionacecae, Rs-K70 termite group, *Nitrosomonas*, and *Oxalobacter* from the Pseudomonadota phylum; *Odoribacter* and *Bacteroides* from the phylum Bacteroidota; Acidaminococcaceae and *Hydrogenoanaerobacterium* (Bacillota phylum); *Mucispirillum* (Deferribacterota phylum); and *Fusobacterium* (Fusobacteriota phylum). The taxa with most connections in this group are Desulfovibrionaceae and Acidaminococcacecae, with 8 links, and they are connected to each other, which makes them the most important pieces of this group. Desulfovibrionaceae, *Bacteroides*, and *Odoribacter* are of great importance too, since they connect to other groups, which makes the net more robust. This is the group with the most links, with a total of 22 and 3 of them connecting to other groups. There are three taxa that are also part of the bacterial core of the control population: *Bacteroides*, *Mucispirillum* and *Fusobacterium*.

The pink group is formed only by 3 taxa grouped in an equal number of phyla: *Candidatus Soleaferrea* (Bacillota phylum); *Desulfovibrio* (Pseudomonadota phylum); and vadinHA49 (Planctomycetota phylum, previously known as Planctomycetes). The total number of links in the group is 2, and there are 2 other links that connect to other groups. Finally, 2 out of 3 taxa are part of the bacterial core of the control population: *Candidatus Soleaferrea* and *Desulfovibrio*. The green group is formed by 2 families of the phylum Bacteroidota: Paludibacteraceae and Dysgonomonadaceae. This group is connected to the yellow one through Dysgonomonadaceae, which is also part of the core bacteria. The clear blue group is formed by 2 taxa of phylum Bacteroidota: *Alistipes* and Tannerellaceae. Both of them are part of the core. The group is not connected to any other group.

Finally, the red group is formed by 6 taxa grouped in 2 phyla: *Lachnoclostridium*, *Lachnospiraceae UCG 010*, *Erysipelatoclostridium*, Lachnospiraceae, and *Breznakia* (Bacillota phylum); and *Raoultibacter* (Actinomycecota phylum). Taxa with most connections in this group are *Lacnoclostridium* and Lachnospiraceae, with 4 links, and they are connected to each other, which makes them the most important pieces of this group. The total number of links of this group is 7. Lachnospiraceae is part of the bacterial core.

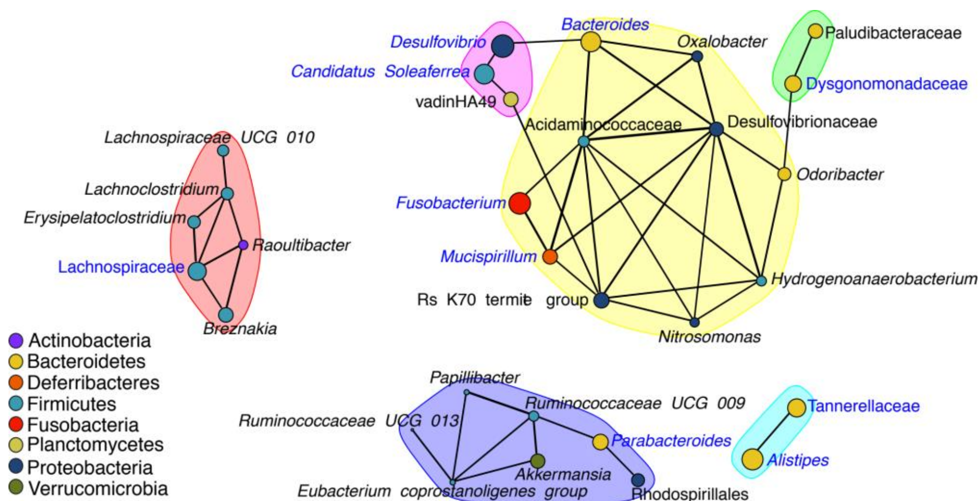

Figure 19: Bacterial co-occurrence plot for the control population. Each node represents a taxon (families and genus), color-coded by phylum. Names in blue represent taxa that are part of the bacterial core in the control population. Size of the circles represent the relative mean abundance of that specific taxon.

4.5.2. Treated population

We also tested this same procedure with treated populations, but we found no co-occurrences with the analysis using the same parameters as with controls. After this result, we decided to use less strict parameters in the analysis. We restricted the *p*-value at 0.05 or lower instead of 0.01 or lower and used only the correlations with an index equal or greater of 0.1 instead of 0.5. Despite these changes, we did not obtain any co-occurrence and thus, we can say that treated populations do not co-occur, at least with our parameters. It seems

that the AB disrupts the equilibrium in the microbiota in a way that bacteria (their abundance) behave in a more random way which seems to prevent the formation of co-occurrence networks.

4.6. Temporal dynamics along the experiment

We wanted to understand not only the state of the microbiome in a given moment, but also how the microbiome adapted and evolved to the changing environment during the experiment. To do so, we used different approaches. First, we checked if our samples followed the Taylors law. As previously said, this law is ubiquitous in nature. We checked it using the complexCruncher software that calculates the fits for this law. Control and treated samples followed the law as shown in Figure 20. Control samples had a slightly better coefficient of determination (R^2 = 0.971) compared to the treated samples (R^2 = 0.969), but both are very high.

The other two parameters, V and β, explained in detail in Materials and Methods, were higher in samples from the treated population. V represents the variability and we expected it to be higher in treatment, since antibiotics are a source of mathematical noise and instability in the microbiome. Its value for control samples equals 0.16 ± 0.1 while it is 0.18 ± 0.2. Margin of error is also higher in treated samples. Lastly, the β parameter value is 0.72 ± 0.1 for control and 0.74 ± 0.1 for treatment. This parameter shows that taxa with more abundance are also more stable, and those with less abundance are more variable. This is more accurate when the β value is higher.

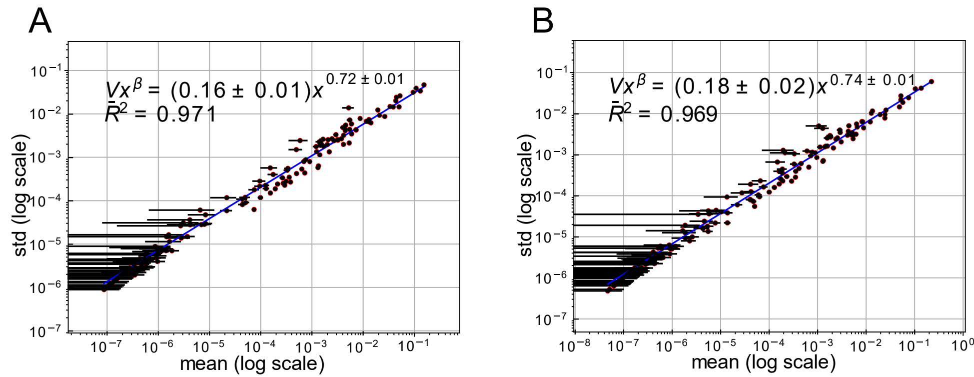

Figure 20: Fits for the Taylor power law. A) Control samples, B) treated samples. Each dot represents one taxa during the longitudinal experiment.

4.6.1. Taxon dynamics

We wanted to assess the abundance of all the taxa in our samples in order to identify possible tendencies. We used the metagenomeseq R package to compare control with treated samples. With this software we obtained the abundance of each bacterial taxa in this study day by day.

We found that, at genus level, there are indeed different groups of taxa, whose abundance increased or decreased similarly. A total of five groups were established based on their abundance dynamics. First, we found *Breznakia, Anaerotruncus* and Lachnospiraceae that increased their abundance during treatment periods (Figure 21 A). We also found a group of five taxa, Micrococcales, *Christensenellaceae,* Odoribacter, *Tyzzerella_3* (shown in Figure 21 B) and Paludibacteraceae that were slightly less abundant at all times in the treated population. The next group is also formed by 5 different genera, *Akkermansia, Paludibacter, Bacteroides* (shown in Figure 21 C), *Fusobacterium* and Bacteroidia. These do not seem to be affected by the AB treatment at any given moment. Three bacterial taxa Rhodospirillales, *Candidatus Endomicrobium* (shown in Figure 21 D) and *Erysipelatroclostridium* from the treatment group kept their abundance permanently at a very low level compared to control

samples. Lastly, and the most interesting group, the taxa that was affected during the first and second exposition to AB, or only the first one, but during the rest of the exposition their abundance were not affected. This is a sign that those may be resilient. We found 5 taxa that followed this criterion: *Lachnoclostridium, Parabacteroides, Christensenellaceae R-7 group* (shown in Figure 21 E), Tannerellaceae and Desulfovibrionaceae. The periods in which control and treatment abundances are divergent are shown in Supplementary Table 2.

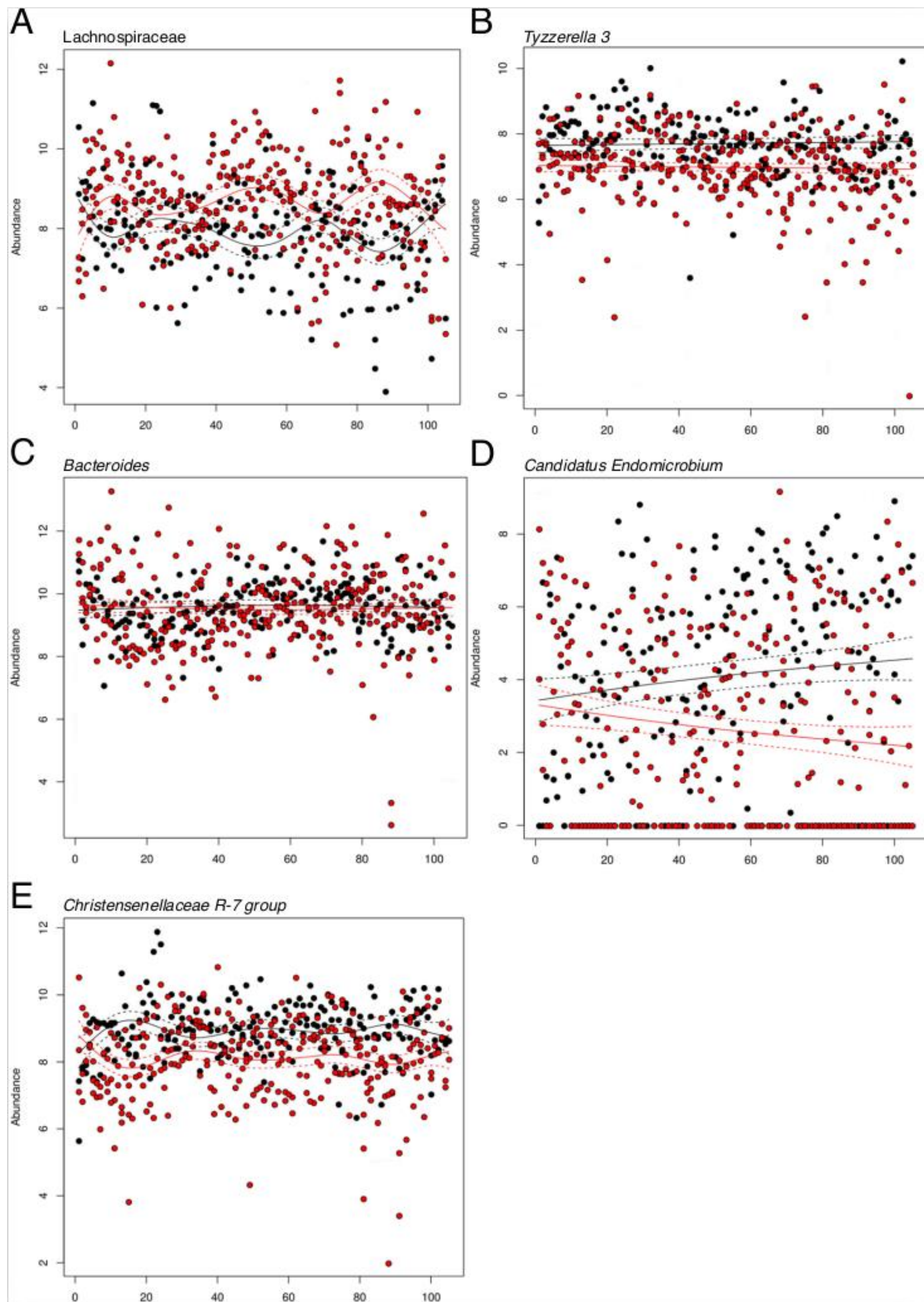

Figure 21: Abundance dynamics of five different bacterial taxa representing the groups of dynamics. A) Lachnospiraceae representing a family of opportunistic bacteria B) Tyzzerella 3 representing mildly affected bacteria C) Bacteroides representing unaffected bacteria D) Candidatus Endomicrobium representing continuously affected bacteria and E) Christensenellaceae R-7 group representing resilient bacteria.

4.6.2. Variability across series

The use of antibiotics is able to induce noise in the bacterial populations since it interacts differently with each one of the species present in the microbiota (Figure 22). We expected that this fact results in an increment in V in the abundance of taxa that are exposed to kanamycin in treated populations.

We studied the V and β parameters in the populations in the three studied periods and found that non-treated populations have less variability compared to the first period of treated populations. Periods 2 and 3 of treated populations have very similar V values compared to control in the 2 and 3 periods, while period 1 of control has the smallest V value and the period 1 of treatment has the highest V value. Regarding β parameter, all populations had a similar value for every period, being the highest the treated population during the period 3 and the lowest the control populations during the period 3.

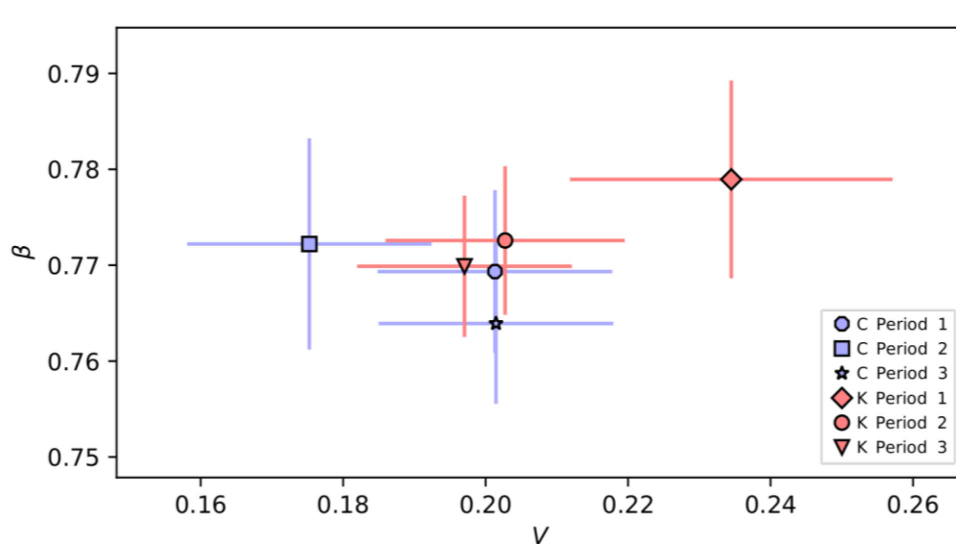

Figure 22: V and β parameters for control (blue) and treated (pink) populations in three different periods.

4.6.3. Rank stability

The ordinal changes that taxa suffer during the experiment is also an interesting factor to study. It is another point of view from which we can assess the dynamics that occur within the gut of *B. germanica*. In this case, we used the complexCruncher software to assess the variation in the rank of each of the 50 most abundant taxa. Rank represents the order in abundance. We have to take into account one of the flaws of this system, even when a specific species of bacteria does not change its rank, its abundance may have suffered a drastic change.

We found that 47 out of the 50 most abundant taxa do not change regardless of AB treatment. As the Taylor law predicted, the top ranks have a higher RSI, that is, they changed their rank less drastically than those of the bottom ranks. *Dysgonomonas* is at the same time the bacterial genus with the highest RSI, with a mean value of 96.8%, and rank nº 1 for all groups (C1, C2, K1, K2, K3). In opposition to Taylor's law, we found some taxa with a low rank that have a high RSI. This is the case of *Oxalobacter, Clostridiales* and *Raoultibacter* that are always above 78.5% RSI and under the rank 38. Every group graph is shown in the Supplementary Figures 1 to 5.

4.7. Functional analysis

4.7.1. Functional profile

We used the Picrust2 program (Langille et al., 2013) to find the most abundant KEGG pathways in control and treated populations as shown in Figure 23. We found that both conditions are very similar in term of abundance of the KEGG pathways. The twenty most abundant pathways are the same for both conditions and have a similar abundance. Metabolic pathways (C: 31.2%; K: 31.44%) represents the most abundant pathway in the experiment, followed by biosynthesis

of secondary metabolites (C: 14.36%; K: 14.42%), microbial metabolism in diverse environments (C: 7.54%; K: 7.57%), biosynthesis of cofactors (C: 6.43%; K: 6.35%), biosynthesis of amino acids (C: 6.29%; K: 6.33 %), carbon metabolism (C: 4.80%; K: 4.77 %), ribosome (C: 3.58%; K: 3.51%), ABC transporters (C: 3.07%; K: 3.14%), two-component system (C: 2.62%; K: 2.65%), purine metabolism (C: 2.35%; K: 2.35%), biosynthesis of nucleotide sugars (C: 2.22%; K: 2.20%), nucleotide metabolism (C: 2.08%; K: 2.08%), amino sugar and nucleotide sugar metabolism (C: 2.02%; K: 2.03%), quorum sensing (C: 2.02%; K: 1.98%), glycolysis / gluconeogenesis (C: 1.98%; K: 1.99%), pyrimidine metabolism (C: 1.95%; K: 1.93%), pyruvate metabolism (C: 1.83%; K: 1.83%), carbon fixation pathways in prokaryotes (C: 1.83%; K: 1.81%), aminoacyl-tRNA biosynthesis (C: 1.58%; K: 1.14%), cysteine and methionine metabolism (C: 0.20%; K: 0.45%).

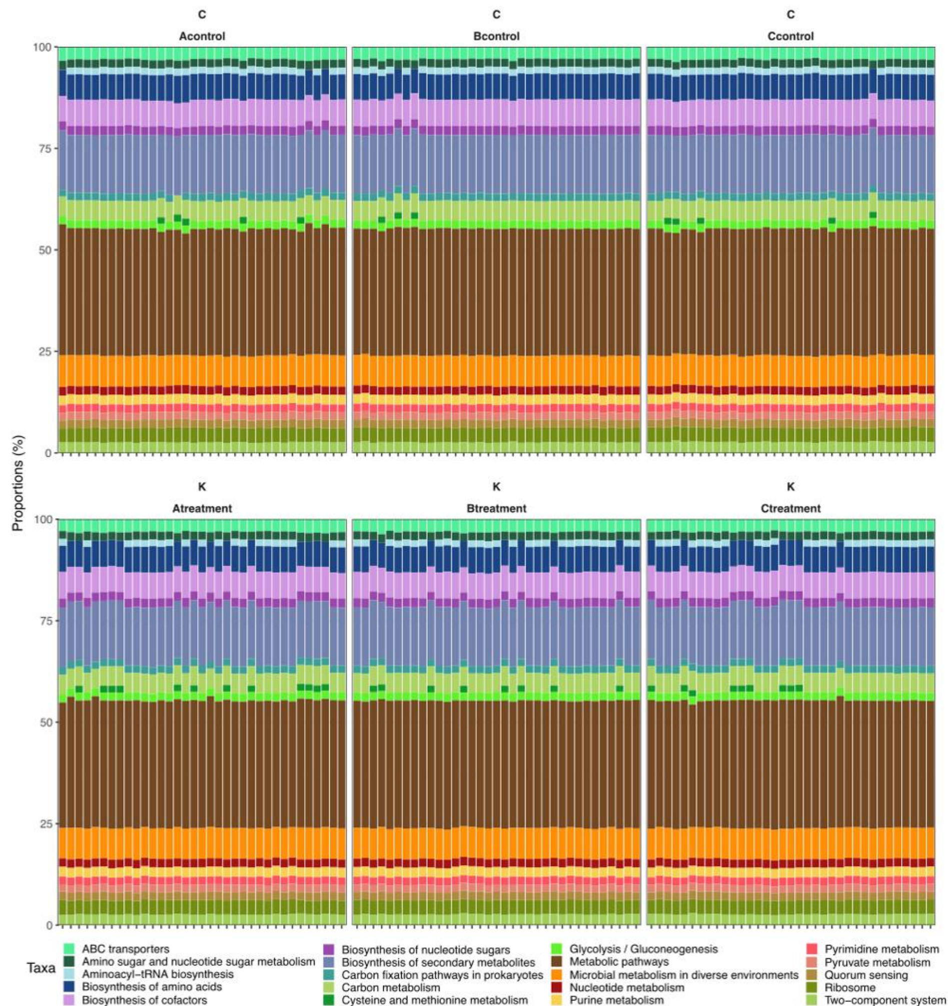

Figure 23: Stacked barplots of the 20 most abundant functions extracted from KEGG Orthology in control (C) and treatment (K) populations. Each condition is divided in three periods (A, B, and C).

We checked if there were significant differences in terms of abundance of the pathways between both conditions. We performed an Adonis test for each of the three periods of the study (A: days 1 to 35; B: days 36 to 70 and C: days 71 to 105). All three comparisons showed significant differences (p-value < 0.05).

4.7.2 Differential pathways

Once we checked that there were significant differences between conditions, we performed a Linear discriminant analysis Effect Size

(LEfSe) to find pathways that were significantly more abundant in each condition.

As shown in Figure 24, there are 10 pathways that are significantly more abundant in treatment conditions than in control. The one with the highest LDA score is metabolic pathways, followed by starch and sucrose metabolism, galactose metabolism, other glycan degradation, pentose and glucuronate interconversions, ABC transporters, sphingolipid metabolism, cyanoaminoacid metabolism oxidative phosphorylation and fructose, and mannose metabolism.

For control populations, we found 6 pathways that were significantly more abundant. The one with the highest LDA score is quorum sensing, followed by carbon metabolism, butanoate metabolism, aminoacyl tRNA biosynthesis, ribosome, and biosynthesis of cofactors.

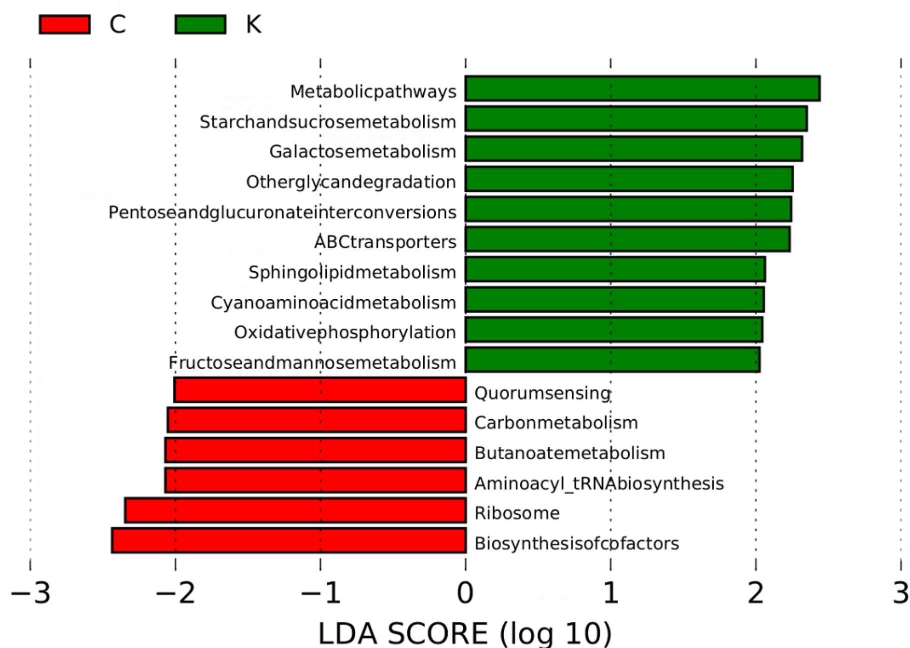

Figure 24: Linear discriminant analysis Effect Size graph showing functions that significantly differ from control (red) to treatment (green) samples.

4.7.3. Kanamycin degradation by kanamycin kinase

First, we checked if there were differences in the abundance of the KEGG enzymes. We performed an Adonis test for each of the three

periods of the study. All three comparisons showed significant differences (p-value < 0.05). Then, we were interested in the enzyme kanamycin kinase (EC: 2.7.1.95) that is responsible for the inactivation of the kanamycin A molecule by phosphorylating it. The reaction is shown in the Figure 25. This enzyme provides resistance against antibiotics of the group of aminoglycosides such as kanamycin, neomycin, paromomycin or neamine among others. Thus, we checked if it was present in our samples and if there were significant differences between conditions (Doi et al., 1968).

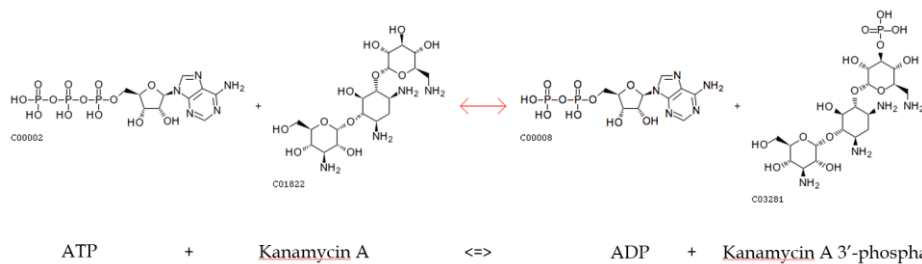

Figure 25: Reaction of inactivation by phosphorylation of the kanamycin A molecule.

We found significant differences in the abundances of this enzyme for the three mentioned periods, with a p-value < 0.05. Interestingly, it is more abundant in control conditions than in treatment conditions. Its abundance in our samples is 0.00030% and 0.00015% for control and treatment, respectively.

We also checked which bacterial taxa carry this enzyme and found that 13 different taxa carry it. The most represented phylum is Bacillota, with 6 taxa, namely *Erysipelatoclostridium, Tyzzerella, Lachnospiraceae UGC-010*, *Agathobacter*, Lachnospiraceae, and *Streptococcus*. The second most presented phylum is Pseudomonadota, with 4 taxa, specifically *Senotrophomonas*, *Pseudomonas*, *Sphingomonas,* and Rhizobiaceae. We also found 2 taxa from the Actinomycecota phylum, *Microbacterium,* and *Mycobacterium* and, lastly, vadinHA49, from the planctomycetota phylum.

4.8 Robustness

To determine the robustness of our samples, we checked the buffering and attenuation in our samples. These two parameters measure how intense the abundance changes should be so that changes in functions are measurable and, how fast these functional changes arise in consequence of the taxonomical fluctuations, respectively.

4.8.1. Robustness comparison by group

First, we compared both parameters with control and treated samples. We did not take into account the three periods for this analysis. We found that there are no significant differences between control and treated populations for the attenuation parameter (Wilcox-test p-value >0.05). On the other hand, we did find significant differences in the case of buffering (Wilcox-test p-value < 0.01). We represented the attenuation and buffering values for treated and control populations in a graph (Figure 26). Three polynomial regressions (simple, quadratic and cubic) were adjusted the data and selected the best model using the Akaike information criterion (AIC). In the case of controls, the simple model was the best one. Meanwhile, in the case of treated populations, the cubic model was the most appropriate.

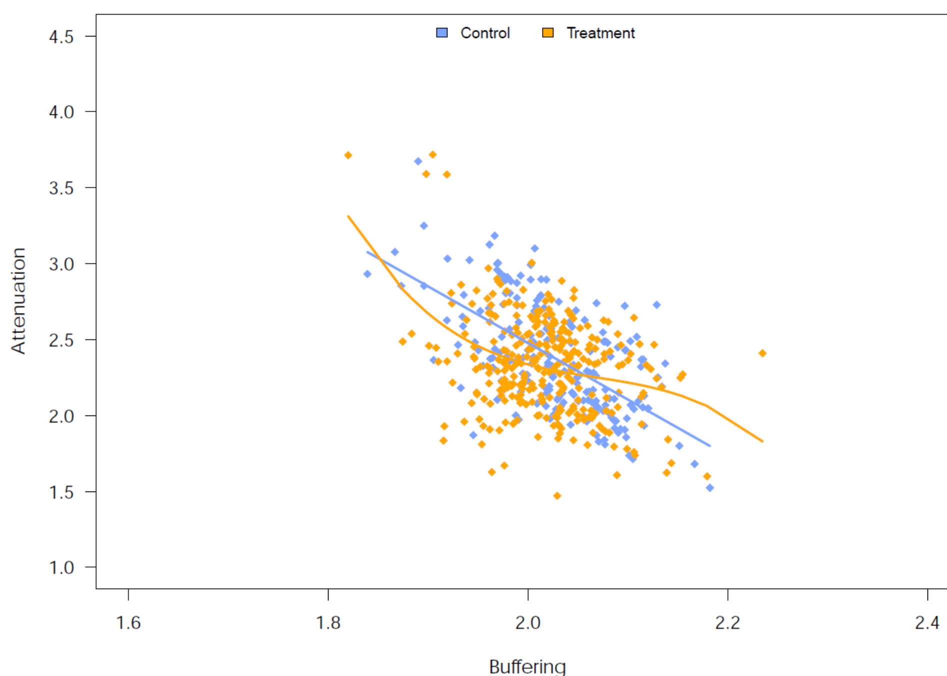

Figure 26: Values of attenuation and buffering for treated and control samples.

Then, we checked if there were differences in attenuation and buffering between control and treated populations by period. And we also made comparisons by dividing each period into two subperiods (i. e. A1, and A2). The first subperiod represents the first 10 days of each period, in which kanamycin was being supplied to treatment populations, while the second subperiod represents the other 25 days, while all samples were treated as controls. The *p*-values are found in Table 1. From all the comparisons, only the second subperiod of C (C2), presented significant differences in buffering.

Period	Attenuation	Buffering
A	0.4259	0.0409
B	0.8533	0.5656
C	0.5056	0.0807
A1	0.4991	0.08568
A2	0.1913	0.1354
B1	0.6885	0.3792
B2	0.594	0.9211
C1	0.3605	0.7761
C2	0.8403	0.0259*

Table 1: Wilcoxon test p-values for control and treatment for the studied periods and subperiods. Significant differences are marked with an asterisk (*).

We also performed the statistical comparisons (Wilcoxson test) by period of time in both conditions and parameters (Table 2). We found that there were statistically significant differences between periods A and C in attenuation for both, control and treatment, and between periods A and B in buffering, but only in the control condition.

	Control		Treatment	
	Attenuation	**Buffering**	**Attenuation**	**Buffering**
A/B	0.1359	0.0213*	0.2841	0.0830
B/C	0.2934	0.3367	0.1350	0.8791
A/C	0.0252*	0.1826	0.0308*	0.3830

Table 2: Wilcoxon test p-values for the comparisons between periods in control and treatment populations for attenuation and buffering. Significant differences are marked with an asterisk (*).

We represented the values of attenuation and buffering by subperiod and the condition in order to visually compare them (Figure 27).

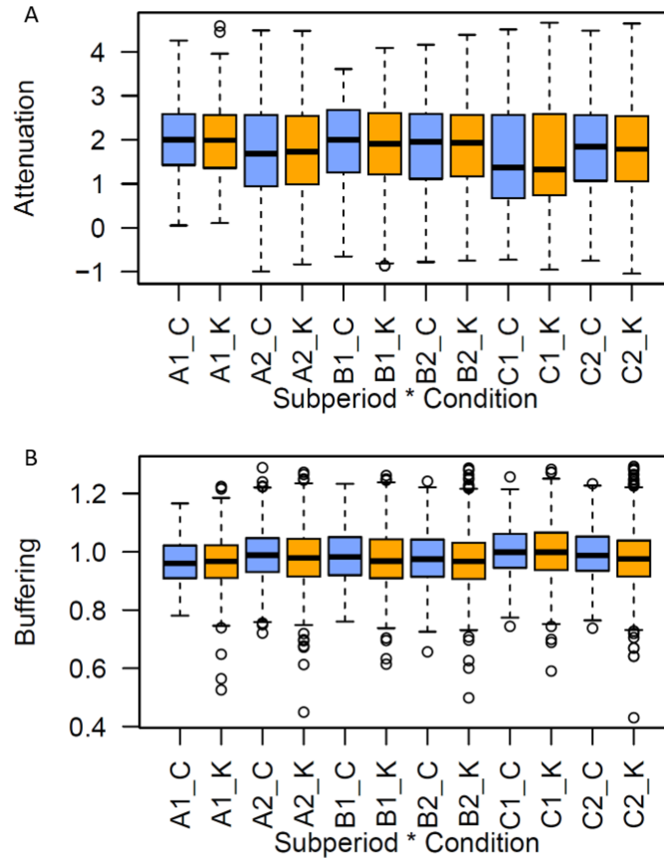

Figure 27: Boxplots of attenuations and buffering values of control and treatment conditions in each subperiod. Ordered by subperiod.

Finally, to have a better understanding of the whole dataset we studied the evolution of attenuation and buffering during the 105 days of the experiment. We calculated the mean of the robustness parameters for control and treatment for each day (Figure 28). We expected attenuation to be higher in control condition since it represents smaller functional shifts, but the values for both conditions are really similar. Regarding buffering, we expected control condition to need bigger perturbations in order to show noticeable functional shifts, since a "healthy" microbiota should be better prepared against external forces than a dysbiotic microbiota. We found that values are similar for both conditions during the whole experiment but the treatment seems to fluctuate less than control.

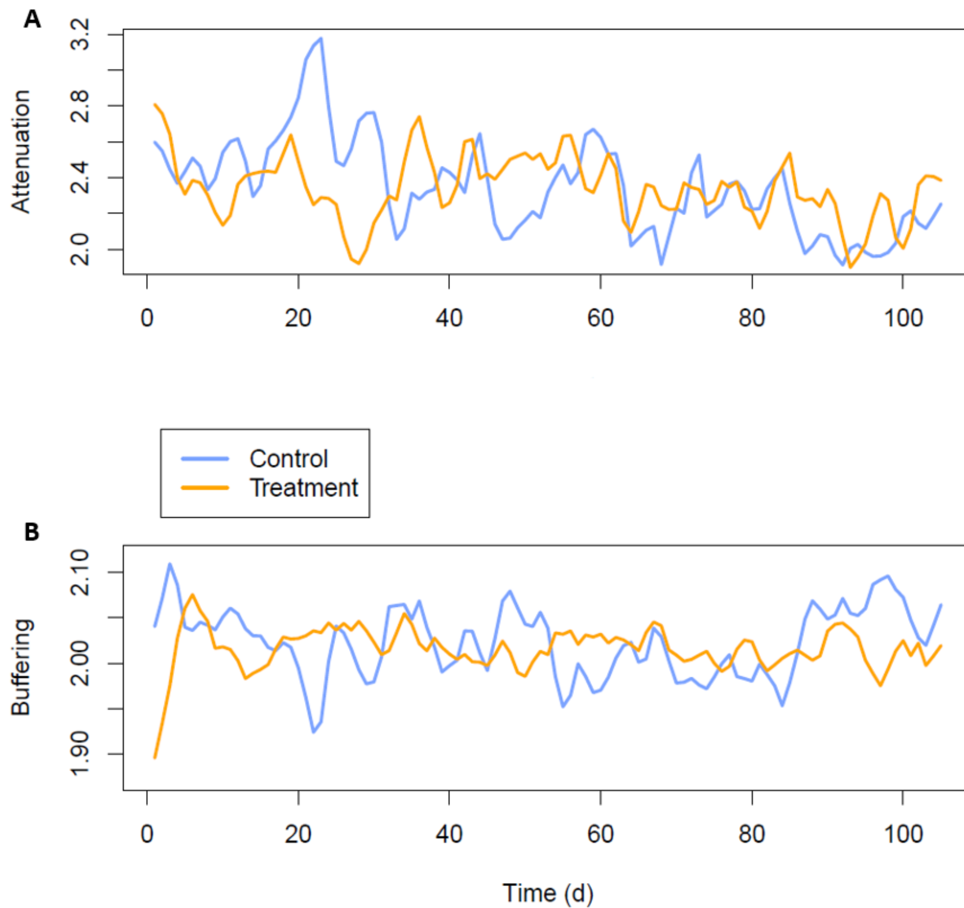

Figure 28: Evolution of attenuation (A) and buffering (B) for control and treatment populations day by day during all the experiment.

We also checked the distribution of attenuation and buffering in our samples for control and treated populations. As shown in Figure 29, values of attenuation are ranging -1 to 4.5, with an average of 2.38 for control and 2.33 for treatment, while buffering shows a range from around 0.5 to 1.3, with an average of 2.02 for control and treatment. Control and treatment distribution are almost identical for both robustness parameters.

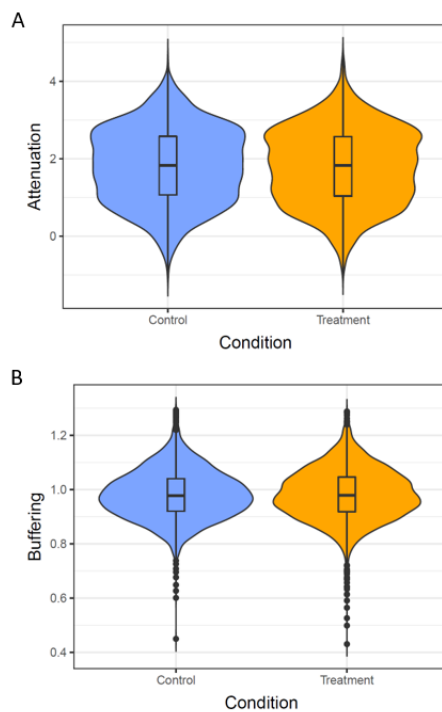

Figure 29: Violin plots representing the distribution of attenuation and buffering for both conditions.

4.8.2. Robustness comparisons by function

We also checked the attenuation and buffering parameters in control and treatment conditions for the KEGG pathways obtained from PICRUSt2. The order of the pathways in the Figure 30 is determined by the mean of attenuation (Figure 30 A) and buffering (Figure 30 B) values.

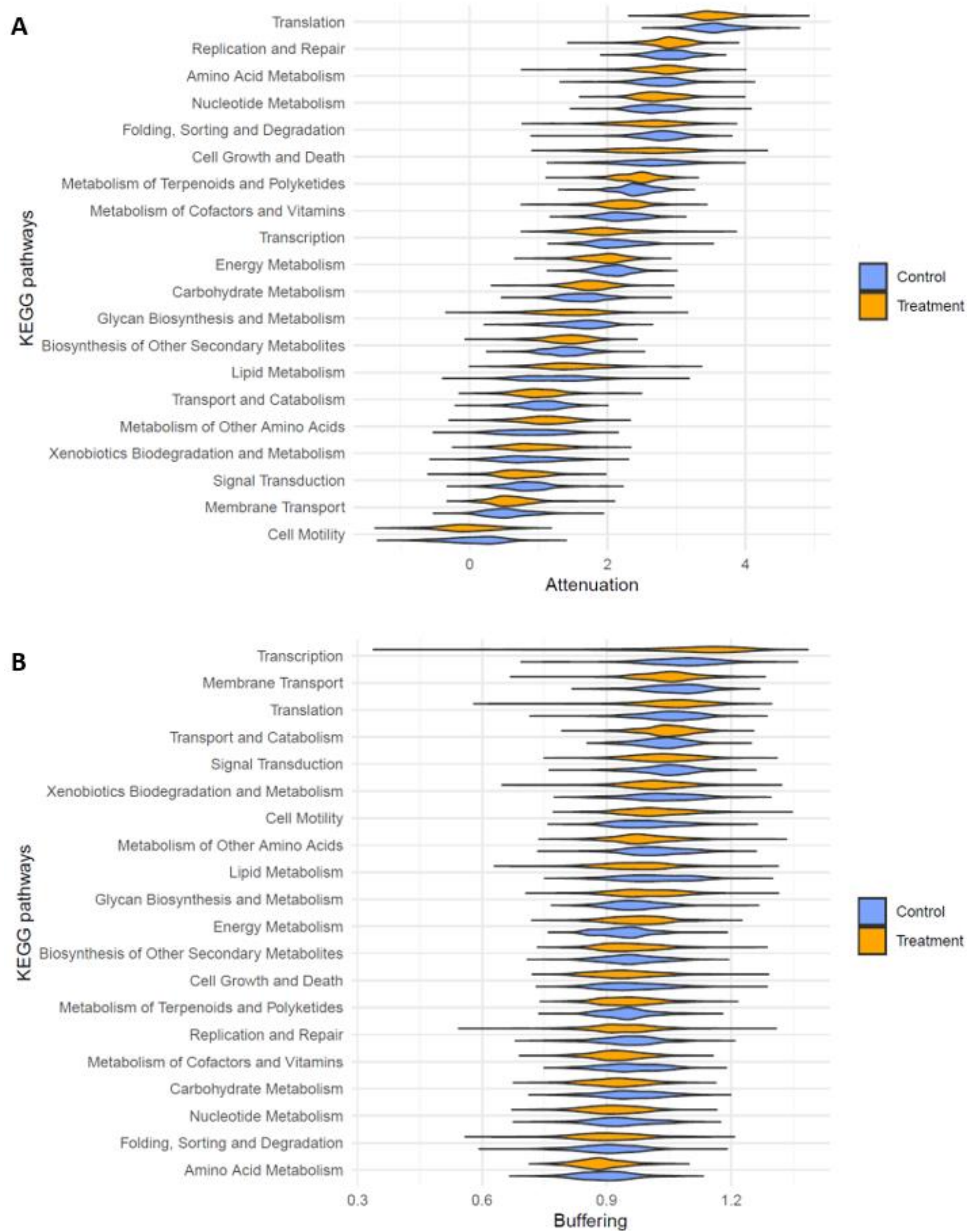

Figure 30: Distribution of Kegg pathways from PICRUSt2 for attenuation (A) and buffering (B) in control and treatment populations.

The values of the robustness parameters for both conditions seem very similar in almost all the studied pathways, although, we performed a Wilcoxon test to compare each pathway in both conditions. The results are shown in the Table 3. In the case of attenuation, 12 out of 20 pathways presented significant differences between control and treatment, while for buffering, the number of significant pathways was 14 out of 20.

Pathway	Attenuation	Buffering
Carbohydrate Metabolism	9,276E-05*	6,666E-07*
Lipid Metabolism	1,773E-07*	8,350E-09*
Metabolism of Cofactors and Vitamins	2,866E-01	7,993E-08*
Energy Metabolism	8,936E-08*	3,280E-09*
Nucleotide Metabolism	2,830E-01	3,566E-03*
Biosynthesis of Other Secondary Metabolites	5,018E-01	4,200E-01
Amino Acid Metabolism	7,493E-02	3,134E-02*
Metabolism of Terpenoids and Polyketides	4,321E-01	4,761E-01
Xenobiotics Biodegradation and Metabolism	1,024E-02*	9,418E-03*
Metabolism of Other Amino Acids	5,725E-08*	6,125E-07*
Glycan Biosynthesis and Metabolism	6,246E-05*	2,662E-05*
Translation	2,522E-01	3,770E-01
Membrane Transport	3,773E-02*	5,875E-04*
Signal Transduction	1,850E-03*	1,490E-01
Cell Motility	1,195E-03*	2,480E-03*
Folding, Sorting and Degradation	5,973E-08*	8,701E-01
Transcription	2,524E-04*	3,357E-04*
Replication and Repair	6,906E-01	2,675E-02*
Cell Growth and Death	4,576E-01	5,015E-02
Transport and Catabolism	1,544E-02*	1,070E-02*

Table 3: Wilcoxon test p-values of each studied pathway for attenuation and buffering between control and treatment populations. Significant differences are marked with an asterisk (*).

4.9. *B. germanica* fitness related to the antibiotic

To find out if the AB treatment is affecting the fitness of the cockroaches, and thus interfering in the interpretation of the observed results of its microbiota, we decided to design an additional experiment with the same conditions. In this experiment, we measured the values of four fitness parameters that we considered indicative of the fitness of these animals: weight, number of oothecae, number of nymphs, and number of dead individuals. We took these measures twice a week for 105 days approximately. For each day of sampling, we took 5 random cockroaches from each population and measured the fitness parameters. To do so, we anesthetized the

cockroaches with CO_2 to select five of them and then kept them anesthetized with ice to measure the parameters. Finally, we returned them to their population until the next day of sampling. We grouped the results depending on sex of the individuals and control/treatment group.

4.9.1. Weight

We found an evident difference between males and females, reflected in the Wilcoxon test that we performed (*p*-value < 0.05). Females are clearly bigger than males; their normal weight is around 0.9 g and 1.14 g during their adulthood while the normal weight of males is around 0.04 g and 0.07 g. The lower limit for weight was 0.054 g for females and 0.032 g for males, while the upper limit was 0.147 g for females and 0.097 g for males. The minimum and maximum weight in both sexes was recorded at the beginning and the end of the experiment, respectively. As shown in Figure 31, both sexes grow at the beginning of their adulthood.

Regarding the evolution of the weight for control and treated populations during the different periods, we compared periods of 35 days namely A, B and C using the Wilcoxon test. We found that there were no differences between treated and control populations during any period of this experiment.

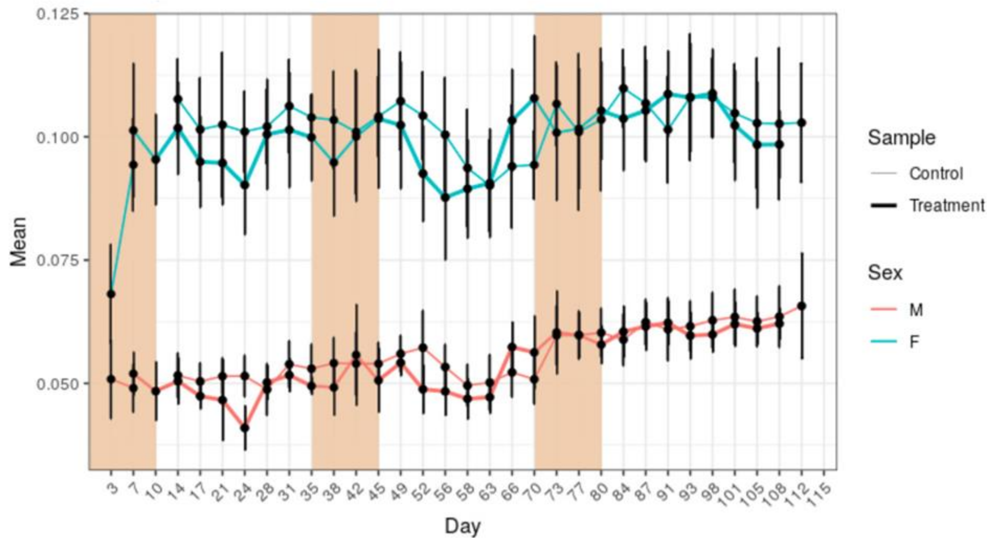

Figure 31: Evolution of the weight of males and females in control and treated populations during the fitness experiment. The three colored bars represent the periods of time where the treated populations were exposed to kanamycin.

4.9.2. Number of oothecae

We also compared the number of oothecae that females held during each sampling time for every population. In both populations, the first oothecae appears during the second week of adulthood (Figure 32). The number of oothecae gradually increases until the nymphs begin to hatch and the females lose them. This cycle is repeated and completed in one month approximately. We compared control and treated populations during three periods, as previously explained, using the Wilcoxon test. We only found differences between treatment and control populations during the last period (p-value = 0.034) in which, counterintuitively, treatment populations showed a higher number of oothecae than control population.

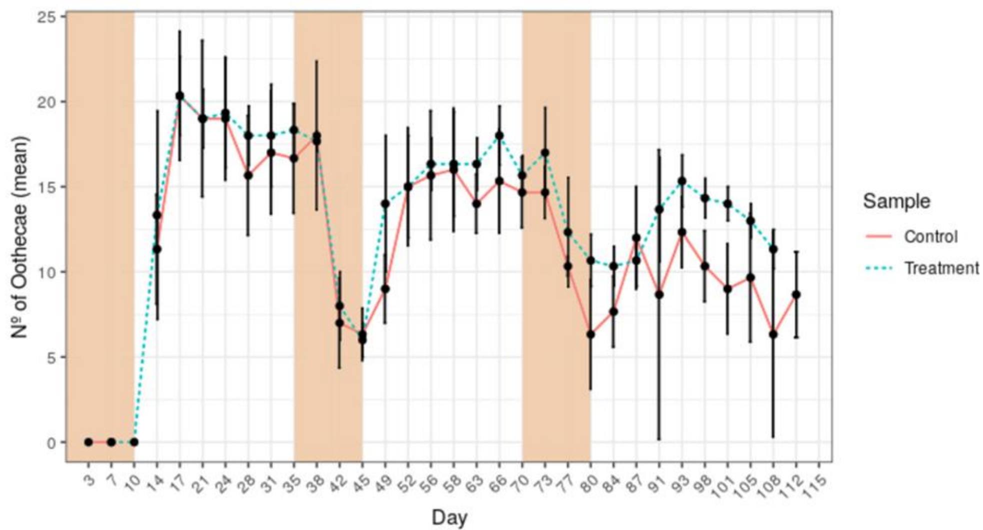

Figure 32: Evolution of the number of oothecae in control and treated samples. The three colored bars represent the periods of time where the treated populations were exposed to kanamycin.

4.9.3. Number of nymphs and deaths

Number of nymphs may be linked to the number of oothecae, but we included this parameter because the number of nymphs per hatching is highly variable and this variability may be induced by the health status of the female. If the antibiotic is affecting the health of the females, we could see a scenario where control and treated populations have the same number of oothecas, but differ in the number of nymphs.

We counted the number of nymphs each sampling time and then discarded them from the experiment in order to not count any nymph twice. When comparing both populations we found that there were no hatchings during the first period as shown in Figure 33. The number of nymphs is almost identical during the rest of the experiment with a few time points where treated populations showed a higher number of nymphs than control ones.

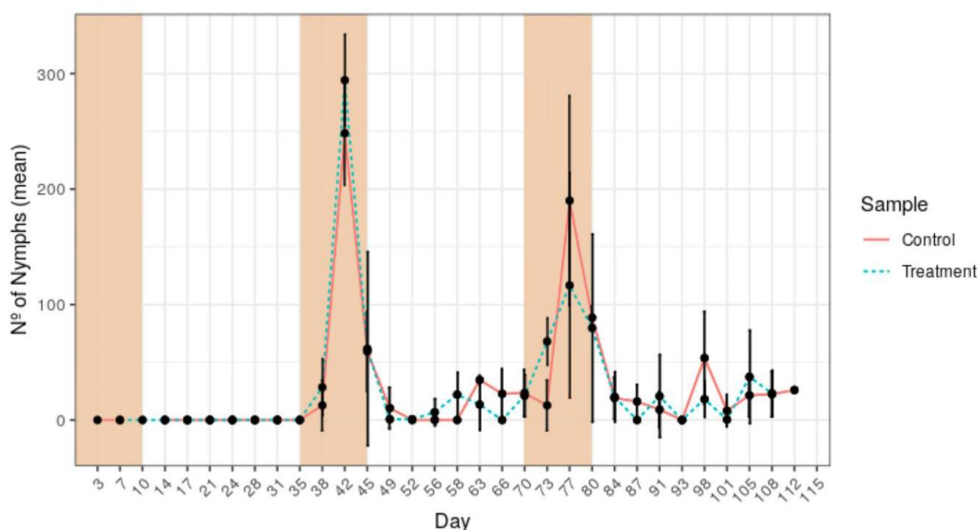

Figure 33: Evolution of the number of nymphs in control and treated samples. The three colored bars represent the periods of time where the treated populations were exposed to kanamycin.

Finally, we measured the number of deaths across the populations. The total number of deaths for males in control populations is 17 and 12 for treated populations. These numbers are 4 and 14 for females respectively. During the period A, 4 males and 8 females died in control populations while 2 males and 1 female died in treated ones. During the period B, 10 males and 2 females died in control populations while 2 males and 1 female died in treated ones. During the period C, 3 males and 4 females died in the control populations while 8 males and 2 females died in treated ones.

5. Discussion

This thesis is focused on the response of the gut microbiota of the German cockroach to the antibiotic kanamycin, during 105 days. This antibiotic and others such as rifampicin, vancomycin, and ampicillin, have been previously tested in the group to see their effect on the gut microbiota (Domínguez-Santos et al., 2020; Domínguez-Santos et al., 2021; Rosas et al., 2018). We needed an AB that did not have direct effects on the endosymbiont or the cockroach itself, but that did affect

the gut microbiota of the cockroaches. Thus, if we observed effects on the biological fitness of the cockroaches, we could suggest that it is due to the absence of important taxa that have been eliminated by the treatment. For this reason, the used AB had to possess three properties. The first one is a broad-spectrum antibiotic, affecting a wide range of different bacteria, including gram-positive and gram-negative. The second property is to not diffuse to the rest of the animal body. And finally, the AB should not affect *Blattabacterium*, the endosymbiont. From the mentioned antibiotics, only kanamycin fulfilled our needs since ampicillin had an unknown diffusion, rifampicin affects the endosymbiont of the following generation (Rosas *et al*., 2018), and vancomycin targets gram-positive bacteria.

The gut is a transient environment. Some bacteria may arrive to it because they usually stay on certain foods (Lang et al., 2014; Erkosar & Leulier, 2014). These bacteria may be not prepared for the traits of this environment (pH, oxygen levels, temperature, etc.), and are dragged out of the system with the feces. Some of them may stay on the gut walls, but they are most probably outcompeted or attacked by the immune system. Other bacteria that access the gut in the same way, through the food, may be adapted to the environment and could rapidly occupy its niche and take part of the ecological community (Zhang et al., 2016). There are plenty of ways for bacteria to arrive in the digestive system, and we cannot control all of them. This means that we cannot know whether a group of bacteria are in symbiosis with the host or just end up in the gut through a stochastic process. To tackle this problem, we followed the following reasoning. Adapted taxa may be present continuously in the gut while normal conditions, since non-adapted taxa cannot outcompete them. Furthermore, the random nature of the arrival of the non-adapted taxa (transient bacteria), may lead to an absence of these taxa during certain time points.

As any other approach, sequencing the 16S rRNA gene has advantages and disadvantages when compared with WGS (whole genome shotgun). Normally, WGS is attributed with better resolution and more diversity recovering, especially when the small subunit is extracted prior to the sequencing (Chan *et al*., 2015; Guo *et al*., 2016). However, in some specific studies, amplicon sequencing shows superior performance, finding more diversity at all taxonomic levels, defying the generalized preference for the WGS over this method (Tessler *et al*., 2017). The main disadvantage of amplicon sequencing is the bias introduced by the amplification by PCR of the gene of study that leads to loss of some rare taxa and overrepresentation of others, depending on the used primers (Poretsky *et al*., 2014).

In our group, we classically used female cockroaches in the experiments. This is due, in part, because they are bigger and thus have more fat body. This makes it easier to perform experiments that involve that tissue. In consequence and to keep the homogeneity, we used female cockroaches for the microbiome experiments too. This led to an unknown male gut microbiota that is now revealed as we used males and females for this experiment. Half of the samples, the ones sampled odd days, come from females, while the even samples come from males. After applying the usual statistical procedures, we concluded that there are no differences between males and females regarding their gut composition in control conditions, and how it responds to kanamycin treatment. This fact may seem logic, but there are examples of species, like mice, where sex is an important factor regarding the composition of the gut microbiota (Yurkovetskiy *et al*., 2019; Markle *et al*., 2013). Mixed results have been obtained in humans where some studies found sex differences (Mueller *et al*., 2006) and others don't (Lay *et al*., 2005). In some cases, finding differences may consist in pointing to specific taxa like in the work of Haro *et al*. (2016)

where general diversity analysis did not find differences, but specific genera gave differential results.

In our experiment we have a great number of time points sampled. This allows us to detect false positive taxa when establishing the bacterial core since the more samples we analyze, the greater the probability that transient bacteria will not appear at some time point, and this, being correctly removed from the core. When determining the core of our samples, we used only the control populations and found that five phyla formed it: Bacteroidota (most abundant genus was *Dysgonomonas*), Bacillota (*Candidatus Soleaferrea*), Pseudomonadota (*Desulfovibrio*), Fusobacteriota (*Fusobacterium*), and Deferribacterota (*Mucispirillum*). Although Deferretibacter was under 1% of abundance, there were other taxa with very low abundances forming the core like *Paracabteroides*, *Tyzzerella 3*, and *Desulfatiferula*. This shows the complexity of the gut microbiota in cockroaches. The aforementioned phyla are the same that are usually found in our group and others, as core in the *Blattella germanica* gut and something similar, although to a lesser extent, happens at the genus level (Dominguez-Santos *et al*., 2020; Pérez-Cobas *et al*., 2015; Rosas *et al*., 2018; Kakumanu *et al*., 2018). Albeit, in Kakumanu's work, Fusobacteria is not found among core bacteria. Core bacteria of other cockroach species is also very similar, such is the case of the *Panchlora* cockroach (Gontang *et al*., 2017). Not surprisingly, some of the abundant families found in our study such as Lachnospiraceae, Ruminococcaeae, Rikenellaceae, Desulfovibrionaceae are present in *Shelfordella lateralis,* another species of cockroach (Schauer *et al*., 2012). The fact that these families are common in different cockroach species may be indicating that they are contributing to the basal metabolism for nutrition, protection, and ecosystem maintenance. We previously remarked that Deferribacterota is under the 1% mark of abundance. Sometimes researchers ignore the least abundant taxa to simplify the

very complex system that the gut microbiota forms, but these kinds of taxa may be performing some unknown essential role.

In this study, we focused on the differences between the gut microbiota of untreated and treated cockroaches with AB. In this respect, we found that AB treatment affected the microbial composition of the insects. This was expected, since the same result was found in another study using the same AB (Domínguez-Santos et al., 2021). We observed that the Bacillota and Pseudomonadota phyla significantly decreased their abundance in the treated samples. Something similar happens at the genus level where *Parabacteroides, Christensenellaceae R-7 group,* and *Tyzzerella 3* are less abundant in treated samples. Some groups, like *Alistipes*, decrease their abundance when we expect them to at least maintain the control levels, as some species of this genus possess kanamycin resistance genes (Parker *et al*., 2020). One possible explanation might be that the most abundant species of this genus in our samples may not harbor AB resistance.

One of our hypotheses was that in treated populations, AB should have a decreasing effect on diversity, especially during the first period of exposure. Since the first exposure to the AB is during the first 48h of adulthood of the cockroaches, the hindgut cuticle has been renewed recently during the moulting process, and resident bacteria is removed. Thus, the microbiota is still forming and is less stable which should make it more sensitive to the AB. As expected, when alpha diversity was studied, control samples had higher Shannon and Chao1 indexes than treated populations. Similar results were obtained in different experiments carried out by our group (Rosas *et al*. 2018; Domínguez-Santos *et al*. 2020; Domínguez-Santos *et al*. 2021), and other groups in other species like humans or mice (Palleja *et al*. 2018; Rodrigues *et al*., 2017).

Regarding beta diversity, treated populations were clearly separated in the PCOA. Only some treated samples were close to the control

samples, but most of these are part of the first days of the experiment, and maybe the AB did not have enough effect at that point of the experiment.

Regarding the co-occurrence results for control populations, we found that 10 out of the 18 bacterial taxa forming the core are present in the co-occurrence clusters and that every major group of occurrences has at least one core taxa. Somethin similar was observed in the work of Domínguez-Santos *et al.* (2021) in which the German cockroach was also treated with kanamycin. In concordance with the same study, taxa forming the networks was mainly from the Bacteroidota, Bacillota and Pseudomonadota phyla. This may be indicating that these taxons are in the gut as aggregations of different bacteria forming communities that interacts and provides stability to the microbial system. We also found that some of the most connected taxons are not necessarily the most abundant. In fact, *Eubacterium coprostanoligenes group* is one of the least abundant taxa but at the same time, it has the highest number of links with other taxons of its own group. This result may be pointing that scarce taxa may be playing important roles in the bacterial communities. This is something to address since normally, less abundant taxa are overlooked in this kind of study. Observing the taxa that form each group it seems that an important factor for the formation of such networks is the phylogenetic signal, as observed in previous studies with the same insect (Domínguez-Santos *et al*., 2021) and in humans (Leung *et al*., 2016), where most of the formed groups are constituted by same phylum taxa. For example, in the dark blue group (Figure 19), half of the involved taxa belong to Bacillota phylum. Something similar happens in the yellow group with 4 out of 10 taxa belonging to the Pseudomonadota phylum. Finally, the red groups with 5 out of 6 taxa from the Bacillota phylum. This reinforces the idea that phylogenetically related bacteria respond similarly to the

environment and interact within their kin. Furthermore, and also in agreement with the previous mentioned study, the two taxa with most links in our study, which is an indicator of the importance of the taxa in the net, are Desulfovibrionaceae and Acidaminococcacecae.

In AB treated populations, we found no co-occurrences for any taxon, either with normal or relaxed parameters. We hypothesize that the antibiotic is disrupting the communities and thus, preventing the formation of co-occurring groups. We have seen that diversity decreases with the AB treatment and maybe some key taxa are either disappearing or only marginally surviving in that environment. This may be the cause of the lack of co-occurrences.

Microbiota followed Taylor's law. This translates as that the most abundant taxa are also more stable than less abundant ones. Moreover, both, control and treated samples had very similar values, only differing by 0.02 ± 0.01 (Control = 0.72 ± 0.01; Treatment = 0.74 ± 0.01). This may indicate that following this power law is an inherent trait of microbial communities. In their work, Kilpatrick and colleagues explained this behavior (Kilpatrick *et al.*, 2003). The variability in the abundance of each taxon is the result of the effect of the changes in the environment and the effect of other microbial competitors. Following this supposition, taxa with higher competitive capabilities in a given environment will have an advantage over less competitive ones and thus, will variate less when competing. Also, other non-competitive microbe-microbe interactions may be playing an important role in the system, influencing the results (Bucci *et al.*, 2016).

When we checked the changes in the ranks of each taxon, calculated from their abundance during the experiment, we found that taxa in the top ranks are less prone to change their rank than the bottom-ranked taxa. This behavior shows that the populations, even in terms of ranks, also follow Taylor's law. We found that 47 out of 50 genera

are the same between treatments. In other words, only 3 genera changed. This is a small change when compared to the LEfSe analysis that we performed using the abundance of each taxon instead of their rank. If we take into account the same taxa in both analyses, we found 4 bacteria that were significantly more abundant in treated populations than in control and 14 more abundant in control than in treatment. We also found some bacteria that, contrary to Taylor's law, were in the bottom-ranks but had a high RSI. This could mean that they are performing some important function for the cockroach and are maintained by the insect in some way. One of these bacteria is *Oxalobacter*. This bacterium is linked to the degradation of oxalate in the gut of different animals such as horses, swine, rats and, cows, but also in humans (Allison *et al.*, 1995). High levels of oxalate are related to hyperoxaluria and the development of kidney stones in humans and other mammals. There are no studies of this compound in insects but it could be harmful at high levels also for them. Oxalate is formed by catabolism in the glyoxylate pathway (Holmes *et al.*, 1998) and is naturally present in different foods such as bread, potatoes, fruits, vegetables and others (Holmes *et al.*, 2000). Oxalobacter plays an important role in preventing diseases in humans (Duncan *et al.*, 2002) and could be doing the same in the German cockroach.

Another important parameter that we measured is *V*. This parameter reflects the noise of the system, the variability. In other words, it shows the fluctuation amplitude of the population over time. As we expected, a higher *V* value for treated samples was found, demonstrating the altering effect of the AB in the cockroach microbiota. But we also expected the microbiota to adapt or recover after the first exposition to kanamycin. We found that after the first AB supply, the variability in treated populations is in the same range that control populations. These results seem to indicate that bacteria are somehow adapting to the AB after only one period of contact, showing

that microbiota populations possess resilience. In this scenario, the AB pressure may be boosting the horizontal gene transfer (HGT) between taxa that harbors kanamycin kinase such as *Pseudomonas* and taxa that does not, such as *Dysgonomonas* or the resilient taxa that we found: *Lachnoclostridium, Parabacteroides, Christensenellaceae R-7 group* (shown in Figure 21 E), Tannerellaceae and Desulfovibrionaceae (Figure 21 E). Thus, potentially favoring the survival of these groups of bacteria. In fact, we found that the abundance of *Dysgonomonas* increased significantly from control to treated condition (C: 15.7%, K: 22.0%). Furthermore, it was found, in other study, that *Pseudomonas* is responsible of HGT in altered gut microbiota environments (Li et al., 2020). In the human gut, there has been reported the transmission of ARGs, especially among the phylum Bacillota (McInnes *et al*., 2020). The transmission of these genes often takes place among bacteria of the same phyla, probably because of differences in the physiological properties of each bacterial group (Porse *et al*., 2018).

We performed the functional analysis of our samples using PICRUSt2, a software that infers the functional profile of microbial samples using taxonomical assignations (Douglas et al., 2020). We found that the functional profile of our samples suffers statistically significant changes in abundance during the three studied periods, in both, control and treatment samples. Although, the most abundant functions present a very similar abundance for all periods and conditions. This fact reinforces the idea that the host might be selecting taxa that carry out specific useful functions. Interestingly, the "ABC transporters" function is significantly more abundant in treated populations, indicating that protective functions gain importance under chemical stress like exposition to AB. In control populations, quorum sensing pathway is significantly more represented, which could be indicating that, in control conditions, communication among the bacterial community is well stablished and AB treatment disrupts

it. Moreover, the oxidative phosphorylation pathway was more repsented in treated populations. This pathway is a signal of oxidative stress in mithocondria and can induce DNA mutations in these plastids. It is also implicated in different neurodegenerative diseases and mithocondria dysfunction (Guo et al., 2013; Singh et al., 2019). This could be an indicator of stress induced by the AB treatment. Finally, we also found that, in treated populations, metabolism of some sugars is overrepresented (galactose, fructose, manose, etc.). It is known that some pathogens can use alternative sugars such as galactose and mannose in order to outcompete commensals (Fabich et al., 2008; Kamada et al., 2013; Le Bouguénec & Schouler, 2011). Thus, kanamycin might be favoring opportunistic taxa to stablish in the gut microbiota. We also specifically checked the abundance of the enzyme responsible for the degradation of kanamycin A, the kanamycin kinase (EC: 2.7.1.95), in both conditions, and, surprisingly, we found that this enzyme is more abundant in control than in treatment conditions. But it is present in a very low abundance in both cases. This might be explained by the fact that the most abundant taxa could be resisting kanamycin by other means virtually decreasing the total representation of taxa harboring this enzyme. Finally, we checked which taxa carried this enzyme and found a total of 13 different taxa carrying it. The enzyme was first described in *Pseudomonas*, which is present in the gut microbiota of *B. germanica.* Kanamycin kinase is capable of inactivating other antibiotics like neomycin and streptomycin (Doi et al., 1968; Poole, 2005; Sindeldecker & Stoodley, 2021)

We also studied the attenuation and buffering differences between control and treatment populations. These are the parameters of robustness, defined by Eng and Borenstein (2018). Robustness expresses the shifts in the functional profile of the microbiota when the community undergo compositional changes. We found that there

were differences between treatment and control conditions for buffering when comparing all periods. However, when inspecting the robustness parameter by periods and conditions, we found that only the last period (C2) of buffering presented significant differences. An interesting result on the evolution of attenuation is that, although there were no differences between conditions, the values had a decreasing tendency, which indicates that the microbiota is less robust as it matures. The opposite result, an increasing attenuation tendency, was obtained by Ma et al. (2020) when studying the effects of antibiotics on the gut microbiota of neonatal calves. Also, the values of attenuation in our experiment are lower than the attenuation values for the human gut (Eng and Borenstein,2018), and the values for buffering are quite similar.

Regarding the robustness associated with specific functional pathways we found that, not surprisingly, housekeeping functions such as translation or replication and repair presented the highest attenuation values while other functions related to transport of molecules and degradation of xenobiotics had an increased buffering value, indicating that these required a bigger perturbation in order to suffer significant changes. This might represent that microbiota, or the host, is preserving essential and protective functions probably by selecting the bacterial species that confers these properties to the community. Furthermore, we found significant differences among the protective and transport functions, such as Xenobiotics Biodegradation and Metabolism, Membrane Transport and Transport and Catabolism, for control and treatment in both attenuation and buffering. Since the buffering values are lower for treated populations in these specific pathways, it might be more likely that smaller taxonomical shifts produce changes in these function in treated populations.

Finally, we studied the effect of the AB not only in the gut microbiota but also in the cockroaches's fitness. The main objective of this study was to ascertain if the changes in the composition of the gut microbiota due to the AB treatment were affecting the survival and reproductive capabilities of the cockroaches. In other words, ascertain if the lack or excess of some bacterial taxa compared to control conditions was affecting the biological fitness of *B. germanica*. We measured four parameters during the experiment (weight, number of ootheace, number of nymphs and deaths). From those, only the number of oothecae seemed to change in treated populations. We hypothesize that the increase in the number of oothecae during the last period of treatment may be similar to the increase in fitness observed of farm animals when treated with AB (Bacanlı *et al.*, 2019; Boeckel *et al.*, 2015). We suspect that the kanamycin is favoring the production of oothecae after 3 expositions in the same way that other ABs improve animal performance in farms, by reducing the physiological effort of suppressing the animal growth in order to fight against some diseases (Council, 1999). Finally, treated farm animals also increase their weight by 4-5% (Witte, 1998), but treated cockroaches did not show this mass increase. This fitness experiment performed in only one generation, was useful to check if the health status of the cockroaches is affected by kanamycin. As it has not been the case, we can state that the changes observed in the composition and function of the microbiota are not due to changes provoked by the AB in the insect. We believe that further research is needed to fully understand how the ABs affects the fitness of *B. germanica* through changes of its gut microbiota. For example, in our lab, we performed an experiment, not yet published, consisting in treating cockroaches with combinations of pairs of ABs. We found that the combination of rifampicyn and kanamycin can produce a decrease in the number of nymphs in one

generation. In any case, future studies are needed to shed light on this topic, which migh be important even for human health.

6. Conclusions

1. There are no statistical differences in the composition and diversity of the gut microbiota of male and female cockroaches. This eases the planification of future experiments with this species of cockroach.
2. Alpha and beta diversity of the gut microbiota is clearly affected by kanamycin when supplied in 10-day frames. These changes are clearer at more specific taxon levels such as family or genus over phylum. The treatment decreases the diversity and changes the microbial composition.
3. In control conditions, phylogenetically related bacteria form co-occurrences groups, forming a network. Furthermore, core bacteria are an essential part of the community, providing stability and working as anchor for the rest of the community. Whereas kanamycin treatment disrupts the co-occurrence networks to a point where we could not find any correlation in treated populations.
4. We observed five main groups of bacteria regarding how the abundance changed over time in response to the treatment with kanamycin: opportunistic, mildly affected, affected, unaffected and resilient. One of the objectives for this thesis was to find resilient bacteria, and we found five taxa that are included in that group: *Lachnoclostridium, Parabacteroides, Christensenellaceae R-7 group*, Tannerellaceae and Desulfovibrionaceae.
5. Treated and untreated microbial communities follow the Taylors law since the β parameter value is always between 0.5 and 1. This means than less abundant bacterial taxa are more prone to suffer changes in their abundance and are less stable than more abundant ones.
6. In control populations, variability V is very similar through the three studied periods. However, in treated populations the first period is clearly more variable than any of the other periods, observing that in the second and third periods the treated levels of variability are

almost restored to control conditions. Then, we conclude that the gut microbiota of *B. germanica*, is able to adapt to kanamicyn after only one first dose of treatment, avoiding the increase of variability in later doses.

7. The bacterial genus with the highest RSI and, thus the most stable one, is *Dysgonomonas,* it being also the most abundant taxon in the study for both conditions. Furthermore, RSI is higher for the more abundant bacteria and lower for more scarce ones, with few exceptions like of *Oxalobacter, Clostridiales* and *Raoultibacter.* These results support the fact that the microbiota follows the Taylor's law.
8. Functional profile suffered changes in the treated populations in the three studied periods. In addition, we found that the enzyme responsible of the kanamycin degradation, kanamycin kinase, is harbored by some taxa such as *Pseudomonas* and *Sphingomonas*, which possess a great metabolic capability.
9. Robustness parameters did not change greatly when comparing periods between controls and treatments. However, attenuation presented a decreasing tendency over time, indicating that the gut microbiota was less robust with time.
10. Regarding fitness parameters, kanamycin does not affect neither the weight nor the number of nymphs of cockroaches. Concerning the number of oothecae females produce, they seem to increase in the last period. We conclude that kanamycin does not affect the health status of the insect in this experiment.

7. Resumen en español

Introducción

La simbiosis está ampliamente extendida en la naturaleza. El término fue acuñado por Anton de Bary en 1879 en su obra "*Erscheinung der Symbiose*" donde la define como "la vida en conjunción de dos organismos disímiles, normalmente en íntima asociación…". Este fenómeno natural puede ser clasificado teniendo en cuenta muchos factores. Si nos centramos en la eficacia biológica de los participantes, nos encontramos con el comensalismo, donde una de las partes se beneficia o incrementa su eficacia biológica al interaccionar con la otra especie, y la otra se mantiene neutra. Si ambas partes se benefician, se trata de mutualismo y si una parte se beneficia y la otra sale perjudicada, entonces es parasitismo. Sin embargo, si nos fijamos en la localización del simbionte, nos encontramos con dos tipos de simbiosis. La ectosimbiosis, donde el simbionte se encuentra en el exterior de las células del hospedador, esto incluye el tracto intestinal en su totalidad, y la endosimbiosis donde el simbionte está confinado en el interior de las células del hospedador. Por último, si ambos necesitan de la simbiosis para sobrevivir, se considera una simbiosis obligada y si no, una simbiosis facultativa.

Lynn Margulis fue quien formuló la teoría del origen de la célula eucariótica. En su obra sostiene que la simbiosis entre arqueas y bacterias podría haber dado origen a los eucariotas y que, tras esto, diferentes procariotas fueron absorbidos por eucariotas para formar los distintos orgánulos.

La endosimbiosis entre bacterias e insectos es muy común y tiene un impacto muy positivo en la capacidad de los insectos para colonizar nuevos ambientes. Comúnmente, los insectos adquieren la capacidad de alimentarse a base de dietas pobres en nutrientes puesto que el

endosimbionte completa la dieta con sus capacidades metabólicas. Por su parte, los endosimbiontes reciben la maquinaria metabólica del hospedador, nutrientes y un ambiente controlado y protegido. En estas relaciones, la bacteria entra en un proceso de reducción del genoma, en el que pierde aquellos genes que no le son útiles en ese ambiente.

Los insectos son el grupo más diverso de animales de nuestro planeta. Estuvieron entre los primeros animales en conquistar la superficie terrestre hace alrededor de 479 millones de años, en el período Ordovícico temprano. Desde entonces, han evolucionado y desarrollado carácteres de todo tipo. Fueron los primeros animales en desarrollar la capacidad de volar hace 406 millones de años en el período Devónico temprano. La clase Insecta posee alrededor de un millón de especies descritas, pero este número aumenta cada año. Estimaciones recientes indican que hay alrededor de 5.5 millones de especies, lo que quiere decir que el 80% de las especies de insectos están todavía por describir. El grupo de insectos más diverso es Coleoptera seguido por Lepidoptera, Diptera e Hymenoptera, representando más del 90% del total de especies.

Las cucarachas (Blattodea) son insectos hemimetábolos, con unas 4500 especies descritas. Forman parte del superorden Dictyoptera junto con termitas (Isoptera) y mantis (Mantodea). Estos insectos tienen grandes capacidades para ocupar nuevos nichos puesto que diferentes especies tienen capacidades muy diversas. Desde vivir en tierra firme a poder volar, trepar o incluso excavar pequeños refugios de hasta 1 metro de profundidad. Los ambientes preferidos por las cucarachas son espacios estrechos, oscuros, húmedos y poco ventilados en la naturaleza o las urbes. En estas últimas, pueden estar involucradas en la transmisión de enfermedades puesto que se alimentan de todo tipo de materia orgánica y se encuentran en

hospitales. La cucaracha alemana, *Blattella germanica*, es una especie que posee un endosimbionte. Este endosimbionte, *Blattabacterium cuenoti*, vive en el interior de unas células especializadas de la cucaracha, llamadas bacteriocitos. Es transmitido verticalmente de las madres a la descendencia. Juega un papel fundamental ya que transforma el ácido úrico que la cucaracha deshecha en amonio para que el insecto lo transforme en glutamina. De esta manera puede almacenarlo y reutilizarlo cuando necesite una fuente de nitrógeno.

El otro sistema biológico que vive junto a la cucaracha es la microbiota intestinal. Podemos estudiar la microbiota en profundidad gracias a que las tecnologías de secuenciación han ido mejorando y abaratándose con el paso de los años desde que en 2005 se comercializó la plataforma 454. Pese a que en la actualidad existen secuenciadores de tercera generación, la mayoría de estudios se siguen realizando con tecnología de segunda generación o NGS por su alta fiabilidad y precio.

Las bacterias son capaces de colonizar casi cualquier parte de un ser vivo, humanos incluídos, pero las dos localizaciones más comunes y estudiadas son la cutícula/piel y el tracto digestivo. Un ejemplo del primer caso es el de los escarabajos ambrosiales, cuya cutícula posee unas invaginaciones membranosas equipadas con glándulas secretoras que favorecen la colonización de bacterias y hongos. En el segundo caso, el intestino de los insectos está dividido en tres secciones: intestino anterior, medio y posterior. En el posterior, es donde se encuentra el mayor número de bacterias, ya que mayormente se da la reabsorción de agua, además los tubos de Malpighi podrían ser un factor positivo debido a los iones y metabolitos que provee. En el intestino medio, se encuentran gran número de enzimas y productos inmunológicos como péptidos antimicrobianos. Esto lo hace un medio difícil de colonizar. Por

último, el intestino anterior de algunos insectos se especializa, como *Bactrocera oleae* que alberga un simbionte que ayuda con la detoxificación de metabolitos secundarios de plantas.

Los principales filos bacterianos que se suelen encontrar en el intestino de los insectos son Actinomycecota, Bacteroidota, Bacillota y Pseudomonadota. Previamente conocidos como Actinobacteria, Bacteroidetes, Firmicutes y Proteobacteria. Pero también se pueden encontrar, aunque en menor medida, arqueas, típicamente relacionadas con la metanogénesis; hongos, relacionados con la fermentación; y otros eucariotas como protistas, representando la mayor fuente de actividad celulolítica y xilanolítica en el intestino de termitas. Por último, puede que los helmintos también tengan un impacto positivo en la estabilidad del microbioma y en la salud del hospedador.

La microbiota del intestino está relacionada con el control de la cantidad y diversidad de un amplio abanico de metabolitos que son de gran importancia en la homeostasis y el funcionamiento normal de animales complejos. Uno de estos metabolitos son los ácidos biliares, sintetizados en el hígado y después conjugados con taurina o glicina antes de ser secretados en la bilis y el intestino delgado. Algunas bacterias del intestino delgado realizan una serie de transformaciones a estos compuestos tales como desconjugación, hidroxilación, deshidroxilación… Tras esto, los ácidos biliares modificados pueden actuar como moléculas de señalización fuera de la circulación enterohepática. Eso significa que las bacterias están regulando el metabolismo del hospedador y son capaces de enviar señales a otras partes del sistema. Otros metabolitos que la microbiota es capaz de transformar o regular son la colina, ácidos grasos de cadena corta.

Otros estudios han relacionado la microbiota intestinal con la expresión de receptores de patógenos como los receptores Toll-like y

de moléculas de presentación de antígenos como el complejo mayor de histocompatibilidad de clase II. Se ha visto que se produce una reducción en el número de componentes del sistema inmune cuando se elimina la microbiota intestinal en ratones.

La microbiota intestinal también está relacionada con algunas enfermedades como la obesidad o enfermedades autoinmunes y neurológicas. Por ejemplo, se ha observado que al transferir la microbiota de pacientes con la enfermedad de Parkinson a ratones puede llegar a inducir sintomatología de dicha enfermedad, sugiriendo que la microbiota está jugando un papel en ella.

Algunos ejemplos del papel de la microbiota intestinal en insectos son la mosca *Bactroera oleae*, que convive con la bacteria *Candidatus Erwinia dadicola*. Esta bacteria permite a las larvas de la mosca alimentarse de olivas inmaduras con altos contenidos de oleuropeína, que es un glicósido fenólico tóxico para los insectos. Otro ejemplo son las termitas, que pueden vivir alimentándose únicamente de madera y cuya microbiota es también responsable de la fijación de nitrógeno y el reciclaje de desechos nitrogenados.

Hasta ahora, hemos visto cómo la microbiota es una parte esencial en la forma de vida del hospedador, pero ¿regula el hospedador qué bacterias forman parte de la comunidad? Estudios en *Drosophila melanogaster* han encontrado que la producción de péptidos antimicrobianos (AMPs) está regulada con dos rutas que se activan por la presencia de bacterias y hongos. Estas rutas no eliminan a la totalidad de las bacterias sino que reducen su número. También han encontrado que, mediante la supresión del gen *Caudal*, la microbiota intestinal es eliminada ya que, en condiciones normales, este gen es responsable de la supresión del factor kappa B, que induce la expresión de AMPs.

La composición de la microbiota intestinal puede variar dependiendo de muchos factores, como la dieta, edad, lugar, genética, o tratamiento entre otros. Otro factor importante es el tiempo, que se estudia con experimentos longitudinales. Este tipo de estudios nos permite tomar instantáneas del estado de la microbiota en diferentes momentos y a partir de estas construir conclusiones más robustas. Con este método, podemos observar tendencias y, por ejemplo, el tiempo que tarda una comunidad en volver a su estado normal tras ser perturbada por algún factor externo, es decir, podemos observar si la comunidad posee resiliencia.

En los últimos años han ido apareciendo nuevas herramientas que nos ayudan con este tipo de estudios. Algunos ejemplos son la prueba de Dickey-Fuller aumentada o el análisis de similaridad local (LSA), además de algunas páginas web como TIME que permiten analizar datos en ellas.

Objetivos

Este trabajo está enfocado al estudio de la dinámica de la microbiota intestinal de la cucaracha alemana cuando es tratada con kanamicina. Una de las mejores maneras de estudiar las dinámicas es con su seguimiento a lo largo del tiempo en forma de serie temporal. *B. germanica* es un modelo animal excelente debido a sus características como su dieta omnívora, lo que nos permite alimentarla según las necesidades del experimento; es fácil de mantener en el laboratorio; tiene un ciclo de vida corto y alta velocidad de reproducción; y lo más importante para este trabajo: esta cucaracha posee una de las microbiotas intestinales más diversas entre los insectos.

El objetivo general de este trabajo es obtener una imagen general de la dinámica de la microbiota intestinal bajo presión de antibiótico y la capacidad de la microbiota para recuperarse. Además, tenemos otros tres objetivos:

1. Determinar la diversidad en condiciones normales y control, para cada punto temporal. Esto nos permitirá observar variaciones en la diversidad durante el tiempo y el posible aumento y disminución bajo el límite detección de algunos grupos taxonómicos que podrían ser importantes.
2. Evaluar la composición y variabilidad de la microbiota intestinal en respuesta al tratamiento periódico con kanamicina. Para ellos, se usará software especializado en el estudio de muestras longitudinales. Además, se realizarán redes de co-ocurrencia con tal de observar el efecto del antibiótico.
3. Observar si la microbiota es capaz de volver a su estado previo antes del tratamiento con kanamicina, es decir, si es resiliente.
4. Comprobar el perfil funcional de las muestras control y tratamiento, así como estudiar la robustez de la microbiota intestinal de la cucaracha; esto es, comprobar si los cambios en la taxonomía son suficientemente grandes como para provocar cambios significativos en las funciones.
5. Por último, queremos determinar si el antibiótico afecta a la cucaracha y cómo. Con esto, nos aseguramos que nuestras conclusiones no están sesgadas debido a que el antibiótico esté afectando no solo a la microbiota, si no a la cucaracha.

Materiales y resultados

Las poblaciones de *B. germanica* se mantuvieron en botes dentro de cámaras climáticas a una temperatura constante de 25º y una humedad del 65%. El ciclo de día fue de 12L:12D de 8:00 a 20:00. Se les alimentó con pienso de perro y el agua fue suministrada en pequeños botes de vidrio tapados con algodón. La limpieza y cambio de medios se realizaron una vez a la semana. Estas poblaciones fueron fundadas

hace 30 años, gracias a la donación del "Institut de Biologia Evolutiva (CSIC-UPF)", en Barcelona.

Las disecciones se realizaron en condiciones estériles con material autoclavado y con una esterilización sistemática durante cada disección. El intestino posterior se abrió y limpió en una placa petri estéril y se almacenó en un tubo Eppendorf de 1,5 mL. Los tubos se mantenían en nitrógeno líquido hasta ser almacenados a -80 ºC en un congelador hasta el momento de la extracción de ADN.

El ADN de todas las muestras se extrajo usando el "JetFlex ™ Genomic DNA Purification Kit" siguiendo el mismo protocolo para evitar diferencias en los resultados posteriores. Tras la extracción, cuantificamos las muestras con el fluorómetro Qubit y se almacenaron a -20º C hasta el momento de enviarlas para la secuenciación.

Un total de 512 muestras fueron enviadas a secuenciar a la Fundación para el Fomento de la Investigación Sanitaria y Biomédica (FISABIO). Allí, se utilizó la tecnología Myseq de Illumina para secuenciar la región V3-V4 del gen de ARNr 16S de las muestras.

Para la realización del experimento del estudio longitudinal de la microbiota intestinal frente al suministro de kanamicina hicieron falta 525 cucarachas.

El diseño experimental se basaba en 5 poblaciones de cucarachas, 2 control y 3 de tratamiento. Cada población constaba de unas 60 cucarachas macho y otras tantas hembras. La kanamicina, a una concentración de 0.2 mg/mL, se suministró a las poblaciones tratadas en tres ocasiones, durante 10 días. En períodos temporalmente equidistantes empezando el día 0. Las disecciones se realizaron todos los días durante 105 días alternando machos y hembras para que también hubiese equidistancia entre muestras.

En cuanto al experimento de *fitness*, en el que comprobamos los efectos del antibiótico en las cucarachas adultas de la misma generación, se utilizaron 6 poblaciones en lugar de 5. La mitad fue control y la otra mitad, tratamiento. El diseño experimental fue el mismo que para el experimento principal. Los parámetros escogidos fueron peso de los individuos, número de ootecas, número de ninfas y número de muertes. Se midieron dos días a la semana durante 105 días. No observamos que el AB afectase a las cucarachas en ningún parámetro excepto para el número de ootecas en el tercer periodo, en el que observamos un aumento de ootecas.

Para los análisis bioinformáticos utilizamos el software especializado QIIME2. Este es un software libre y desarrollado por la comunidad que se especializa en el análisis de muestras de 16S. Utilizamos el plugin DADA2 para el control de calidad de las lecturas. Recortamos 22 nucleótidos en el extremo 5′ para eliminar los *primers*. Además, sabiendo que la calidad de las lecturas disminuye drásticamente al final de las mismas, se truncaron. Para las lecturas *forward*, a 270 nucleótidos mientras que las para las *reverse,* a 210 nucleótidos. Se comprobó si había efecto de *run*, ya que hicieron falta 5 *runs* para poder secuenciar las 512 muestras. También se eliminaron aquellas muestras con menos de 5000 lecturas, que resultaron ser un total de 9 muestras. Para eliminarlas, utilizamos R en lugar de QIIME2. Tras esto, se asignaron taxonómicamente las lecturas con la base de datos SILVA 132.

Se calculó la alfa diversidad total y en tres periodos de misma longitud de los grupos utilizando el índice de Shannon y la riqueza utilizando el índice de Chao1. Se observó que la diversidad y la riqueza no variaba entre sexos pero que las muestras tratadas presentaban una disminución en ambos parámetros. Para la beta diversidad, se utilizaron las distancias de Jaccard para observar en un

PCoA la distancia entre los grupos. Otra vez se observó que el sexo no es un factor diferencial de la microbiota intestinal, sin embargo, el tratamiento la moldea claramente. Además, se hizo un test de ADONIS para confirmar que estadísticamente sí existían diferencias entre los grupos estudiados.

Se obtuvo el *core* de la microbiota, es decir, qué bacterias presentan una abundancia superior a 0 durante los 105 días que dura el experimento en las poblaciones control. De esta manera se obtuvieron 18 bacterias. Las 4 más abundantes son: *Dysgonomonas*, *Alistipes*, *Bacteroides* y Rikenellaceae,

También realizó un "Linear discriminant analysis Effect Size" o LEfSe, para determinar qué bacterias estaban más presentes en condición control con respecto al tratamiento y viceversa. Con este análisis se encontraron 4 bacterias cuya abundancia era significativamente superior en tratamiento y 24 cuya abundancia era significativamente superior en controles.

Con tal de conocer cómo afectó el antibiótico a las relaciones entre bacterias en la comunidad, se realizó un análisis de redes de co-ocurrencia. En estos estudios se pudo ver qué bacterias se agrupaban dependiendo de sus dinámicas de abundancia. En el grupo control, se encontraron 6 grupos de bacterias que formaban una red robusta mientras que para tratados no se encontraron grupos de bacterias, incluso tras relajar los parámetros del software.

Para los análisis temporales se utilizó el paquete de R, metagenomeseq, gracias al cual pudimos ver la dinámica de cada bacteria comparando la condición control con la del tratamiento. Con este análisis pudimos observar que en nuestra comunidad existen 5 tipos de comportamiento con respecto al antibiótico. Uno de los cuales es precisamente lo que buscábamos, bacterias resilientes.

Con tal de estudiar la dinámica de las poblaciones también empleamos otro método. Comprobamos si nuestras muestras seguían la ley de Taylor utilizando el software complexCruncher realizando los ajustes apropiados. Observamos que nuestras muestras se ajustaban estrechamente al modelo con un R^2 superior a 0.96. Cuando una población sigue esta ley, podemos interpretar que las bacterias con mayor abundancia son los más estables, y los menos abundantes, son más variables. Se observó que, al dividir los grupos tratados y control en tres periodos, igual que se hizo anteriormente, el primer periodo del grupo tratamiento presentaba una variabilidad (variable *V*) mayor que todos los demás grupos. Sin embargo, los periodos 2 y 3 del tratamiento tenían una variabilidad muy similar a la de los tres periodos control. Esto podría querer decir que la microbiota es capaz de adaptarse a los pulsos de antibiótico tras el primero.

También estudiamos el índice de estabilidad de rango nos ayuda a ver qué bacterias son aquellas más estables basándonos en su posición en cuanto a abundancia, su rango. Para este análisis utilizamos las 50 bacterias más abundantes y observamos que *Dysgonomonas* es la bacteria más estable, en ambas condiciones. En general, confirmamos que nuestras muestras seguían la ley de Taylor ya que observamos que se cumplía su comportamiento con respecto a la abundancia y la estabilidad. Algunas bacterias que no seguían este patrón fueron *Oxalobacter*, *Clostridiales* y *Raoulobacter*, que fueron poco abundantes pero muy estables.

Con tal de estudiar el perfil funcional de nuestras muestras, utilizamos el software PICRUSt2 que, a partir de las secuencias del gen 16S de rRNA, nos permitió inferir las funciones de la microbiota intestinal en ambas condiciones y a lo largo de todo el experimento. Observamos que el antibiótico provocaba diferencias significativas realizando un test de ADONIS. Además, comprobamos la presencia

de la kanamicina quinasa, la enzima responsable de la degradación de la kanamicina en bacterias, en taxones bacterianos con un metabolismo muy diverso.

Por último, estudiamos la magnitud de los cambios en las funciones provocados por los cambios en las abundancias de los taxones bacterianos al ser tratados. En otras palabras, estudiamos la robustez de la microbiota intestinal de la cucaracha. Uno de los parámetros de la robustez es la atenuación que representa la velocidad de los cambios en el perfil funcional en relación con los cambios en las abundancias taxonómicas. Cuanto mayor sea el valor de la atenuación, mayor será la robustez de la muestra. En nuestras muestras, pudimos observar que la atenuación tenía una tendencia a disminuir con el paso del tiempo, teniendo sus valores más bajos hacia el final del experimento. También estudiamos 20 rutas metabólicas diferentes y observamos que en 14 de ellas había diferencias significativas entre control y tratamiento. Algunas de esas rutas son "*Xenobiotics biodegradation and metabolism*" y "*Transport and Catabolism*", que podrían estar relacioandas con el estrés provocado por el antibiótico.

Conclusiones

1. No existen diferencias estadísticas entre la composición y diversidad de la microbiota intestinal de cucarachas macho y hembra. Ese hecho facilita la planificación de futuros experimentos con esta especie de cucaracha.
2. Tanto la diversidad alfa como beta de la microbiota intestinal se ve claramente afectada cuando se le suministra kanamicina en ventanas temporales de 10 días. Estos cambios se hacen más claros a niveles taxonómicos más específicos tales como familia o género antes que filo. Además, el tratamiento disminuye la diversidad y cambia la composición del sistema.

3. En condiciones control, las bacterias que son similares filogenéticamente forman redes de co-ocurrencia en forma de red. Además, las bacterias que forman esas redes son una parte esencial de la comunidad, ya que proporcionan estabilidad y funcionan como ancla para el resto de miembros de la microbiota. El tratamiento con kanamicina disrumpe las redes de co-ocurrencia hasta el punto en que no hemos podido encontrar ninguna correlación en nuestras poblaciones.
4. Hemos encontrado cinco grupos de bacterias con respecto a cómo su abundancia varía a lo largo del tiempo en respuesta al tratamiento a la kanamicina. Estos son: bacterias oportunistas, ligeramente afectadas, afectadas, resistentes y resilientes. Uno de los objetivos de esta tesis era encontrar bacterias resilientes, y hemos encontrado cinco taxones incluidos en este grupo: *Lachnoclostridium, Parabacteroides, Christensenellaceae R-7 group*, Tannerellaceae and Desulfovibrionaceae.
5. Las comunidades bacterias tratadas y no tratadas siguen la ley de Taylor ya que el parámetro β siempre se encuentra entre 0.5 y 1. Esto se traduce en que, en nuestras muestras, las bacterias menos abundantes son más proclives a sufrir cambios en su abundancia que las bacterias más abundantes, que son más estables.
6. En poblaciones control, la variabilidad es muy similar a lo largo de los tres periodos estudiados. Sin embargo, cuando estudiamos las poblaciones tratadas, el primer periodo es el más variable de todo el experimento, incluyendo ambas condiciones. Los periodos segundo y tercero de las poblaciones tratadas practicamente recuperan la variabilidad de las condiciones control. Por lo tanto, podemos concluir que la microbiota, en conjunto, es capaz de adaptarse a la kanamicina tras ser expuesta una única vez y evitar una mayor variabilidad en el sistema tras repetidas exposiciones.

7. La género bacteriano con un RSI más alto y por tanto la más estable es *Dysgonomonas,* siendo este también el taxón de mayor abundancia en ambas condiciones. Además, en este análisis, el RSI es mayor en taxones bacterianos con rangos altos, mientras que es menor para taxones en posiciones más bajas. Existen algunas excepciones como *Oxalobacter, Clostridiales* y *Raoultibacter* con un RSI alto teniendo una abundancia baja. Estos resultados sostienen el hecho de que la microbiota intestinal de cucarachas sigue la ley de Taylor.
8. El perfil funcional de la microbiota intestinal de *B. germanica* sufre cambios en poblaciones tratadas en los tres periodos. Además, mediante la inferencia de funciones, se ha encontrado la enzima responsable de la degradación de la kanamicina, la kanamicina quinasa, en algunos taxones de nuestras muestras, como *Pseudomonas* y *Sphingomonas*, bacterias con una gran capacidad metabólica.
9. Los parámetros de robustez no cambian cuando son estudiados periodo por periodo entre control y tratamiento, pero la atenuación presenta una tendencia a disminuir con el paso del tiempo, lo que indica que la robustez en estas muestras disminuye con él.
10. La kanamicina no afecta ni al peso de los insectos ni al número de ninfas en el experimento. En cuanto al número de ootecas, parece incrementarse en el último periodo del experimento. Concluímos que la kanamicina no afecta al estado de salud de los insectos en este experimento.

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9. Appendix

9.1 Additional Tables

Taxa (Genus)	Control	Treatment	*P*-value
g__Dysgonomonas	15,67	22,02	5,71E-04
g__Desulfovibrio	13,1	13,71	0,274145
g__Fusobacterium	11,01	10,75	0,430772
g__Alistipes	9,22	7,2	9,80E+04
g__Bacteroides	5,26	5,25	0,061734
g__Candidatus_Soleaferrea	5,17	4,89	0,194579
g__Christensenellaceae_R-7_group	3,33	1,96	2,66E-06
g__Tyzzerella_3	1,43	1,22	6,36E-03
g__Ruminiclostridium_5	1,29	0,89	0,402925
g__Parabacteroides	1,13	0,8	1,17E+07
g__Paludibacter	1	0,67	0,006787
g__Desulfatiferula	0,71	0,62	0,001707
g__Mucispirillum	0,6	0,61	0,587346
g__Incertae_Sedis	0,48	0,55	1,40E-61
g__Pseudomonas	0,45	0,42	1,46E-46
g__Akkermansia	0,44	0,41	0,269988
g__Odoribacter	0,44	0,29	2,64E+07
g__Erysipelatoclostridium	0,4	0,25	3,77E+01
g__Candidatus_Endomicrobium	0,38	0,2	5,00E+05
g__Ruminococcaceae_UCG-009	0,33	0,18	3,02E-05
g__Lachnoclostridium	0,31	0,18	5,47E+09
g__Anaerotruncus	0,25	0,15	1,84E+05
g__Hydrogenoanaerobacterium	0,22	0,15	8,53E-14
g__Elusimicrobium	0,21	0,14	7,41E+08
g__Breznakia	0,2	0,13	2,05E-25
g__Lachnospiraceae_UCG-010	0,17	0,13	0,463201

g__Sanguibacteroides	0,15	0,12	0,846001
g__Robinsoniella	0,15	0,11	1,05E-29
g__Harryflintia	0,14	0,1	0,448438
g__Raoultibacter	0,14	0,09	5,73E-13
g__Candidatus_Saccharimonas	0,13	0,08	2,97E+09
g__Oxalobacter	0,13	0,07	0,0901
g__Enterococcus	0,12	0,06	6,13E-09
g__Blattabacterium	0,12	0,06	0,037733
g__Nitrosomonas	0,07	0,04	0,002103
g__[Eubacterium]_coprostanoligenes_group	0,07	0,03	2,13E-13
g__Fournierella	0,07	0,03	9,45E-15
g__Ruminococcaceae_UCG-013	0,05	0,03	1,12E-18
g__Anaerosporobacter	0,05	0,02	0,004112
g__Tyzzerella	0,04	0,02	5,15E+08
g__Intestinimonas	0,04	0,02	0,152639
g__Ruminococcaceae_UCG-014	0,03	0,02	2,07E-10
g__Ruminiclostridium_9	0,03	0,02	2,19E+08
g__Blattella_germanica_(German_cockroach)	0,03	0,02	3,52E+05
g__GCA-900066225	0,03	0,02	2,27E-22
g__Papillibacter	0,03	0,02	3,27E+00
g__Erysipelothrix	0,03	0,01	0,001711
g__Candidatus_Tammella	0,02	0,01	0,390631
g__Ruminococcaceae_NK4A214_group	0,02	0,01	3,56E-11
g__Butyricicoccus	0,02	0,01	5,65E+05
g__Anaerovorax	0,02	0,00	1,32E-27
g__Serratia	0,01	0,00	0,360558
g__[Eubacterium]_brachy_group	0,01	0,00	0,256894
g__Lachnospiraceae_NK4A136_group	0,01	0,00	1,48E+07
g__Ruminococcaceae_UCG-010	0,00	0,00	5,26E-03

g__Anaerofustis	0,00	0,00	0,180943
g__Veillonella	0,00	0,00	0,632719
g__Delftia	0,00	0,00	0,388877
g__Corynebacterium_1	0,00	0,00	0,088042
g__Tannerella	0,00	0,00	0,601985
g__bacterium_PM5-3	0,00	0,00	9,16E-07
g__SP3-e08	0,00	0,00	0,158752
g__Cutibacterium	0,00	0,00	0,384249
g__Streptococcus	0,00	0,00	0,859584
g__Lawsonella	0,00	0,00	0,205127
g__Brachybacterium	0,00	0,00	0,786414
g__Renibacterium	0,00	0,00	0,228012
g__Rothia	0,00	0,00	0,786414
g__Staphylococcus	0,00	0,00	0,939357
g__Stenotrophomonas	0,00	0,00	0,515428
g__rumen_bacterium_YS2	0,00	0,00	0,790852
g__Lachnospiraceae_UCG-004	0,00	0,00	0,982493
g__Capnocytophaga	0,00	0,00	0,802788
g__Shewanella	0,00	0,00	0,786414
g__Paracoccus	0,00	0,00	0,489335
g__Sphingomonas	0,00	0,00	0,370234
g__Anaerobacillus	0,00	0,00	0,015335
g__Acinetobacter	0,00	0,00	0,959578
g__Anaerofilum	0,00	0,00	0,205127
g__Subdoligranulum	0,00	0,00	0,071958
g__Massilia	0,00	0,00	0,087374
g__Enhydrobacter	0,00	0,00	0,802788
g__Roseomonas	0,00	0,00	0,228012
g__Bifidobacterium	0,00	0,00	0,015335
g__Hymenobacter	0,00	0,00	0,228012
g__Aggregatibacter	0,00	0,00	0,799146

g__Lysinibacillus	0,00	0,00	0,015335
g__Lactobacillus	0,00	0,00	0,036004
g__Escherichia-Shigella	0,00	0,00	0,38008
g__Corynebacterium	0,00	0,00	0,228012
g__Ruminococcus_2	0,00	0,00	0,087374
g__Streptomyces	0,00	0,00	0,228012
g__Truepera	0,00	0,00	0,228012
g__Micrococcus	0,00	0,00	0,228012
g__Gemella	0,00	0,00	0,228012
g__Curvibacter	0,00	0,00	0,228012
g__Agathobacter	0,00	0,00	0,228012
g__Prevotella_9	0,00	0,00	0,228012
g__Marinomonas	0,00	0,00	0,228012
g__Bacillus	0,00	0,00	0,786414
g__Wolbachia	0,00	0,00	0,228012
g__Neisseria	0,00	0,00	0,795508
g__Halomonas	0,00	0,00	0,799146
g__Mitsuokella	0,00	0,00	0,228012
g__Prevotellaceae_NK3B31_group	0,00	0,00	0,228012
g__Kocuria	0,00	0,00	0,795508
g__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	0,00	0,00	0,228012
g__Dolosigranulum	0,00	0,00	0,228012
g__Ruminococcaceae_UCG-005	0,00	0,00	0,228012
g__Gardnerella	0,00	0,00	0,410696
g__Mycobacterium	0,00	0,00	0,410696
g__Williamsia	0,00	0,00	0,410696
g__Blastococcus	0,00	0,00	0,243018
g__Leucobacter	0,00	0,00	0,410696
g__Microbacterium	0,00	0,00	0,410696
g__Pseudoglutamicibacter	0,00	0,00	0,410696
g__Pseudopropionibacterium	0,00	0,00	0,410696

g__Porphyromonas	0,00	0,00	0,410696
g__Flavisolibacter	0,00	0,00	0,410696
g__Nibribacter	0,00	0,00	0,410696
g__Jeotgalicoccus	0,00	0,00	0,410696
g__Anaerococcus	0,00	0,00	0,410696
g__Finegoldia	0,00	0,00	0,410696
g__[Eubacterium]_nodatum_group	0,00	0,00	0,410696
g__[Eubacterium]_oxidoreducens_group	0,00	0,00	0,410696
g__Lachnoclostridium_5	0,00	0,00	0,410696
g__Lachnospiraceae_NC2004_group	0,00	0,00	0,410696
g__Ruminococcaceae_UCG-011	0,00	0,00	0,410696
g__Selenomonas_3	0,00	0,00	0,410696
g__Skermanella	0,00	0,00	0,410696
g__Brevundimonas	0,00	0,00	0,243018
g__Caulobacter	0,00	0,00	0,410696
g__Microvirga	0,00	0,00	0,243018
g__Shinella	0,00	0,00	0,410696
g__Rubellimicrobium	0,00	0,00	0,243018
g__Bdellovibrio	0,00	0,00	0,243018
g__Candidatus_Adiutrix_intracellularis	0,00	0,00	0,410696
g__Aeromonas	0,00	0,00	0,410696
g__Acidovorax	0,00	0,00	0,410696
g__Aquabacterium	0,00	0,00	0,243018
g__Castellaniella	0,00	0,00	0,410696
g__Oligella	0,00	0,00	0,410696
g__Pelomonas	0,00	0,00	0,410696
g__Eikenella	0,00	0,00	0,410696
g__Steroidobacter	0,00	0,00	0,243018
g__Pseudofulvimonas	0,00	0,00	0,410696
g__Pseudoxanthomonas	0,00	0,00	0,410696

g__Treponema_2	0,00	0,00	0,410696

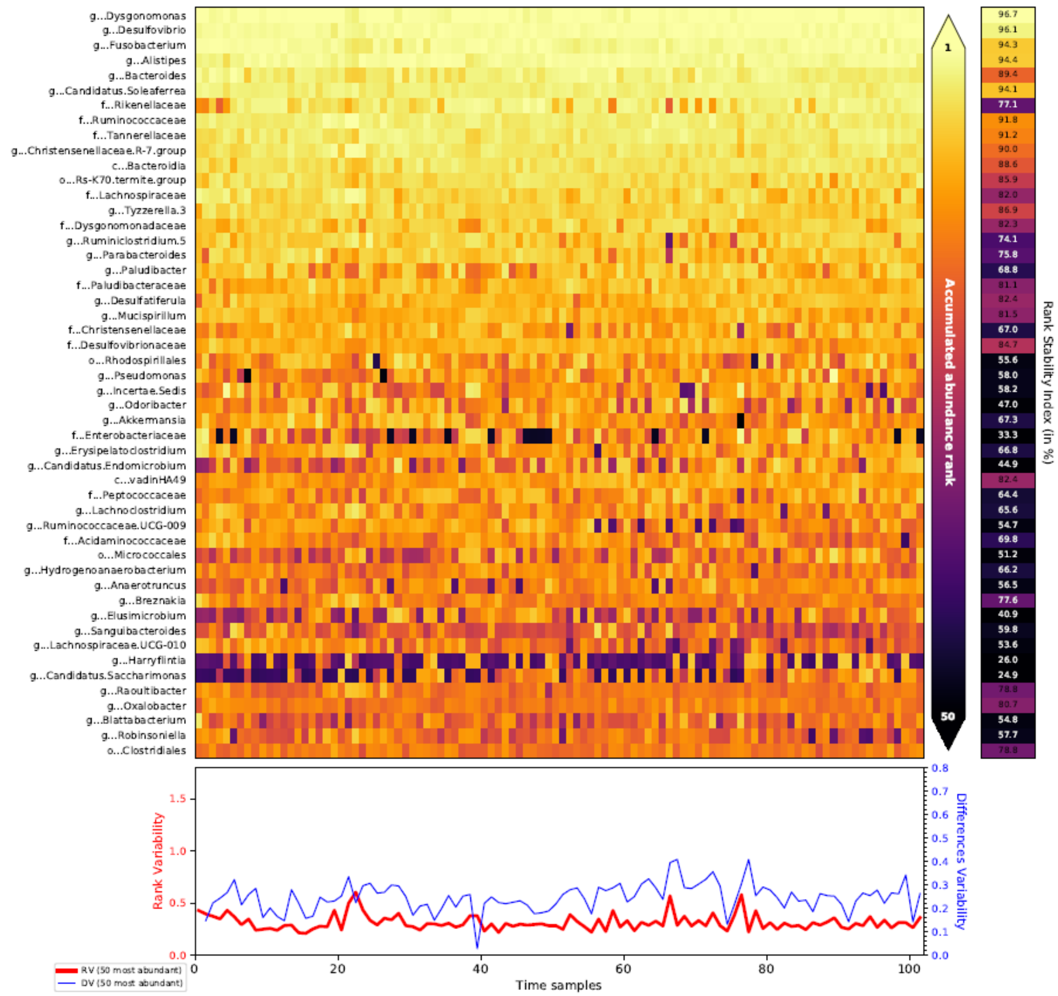
Supplementary Table 1. Relative abundance of each bacteria at genus level in both conditions with the associated p-value.

Day 1 to 35	**Interval start**	**Interval end**	**Area**	**p.value**	**difference**
f__Enterobacteriaceae interval:1	15	35	49221	0,091	115043
g__Paludibacter interval:1	6	13	-9116	0,455	47933
o__Rs-K70_termite_group interval:1	2	30	-34300	0,455	41458
g__Parabacteroides interval:1	1	4	4632	0,091	38027
g__Parabacteroides interval:2	11	26	-16814	0,091	38027
f__Rikenellaceae interval:1	6	26	12400	0,636	34180
f__Lachnospiraceae interval:1	6	17	13855	0,091	31266
f__Lachnospiraceae interval:2	29	34	3828	0,091	31266
g__Christensenellaceae_R-7_group interval:1	7	26	-18879	0,091	29736
g__Ruminiclostridium_5 interval:1	34	35	-1630	0,727	29314
f__Ruminococcaceae interval:1	1	35	22200	0,636	29133
g__Bacteroides interval:1	11	13	802	0,091	28786
g__Mucispirillum interval:1	6	28	17740	0,273	28625
g__Dysgonomonas interval:1	6	33	20293	0,182	27221
g__Fusobacterium interval:1	20	30	9044	0,091	26041
g__Fusobacterium interval:2	1	3	-3400	0,636	26041
g__Tyzzerella_3 interval:1	9	32	-15504	0,182	21952
c__Bacteroidia interval:1	13	23	4906	0,273	21552
f__Tannerellaceae interval:1	1	24	-11455	0,091	18725
g__Desulfatiferula interval:1	1	20	8445	0,545	18312
g__Candidatus_Soleaferrea interval:1	10	17	4074	0,182	17684
g__Candidatus_Soleaferrea interval:2	27	34	4042	0,091	17684
g__Alistipes interval:1	22	25	1111	0,091	16808
g__Desulfovibrio interval:1	18	31	5798	0,091	15051
Day 36 to 70					

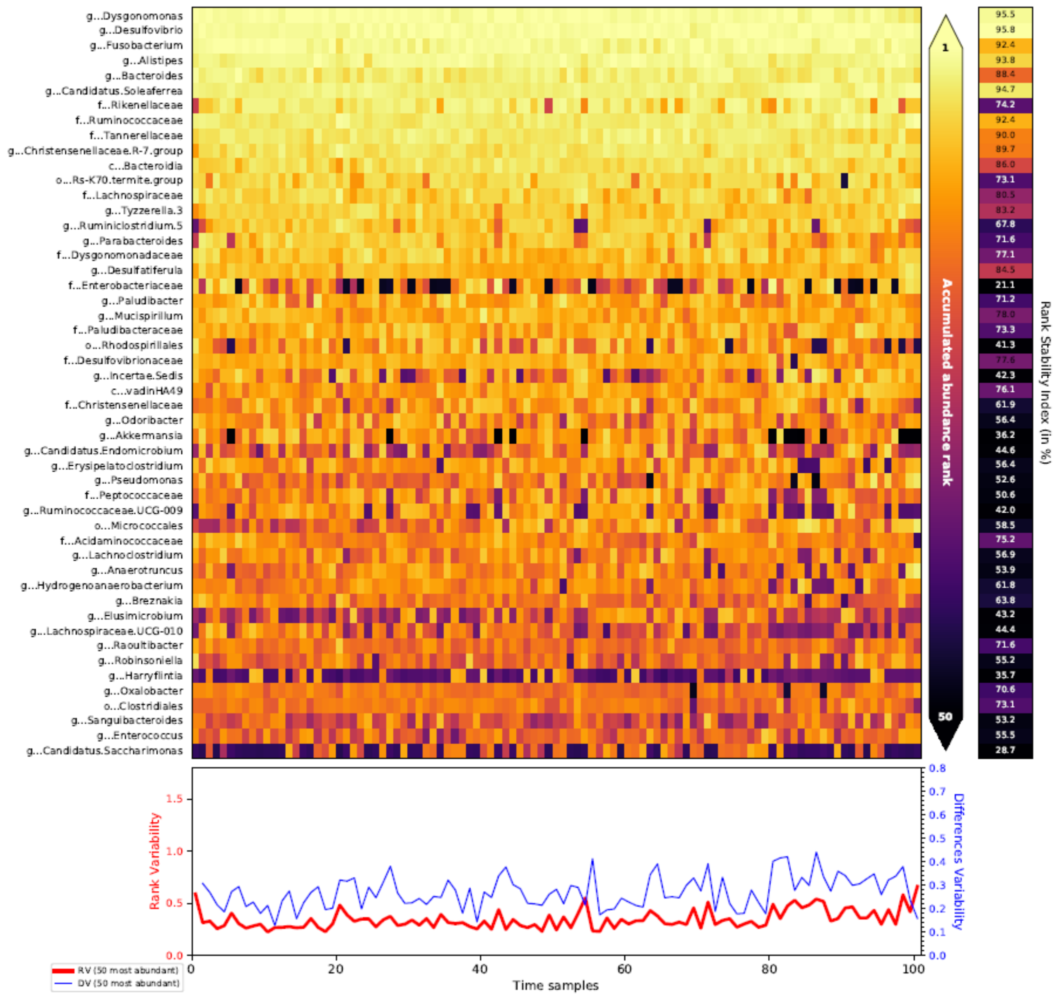
f__Enterobacteriaceae interval:1	41	70	63118	0,182	111557
o__Rs-K70_termite_group interval:1	37	67	-90596	0,636	96319
g__Ruminiclostridium_5 interval:1	44	61	18565	0,091	43620
f__Lachnospiraceae interval:1	36	68	38093	0,182	43591
f__Rikenellaceae interval:1	48	70	25167	0,091	41493
g__Dysgonomonas interval:1	36	70	31692	0,091	38100
f__Ruminococcaceae interval:1	36	64	23943	0,545	30654
f__Tannerellaceae interval:1	46	70	-21345	0,455	29637
g__Christensenellaceae_R-7_group interval:1	43	70	-19043	0,727	25392
g__Tyzzerella_3 interval:1	49	70	-10641	0,455	22775
g__Desulfovibrio interval:1	37	70	18349	0,455	19404
g__Alistipes interval:1	55	70	-5605	0,364	18915
g__Candidatus_Soleaferrea interval:1	36	57	8325	0,455	17423
c__Bacteroidia interval:1	45	59	4317	0,545	15204
Day 71 to 105					
f__Enterobacteriaceae interval:1	85	94	9378	0,091	113992
o__Rs-K70_termite_group interval:1	73	102	-66845	0,636	91000
f__Rikenellaceae interval:1	71	97	23635	0,727	48493
g__Dysgonomonas interval:1	72	105	29473	0,091	43928
f__Lachnospiraceae interval:1	76	97	26234	0,273	40485
g__Tyzzerella_3 interval:1	76	105	-24665	0,091	36300
g__Christensenellaceae_R-7_group interval:1	77	101	-24362	0,273	33756
f__Tannerellaceae interval:1	71	87	-7945	0,636	27709
f__Ruminococcaceae interval:1	71	99	18504	0,182	27607
g__Desulfatiferula interval:1	91	96	-1534	0,364	19600
g__Desulfovibrio interval:1	72	86	4294	0,91	16510

Supplementary Table 2. Periods in which control and treatment are divergent.

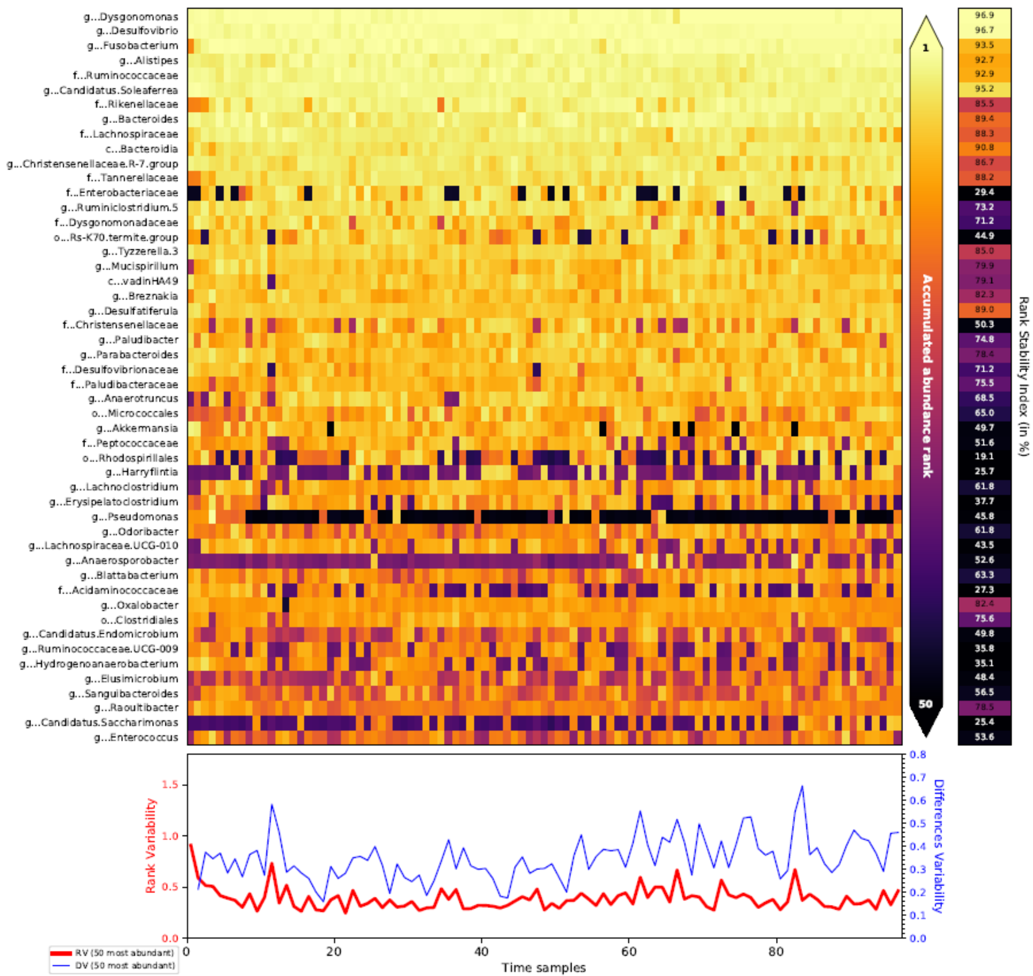
9.2 Additional Figures



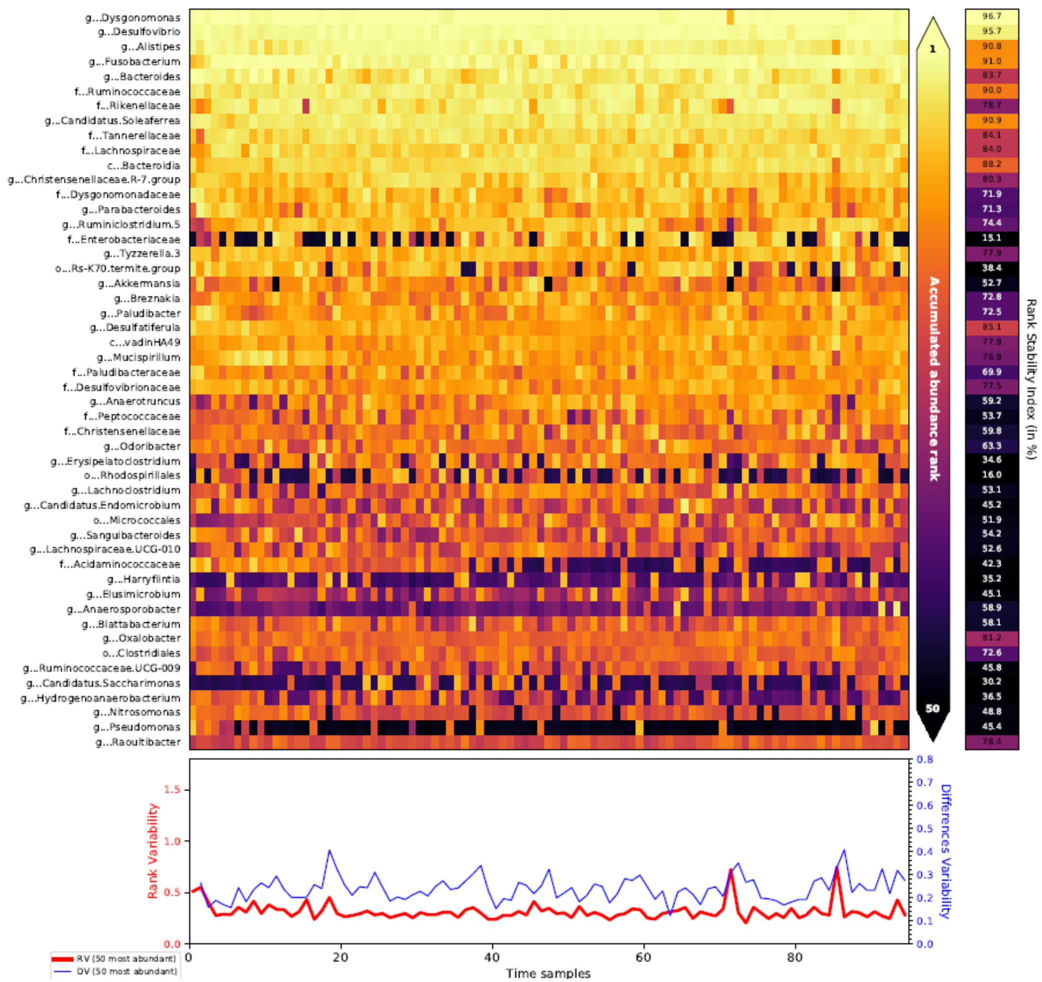
Supplementary Figure 1. Rank matrix and stability for population C1.



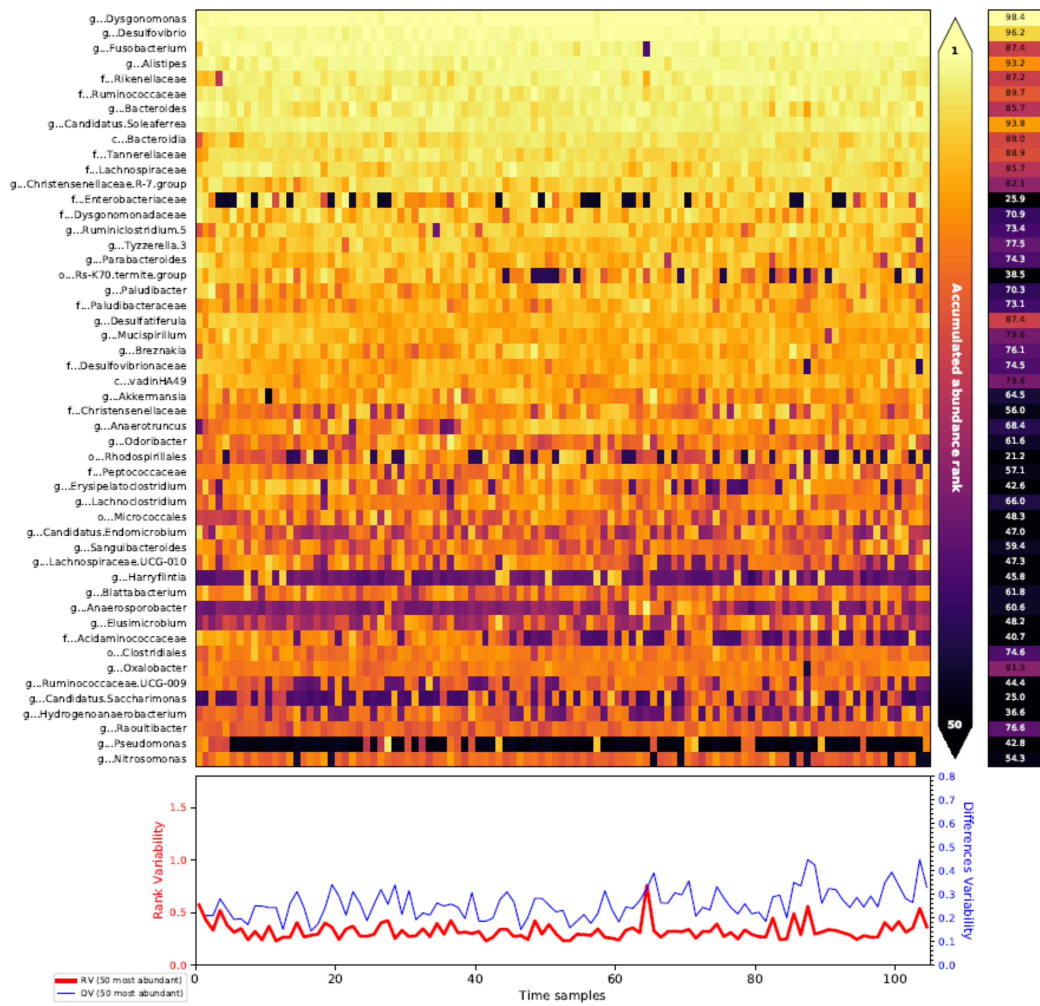
Supplementary Figure 2. Rank matrix and stability for population C2.



Supplementary Figure 3. Rank matrix and stability for population K1.



Supplementary Figure 4. Rank matrix and stability for population K2.



Supplementary Figure 5. Rank matrix and stability for population K3.