IDENTIFICATION OF MICROBIOTA PROFILE USING DIFFERENT MOLECULAR METHODS, AND INVESTIGATION OF INTERACTIONS BETWEEN MICROBIOTA, HOST GENETICS AND HOST METABOLISM

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ABSTRACT

IDENTIFICATION OF MICROBIOTA PROFILE USING DIFFERENT MOLECULAR METHODS, AND INVESTIGATION OF INTERACTIONS BETWEEN MICROBIOTA, HOST GENETICS AND HOST METABOLISM

Understanding the microbiota profile, including its interactions with host genetics and metabolism, is critical for understanding host physiology and creating targeted therapeutics. The gut microbiota, which consists of many different kinds of bacterial and fungal species, is essential for nutrient absorption, immune function, and metabolic regulation. In this study, microbial species within the gut of the model organism *Drosophila melanogaster* were identified and quantified using NGS, qPCR, and LAMP molecular methods. Notably, *A. pomorum* and *L. brevis* were highly prevalent and significantly correlated with host lipid metabolism negatively ($p < 0.001$ and $p < 0.01$, respectively). Further contributing to the bacterial microbiota, a fungal microbiota composed primarily of Ascomycota and Basidiomycota phyla was discovered. Interestingly, the presence of the *M. restricta* demonstrated a negative correlation with triglyceride levels (p=9.4e-05), emphasizing the unique roles of fungi in metabolism.

GWAS was employed to discover host genetic variants that influence microbiota composition and metabolic profiles. Important genes such as *pyd* and *Myd88*, which are required for intestinal barrier integrity and immune-mediated signaling, were revealed. Our research, which integrates modern molecular methods and genetic analyses, may contribute to the development of personalized therapies to reveal microbiota composition, and aimed improve metabolic health, disease management. Additionally, in this study, LAMP was successfully performed amplification of microbial species exist in the DNA obtained from very small *Drosophila* intestinal samples, with high sensitivity. Therefore, LAMP can facilitate microbiota-related diagnostics by minimizing time and technical requirements and simplifies detection strategies with wide applicability in microbiota research across various species and sample types.

ÖZET

FARKLI MOLEKÜLER YÖNTEMLER KULLANARAK MİKROBİYOTA PROFİLİNİN BELİRLENMESİ VE MİKROBİYOTA, KONAK GENETİĞİ VE KONAK METABOLİZMASI ARASINDAKİ ETKİLEŞİMLERİN ARAŞTIRILMASI

Mikrobiyota profilinin, konakçı genetiği ve metabolizması ile olan etkileşimleri ile anlaşılması, konak fizyolojisini anlamak ve hedefe yönelik tedaviler oluşturmak için kritik öneme sahiptir. Birçok farklı türde bakteri ve mantar türünden oluşan bağırsak mikrobiyotası, besinlerin emilimi, bağışıklık fonksiyonu ve metabolik düzenleme için gereklidir. Bu çalışmada, model organizma *Drosophila melanogaster'*in bağırsağında yer alan mikrobiyal türler NGS, qPCR ve LAMP moleküler yöntemleri kullanılarak tanımlandı ve miktarları belirlendi. Özellikle *A. pomorum* ve *L. brevis* oldukça yaygındı ve konakçı lipit metabolizması ile negatif yönde anlamlı korelasyon gösterdi (sırasıyla $p < 0.001$ ve $p < 0.01$). Ayrıca bakteriyel mikrobiyotaya katkıda bulunan, öncelikle Ascomycota ve Basidiomycota filumlarından oluşan bir mantar mikrobiyotası keşfedildi. İlginç bir şekilde, *M. restricta*'nın varlığı, trigliserit seviyesi ile negatif bir korelasyon gösterdi ($p=9.4e-05$), bu da mantarların metabolizmadaki eşsiz rollerini vurgulamaktadır.

GWAS, mikrobiyota kompozisyonunu ve metabolik profilleri etkileyen konakçı genetik varyantlarını keşfetmek için kullanıldı. Bağırsak bariyer bütünlüğü ve immün aracılı sinyalleme için gerekli olan *pyd* ve *Myd88* gibi önemli genler ortaya çıkarıldı. Modern moleküler yöntemler ile genetik analizleri birleştiren araştırmamız, mikrobiyota kompozisyonunu ortaya koyan, metabolik sağlığı ve hastalık yönetimini iyileştirmeyi amaçlayan kişiselleştirilmiş tedavilerin geliştirilmesine katkıda bulunabilir. Ayrıca bu çalışmada LAMP, çok küçük *Drosophila* bağırsak örneklerinden elde edilen DNA'da bulunan mikrobiyal türlerin amplifikasyonunu yüksek hassasiyetle başarıyla gerçekleştirdi. Bu nedenle LAMP, zaman ve teknik gereksinimleri en aza indirerek mikrobiyota ile ilgili teşhisleri kolaylaştırabilir, çeşitli türler ve numune türleri genelinde mikrobiyota araştırmalarında geniş uygulanabilirliği ile tespit stratejilerini basitleştirebilir.

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CHAPTER 1

INTRODUCTION

1.1. Significance of Gut Microbiota and Factors Shaping Its Composition

Microbiota refers to the different community of microorganisms exist in multiple regions of the body, including the skin, mouth and, most notably, the gastrointestinal tract. The gastrointestinal track, particularly the colon, has the biggest and most diversified population of microbiota, with billions of microorganisms coexisting in symbiosis with their hosts (Dieterich, Schink, and Zopf 2018). Organisms live with complex communities of microorganisms that affect their physiology and health within their intestines. These microorganisms have a strong impact on the physiology of the host, including their development, diet, immunity, and behavior. The gastrointestinal (digestive) system is one of the ecosystems with the richest microbiota in an organism. In the gut microbiota, which hosts more than 1500 bacterial species, approximately five hundred dissimilar bacterial species are effective in the formation of an individual human microbiota. The intestine acts as a crucial interface between the external environment and the host because it is the primary habitat where host-microbiota interactions take place (Pais et al. 2018).

There are several methodologies and strategies for studying the intestinal microbiota composition and diversity, as well as their functional contribution to host health and illness. Since most intestinal microorganisms are anaerobic, their isolation success rate with culture-based approaches is significantly lower. On the other hand, the utilization of gene sequencing technologies gives detailed information by increasing efficiency. The first of these approaches is sanger sequence analysis, which can be used for targeted sequencing of interested genomic regions (Adak and Khan 2019). However, it is not as popular as new generation sequencing technologies in microbiota research.

Next-generation sequencing technologies provide high-throughput data by utilizing approaches like amplicon metagenomic sequencing and shotgun sequencing (Jandhyala et al. 2015). Shotgun sequencing is more costly, with the advantages of providing both taxonomic composition and gene prediction. With the aid of next-generation sequencing, it has been observed that all vertebrates and invertebrates have a rich microbiota consisting of bacteria, fungi, archaea, and viruses in their bodies. Microbial community in humans is dominated by bacteria, with *Firmicutes* and *Bacteroidetes* accounting for more than ninety percent of species (Mariat et al. 2009). On the other hand, both within and between species microbiome composition is characterized specifically for each organism. In addition to determining the composition of the microbiota, germ-free animal models can be used to examine the impact of microbial colonization on physiological functions in host. For example, germ-free (axenic) mice are a unique experimental model created by excluding all microorganisms, including bacteria, viruses, and fungus. These animals are preferred for studying the influence of the gut microbiota on various aspects of host physiology, including metabolism, immunological function, neurodevelopment, and behavior (Smith, McCoy, and Macpherson 2007).

The gut microbiota contributes to host health by maintaining mucosal integrity. Therefore, it has undeniable benefits for host metabolism. They mediate important events such as influencing the development of our immune system, regulating the barrier function of the intestinal epithelium, providing direct protection against pathogens, nutrient absorption, and vitamin production like B and K. A large number of complex carbohydrates and plant polysaccharides cannot be broken down by human enzymes. By breaking down carbohydrates, proteins, and fats, they constitute metabolites that are both digestible and act as a signal in the metabolic regulation of the host. These processes create short-chain fatty acids, such as acetate, propionate, and butyrate, which serve as important energy substrates for host cells and help to maintain the intestinal barrier (Fan and Pedersen 2021). While butyrate is absorbed in the colon and used as an energy source by the cells in that location, acetate is utilized in lipogenesis and propionate in gluconeogenesis by transporting to the liver (Coppola et al. 2021). These short-chain fatty acids also result in increased insulin and reduced glucose by interact with G protein-coupled receptor on the intestinal cells. Butyrate also contributes to vitamin K production, anti-inflammatory responses, improvement of insulin activity by acting as an inhibitor of histone deacetylase enzymes, and the maintenance of an anaerobic environment in the

intestine (Pushpanathan et al. 2019). Microbiota also participates in endothelial tissue repair, regulation of intestinal endocrine functions by specific changes in hormone levels (Neuman et al. 2015), energy production, weight gain, and development of insulin resistance (Canfora, Jocken, and Blaak 2015; Cho et al. 2012). In addition, by preventing the proliferation of harmful bacteria, anaerobic bacteria in the gut constitute a crucial defensive mechanism (Lopez-Medina and Koh 2016). They defend the body by producing several antimicrobial peptides like defensin and C-type lectin. Thus, they affect host metabolism and physiology. These symbiotic interactions, both among themselves and with the host organism, are thought to be very important for the health of the host but the interactions between different taxa have not yet been understood. That's why investigating the functions of bacteria and fungi, which are an important part of the microbiota in the digestive system, the changes in their types and abundance, and how they interact with the host genome and each other, are of great importance in understanding how the microbiota plays a role in human health. On the other hand, some irregularities or disruption of host-microbiota interactions called dysbiosis plays a role in the occurrence of many diseases, including inflammatory bowel diseases (IBD), obesity, cardiovascular diseases, and even cancer (Blum et al. 2013). The reasons for the development of dysbiosis and its negative impact on host health are one of the most important issues in today's health research. Through the production of proteases and chitinases, pathogens impair and weaken the peritrophic membrane. Therefore, they allow entrance of pathogens into intestinal epithelium more easily. Diet, another factor, has a significant impact on the diversity of the intestinal microbiota (Scott et al. 2013). Abundance of *Bacteroidetes* rises as weight is lost on diets. In species level, *Faecalibacterium prausnitzii*, a member of the Firmicutes phylum, may help in diabetes recovery (Tremaroli and Bäckhed 2012). With the increased microbial activity, concentration of ethanol has been reported in obese individuals than thin people (Nair et al. 2001). Investigating interactions between microorganisms will play a major role in understanding the underlying causes of dysbiosis.

In addition to dietary factors, the diversity of the gut microbiota is also significantly influenced by the host genetics. The host genome controls critical factors that affect microbial colonization and interaction in the gut environment. Genetic variations in genes involved in pathways such as epithelial barrier integrity, antimicrobial peptide synthesis, pathogen recognition may impact pathogenic susceptibility, thereby they can alter the overall composition of the gut microbiota. Members of the Nucleotide-

binding and leucine-rich repeat–containing receptors (NLRs) family, are fundamental regulators of pathogen identification, host immunity, and inflammation. They play a role in innate immunity and related in some human disorders. *NOD2*, one of the best researched members of this family, is reported important genetic risk factor for Crohn's disease (Guo, Gibson, and Ting 2020). *NOD2* directs *ATG16L1*, an autophagy gene, to the infection site to activate the autophagy mechanism. However, in *NOD2* mutant cells, the autophagy pathway responsible for removing intracellular pathogens has been impaired (Travassos et al. 2010). Enterocytes express another member, *NLRP6*, which regulates intestinal homeostasis and responds to infection. However, levels of *NLRP6* reduce in obese rats (Wang et al. 2020). Multiprotein complexes called inflammasomes are important to the innate immune response and help to defend against microbial infections. Upon activation, the inflammasome stimulates the development and production of proinflammatory cytokines like interleukin-18 (*IL-18*) (Dai, Zhou, and Shi 2023). *NLRP6* activation causes the inflammasome assembly, which results in the cleavage and activation of pro-inflammatory cytokines. Metabolites generated by gut microbiota influence host-microbiota interactions by modulating *NLRP6* inflammasome signaling and *IL-18* production (Levy et al. 2015). At that point, inflammasome deficiency results in dysbiosis. Mucin proteins are essential components of the mucus layer that lines the intestinal epithelium and may influence microbial adhesion (Rodríguez-Piñeiro and Johansson 2015). *MUC2*, the first secretory mucin discovered in humans, sustains homeostasis by protecting the gut. Probiotics can strengthen the mucus barrier against infections by increasing *MUC2* production. As with ulcerative colitis patients, intestinal mucus in axenic mice has been found to be thinner than that of normal mice (Liu et al., 2020).

1.2. Model Organisms: Benefits and Drawbacks for Biological Studies

In biological studies, model organisms are frequently utilized to comprehend diseases and developmental processes since they minimize the requirement for substantial experimental labor and make easier to understand the molecular etiology of diseases. Their genomes are fully characterized, therefore they allow elucidation and understanding

of the functions of genes or proteins which participate in biological pathways, as well as development of new methodologies for biological research. These organisms are selected because of their phylogenetic proximity to humans and many of their genes are orthologous to human disease-associated genes. Because the fundamental biological pathways are often conserved among the species, this genetic homology facilitates the adaptation of any findings to human biology. Controlled experiments can be carried out with genetic modifications such as creating mutant strains and genome editing in model organisms. Thanks to their short life cycles, they increase experimental efficiency by shortening the observation time for these applied phenotypic and genotypic changes. In this process, it helps avoiding ethical concerns regarding experiments on complex animals or humans. Since the maintenance and cultivation conditions of model organisms are more cost-effective, experiments can be designed comprehensively with larger sample volumes (Boettcher and Simons 2022).

Drosophila melanogaster, *Caenorhabditis elegans*, Zebrafish (*Danio rerio*) and mice (*Mus musculus*) or rats (*Rattus norvegicus*) are the most commonly utilized model organisms in the biological investigations (Irion and Nusslein-Volhard 2022). Each model organism has individual benefits and drawbacks in different field of biology (Figure 1.1). For example, *C. elegans* is a key model organism, especially for developmental biology. The transparent nature of *C. elegans* allows researchers to observe and follow individual cells throughout development. This transparency is particularly useful for studying tissue differentiation and organ development. It also enables discovery of how and why certain cells undergo apoptosis. Nevertheless, as a remarkable difference from mammals, *C. elegans* does not have an adaptive immune system. This limitation makes the *C. elegans* unsuitable for studying certain aspects of immune function, particularly those involving adaptive immune responses (Liu and Sun, 2021). Additionally, there have no existed male and female gender, so this is the another limiting feature of the *C. elegans* in the studies (Pandey and Nichols 2011). *D. melanogaster* is a useful model organism for morphogenesis studies, due to its four different developmental stages (embryo, larva, pupa, and adult). Unlike *C. elegans* (42%), almost 75% of its genes are orthologous to human disease genes, and this relationship makes it more advantageous in terms of studying disease mechanisms (Boettcher and Simons, 2022; Kim et al., 2020). *D. melanogaster* is also preferred for neurobiology studies because it exhibits complex behaviors such as mating, learning, and memory. Most importantly, because of the fact that innate immune system genes are highly conserved between *D. melanogaster* and

humans increases their preference for immune system studies. For example, the identification of Toll and immune deficiency (Imd) signaling pathways in *D. melanogaster* has been led to the discovery of receptors involved in these pathways (such as Toll-like receptors) in mammals (Kim et al., 2012).

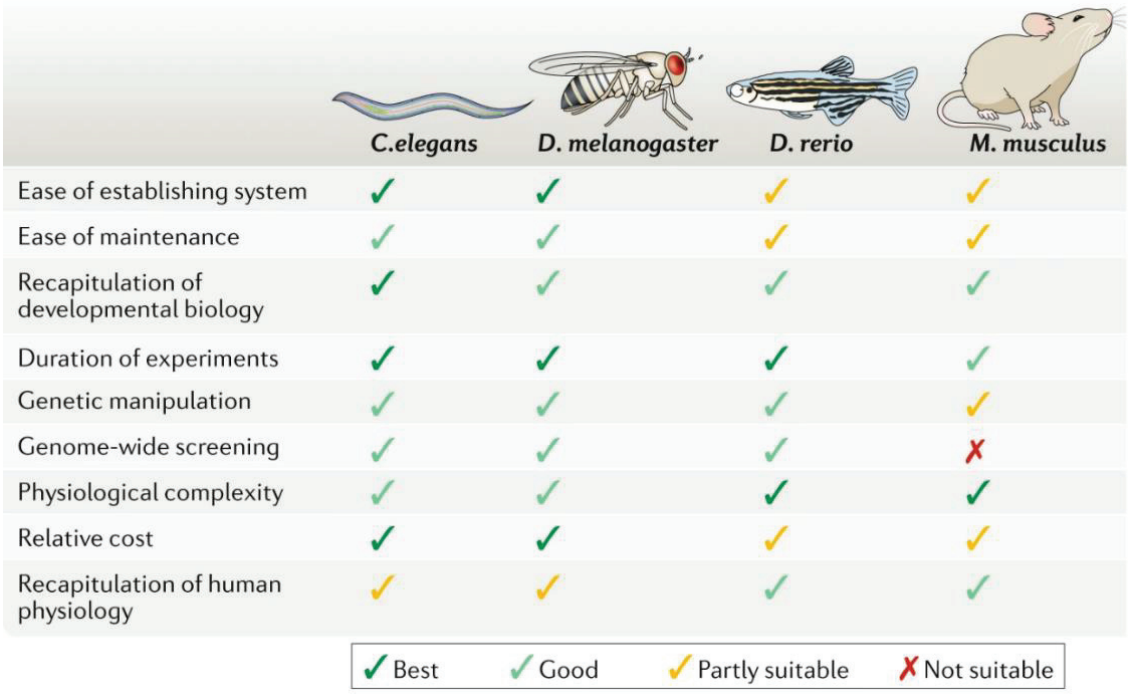

Figure 1. 1. Pros and cons of model organisms in biological research. (Source: adapted from Kim et al. 2020).

Like *D. melanogaster*, *D. rerio* has genetic similarity with humans over the percentage of seventy. Like other model organisms, it has a short life cycle, easy cultivation, and the ability to produce large numbers of offspring. It provides a great advantage for monitoring organ development in vertebrates (Pandey and Nichols 2011). The transparency of embryos during early development provides the opportunity to easily observe biological processes. Because they absorb chemicals directly from water, this makes them suitable models for studying the effects of waterborne chemicals (Zhong et al. 2022). In drug discovery, pharmacology, toxicology, neurodegeneration and cancer studies, zebrafish has been successfully utilized (Bandmann and Burton 2010; Hason and

Bartůněk 2019). However, zebrafish does not cost effective in terms of requirement either infrastructure and maintenance as compared to both flies and worms. Also due to dominant relative abundance of Proteobacteria approximately 80%, it becomes limited for microbiota studies (Zhong et al. 2022). *M. musculus* has the highest genetic similarity to humans (almost 99%), unlike other model organisms. It is one of the most appropriate models for examining physiological responses with its high similarity in tissue and organ structures and cellular functions. Genetically modified transgenic and knock-out mice and genetically uniform inbred strains have been created and available commercially for scientific studies (Han et al., 2017). Up to now, mice have been utilized in many studies, such as cancer, immunology, neuroscience, cardiovascular, aging, drug development and reproductive (Céspedes et al. 2006; Rydell-Törmänen and Johnson 2019; Vanhooren and Libert 2013). However, its limitations include the high cost of cultivation and ethical requirements. In conclusion, although these drawbacks, model organisms continue to be substantial choices for biological studies since they help to elucidation of mechanisms underlying diseases and the development of treatments.

1.3. Utilizing *Drosophila melanogaster* as a Model Organism in Biological Studies

Despite the fact that the relationship between microorganisms and disease have been explained in detail in studies conducted with humans and model mammalian organisms, the complexity of host-microbiota interactions requires a genetically, physiologically, and metabolically simpler model organism system. For this reason, *Drosophila melanogaster* has emerged as an effective model organism for microbial dynamics, gut homeostasis, and interactions among microbiota and intestinal environment.

Drosophila melanogaster (DM), also known as fruit fly, is one of the most important model organisms used in biological studies to examine the dynamics and consequences of host-microbial interactions. Because of their straightforward and affordable cultivation, and quick reproduction, fruit flies not only facilitate experimental procedures but also can provide researchers with the ability to conduct large-scale studies

efficiently (Chandler et al., 2011; Douglas, 2018). The small size of the DM allows spending less laboratory source, so making them a cost-effective model organism for experiments. From an ethical perspective, the use of fruit flies in research significantly reduces the ethical obligations that necessary working with more complex organisms (Sahlgren et al. 2017). Genetic manipulation of DM owing to a well and fully characterized genome, lets researchers to investigation in depth the molecular mechanisms underlying various biological processes. This property not only increases the precision of experiments but also allows targeted investigation of specific genes and their functions. Additionally, the experimental traceability contributes to the reliability and reproducibility of research results. The ease of following genetic changes and observing phenotypic differences across generations facilitates a comprehensive understanding of the factors affecting the characteristics under investigation. Moreover, with the short life cycle (ten days at 25°C) consists of embryo, larva, pupa, and adult stages, it can be modeled of physiological processes in many behaviors such as neuronal development, sleep, learning and memory, courtship, and nutrition (Pandey and Nichols 2011).

D. melanogaster has four pairs of chromosomes called X-Y, 2L-2R, 3L-3R, and 4, consisting of approximately 14,000 protein-coding genes (Wolf and Rockman 2008). Following the completion of *Drosophila* whole genome sequencing in 2000 (Myers et al. 2000), its transcriptome and proteome have been fully characterized, and between fruit fly and humans, 60% genetic identity has been reported. Even though this percentage seems to be low, over 70% of the genes that related with human disease have been reported highly conserved (Ferrero 2021). *D. melanogaster* a precious model organism for understanding fundamental biological processes and their relationship with human biology and disease. Due to the conservation of gene functions mostly, the genetic homology between humans and DM speeds up the application of scientific discoveries to human health. The DM has an analogous system to nearly every organ system in humans. For instance, despite the morphological differences among the DM and human brains, several fundamental neuronal functions, including voltage-gated ion channels, and neurotransmitter receptors, are highly preserved (Fischer et al. 2023). Because of these either genetic or functional gene conservations, and genetically modifications applicability, studies related with cardiovascular diseases, neurodegenerative and metabolic disorders, cancer have been successfully developed (Mirzoyan et al. 2019; Salim et al. 2021). *D. melanogaster* is also advantageous to examine toxicological effects due to its similar anatomical and physiological features with humans. The small size of

D. melanogaster makes it easier to screening toxicological profile of especially nanomaterials that intended the usage in humans (Severino et al. 2023). Reiter and coworkers identified matching between nearly seven hundred human disease-related genes and over the five hundred *D. melanogaster* genes (Reiter et al. 2001). Because of this similarity, it has become simpler to identify the mammalian counterparts of the tumor necrosis factor (TNF) and Toll-like receptor pathways after the discovery of the Toll and Imd signaling pathways, which are act as mediators of bacterial and fungal infections in *Drosophila* (Kim et al., 2012).

1.4. Structure of the *Drosophila melanogaster* gut

Gastrointestinal (GI) track has been known as a crucial source of signals for modulating feeding behaviors, nutrient intake, absorption and processing, host metabolism like energy balance or homeostasis, microbial symbiosis in *Drosophila* life (Capo, Wilson, and Di Cara 2019). The gastrointestinal tract of DM is partitioned into three parts called foregut, midgut, and hindgut as similar to esophagus, small and large intestines in humans respectively (Figure 1.2). The foregut provides physical and chemical processing of the consumed food with the help of enzymes in saliva and transfers them to the crop (like stomach in humans) (Kitani-Morii et al. 2021). Cardia links foregut and midgut, controlling the passage of food into the midgut. The midgut carries out the chemical digestion and absorption of nutrients by the different types of digestive enzymes such as protease, lipase, and carbohydrase within its' environment. The final part of the gut, hindgut, is responsible for the absorption of water, and ions. Malpighian tubules located between the midgut and hindgut, are not a part of the gut by itself. However, they play role in excretion, osmoregulation, detoxification and therefore have a similarity with kidney in humans (Douglas 2018).

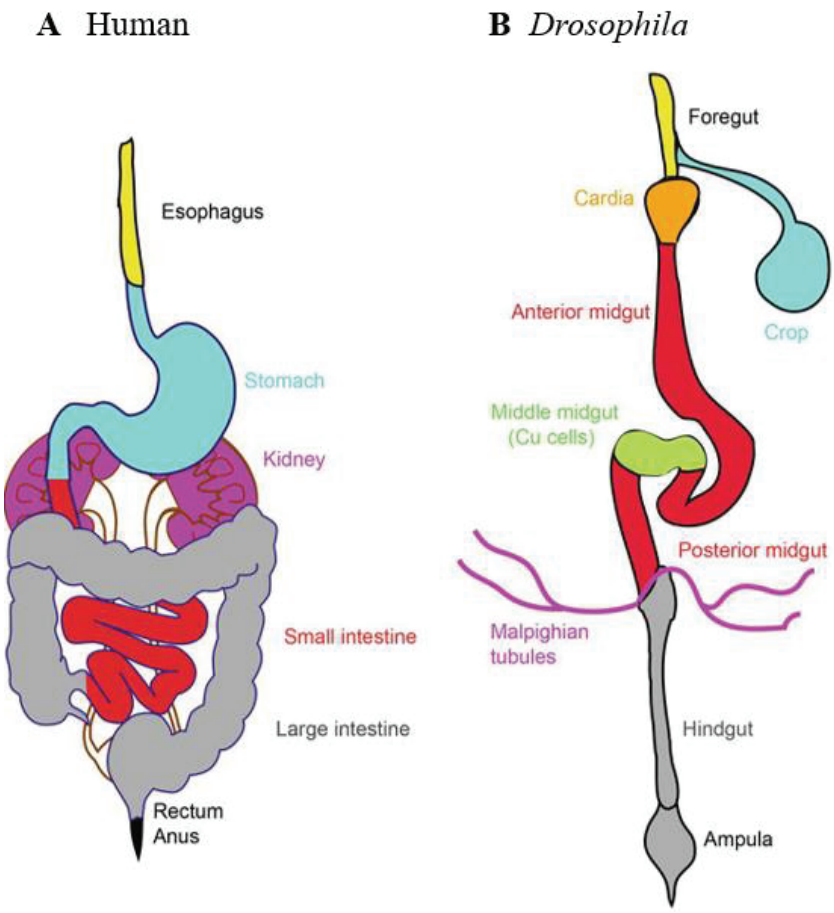

Figure 1. 2. Schematic visualization of human (A) and *Drosophila* (B) gastrointestinal track structure. Similar parts of among human and *Drosophila* is representing by same color. (Source: adapted from Sadaqat et al. 2022).

The intestinal epithelium contains different cell types with diverse functions. Mammalian intestinal epithelium consists of enterocytes, enteroendocrine, Paneth, goblet, and stem cells (Figure 1.3A). Enterocytes are primarily responsible for the absorption of nutrients, and their luminal surface is covered with microvilli, which increases the surface area. The taste organs of the digestive system are known to be nutrient-sensing enteroendocrine cells in the intestinal lumen. These cells generate bioactive peptides in response to signals from nutrients and commensal bacteria. These peptides regulate local and systemic metabolic responses by modulating carbohydrate and lipid metabolisms. Paneth cells release antimicrobial peptides, while goblet cells coat and protect the epithelial surface by producing mucus. The function of stem cells is to regenerate the epithelium, those cells specifically located in the +4 position are triggered

in case of intestinal damage or infection (Wong et al., 2016). The surface of the *Drosophila* intestine is unconvoluted, in contrast to the intestinal epithelium of mammals. The midgut epithelium of *Drosophila* is protected from infections and abrasive food particles by a mucus layer and a peritrophic matrix (PM) composed of chitin and glycoproteins like peritrophins and drosocrystallin (Kuraishi, Hori, and Kurata 2013). Some special cells belong to cardia, constantly produce type II PM that is acting similarly to the mucus secretions of vertebrate (Kuraishi et al. 2011). Additionally, visceral muscle cells and a basal lamina envelop the epithelium (Liu et al., 2017).

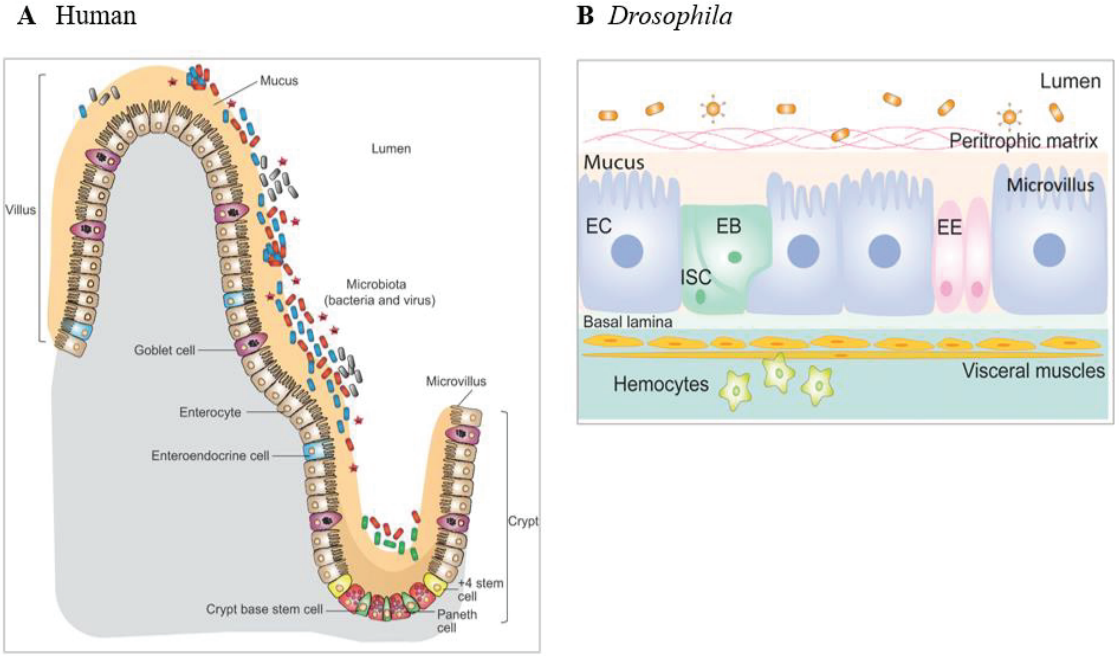

Figure 1. 3. Structural comparison of the *Drosophila melanogaster* intestine with mammalian intestine. (Source: adapted from Liu et al. 2017; Wong et al. 2016).

Four types of epithelial cells are responsible for the formation of the epithelium: intestinal stem cells (ISCs), enteroendocrine cells (EEs), enterocytes (ECs), and enteroblasts (EBs) (Figure 1.3B). ISCs are self-renewing cells and when they perceive injury, stimulate intestinal regeneration (Lian et al. 2018). Hereby, by modifying the size of the epithelium in response to alterations in dietary circumstances or stressors, they help sustain the integrity of the intestinal barrier. ISCs firstly differentiate to enteroblasts, and

those EBs then transformed into secretory enteroendocrine cells and absorptive enterocytes so that continuous renewal of the intestinal epithelium is ensured (Royet 2011). The most prevalent cell type in the midgut epithelium is the enterocytes. They are necessary to digestion and in charge of absorbing nutrients. On the other hand, enteroendocrine cells are essential to secretion of hormones that take role in gut mobility and defense. They help to coordination various physiological processes, including feeding behavior, appetite, and metabolism, by releasing different types of neuropeptides or neurotransmitters in response to specific stimuli. Hemocytes provide the clearance of pathogens or foreign substances that cross the epithelial barrier, by phagocytosis and encapsulation (Garriga et al. 2020).

The structure of the *Drosophila* gut indicates similarity with the mammalian gut. Digestive system is divided into three main regions with an endothelial origin in both: foregut, midgut, and hindgut (Chopra, Kaushik, and Kain 2022). They both have similar intestinal anatomy which consist of a monolayer of epithelial cells called ECs, enteroendocrine cells to hormone production, and ISCs for controlling processes of intestinal regeneration. Also, to increase surface area, epithelial cells contain microvillus in both. Therefore, the midgut epithelium of the fruit fly has been utilized to explore the importance of signaling pathways to the renewal of ISCs as a result of the physiological similarities among the *D. melanogaster* and mammalian gut. EEs coordinate a systemic response to nutrients or metabolites in the gut like mammalian enteroendocrine cells with the help of vesicles filled by small peptides. These vesicles help regulation of lipid and carbohydrate metabolisms in the host. Apart from the jointly shared cell types, a cell type of human intestine, Paneth cells that play role in immune defense by secreting antimicrobial peptides, are not found in *Drosophila* (Sadaqat, Kaushik, and Kain 2022). However, because the fruit fly feeds on rotting food, it has developed an advanced defense system against microbes. The inner wall of the *Drosophila* intestine is surrounded by a peritrophic membrane in addition to the mucosal layer of mammals. This membrane acts as a protective barrier among the intestinal lumen and epithelial cells. Goblet cells, another type of cell of the gastrointestinal system in mammalian, are responsible for mucus production and secretion. Cardia cells in *Drosophila* are similar to goblet cells due to their mucin-secreting properties (Hung et al. 2020). For this reason, it is a perfect model organism to simulate several features of intestinal pathology in humans. On the other hand, compared to mammalian genomes, the *Drosophila* genome contains fewer genes controlling intestinal regeneration. The use of fruit fly offers a true intestinal environment,

which makes it a better option for experiments than cell culture experiments, particularly in drug toxicity investigations (Apidianakis and Rahme 2011).

Significant conservation among the signaling pathways that control intestinal pathophysiology and regeneration have been also seen among the fruit fly and mammalian. For example, Wingless (Wg) signaling pathway is a conserved pathway in *Drosophila* that is homologous to the Wnt pathway in humans. There are seven Wnt genes in *Drosophila*, and among these genes *Wg* that is secreted more frequently in muscle cells connected with basal lamina, and intestinal stem cells, contributes the ISC maintenance (Tian, Benchabane, and Ahmed 2018). The other signaling pathway which is counterpart of nuclear factor-κB (NF-κB) in mammals, includes Toll and Imd pathways. NF-κB is activated when intestinal damage occurs or in response to infection by microorganisms. In fruit flies, one of two NF-κB pathways is activated during infection. While the Toll pathway involves for responses to gram-positive bacteria or fungi via two NF-κB proteins, Dorsal and Dorsal-related immunity factor; the Imd pathway is responsible for the production of antimicrobial peptide (AMP) against gram-negative bacteria via other NF-κB protein, Relish (Chen et al. 2010; Hetru and Hoffmann 2009). Furthermore, human innate immunity is regulated by relatives of the Toll receptor proteins, which are present on the cell surface of *Drosophila*. Seven peptide classes have been identified as antimicrobial peptides in fruit fly. Four of them called Diptericin, Attacin, Drosocin, Cecropin are produced against gram-negative and Defensin is for gram-positive bacteria (Lemaitre and Hoffmann 2007). The remaining two peptides Drosomycin and Metchnikowin, is known as antifungal AMPs (Hultmark 2003). Consequently, immune responses and the *Drosophila* host defense mechanism are regulated through the development of a response to diverse extracellular signals.

1.5. Gut microbiota composition of the *Drosophila melanogaster*

Drosophila coexists with the microbial ecosystem, which has a significant impact on many physiological processes including host development, feeding, and immune responses. On the contrary a random arrangement, the gut microbiota is a complex community of microorganisms that maintain a mutualistic, symbiotic relationship with

host. Apart from the different species, the composition of the microbiota alters based on environmental factors such as age, diet, and stress, even within the same species. Gut microbiota provides digestible byproducts by catabolizing the consumed nutrients. However, during infection, pathogens have ability to outcompete beneficial microbiota members results in either immune response or disrupted commensal development. Toxins secreted by intestinal pathogens can prevent the translational response to foreign microorganism in host defense mechanism (Wong et al., 2016). As an alternative, after their consumption compounds released from commensal microbiota, they can stop the host-microbiota communication. As a result, it might lead to formation of long-term metabolic disorders like obesity and diabetes.

Drosophila melanogaster gut microbiota, which can be represented by about thirty taxa, has a simpler composition compared to mammalian microbiota but has a strong impact on host development and physiology (Pais et al. 2018). The simpler microbiota composition of the *Drosophila* provides the advantage of experimental traceability. Fruit flies in the laboratory feed on their food, which provides a rich substrate for microbial development. Microorganisms consumed by both larval and adult flies grow on nutrients in the vials used for *Drosophila* production in the laboratory. Therefore, frequent transfer of flies to a new vial has reduced the abundance of bacterial members, but composition has reached higher values when flies have been kept on the same vial up to three days (Blum et al. 2013). Upon this, it can be concluded that *Drosophila* generates and sustains its microbiota through regular consumption (Figure 1.4A). These microorganisms can alter the composition of the existing media contributing to host nutrition (Figure 1.4B). Either peritrophic matrix or AMP production, protect the host against pathogens, but they can invade and impair the intestinal cells when immune system is impaired (Figure 1.4 C and D).

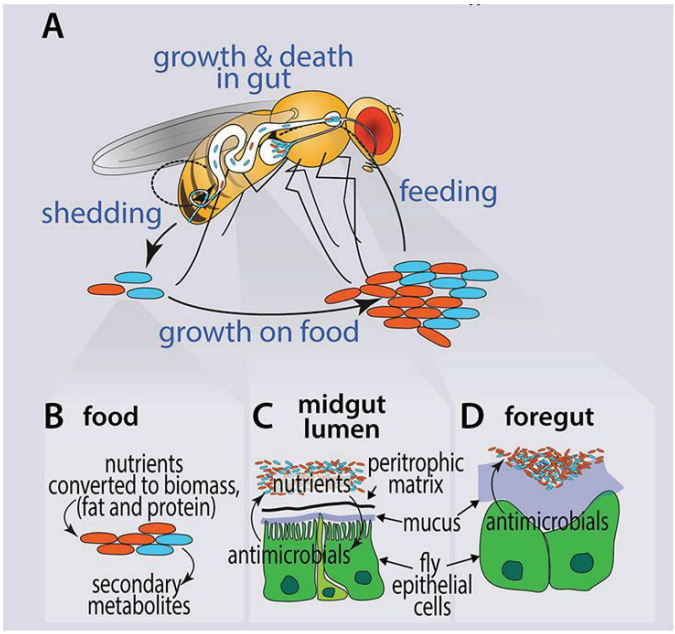

Figure 1. 4. Regulation of the gut microbiota of fruit fly. (Source: Ludington and Ja, 2020).

1.6. Gut microbiota interactions among different taxa

The complex network of interactions between various microbiota members in the intestine has a major impact on the host immune system and health. *Drosophila* interacts with both fungal and bacterial species in its intestine. These interactions include various relationships such as commensalistic, mutualistic, antagonistic among bacteria and fungus species, as well as within the same taxon. Either bacterial or fungal species can contribute immune system regulation, nutrition absorption, and digestive functions. Each member of the microbiota produces metabolites that may have either favorably or adversely impact on the existence other species or may be effective in the host-microbiota relationship. At this point, the relationship between the composition and function of the host microbiota is investigated using gnotobiotic animal models. Gnotobiotic models allow researchers to control the composition of microbiota as required. When compared to other animal models, gnotobiotic DM is more advantageous with its large production volume and cost-effective, easy cultivation (Grenier and Leulier 2020). For this purpose, the microbiota members to be investigated are selectively colonized in the host organism

by experimental methods. Thus, researchers can easily investigate how each species or combinations of species affect the host immune response, metabolism, or other physiological processes.

Dietary decisions have been known have an impact on the composition of the microbiota. For example, complex polysaccharide diets in fruit fly led to an increase in *Lactobacillus* spp. abundance, whereas high-sugar diets promote *Acetobacter* and *Gluconobacter* species in particular (Yun, Hyun, and Seogang 2023). Studies on *D. melanogaster* have been provided important insights into the interspecies relationships within the gut. For example, when combining several species of bacteria, the reduction in glucose and lipid levels has been greater than when *Drosophila* samples were colonized with a single species (Newell and Douglas 2014). In fact, among all the bacterial combinations, samples containing both *Lactobacillus brevis* and *Acetobacter tropicalis* have shown the highest rate of decline. These results suggest that interactions among bacterial species have an impact on metabolism. The beneficial effect of *Lactobacillus* on *Acetobacter* was also demonstrated in this study by the finding that the abundance of both bacteria was greater when colonized together than was separately. This conclusion is supported by research that demonstrated how *Acetobacter pomorum* and *Lactobacillus plantarum* collaborate to alter the eating tendency of flies grown in an isoleucine-free environment (Henriques et al. 2020) (Figure 1.5). *A. pomorum* has used the lactate that *L. plantarum* generated to synthesis essential amino acids in the present environment. The amino acids generated have enhanced egg laying, decreased *Drosophila*'s demand for protein, and supported the growth of *L. plantarum*. In another study, it has indicated that female flies have preferred medium containing acetic acid to medium non-containing for egg laying (Kim et al., 2018). As a consequence, *Drosophila*, with its both resident and transient commensal microbiota, is a powerful model organism for investigating effects of microbiota on host physiology. Commensal microorganisms can also stimulate signaling pathways such as TOR and insulin, resulting in faster fly development (Yamada et al. 2015). When we consider effect of microbiota on *Drosophila* behaviors, the ability of the microbiota to impact brain function in *Drosophila* demonstrates the importance of gut-brain interactions in determining host behavior and physiology. Exposing DM to *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* has been proven to improve olfactory memory capabilities in flies by raising the amount of lactate dehydrogenase in the brain; moreover, this effect has been demonstrated to be synergistic (Ho et al. 2024).

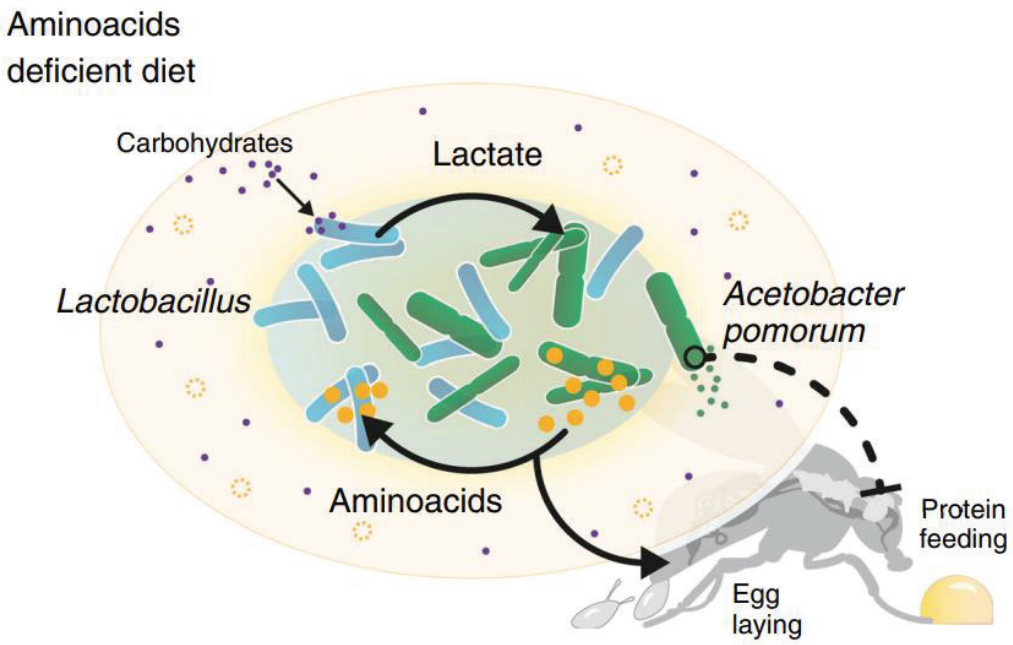

Figure 1. 5. Cooperation between *L. plantarum* and *A. pomorum* in amino acid deficient environment. (Source: Henriques et al. 2020).

Preserving the host health and controlling the immune system depend heavily on our ability to comprehend the interactions between various microbiota members. Imbalances among microbiota members can cause health problems such as intestinal diseases, inflammation, and metabolic disorders. There are several possible reasons of dysbiosis in the gut microbiota of *Drosophila*, including as genetic or nutritional modifications, and stress factors. Inflammation and oxidative stress caused by dysbiosis can disrupt insulin signaling pathways and leading to metabolic disorders such as insulin resistance. However, *Acetobacter* and *Lactobacillus* have been shown potentially to reduce insulin resistance together, in fruit fly (Meng et al. 2024). Since fungal microbiota studies are very limited, the interaction between the fungal and bacterial flora could not been investigated deeply and focused on mostly *Saccharomyces* and *Candida* species (Figure 1.6). These interactions can be mutualistic, competitive, or neutral, depending nutrient availability, environmental factors, or microbial composition. For example, Ponomarova and colleagues have investigated commensal interaction between *Saccharomyces cerevisiae* and lactic acid bacteria. While *S. cerevisiae* is producing metabolites for bacterial growth, *Lactobacillus lactis* provides lactose, carbon

source for fungi (Ponomarova et al. 2017). Also, reduction in bacterial and fungi abundance with antifungal treatment has suggested that fungal species in the intestinal environment could positively affect bacteria (McFrederick, Mueller, and James 2014). On the other hand, utilizing antibiotics has affected the gut mycobiota by decreasing bacterial abundance and increasing relative abundance of *Candida*, also resulted in higher fungal diversity (Ventin-Holmberg et al. 2022). In another study, it has been shown that short-chain fatty acids, such as butyric acid produced by lactic acid bacteria has maintained the gut homeostasis by inhibiting the growth of the *Candida albicans* (Noverr and Huffnagle 2004).

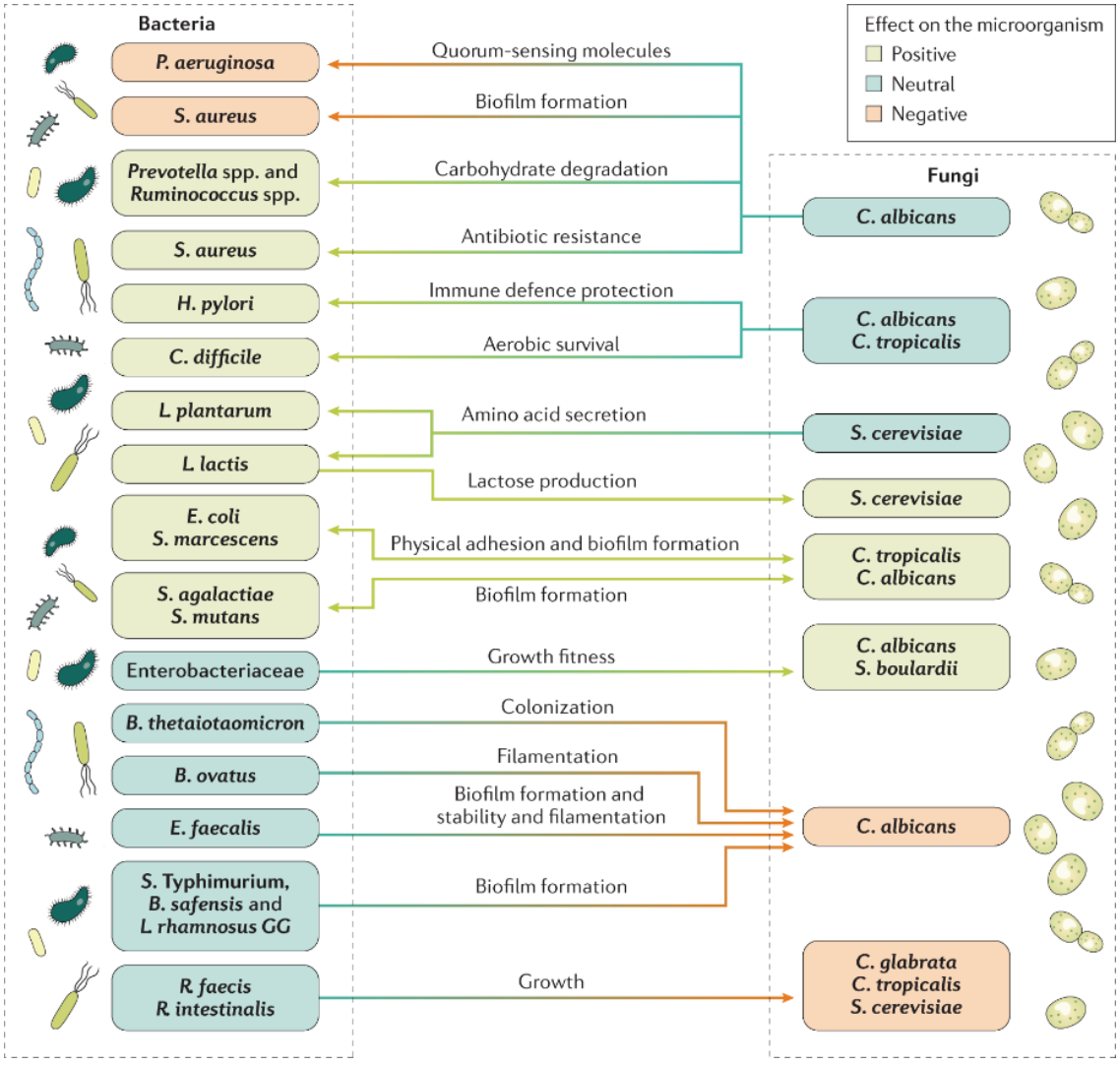

Figure 1. 6. Relationship between bacteria and fungi. (Source: Richard and Sokol 2019).

1.7. *Drosophila* host metabolism

In *D. melanogaster*, host metabolism has significant importance for ensuring various physiological processes such as survival, growth, development, and reproduction. Since it needs to effectively extract, process, and utilize nutrients, the host metabolism is essential to maintaining energy homeostasis. While nutrients are utilized to serve energy requirements, excess resources are stored at the same time to conduct essential functions. Diet of *Drosophila* is more restricted than that of mammals, mostly consists decaying plants, fungus, and fruits and vegetables. Although larvae constantly consume nutrients in order to maintain protein synthesis, triglyceride and glycogen storage, and growth; embryonic and pupal stages are not fed. Through this process, DM adapts to shifting environmental factors and life stages, such as the pupal stage, and is ensured constant energy availability for vital body functions even in times of limited nutritional intake. Because adult flies utilized most of their energy for reproduction, they feed much lower and store less levels of glycogen and triglycerides than larvae. Nutrients containing yeast or yeast extract, together with a source of sugar and protein, are used to cultivate *Drosophila*. Yeast extracts are a major source of proteins, lipids, carbohydrates, and B vitamins (Wong et al., 2016). The ratios of sucrose and yeast, the two main components of the laboratory fruit fly diet, dissimilarly influence nutrient storage, fertility, or lifespan. For instance, limiting the amount of yeast in the diet, however not carbohydrate, has been improved the average survival rate of adult DM infected with *Staphylococcus aureus,* by reducing target of rapamycin (TOR) signaling network (Lee et al., 2017). Additionally, it has been demonstrated that reduction in the quantity of yeast supplement results in delayed growth because it reduces signaling via again the highly conserved TOR pathway, which is also present in mammals.

1.8. Insights of shared pathway similarities in human and fruit fly metabolism

When nutritional requirements are compensated, additional nutrients are stored for later circumstance, like stress or illness. However, distruption in energy balance results in the development of metabolic diseases including obesity and diabetes. Humans and flies have many genes and metabolic pathways that are significant and conserved in metabolic disorders. *Drosophila* has become a frequently preferred model organism for metabolism studies as well as developmental studies due to its high similarity to the human genome. In contrast to mammals, which transport both nutrients and oxygen through the cardiovascular system, *Drosophila* transports nutrients via hemolymph an open circulatory system, while oxygen is transported to the organs via the trachea (Rajan and Perrimon 2013). Fruit fly digests and absorbs nutrients mainly through its digestive system alike mammals. Genes related with the carbohydrate digestion are abundant in the anterior midgut, as opposed to genes related with protein digestion which are more prevalent in the posterior (Miguel-Aliaga, Jasper, and Lemaitre 2018). *Drosophila* insulin-like peptides (DILPs) and adipokinetic hormone (AKH) are insulin and glucagon homologous peptides, respectively, that play a role in the regulation of glucose and lipid metabolism in *Drosophila* (Bharucha 2009). In fruit flies feeding with high fat diet have been showed cardiac problems resembling diabetic cardiomyopathy in humans in addition to disrupted glucose homeostasis and higher triacylglycerol levels (Birse et al. 2010). Additionally, since generating mutations in the insulin pathway, have been resulted in symptoms observed in diabetes, such as fat accumulation or hyperglycemia, *Drosophila* is a suitable model organism for diabetes research (Brogiolo et al. 2001). Also, high-sugar diets have been applied for *Drosophila* type 2 diabetes research. These diets in *Drosophila* have resulted in insulin resistance, hyperglycemia and cardiovascular problems which represent typical symptoms of type 2 diabetes (Na et al. 2013). Applying high-fat diet also causes increased triglyceride storage, insulin resistance and shortened lifespan in the fruit flies. Since balance in triglyceride metabolism is critical for healthy growth and development, metabolic disorders such as lipodystrophy, non-alcoholic fatty liver disease (NAFLD), and obesity are developed by both defective and abnormal triglyceride production (Heier et al. 2021). Genetic

traceability of fruit flies allows for the identification of evolutionarily conserved regulators of triglyceride metabolism by helping of various metabolic phenotyping and genetic screening methods. These characteristics make fruit fly an appropriate model for investigating the developmental mechanisms and activities of metabolic pathways. Thus, investigating the host metabolism of *Drosophila* improves our knowledge of fly physiology and offers important insights into conserved metabolic pathways, including human health and illness.

1.9. Genomic approaches to microbiota investigation

Despite the fact that culture methods are inexpensive in order to identification, they are insufficient to reveal the microbial community within the sample since less than thirty percent of the intestinal microbiota can be cultured (Fraher et al. 2012). DNA-based approaches include analyses that enable the identification of microbial taxa. It reveals microbiota composition by analyzing microbial variation, including bacteria, archaea, fungi, and viruses. It is also useful since it identifies novel microorganisms that might play an important role in host health and illness.

1.9.1. Sequencing based techniques

When intestinal microorganisms analyzed using culture-dependent techniques, results can be limited just cultivable species. With the use of DNA-based techniques since the 1980s, a new era has been started in microbiota research. Sanger sequencing method is beneficial due to its ability to sequence lengthy fragments of DNA in a per reaction. As seen in Figure 1.7A, Sanger sequencing constructs new DNA strands by utilizing dideoxynucleotide triphosphates (ddNTPs). With the addition of ddNTP, which lacks a 3’-OH group, DNA synthesis is terminated, and various-length DNA fragments are produced while DNA polymerase is still continue synthesizing the new DNA strands. Gel electrophoresis is used after the reaction to separate DNA fragments based on their size.

This approach is still regarded the gold standard for the sequencing of reads less than five hundred base pairs (Singh 2021). However, it is less useful since it is relatively slow and requires intensive labor. Also, it may not fully capture the microbial communities within the gut microbiota due to limited depth of coverage.

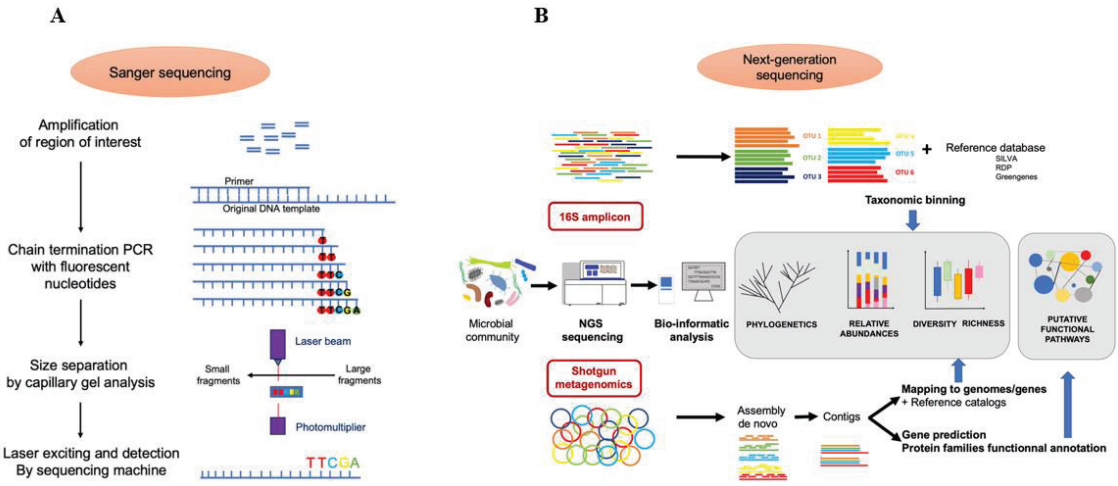

Figure 1. 7. Explanation of Sanger Sequencing and Next-Generation Sequencing methods. (Source: adapted from Szychowiak et al. 2022).

Over the last decade, methods for sequencing have shifted from Sanger sequencing to next-generation sequencing (NGS), and NGS devices developed by Illumina, Pacific Bioscience, Roche, and Thermo Fischer Scientific all have been utilized successfully for examining complex biological samples (Figure 1.7B). As opposed to Sanger sequencing, NGS generates a greater amount of reads in a single run, enabling for the rapid and cost-effective analysis of the sample. It also allows detection of the dominant microbial community within the sample. This provides for a better understanding of the affect or contribution of microbiota to host physiology in both health and illness. The most often utilized NGS approach for taxonomic and phylogenetic assessment is metagenomic sequencing (targeted amplicon sequencing) analysis. This approach identifies the microbiota within a sample by targeting, amplifying, and sequencing a specific genomic region. Samples to be used in microbiota analysis are generally collected with few amounts because of the source limitations. Therefore,

nucleic acid extraction from samples becomes a critical step. The microorganisms in the sample must be acquired as efficiently as possible with the method chosen. Unlike enzymatic digestion, applying homogenization with beads can better degrade the peptidoglycan cell wall, thus increases the amount of microbial DNA obtained (Lourenco and Welch 2022).

16S rRNA gene for both bacteria and archaea, ITS (Internal Transcribed Spacer) region for fungal species are the most targeted genetic regions in NGS studies (Massart, Martinez-Medina, and Jijakli 2015). Due to both ITS region and *16S rRNA* gene comprises highly conserved and variable regions in fungus and bacteria-archaea respectively, they are valuable molecular marker regions for identifying bacterial and fungal diversity. Targeting conserved regions enables for simultaneous amplification of more DNA comes from different microorganism by developing universal primers, whilst variable regions are employed for taxonomic classification. Especially hypervariable regions, provides obtaining higher taxonomic levels like genus, species and improves the phylogenetic resolution. Ribosomal RNA, which consists of 30S small subunit (SSU) and 50S large subunit (LSU), is greatly conserved among bacterial species. The *16S rRNA* gene, found in the 30S subunit (Figure 1.8A), is preferred to *5S* and *23S* genes, for phylogenetic identification due to its conservation among species, variable region content, and enough length to polymerase chain reaction (PCR) amplification (Fraher et al. 2012). Although it is achievable to sequence the full length of *16S rRNA* gene, which is around 1500 bp with a total of nine hypervariable regions (V1-V9), a few hypervariable sections are chosen to reduce cost and ease bioinformatic analysis (Abellan-Schneyder et al. 2021). Among the region combinations, targeting the V3-V4 region of the *16S rRNA* gene has been shown to promote diversity and provide the most (Operational Taxonomy Units) OTUs (Thijs et al. 2017). The ITS region is characterized by highest possibility of identification among fungal taxa, making it a valuable marker for fungal classification. The eukaryotic rRNA is divided by two subunits; SSU (40S) consists of the *18S*, while LSU (60S) comprises *5S*, *5.8S* and *25S/28S rRNA* genes (Schoch et al. 2012). As seen in Figure 1.8B, the ITS region is divided into two subregions: ITS1, found between the small subunit rRNA and the *5.8S rRNA* genes, and ITS2, located between the *5.8S rRNA* and the large subunit rRNA genes (Fathy et al. 2023). The ITS region is more effective for phylogenetic analysis and species-level identification because it contains more hypervariable regions compared to other gene sections, has a higher evolutionary rate, and can easily amplified and sequenced with a length of around 700 bp (Baldwin et al.

1995; Irinyi et al. 2016; Kauserud 2023). Following metagenomic sequencing targeting either *16S rRNA* or ITS regions, raw data from metagenomic sequencing is processed by differ software such as QIIME (Quantitative Insights Into Microbial Ecology) (Estaki et al. 2020), Mothur (Schloss et al. 2009), DADA2 (Callahan et al. 2016) to filtering, quality control, or remove errors. After that, effective tags are classified as OTUs, which represent species of closely related microorganisms. These OTUs are assigned at their taxonomic level utilizing reference databases like SILVA (Quast et al. 2013), Greengenes (Desantis et al. 2006), Ribosomal Database Project (RDP) (Cole et al. 2005), and User-friendly Nordic ITS Ectomycorrhiza Database (UNITE) (Nilsson et al. 2019). Then, applying alpha and beta diversity analyses, a summary of species diversity within and between groups are obtained. Another NGS approach Shotgun metagenomic sequencing can reveal both known and new microbial species, as well as identify functional genes and pathways in the microbiota. However, the huge amount of data collected demands extensive bioinformatics efforts for analysis (Boers, Jansen, and Hays 2019).

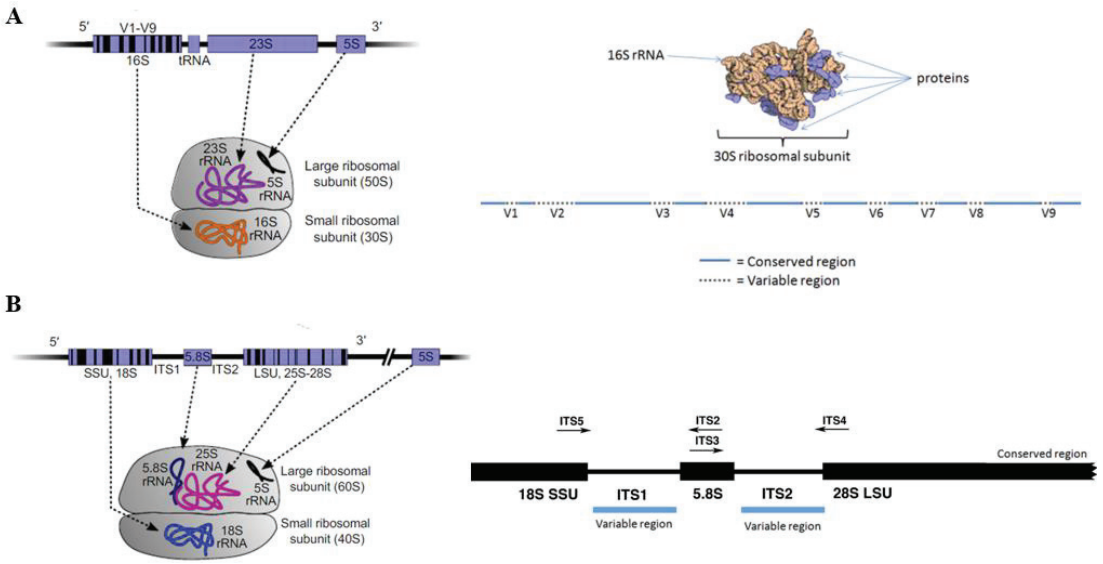

Figure 1. 8. Structure of the prokaryotic (A) and eukaryotic (B) ribosomes and illustration of the marker regions (*16S rRNA* and ITS). (Source: adapted from Gibbens et al. 2015; Lavrinienko et al. 2021; Raja et al. 2017).

1.9.2. PCR-based techniques

PCR enables the qualitative identification of targeted microbial taxa and indicating the presence or absence of selected microbial species. It is relatively straightforward and inexpensive, due to require basic laboratory equipment and reagents. Real-time PCR (qPCR, Quantitative Polymerase Chain Reaction), which has better sensitivity and specificity than PCR, can identify and quantify low amounts of microbial taxa. It represents the relative or absolute abundance of microbial species in the gut microbiota. The relative quantification approach compares gene expression profile between samples or groups, whereas the absolute quantification method uses a standard curve constructed with known positive control concentrations to predict samples quantification. TaqMan and hybridization probes or intercalating dyes like SYBR-Green added into the qPCR reaction mixture, provide a fluorescent signal proportional to the quantity of target DNA (Harshitha and Arunraj 2021). Amplification curves plotted by fluorescence emission (ΔRn) against cycle number, indicate the reaction is not inhibited by following the "S" pattern (Figure 1.9A). Probe-based qPCR improves the specificity and sensitivity of microorganism detection by utilizing sequence-specific probes that hybridize to the target DNA sequence but designing the probes are laborious and costly. Although SYBR-Green is less costly than probes, it produces fluorescence by binding to both specific and non-specific double-stranded DNA molecules. However, to ensure that interested DNA is amplified, melting curve analysis is utilized, which provides information about the amplicon specificity. Melting curve analysis, a thermodynamic analysis, involves gradually increasing the temperature across a specific range around 50 to 95°C (Isaac 2009). This process separates the two DNA strands to form single-stranded DNA (ssDNA) molecules. Melting temperature (Tm) refers half of the double-stranded DNA molecules are denatured into ssDNA. The melting curve plots are drawn as the negative derivative of fluorescence (dF/dT) against temperature. The position and shape of the Tm peak indicate the stability of the targeted DNA, as well as the existence of secondary structures (Figure 1.9B). Nonspecific amplification products or primer dimers can be identified by the formation of extra peaks or shoulders in the melting curve.

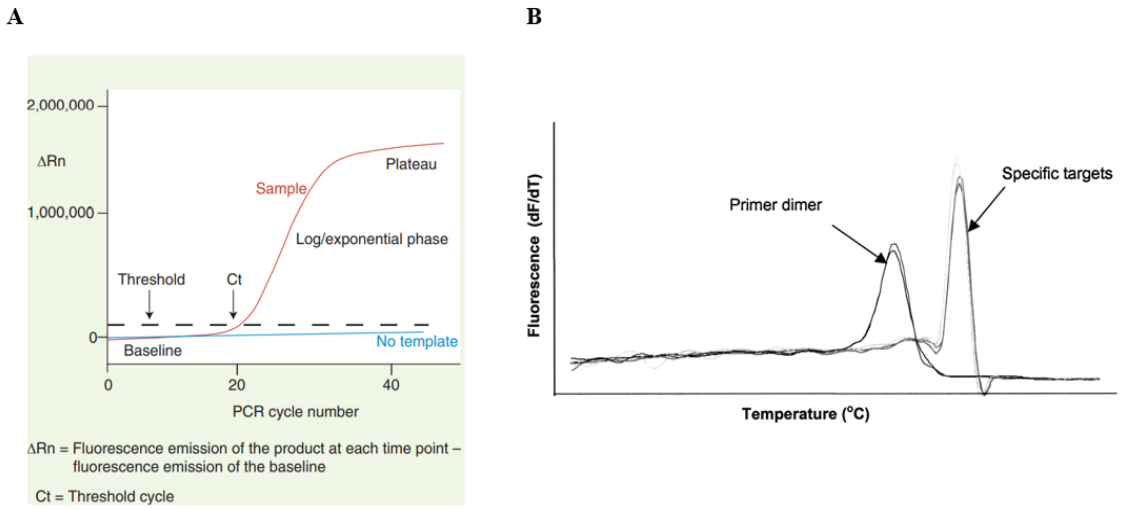

Figure 1. 9. Schematic representation of amplification curve (A) and melting curve (B) plots. (Source: adapted from Arya et al. 2005; Isaac 2009).

1.10. Hypothesis and Aims of the Thesis

Main hypothesis of this thesis is that both bacterial and fungal microbiota can influence host metabolism, and host genetics can modify the interactions between the host microbiota and host metabolism. To test this hypothesis, five main aims were pursued. The first aim was to identify the fungal and bacterial microbiota composition in DGRP lines and identify dominant fungal and bacterial taxa. To address this aim 16S rRNA and ITS amplicon sequencing on ten randomly chosen DGRP lines were performed. Using amplicon sequencing results of the ten randomly chosen DGRP lines, dominant bacterial and fungal taxa were determined. Then, by focusing on targeted species and phyla, their relative abundance in all samples was determined by qPCR.

The second aim was to measure the metabolic pools of DGRP lines. The second aim was to measure the metabolic pools of DGRP lines. Therefore, after revealing the gut microbiota composition of the DGRP lines, measurements of protein, glucose, trehalose, glycogen, and triglyceride were determined in all samples. Protein measurements were utilized for standardization of other metabolic pool measurements. The third aim was to determine interactions among the gut microbiota and metabolic pools. So, in the next step, principal component analysis (PCA) and correlation analysis (linear regression) were

utilized. The statistical significance of relationships either negative or positive, revealed by PCA, were determined through correlation analyses. The symbiotic interaction of microorganisms from different taxa is very important for host health and survival. It is still an active research area because what kind of symbiotic relationship arises between different taxa have in the digestive tract has not been understood. Investigating the interactions between microorganisms will help to understand the underlying causes of dysbiosis and also their role in the etiopathogenesis of chronic diseases.

Considering that environmental and genetic factors can affect the microbiota, it is expected that host genetic factors also affect the interactions of the microbiota. Host genetic factors can influence microbiota diversity by regulating the interactions of microorganisms with each other. The fourth aim of the study was to uncover host genetic factors associated with microbial taxa. With the help of Genome-Wide Association Studies (GWAS), Gene Ontology (GO) enrichment and STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analyses, host genetic factors associated with intestinal taxa were determined. Particularly, we focused on immune system and metabolism-related genes because of their relevance to gut microbiota.

At the end of the thesis, as our last aim, the efficacy of loop-mediated isothermal amplification method and qPCR in terms of identifying two bacterial and one fungal species in DGRP gut samples were assessed. Hereby, besides the bacteria it was observed that even fungal species could be effectively detected with LAMP technique in low sample volumes (DGRP gut sample). Most importantly, this study is the first study to examine the fungal microbiota in the *Drosophila melanogaster* model metabolically and genomically, aim to fill the lack of knowledge in this area and contribute to the human health literature. Additionally, regarding to applying LAMP technique in detection of gut microbiota in *Drosophila*, it was again the first study.

CHAPTER 2

SCREENING THE RELATIVE ABUNDANCE OF SELECTED BACTERIA AND FUNGUS IN *DROSOPHILA MELANOGASTER*

2.1. Introduction

2.1.1. Bacterial taxa within the *Drosophila* gut environment

Bacteria are effective in regulating many different biological pathways, including development, behavior, lifespan, and disease resistance. Even though there are significant differences at the species level, Proteobacteria and Firmicutes are the prevalent phyla (Figure 2.1); *Lactobacillus* and *Acetobacter* are the most common genus (Arias-Rojas and Iatsenko 2022; Buchon, Broderick, and Lemaitre 2013). Furthermore, *Lactobacillus* spp. which is gram-positive anaerobe and *Acetobacter* spp. that are gram-negative aerobe represent ninety-four percentage of the microbiota composition of *Drosophila* grown in the laboratory (Blum et al. 2013). The most common species observed in studies are *Lactobacillus plantarum*, *Lactobacillus brevis*, *Acetobacter pomorum*, and *Enterococcus faecalis* bacteria (Arias-Rojas and Iatsenko 2022).

Lactobacillus spp. are gram-positive bacteria of the Firmicutes phylum that are responsible for lactic acid synthesis. The most prevalent bacteria found in fruit flies are *L. plantarum*, *L. brevis*, and *Lactobacillus fructivorans*. *Lactobacillus* strains are also utilized as human probiotics (Abdelazez et al., 2018). *L. plantarum* and *L. brevis* constitute approximately 8% and 10% of the total bacterial microbiota in *D. melanogaster*, respectively, however only *L. plantarum* has been observed among all wild fruit fly samples (Chandler et al., 2011). *Lactobacillus plantarum* is a gram-positive lactic

acid bacterium with several health benefits and present in GI track in humans (De Vries et al., 2006). Besides the probiotic characteristics, they are successful to reduction effects of autoimmune diseases like Inflammatory Bowel Disease (IBD) (Le and Yang 2018). This bacterium reduces cholesterol and regulates the immune system. *L. plantarum* produces exopolysaccharides (EPS) which are the extracellular polymers, and extremely important in therapeutic applications such as antibacterial, antifungal, anti-inflammatory, and anticancer (Angelin and Kavitha 2020). Many microorganisms form EPS, which is more useful in commercial and medicinal applications than other metabolic byproducts. EPS that is generated by probiotic bacteria like *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, have a lot of interest, particularly for their medical usage (Netrusov et al. 2023). EPS also has an antagonistic impact on pathogenic microorganisms in the intestine. In fruit fly, *L. plantarum* and *Lactobacillus rhamnosus* GG that are commonly utilized as human probiotics, have been demonstrated to protect the fly against pathogen microorganisms such as *Pseudomonas aeruginosa* (Blum et al. 2013). Commensal microorganisms support larval development through a number of processes in *Drosophila*. For example, *L. plantarum* stimulates amino acid absorption by increasing peptidase activity in the fly gut (Matos et al. 2017). *L. plantarum* has also enhanced the production of hormones which accelerated larval development and growth by activating the TOR signaling pathway in flies grown in restricted diet (Storelli et al. 2011). *L. brevis* has been indicated to increase glucose content in male flies (McMullen et al. 2020).

Another prevalent bacterial group in the gut microbiota of *D. melanogaster* is acetic acid bacteria, more especially members of the *Acetobacter* genus. These gram-negative microorganisms convert ethanol that is a byproduct of fermentation, to acetic acid. This metabolic activity not only performs the oxidation of ethanol, but also contributes to the production of acetic acid, which flies can use as a nutrition supply in situations where they have no other food source (Devineni et al., 2019). Acetate is a short-chain fatty acid (SCFA) composed of two carbon atoms. SCFAs produced by microbiota in *Drosophila* crucial for regulating lipid and carbohydrate metabolisms (Neophytou and Pitsouli 2022). The gut microbiota of DM is mainly composed of *A. pomorum*, *Acetobacter pasteurianus*, *Acetobacter tropicalis*, and *Acetobacter aceti* in terms of *Acetobacter* species. (Yun, Hyun, and Seogang 2023). Following its absorption by the intestinal enteroendocrine cells, acetic acid is converted into acetyl-CoA, and then is processed by the tricarboxylic acid cycle (TCA, known as the Krebs Cycle). Continuity of the metabolism of carbohydrates is accomplished by thiamine (vitamin B). Thiamine

is a cofactor of pyruvate dehydrogenase, an enzyme that converts pyruvate to acetyl-CoA (Rapala-Kozik 2011). Thiamine promotes growth and survival in *D. melanogaster*. *A. pomorum* is a symbiotic microorganism that provides thiamine to fruit fly. Moreover, by generating thiamine in a thiamine-deficient feeding medium, it has facilitated the growth of axenic flies (Sannino et al. 2018). On the other hand, independently from thiamine and acetic acid production, *A. pomorum* by itself supports larval development in flies that are germ-free and have mutated Imd-related genes. Furthermore, this impact was species specific since it was not seen in flies with Toll pathway genes mutants or fed with *L. plantarum* (Lee et al., 2023). Among the bacterial species isolated from wild-caught flies, *Acetobacter thailandicus* is mutually benefit the DM by promoting rapidly host growth and increased fertility (Pais et al. 2018). *Acetobacter fabarum* has decreased development time of the DM compared to axenic flies.

The bacterial phyla Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria constituent of the largest proportion of the gut microbiota in humans (Sasaki and Klapproth 2012) (Figure 2.1). The most common probiotic bacteria used in humans are *Lactobacillus* (from the Firmicutes phylum) and *Bifidobacterium* (from the Actinobacteria phylum) spp. While *Lactobacillus* spp. produces lactic acid following fermentation, *Bifidobacteria* spp. are significant producers of short-chain fatty acids (Vlasova et al. 2016). Simple microbiota composition of *Drosophila* allows to generation a high number of mono-associated or germ-free offspring. It is straightforward to cultivate or remove the *Acetobacter* and *Lactobacillus* species that are predominant in the gut environment of DM and resulting in axenic or germ-free animal formation. This advantage provides a high sample volume to investigate the action mechanisms of probiotic microorganisms. Furthermore, the effects of probiotic species can be modulated by including prebiotic compounds into fly nutrition at varying rates (Trinder et al. 2017). The probiotic *Lactobacillus reuteri*, which is present in human breast milk, has increased the lifespan of *Drosophila* by producing the secondary metabolites such as reuterin and inhibiting the insulin/IGF-1 signaling (IIS) pathway (Lee et al., 2023).

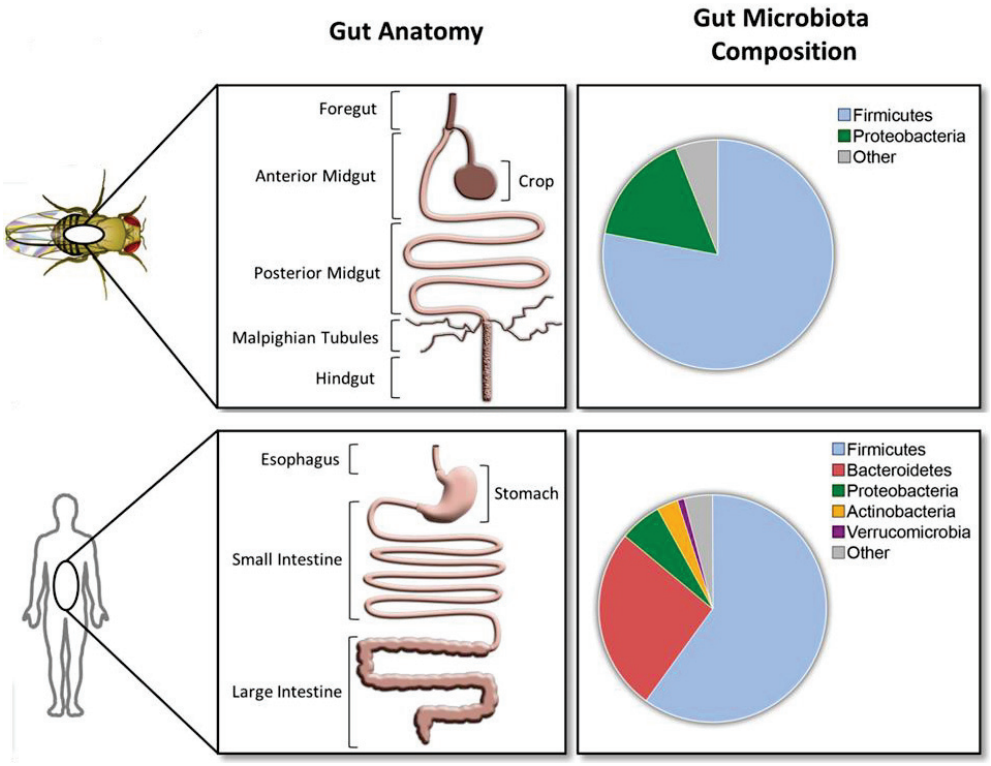

Figure 2. 1. Structure of GI track and taxonomical distribution of gut microbiota in *Drosophila melanogaster* (top) and human (bottom). (Source: Trinder et al. 2017).

Dysbiosis in *Drosophila* can be displayed by a variety of reasons, including nutritional changes, stress, and pathogen invasion. In this case, changes may occur that have detrimental effects on host health and homeostasis, resulting in a reduction in the relative abundance and diversity of microbes. Returning to homeostasis is achieved by removing harmful bacteria from the gut and renewing the epithelium in order to repair the damage caused by dysbiosis. In fruit fly, the innate immune system is critical for protection against microbial infections. Pattern recognition receptors (PRRs) are essential components of the innate immune system that identify pathogen-associated molecular patterns (PAMPs), such as bacterial peptidoglycan. *D. melanogaster* encodes seventeen peptidoglycan recognition proteins (PGRPs), which are responsible for recognizing PAMPs (Kaneko et al. 2006). *Drosophila* responds to microbial infections via two distinct pathways called the Toll and Imd pathways. A kind of intracellular receptor PGRP-LE and transmembrane receptor PGRP-LC recognize diaminopimelic acid-containing (DAP-type) peptidoglycans from gram-negative bacteria, activating the Imd pathway. PGRP-

SA and PGRP-SD detect the gram-positive bacterial lysine-containing peptidoglycans, which activate the Toll pathway. After receptor-ligand interaction, the host generates immune responses such as AMP synthesis or reactive oxygen species (ROS). PGRP-LB, a kind of amidase, acts as a negative feedback regulator of the Imd pathway by eliminating DAP-type peptidoglycans in extracellular environment (Yanagawa et al. 2017). Aside from pathogens, Imd pathway can be activated by commensal bacteria *A. pomorum* and *L. plantarum* (Yun, Hyun, and Seogang 2023). Lactate generated by the commensal bacteria *L. plantarum* is transported into intestinal cells and metabolized by lactate dehydrogenase (LDH) to yield nicotinamide adenine dinucleotide (NADH). The generated NADH is utilized to make ROS, that encourages the proliferation of ISCs and differentiation of EB, EC cells in order to maintain tissue homeostasis (Arias-Rojas and Iatsenko 2022).

2.1.2. Fungal taxa (mycobiota) within the *Drosophila* gut environment

As a result of its diet of decaying fruits, DM lives in an environment rich in fungi from the larval stage. Yeasts interact with the host's immune system and affect *D. melanogaster* behavior in addition to providing an important food supply. One of the major shortcomings in understanding microbiota-host interactions is that almost all microbiota studies are bacteria focused. On the other hand, eukaryotic microorganisms (e.g. fungi) are expected to have greater effects with their larger genomes, genetic richness, and metabolic pathways closer to the cell physiology of the multicellular eukaryotic organisms (Mohanta and Bae 2015). As in humans, eukaryotes in *Drosophila* continue to play a significant role in the microbiome despite getting more attention than bacteria in the research. Fungal microbiota studies are very limited in *D. melanogaster* model. Using next-generation sequencing in eukaryotic microbiota research, it has been observed a temporally stable and individually specific fungal microbiota lower than bacteria amount in the human and mouse intestine. Yeasts present in the *Drosophila* gut may have different metabolic activities compared to the bacterial microbiota or there may be some overlap.

Essential substances such as fatty acids, amino acids, sterols, and B vitamins, can be obtained with the help of yeast for efficient development in fruit fly (Broderick and Lemaitre 2012). The chance of larvae grown in a yeast-free environment is extremely low forming pupae and reaching the adult stage (Anagnostou, Dorsch, and Rohlfs 2010). Ascomycota and Basidiomycota are the two phyla that constitute major mycobiota abundance of the GI tract, respectively. More than fifty-six yeast species have been obtained in a study to investigate gut mycobiota in different *Drosophila* species (Lachance, Gilbert, and Starmer 1995). At the species level, the dominant yeast taxa have been reported as *Hanseniaspora*, *Pichia* and *Candida* species belong to Ascomycota phylum (Douglas 2018). *Hanseniaspora uvarum* was shown to be the most prevalent fungus species among different *Drosophila* species including *D. melanogaster*, *D. simulans*, *D. hydei, D. suzukii* (Huang and Gut, 2021; Lam and Howell, 2015). *H. uvarum* has enhanced triacylglycerol levels while lowering glucose content in male flies (McMullen et al. 2020). When the effects of yeasts on the development of *D. melanogaster* have been investigated, although *Saccharomyces cerevisiae*, *Pichia toletana*, and *Kluyveromyces lactis* have positive impacts, *Metschnikowia pulcherrima* has a weaker effect than the other three yeasts (Anagnostou, Dorsch, and Rohlfs 2010). In addition to its beneficial aspects, such as reducing DM development time and increasing survival rate, *P. toletana* has also enhanced host immunity against bacterial infections (Meshrif, Rohlfs, and Roeder 2016). Alike *P. toletana*, survival rates and body sizes of flies have increased in diets containing *S. cerevisiae* while developmental periods have been shortened (Lewis and Hamby 2019). *K. lactis*, which is one of the important yeast species for industrial biotechnology and used in the dairy industry, has ability to process lactose (Spohner et al. 2016). Fruit flies have a highly developed sense of smell and are attracted to the volatile compounds released by fruits. Volatile aroma-active esters can be synthesized by *K. lactis* and *S. cerevisiae*, therefore smell of these compounds may contribute to the selection of *K. lactis* by flies (Van Laere et al. 2008). Both *K. lactis* and *S. cerevisiae* have Generally Regarded as Safe (GRAS) status which determines whether it is safety for biotechnological administrations (Karim et al., 2020). One of the other commensal yeasts, *Issatchenkia orientalis*, has been indicated to extend the lifespan of fruit flies fed with reduced yeast diet (Yamada, et al., 2015). In the presence of ROS, which is a key component of the immune response in *Drosophila melanogaster*, *Hanseniaspora occidentalis* can survive in the intestine, while the growth of *S. cerevisiae* can be inhibited (Hoang, Kopp, and Chandler 2015). In yeast deficient diet, flies body

mass and metabolic rate have reduced, glucose and glycogen levels, and larval development time have increased. Furthermore, antibiotic administration has resulted in a major reduced abundance of bacteria than yeast in flies (Henry, Overgaard, and Colinet 2020). These findings indicate that yeasts, in addition to serving as a basic food supply, play an important role in host physiology. Medium content is also a factor affecting yeast abundance. For instance, flies have been fed a diet with a high salt concentration (up to 4%), their yeast abundance have increased compared to the controls (Dmitrieva et al. 2021).

According to research conducted on humans, over 83% of the fungus found in the gut fungal microbiota are yeasts. *Fusarium*, *Malassezia*, *Penicillium*, *Aspergillus*, and *Candida* are the most common genus within the human gut mycobiota (Suhr, Banjara, and Hallen-Adams 2015). Even if *Candida* species represent the majority, the most prevalent fungi also include *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Cladosporium cladosporioides*, and *Malassezia restricta* (Suhr and Hallen-Adams 2015). Despite *C. albicans* is a commensal fungus, in case of dysbiosis it increases mucosal permeability and invade the body. Therefore, an excessive increase in its abundance is linked to the pathology of inflammatory bowel disease (Jawhara 2022). Proteases generated by *Candida albicans* in flies have activated the Toll signaling pathway and promoted the formation of AMP (Glittenberg et al. 2011).

Pathogenic fungi trigger the immune system via the Toll signaling pathway by activating Dorsal and Dorsal-related immunity factor proteins, and lead to AMP synthesis. Mutations in the Toll signaling pathway significantly impair survival of flies following fungal infection (Lemaitre et al. 1996). Gram-negative binding protein 3 (GNBP-3) is critical for recognizing fungal infections and activating immune responses (Mpamhanga and Kounatidis 2024). This protein is a kind of PRR that recognizes β-1,3-glucan, a fungal cell wall component. The activation of GNBP-3 promotes the formation of AMPs such as drosomycin and metchnikowin. GNBP-3 mutant flies have been found to be vulnerable to *Candida albicans* and *Aspergillus* infections (Hamilos, Samonis, and Kontoyiannis 2012). GNBP-3 also initiates melanization, a defensive response that encapsulates and immobilizes pathogens by depositing melanin at the infection site, restricting their spreading inside the host (Matskevich, Quintin, and Ferrandon 2010; Tang 2009). In pharmacological studies, *Drosophila* has been used successfully. Utilizing Toll-mutant flies infected with *Aspergillus fumigatus*, the efficacy of voriconazole antifungal drug has been indicated (Lionakis et al. 2005).

2.2. Materials and Methods

In this chapter, to evaluate the intestinal microbiota composition within DGRP lines in terms of bacteria and fungi, different molecular approaches were utilized. All primers used in microbiota studies were purchased from Macrogen (Korea).

2.2.1. *Drosophila* Samples

In this study, 120 strains from the Drosophila Genetic Reference Panel (DGRP) were utilized, purchased from the Bloomington Drosophila Stock Center in Indiana, USA (Mackay et al. 2012). The stocks were grown in tubes with a standard medium composed of agar, cornmeal, sugar, and non-living yeast, optimized for the development and maintenance of *Drosophila melanogaster*. The stocks were kept in a climate-controlled room at 25°C with 65% relative humidity and a twelve-hour light/dark cycle.

2.2.2. DNA isolation from DGRP samples

For the microbiota analyses, 5 male flies 5-7 days old (adult) from each DGRP line were used. Each fly sample was sterilized by washing with 10% sodium hypochlorite, then rinsed with distilled sterile water three times. The abdomen was then separated from the rest of the body using a sterile scalpel and tweezers in sterile Ringer's solution, under a light microscope (Figure 2.2). DNA isolation was performed from 120 DGRP male gut samples using the High Pure PCR Template Preparation Kit (Roche Applied Science, Germany) according to the manufacturer protocol and following optimized steps.

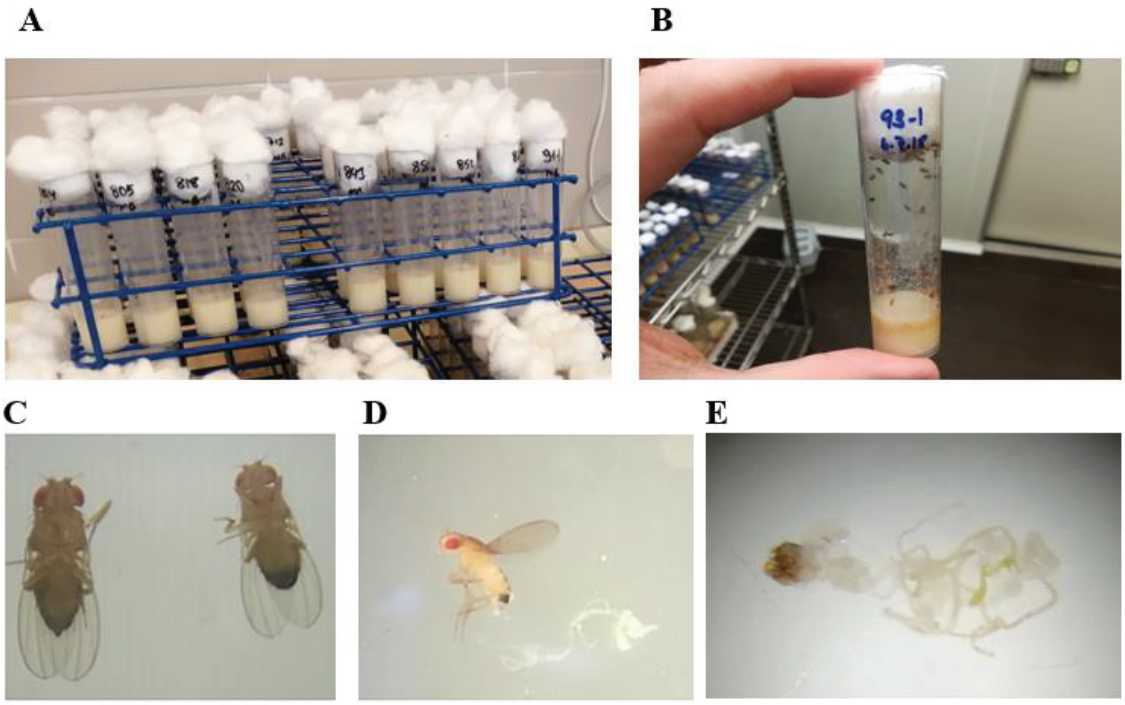

Figure 2. 2. DGRP stocks within the tubes (A, B), fruit fly samples female (left) and male (right) (C), and gut sample extraction from male fly sample (D, E).

First of all, five intestinal samples were placed in eppendorf tubes, 200 µl of Tissue Lysis Buffer and 40 µl of Proteinase K were added and mixed thoroughly. Tubes were incubated at 55°C for an hour. Then ten µl of lyticase (for fungal DNA) or five µl of lysozyme (for bacterial DNA) was added to each tube and incubated to obtain fungal and bacterial DNA at 37°C for 30 minutes and 15 minutes respectively. Afterward, 200 µl of Binding Buffer was added and mixed well. Incubation at 70°C for ten minutes was applied. 100 µl of isopropanol was added to each tube after incubation and the DNA was precipitated by mixing with a pipette. These prepared mixtures were transferred to filter tubes and centrifuged at 8000 x g for 1 minute. By addition 500 µl of Inhibitor Removal Buffer into the filter tubes, 8000 x g for 1 minute centrifugation was applied. Then, each filter tube was placed into new collection tube, and 500 µl Wash Buffer was added and centrifuged at 8000 x g for 1 minute. After repeating this step two times, tubes were centrifuged for 10 second at full speed. In final step, DNA samples were eluted with utilizing 100 µl of Elution Buffer that warmed up to 70°C previously.

Isolated DNA samples were analyzed in terms of both concentration (ng/µl) and purity (A260/A280 and A260/A230) using the Nanodrop 8000c Spectrophotometer (Thermo Fisher Scientific, USA). A260/A280 ratios were measured between 1.80 and 2.05. DNA samples were stored at -20°C for further studies.

2.2.3. Sample preparation for *16S rRNA* and ITS metagenomic sequencing

Before next-generation sequencing, *16S rRNA* and ITS specific primers were designed in order to determine whether bacterial or fungal DNA was present in the isolated DNA samples. For fungal microbiota determination in the gut environment, the ITS region was selected whereas for bacterial microbiota determination *16S rRNA* region was chosen. Primers were designed using NCBI (https://www.ncbi.nlm.nih.gov/), BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) databases and IDT SciTools (https://www.idtdna.com/pages/tools), Primer 3 (http://primer3.ut.ee/) programs.

PCR reactions were performed by utilizing FastStart High Fidelity PCR System, dNTPack kit (Roche Applied Science, Germany) with randomly selected samples. Primer pairs for ITS region, ITS1-Forward:TCCGTAGGTGAACCTGCGG and ITS4-Reverse:TCCTCCGCTTATTGATATGC; for *16S rRNA* gene, 331-Forward:TCCTACGGGAGGCAGCAGT and 797-Reverse:GGACTACCAGGGTATCTAATCCTGTT were utilized. Primers were diluted by adding the necessary amount of water for 100 µM (i.e., 100 pmol/µl) as directed in the user manuals to obtain main stock. By taking 10 µl from these stock solutions, interim stocks for both forward and reverse primers, were prepared as 10 µM (10 pmol). For a single reaction, 17.25 µl of PCR-grade water, 2.5 µl reaction buffer, 0.5 µl nucleotide mix, 0.5 µl DMSO (dimethyl sulfoxide), 0.5 µl of ITS or *16S rRNA* specific forward and reverse primers, and 0.25 µl of FastStart High Fidelity Enzyme Blend were mixed into sterile 0.2 ml PCR tubes and total volume was set to 22 µl without the DNA sample. Three µl of sample DNA was loaded in each tube and the final volume was completed to 25 µl. Then, the prepared tubes were run with the SimpliAmp Thermal Cycler machine (Applied Biosystems (ABI), USA). The PCR amplification was as follows: initial

denaturation of 10 minutes at 94°C, 35 cycles of 2 minutes at 94°C, 30 seconds at 57°C, 1 min at 72°C, final elongation of 7 minutes at 72°C, and cooling at 4°C. At the end of PCR analysis, agarose gel electrophoresis at 1.4% concentration with 0.5X TBE (Tris/Borate/EDTA) Buffer was utilized for observation of the band formation in the PCR products. For 10X TBE stock buffer, 108 g of Tris base, 55 g of boric acid, and 7.5 g of EDTA (Sigma-Aldrich) were dissolved in 800 ml of dH_2O and pH of the solution was adjusted 8 by completing total volume to 1000 ml. Lastly, the gel was imaged with UV Transilluminator (Uvitec, UK) at the end of the execution (Figure 2.3). After visualization of band formation on agarose gel, samples were prepared in separate tubes for NGS analysis with adjusting approximately 30 ng DNA per microliter.

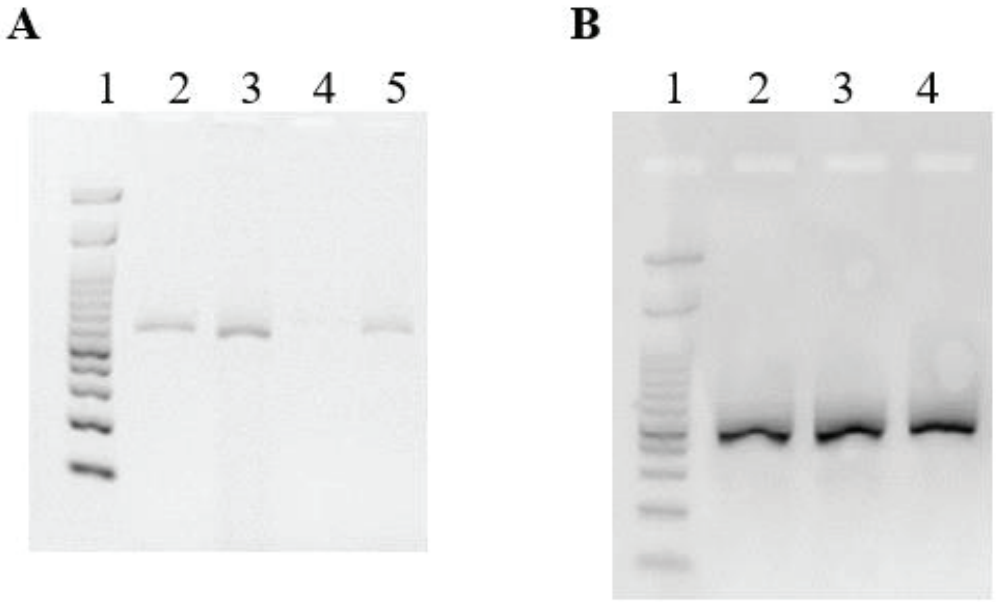

Figure 2. 3. Display of ITS (A) and *16S rRNA* (B) specific PCR results on agarose gel. First well in each figure represents 100 bp DNA ladder. Samples are 2-5 (A) and 2-4 (B).

2.2.4. Identification of fungal and bacterial microbiota by ITS region and *16S rRNA* gene amplicon sequencing

Amplicon sequencing mainly involves steps of DNA extraction, PCR amplification, library preparation, high-throughput sequencing, data analysis and interpretation. Markers genes ITS and *16S rRNA* amplified with PCR by utilizing primers contain barcodes and adapters. Then obtained PCR products are purified, quantified and libraries are prepared. After sequencing these libraries by Illumina or other platforms, raw data for our sample is obtained. Raw reads are then filtered to get effective tags and clustered as operational taxonomic units (OTUs). Using bioinformatics tools like QIIME, DADA2, taxonomic classification of OTUs is done by reference databases. Finally, OTU table is utilized to perform downstream analyses, such as alpha diversity, or correlation analysis.

Table 2. 1. Targeted and amplified regions in amplicon sequencing.

Target gene/region	**DNA sequences of the primers (5'-3')**		**Amplified region**
Bacterial *16S rRNA*	341F	CCTAYGGGRBGCASCAG	V3-V4
	806R	GGACTACNNGGTATCTAAT	
Fungal ITS	ITS5-1737F	GGAAGTAAAAGTCGTAACAAGG	ITS1
	ITS2-2043R	GCTGCGTTCTTCATCGATGC	
	ITS3-2024F	GCATCGATGAAGAACGCAGC	ITS2
	ITS4-2409R	TCCTCCGCTTATTGATATGC	

In this study, primers listed in Table 2.1 were used to amplification of the ITS region and *16S rRNA* gene in 10 randomly chosen DGRP samples (line numbers 138,

235, 26, 354, 370, 439, 837, 900, 217, 705) in order to determine the fungal and bacterial microbiota.

Following the purification of PCR products, sequencing libraries were constituted by adding adapters with Nextera XT DNA Library Preparation Kit (Illumina, USA). The libraries concentrations were then normalized to 4nM by qPCR. Amplicons were sequenced on Illumina NovaSeq 6000 by paired-end (2 × 250 bp) to generate 250bp paired-end raw reads by Novogene Company Ltd. (Cambridge, UK). All statistical analyzes were completed with R software (Version 4.3.1) (https://www.r-project.org). To visualize relative abundance of OTUs, the "ggplot2" package was used.

2.2.5. Analysis and quantification of dominant bacterial and fungal taxa by Real-Time PCR (qPCR)

DGRP gut samples were screened in terms of abundant species obtained in NGS data. However, since the fungal relative abundance is a quite less than bacterial composition within the gut environment, qPCR analyses were performed by firstly targeting three major fungal phylum Ascomycota, Basidiomycota, and Mucoromycota. First of all, ITS region sequence data revealed by NGS were aligned with the multiple sequence alignment using the Clustal algorithm in Unipro UGENE (version 46.0) software for taxa Ascomycota, Basidiomycota, and Mucoromycota separately. Primers for Ascomycota (GAATTGCAGMMWTCMGTGAATC-GCCTGTYTGAGCGTCRTTTC), Basidiomycota (CGAATCTTTGAACGCAMCTTG-GCCTGTTTGAGTATCATGA), Mucoromycota (GCTGAGTATCATCTGGAA-TGAATCATCGAATCTTTGAACGC) were designed that could amplify taxa in each phylum but would not amplify taxa in another phylum. Samples were analyzed with the Roche LightCycler 480 II system. 5 µl of LightCycler® 480 SYBR Green I Master enzyme (Roche Applied Science, Germany), 0.3 µl of each forward and reverse primer pairs, and 1.9 µl of PCR-grade water were mixed together for a single reaction. The total volume was then adjusted to 7.5 µl in without the inclusion of the DNA sample. Reaction mixture was made separately for each three primer pairs. *Saccharomyces cerevisiae* for Ascomycota, *Agaricus bisporus* for Basidiomycota, and *Rhizopus oryzae* for

Mucoromycota were the positive controls. This reaction mixture was loaded into 96-well plate in duplicates. After adding 2.5 µl of sample DNA into the mixture in the wells, the volume was completed to 10 µl. Then, the prepared plate was run with the Roche LightCycler® 480 II Real-Time PCR System in Biotechnology and Bioengineering Application and Research Center (BIOMER) within İzmir Institute of Technology (IZTECH) Integrated Research Centers, according to the protocol indicated in Table 2.2. Annealing and melting temperatures for each taxon were indicated under the table. The analysis of the data was performed by utilizing Absolute Quantitation Second Derivative and Tm Calling (determination of melting temperature) analyzes in LightCycler® 480 II software and relative abundance of Ascomycota and Basidiomycota were calculated for each DGRP sample. To complement and confirm the results of qPCR analyses, agarose gel electrophoresis (1.4%) was performed.

Table 2. 2. Device protocol of Real-Time PCR for fungal taxa.

Program Name	**Denaturation**	**Amplification**			**Melting Curve**			**Cooling**
nalysis mode	None	Quantification			Melting curves			None
Cycles	1	50			1			1
Target [°C]	95	95	*[1]	72	95	*[2]	97	40
Hold	5 min	10 sec	20 sec	10 sec	5 sec	1 min	-	30 sec
Ramp rate (°C/s)	4,8	4,8	2,5	4,8	4,8	2,5	0,11	2,5
Acquisition mode	None	None	None	Single	None	None	Continuous	None

**[1]: 56°C for the Ascomycota primer pair; 52°C for the Basidiomycota primer pair were used.*

**[2]: 61°C for the Ascomycota primer pair; 57°C for the Basidiomycota primer pair were used.*

Following the representation of both Ascomycota and Basidiomycota phyla in all samples, the presence and relative abundance of the common bacterial and fungal species selected from NGS data, were determined by qPCR in 120 DGRP lines. Primer sequences of each targeted species were indicated in Table 2.3. All qPCR reaction mixes were prepared as described above and run through protocol showed in Table 2.4. In each run, distilled water was used as a negative control. Melting curve analysis was utilized to determine the achievement of qPCR reactions with a ramp rate of 0.11°C per second. Due to specific results were not obtained with SYBR Green for fungal species (*Malassezia restricta* and *Pleurotus ostreatus*), LNA (Locked Nucleic Acid)-based TaqMan probes were utilized. These hydrolysis probes improve amplification efficiency owing to their highly sensitive and unique chemistry (Montone and Feldman 2009). For a single reaction, 2.5 µl of PCR-grade water, 1 µl of species-specific forward and reverse primers, 0.5 µl of LNA-TaqMan probe, and 10 µl of LightCycler® 480 Probes Master 2x enzyme (Roche Applied Science, Germany) were mixed and total volume was adjusted to 15 µl without the DNA sample. With 5 µl of DGRP DNA sample, the final volume was completed to 20 µl. The qPCR reaction was carried out to the following conditions: 95°C for 10 min with denaturation followed by 45 cycles of 95°C for 10 s, annealing at 60°C for 30 s, and 72°C for 1 s.

In LightCycler® 480 II Software v.1.5, utilizing the Absolute Quantitation/Second Derivative and Tm calling analysis modes, amplification and melting curves for each sample were obtained. The threshold value needed for assessing the quantity of fluorescence signal acquired through Real-Time PCR analysis is indicated by Ct values (Cycle Threshold). These values inversely proportional with the target content in the DNA sample. As the amount of the target DNA increases, the Ct value lowers. Relative abundance of targeted microorganisms was indicated as $2^{-\Delta Ct}$ and calculated according to the following formulas:

$$2^{-\Delta Ct} = 2^{-} \text{(Ct of target bacteria/fungal species- Ct of total bacteria/fungi)}$$

Table 2. 3. Primers utilized for detection and quantification of targeted bacterial and fungal species.

Target microorganism	**Genome region (size)**	**DNA sequences of the primers (5′–3′)**		**References**
Lactobacillus plantarum	*16s rRNA* (153 bp)	Forward	CGAACGAACTCTGGTATTGATTG	(Obata, Fons, and Gould 2018)
		Reverse	ACCATGCGGTCCAAGTTG	
Acetobacter pomorum	*16s rRNA* (204 bp)	Forward	CTAGATGTTGGGTGACTTAGTCA	(Wong et al. 2015)
		Reverse	CGGGAAACAAACATCTCTGCTTG	
Enterococcus faecium	Genomic location (73 bp)	Forward	GACGGCGAAATGGGTGACT	This study
		Reverse	CAGAGAGTTTACGCAATGCTTGA	
Lactobacillus brevis	*recA* (64 bp)	Forward	GCAGTTGCCGAGGTCCAA	(Xu et al. 2020)
		Reverse	CCAACGCATTTTCAGCATCA	
Acetobacter persici	Genomic location (132 bp)	Forward	GGAGCCAGAAGCGGATTT	This study
		Reverse	GGTCACATACGTCATACCTGAG	
Total bacteria	*16s rRNA* (466 bp)	Forward	TCCTACGGGAGGCAGCAGT	(Dantoft et al. 2016)
		Reverse	GGACTACCAGGGTATCTAATCCTGTT	
Malassezia restricta	Genomic location (118 bp)	Forward	TTCATGTTCCCATGTTTCCTTTG	This study
		Reverse	GTGAGTCCCTTCACTTCTTTCT	
Pleurotus ostreatus	*pyrG* (89 bp)	Forward	AGATCACGCGCTTAGATGATAG	This study
		Reverse	CCTTCGGGAAGTGGATGAA	
Fungi	ITS (200-400 bp)	Forward	GGAAGTAAAAGTCGTAACAAGG	(Abliz et al. 2003)
		Reverse	GCTGCGTTCTTCATCGATGC	

Table 2. 4. Run protocol of Real-Time PCR for targeted species.

Program Name	**Denaturation**	**Amplification**			**Melting Curve**			**Cooling**
Analysis mode	None	Quantification			Melting curves			None
Cycles	1	50			1			1
Target [°C]	95	95	58	72	95	63	97	40
Hold	10 min	10 sec	15 sec	15 sec	5 sec	1 min	-	30 sec
Ramp rate (°C/s)	4,8	4,8	2,5	4,8	4,8	2,5	0,11	2,5
Acquisition mode	None	None	None	Single	None	None	Continuous	None

2.3. Results

2.3.1. ITS region amplicon sequencing

For the veracity of sequencing data analysis, firstly, quality control of raw data was checked by FastQC. After that, effective tags were obtained by removing chimeric reads, primer, and barcode sequences, and reads with a Phred Score of less than 20, using DADA2. To determine taxonomic knowledge of each OTU, QIIME2 was utilized. Representative sequences for each OTU were created using ≥ 97% similarity against the reference databases of Greengenes (v13_8) and SILVA (v138.1) for bacteria and UNITE (v8.3) for fungi. The relevance of sequencing depth for samples were assessed using rarefaction curves that plotted the depth of sequencing versus the number of identified taxa. Additionally, the most prevalent OTU representative sequences were blasted and checked through reference sequences by using the National Center for Biotechnology Information (NCBI). Finally, each OTU was represented at taxonomic rank (kingdom,

phylum, class, order, family, genus, species). OTU table that indicates the family level of fungal microbiota in each sample is provided in Appendix A, Table A.1.

In the nine DGRP gut samples, an average of 122,684 reads (range 60,766–253,297) were obtained for each sample. In accordance with a 97% similarity criterion, 3 phyla, 15 classes, 46 orders, 87 families, 113 genera, and 140 species were found out in all sequences. Unidentified reads, which constitute approximately 6% of all reads, were excluded from the analyses. Ascomycota (97.5%), Basidiomycota (2.4%), and Mucoromycota (0.1%) were the three most prevalent phyla in the samples (Figure 2.4). *Saccharomycetes* was the dominant fungi class in the *Drosophila* intestine, representing 87% of the ITS sequences in our samples. The most common species were found as *Pichia manshurica*, belongs to *Saccharomycetes*. The top ten most common yeast species were *P. manshurica*, *Tuber rufum*, *Saccharomyces paradoxus*, *Malassezia restricta*, *Malassezia globosa*, *Pleurotus ostreatus*, *Kluyveromyces marxianus*, *Beauveria bassiana*, *Lecanicillium muscarium, Xenopolyscytalum pinea.*

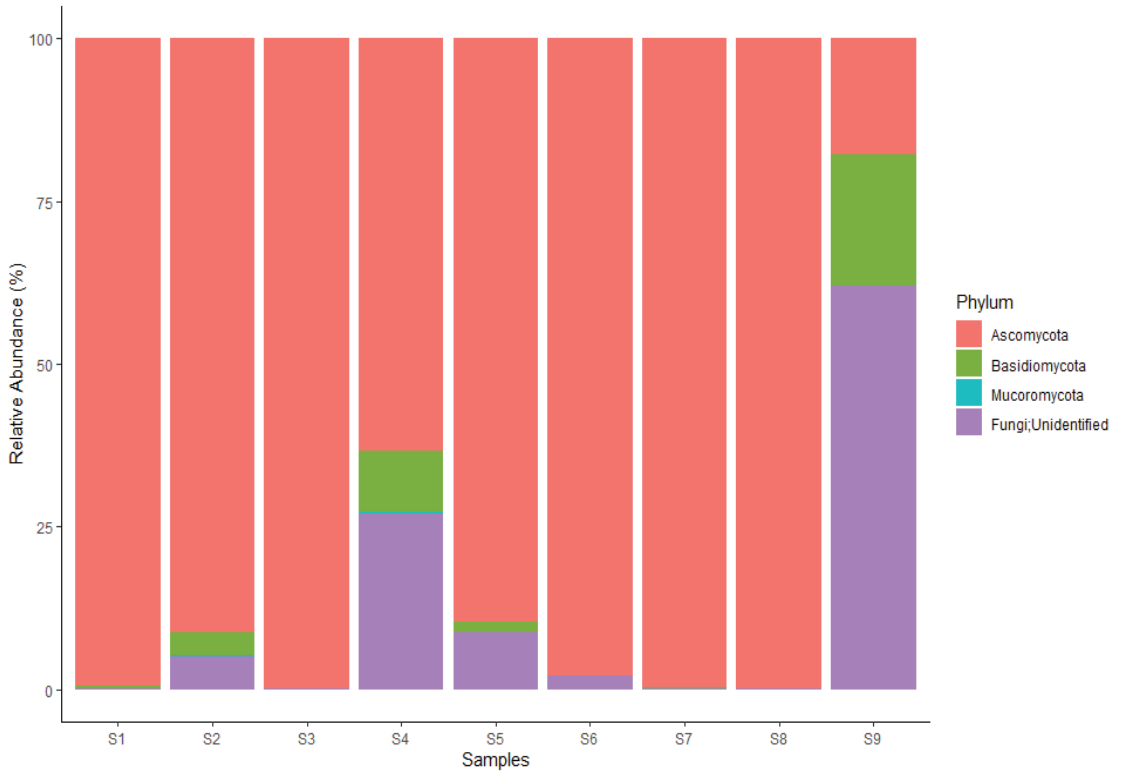

Figure 2. 4. Relative abundance of fungi at phylum taxonomic level in DGRP samples.

Alpha diversity is defined to microbial diversity within samples. There are different alpha diversity indices such as Observed taxa, Chao1, Shannon, Simpson, and ACE (Abundance-based Coverage Estimator), which identify decreasing or increasing diversity. The Observed taxa index indicates the microorganism diversity of the sample by accounting number of different species (OTUs). Observed taxa index between samples ranged from 9 to 166. One of the most used alpha diversity indices in ecological studies is Shannon (H) and Simpson (D) indices. The Simpson index focuses on common species in samples. However, Shannon index is more useful for assessing alpha diversity, because it is more susceptible to rare taxa. Shannon index values were obtained between 0.03 and 2.32 in samples (Figure 2.5).

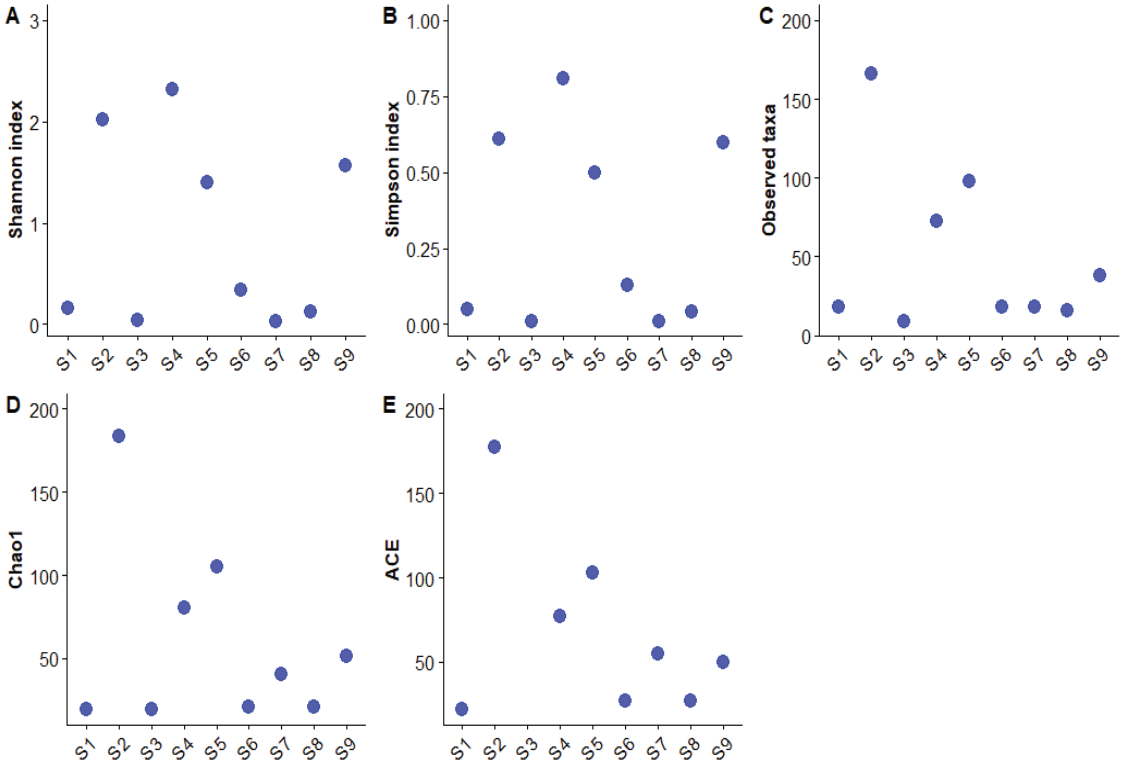

Figure 2. 5. Alpha diversity indices of the fungal microbiota. While y axis represents each matrix, x axis indicates the sample numbers.

2.3.2. *16S rRNA* amplicon sequencing

In the ten DGRP gut samples, an average of 129,585 reads (range 47,575–236,311) were obtained for each sample. In accordance with a 97% similarity criterion, 6 phyla, 19 classes, 42 orders, 93 families, 276 genus, and 614 species were identified in all sequences. The most prevalent phyla were Firmicutes (70.5%), Proteobacteria (21.8%), Bacteroidetes (4.8%), Actinobacteria (2.7%), and Fusobacteria (0.1%) in the samples (Figure 2.6). Most dominant species in the gut environment of DGRPs were *Acetobacter persici, Escherichia coli, Lactococcus lactis, Enterococcus faecium, Lactobacillus plantarum, Acetobacter indonesiensis, Prevotella copri, Faecalibacterium prausnitzii, Bacillus filamentosus,* and *Bacteroides vulgatus.* OTU table that indicates the family level of bacterial microbiota in each sample is provided in Appendix A, Table A.2.

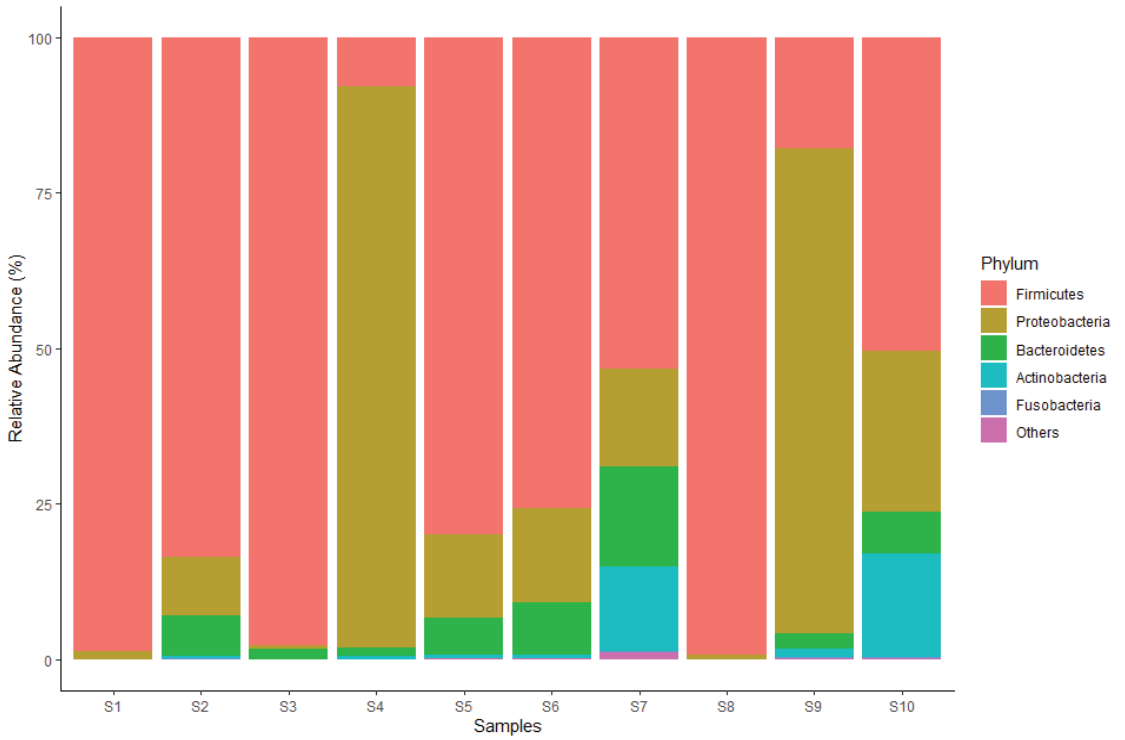

Figure 2. 6. Relative abundance of bacteria at phylum taxonomic level in DGRP samples.

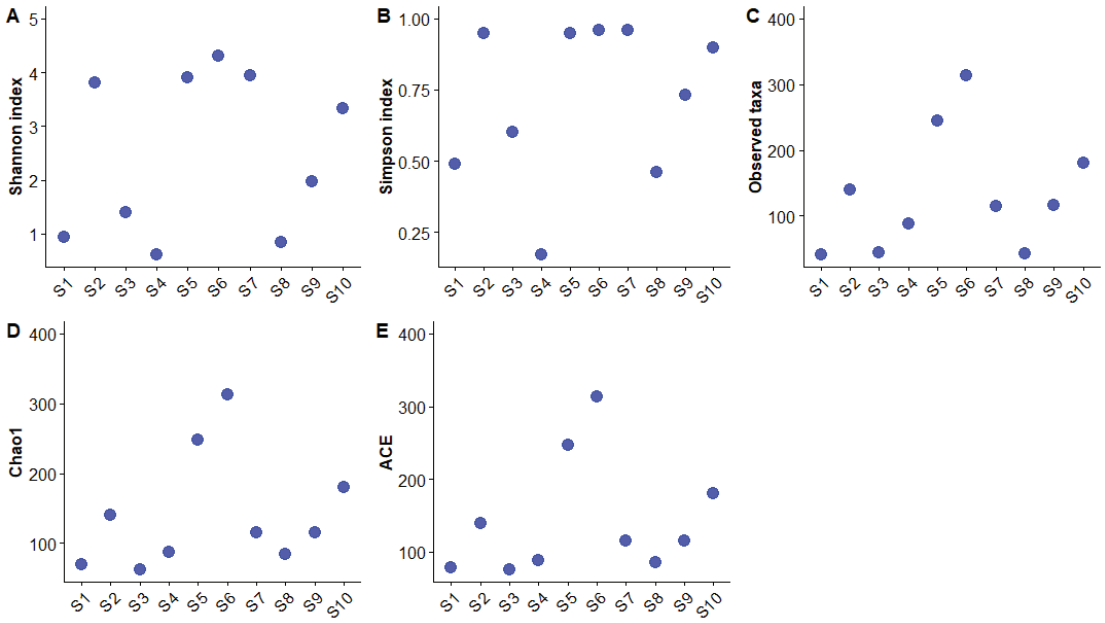

Figure 2. 7. Alpha diversity indices of the bacterial microbiota. While y axis represents each matrix, x axis indicates the sample numbers.

Alpha diversity indices are shown in the Figure 2.7. Observed taxa between samples ranged from 41 to 245. Also, Shannon index values were obtained between 0.17 and 4.31 in samples. In the DGRP samples, alpha indices for fungi were lower than for bacteria (Figure 2.8). These findings indicate that the gut fungal flora was statistically significantly less varied than the bacterial population for Shannon (p, 0.025) and Simpson (p, 0.015) indexes. For observed taxa, bacteria again showed a higher median value than fungi, although the difference was not reached statistical significance (p, 0.054). After that, to assess whether there was a relationship between bacterial and fungal microbiota in terms of Shannon, Simpson, and Observed taxa alpha diversity indices, a non-parametric Spearman correlation test was utilized. The Shannon and Simpson alpha diversity indexes were reflected Spearman correlation coefficients (rho values) of -0.2 (p, 0.613) and -0.18 (p, 0.640), respectively (Figure 2.9A and B). While these results indicate a weak negative relationship; rho value of the Observed taxa was indicated a positive relationship with 0.56 (p, 0.117) (Figure 2.9C). However, neither negative nor positive relationships were not statistically significant.

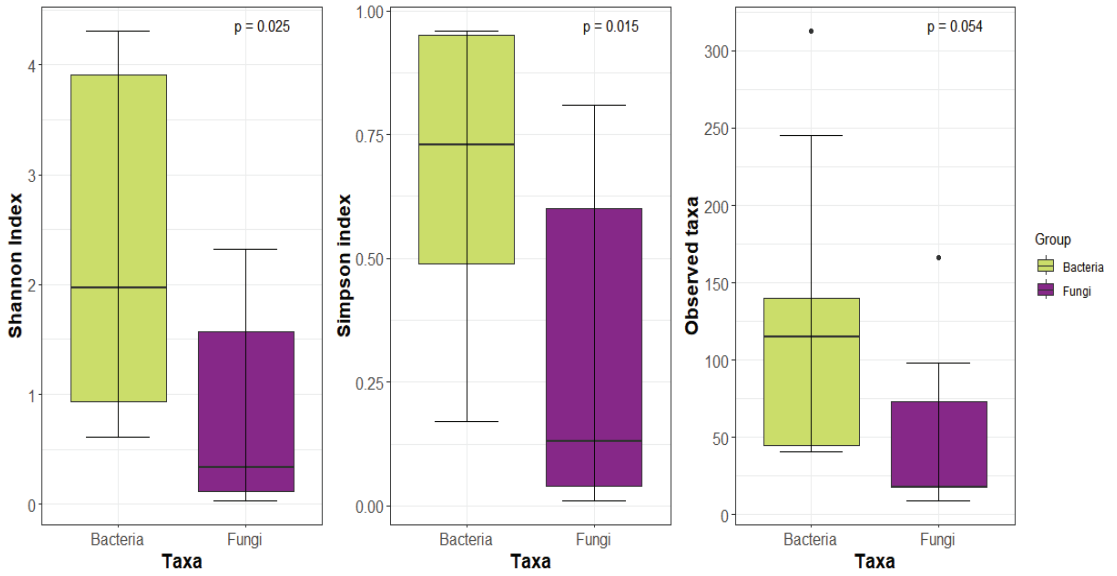

Figure 2. 8. Box plots of alpha-diversity indexes of the DGRP samples (whisker: minimum to maximum, horizontal line: median, box: 25th to 75th percentile).

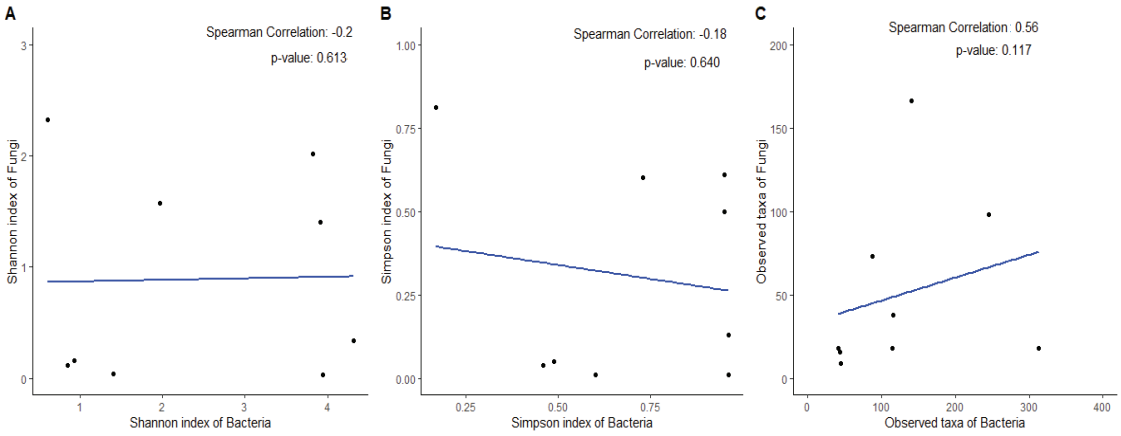

Figure 2. 9. Spearman correlation plots of Shannon (A), Simpson (B), and Observed taxa (C) alpha diversity indices.

2.3.3. Scanning of relative abundance of selected bacteria and fungus species by qPCR

In accordance with literature and based knowledge from our NGS data, by focusing on specific bacterial and fungal taxa, detailed scanning of our dataset consists of 120 DGRP samples, was performed.

Because species level identification for fungal taxa would be challenging for most readings, qPCR analyses were carried out by first constructing primer sets for the Ascomycota, Basidiomycota, and Mucoromycota phyla. Utilizing Absolute Quantitation Second Derivative and Tm calling analyses, Ct values and melting peaks (Figure 2.10) which validate the amplification specificity were obtained for each sample. qPCR analysis revealed that primers designed for Ascomycota and Basidiomycota efficiently amplified the targeted taxa (Figure 2.11). However, considering the relative abundance percentage obtained in ITS metagenomic sequencing (0.1%), observations for the Mucoromycota phylum were not obtained due to limitations with DNA amplification efficiency.

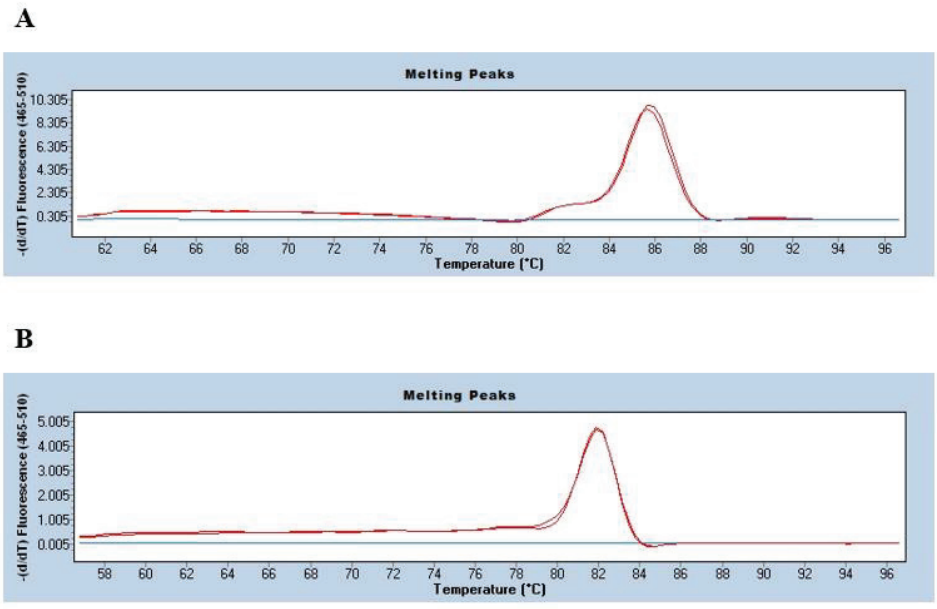

Figure 2. 10. Melting peaks of Ascomycota (A) and Basidiomycota (B) primer sets.

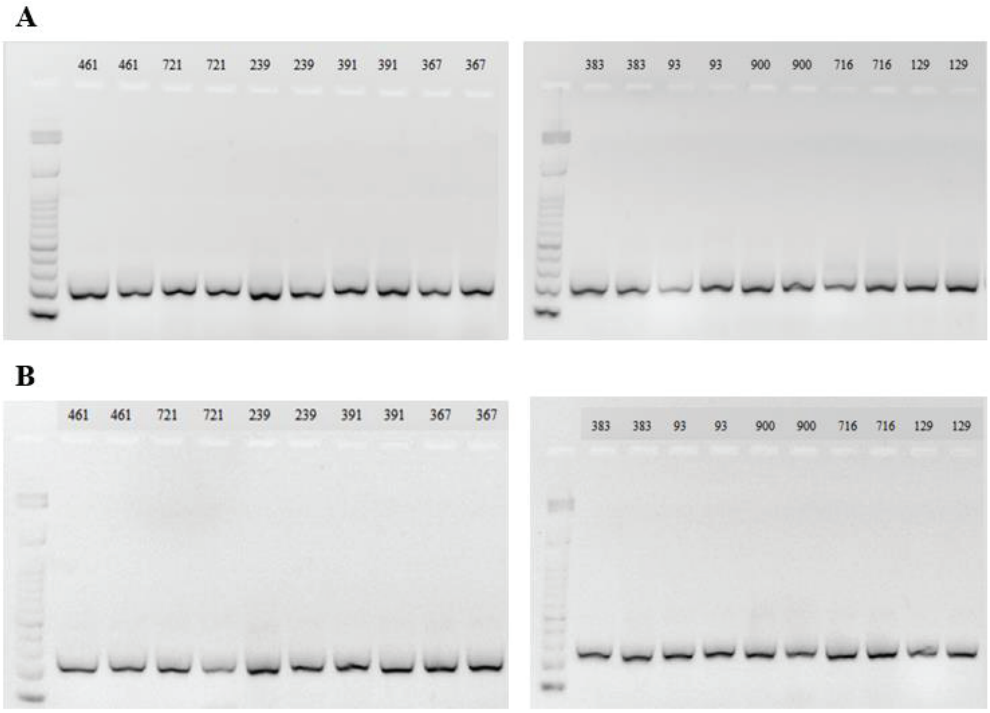

Figure 2. 11. qPCR results with Ascomycota (A) and Basidiomycota (B) specific primers. Numbers on the figure indicate DGRP lines.

The delta delta Ct ($2^{-\Delta\Delta Ct}$) method is utilized to analyze qPCR results and calculate fold changes (Livak and Schmittgen 2001). In this approach relative gene expression of the target gene are calculated using the reference (housekeeping) gene. In this study, analyses were performed using each taxon as a target and total bacteria/fungi as a reference (Navidshad, Liang, and Jahromi 2012). The $\log_2 2^{-\Delta Ct}$ formula was utilized to express the correlation between samples (Feng et al. 2010). Ascomycota had a greater relative abundance in the samples than Basidiomycota. Performed pairwise comparison utilizing the Wilcoxon rank-sum test, it was found a statistically significant difference with p-value of less than 2e-16 in the relative abundance of Ascomycota and Basidiomycota among the samples (Figure 2.12).

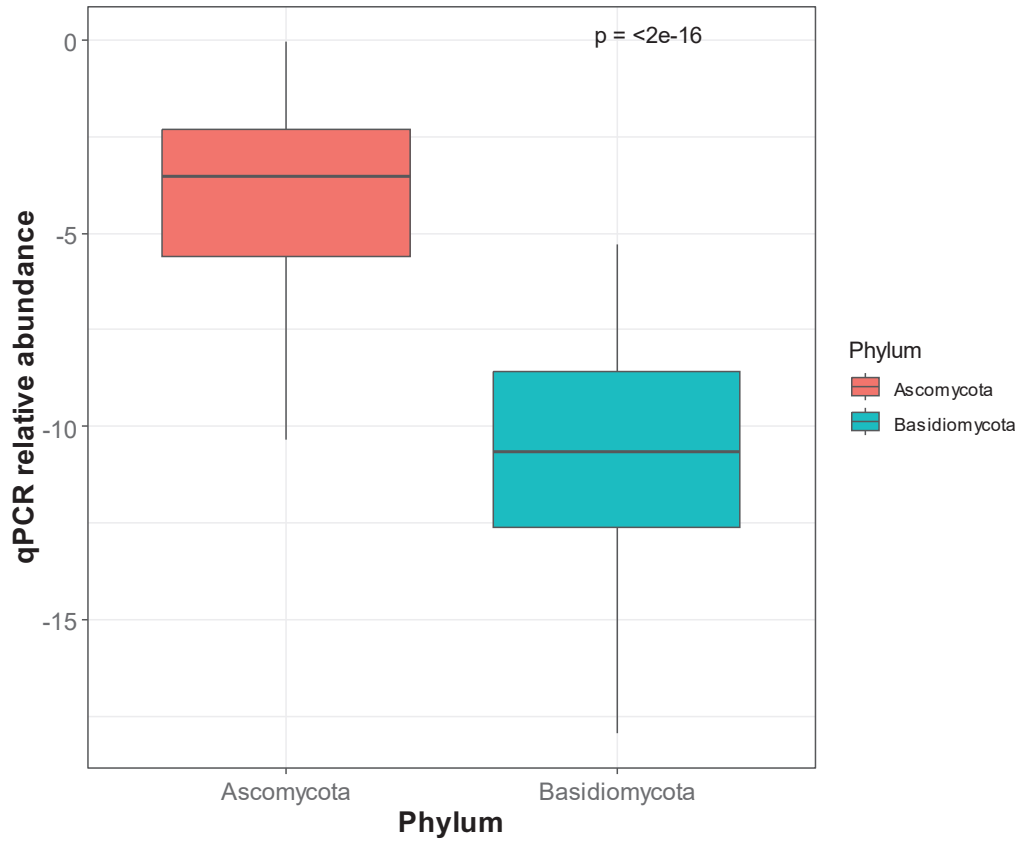

Figure 2. 12. Boxplot comparison of the relative fungal phyla abundance between DGRP samples.

For bacterial screening *A. persici, A. pomorum, E. faecium*, *L. brevis*, and *L. plantarum* species were targeted. In qPCR analyses of all DGRP samples, the bacteria with the highest relative abundance were *E. faecium, A. pomorum, L. plantarum, L. brevis,* and *A. persici* respectively (Figure 2.13). The Kruskal Wallis rank-sum test was used to evaluate the abundance of *E. faecium, A. pomorum, L. plantarum, L. brevis*, and *A. persici* species in 120 DGRP samples and statistically significant difference was observed between samples in accordance with bacterial abundance (p-value = 1e-05). The pairwise comparisons conducted with Wilcoxon rank-sum test revealed five statistically significant observations. Firstly, the abundance of *A. persici* was significantly different from *E. faecium* (Wilcoxon rank-sum, p-value = 2e-6), suggesting a notable distinction in their prevalence within the DGRP samples. Likewise, the abundance of *L. plantarum* and *L. brevis* differed significantly from the abundance of *E. faecium* (Wilcoxon rank-

sum, p-value = 2.6e-4 and 2.8e-4, respectively). Additionally, there was a significant difference among *A. persici* and *A. pomorum* (Wilcoxon rank-sum, p-value = 0.008). Finally, *A. pomorum* displayed a significant difference in abundance compared to *E. faecium* (Wilcoxon rank-sum, p-value = 0.003).

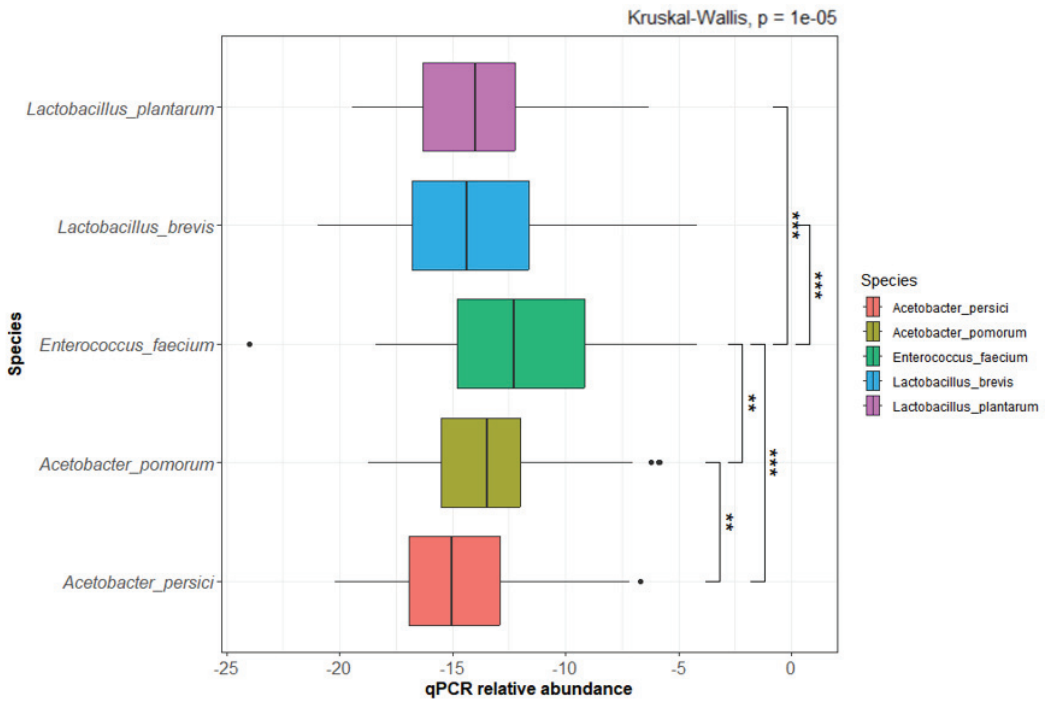

Figure 2. 13. Boxplot comparison of the relative bacterial species abundance between DGRP samples.

2.4. Discussion

This chapter aimed to comprehensively analyze the microbial diversity within *Drosophila* gut samples using ITS region and *16S rRNA* amplicon sequencing. Our results highlighted the significant presence of fungal and bacterial taxa and their relative abundance in the DGRP samples. In recent years, *Drosophila* has become a powerful model organism for research on the host-microbiota relationship. A lot of microbiota studies have been carried out by the development and widespread application of next-generation sequencing methods. Compared to mammals, *D. melanogaster* has a simpler

gut microbiota consisting of 1-30 species. The majority of the bacteria in the gut microbiota of *Drosophila* larvae, pupae, and adults are *Firmicutes* and *Proteobacteria* (Wong et al., 2015). Han and coworkers, using different *Drosophila* strains revealed that the gut microbiota was dominated by the genera *Acetobacter*, *Lactobacillus*, *Enterococcus*, and *Leuconostoc*. The most common bacteria in male flies have been *A. pasteurianus* and *L. plantarum*. When the effect of host age was examined, the abundance of *Lactobacillus* increased with age (Han et al., 2017). In our samples, the prevalence of Firmicutes and Proteobacteria revealed by *16S rRNA* amplicon sequencing is consistent with other gut microbiota studies. The families of the gut microbiota in fruit flies, both wild and grown in laboratories, have been discovered to be often connected with the *Enterobacteriaceae*, *Acetobacteraceae*, *Lactobacillaceae*, and *Enterococcaceae* (Vacchini et al. 2017). The genera *Lactobacillus* and *Acetobacter* include a large proportion of known bacterial species (Buchon, Broderick, and Lemaitre 2013; Lesperance and Broderick 2020). They promote systemic growth of fly larvae under nutrient-limiting conditions (Storelli et al. 2011). In addition, *L. plantarum* protects flies by reducing the toxicity and mortality rates caused by pathogenic fungi (Su et al., 2019). Considering the first five bacterial species most frequently seen in NGS data, *Acetobacter* and *Lactobacillus* bacteria dominate the gut environment of our DGRPs. This highlights the critical role these genera play in shaping the gut microbiota. Because of their ability to produce acetic acid, *Acetobacter* species play a key role in maintaining an acidic environment. Widely known for their probiotic properties, *Lactobacillus* spp. contribute gut health by enhancing the immune system of the host and supporting digestion. Their ability to generate lactic acid and other antimicrobial substances may stimulate the growth of advantageous microorganisms and inhibit pathogenic bacteria. Within the most prevalent species, the existence of other lactic acid bacteria *Lactococcus lactis* and *Enterococcus faecium* further emphasizes the importance of these genera in the gut ecosystem. *Enterococci* are lactic acid bacteria containing both pathogenic and commensal microorganisms that are more abundant in laboratory-grown *Drosophila* than in wild-type. *E. faecium* constitutes 22.4% of *Enterococcus* in the gut of *Drosophila* (Cox and Gilmore 2007). In our samples, *E. faecium* composed of 81% of *Enterococcus*. Numerous studies have been conducted to assess the probiotic properties of *Enterococcus* strains, particularly *E. faecium*, and have been demonstrated that *E. faecium* enhances absorption capacity in the small intestine and improves intestinal barrier integrity (Hanchi et al. 2018). Li and colleagues have isolated *E. faecium* from the *Drosophila* intestinal

environment for the first time and investigated its effect for host (Li et al. 2023). They have demonstrated that *E. faecium* as a symbiont, increases growth hormones, supports *Drosophila* development through the insulin signaling pathway, and provides immune activity by enhancing the proliferation of intestinal cells.

Although the majority of studies on microbiota have been on bacterial species, the gut also contains a smaller number of individually stable fungal microbiota. Even fungal microbiota studies are very limited in the *D. melanogaster* as in humans and other model organisms, intestine predominantly has Ascomycota. Predominance of Ascomycota found in this study, is consistent with metagenomic studies in *Drosophila* (Chandler et al., 2012; Jiménez Padilla et al., 2020). *Aspergillaceae*, *Saccharomycetaceae*, *Chaetomiaceae*, *Mycosphaerellaceae*, and *Pleosporaceae* have been found the most abundant families that belong to Ascomycota after the ITS amplicon sequencing in *D. suzukii* (Jiménez Padilla et al. 2020). *Drosophila* consumes some yeasts as protein sources, and yeast nutrients have been shown to influence the growth, lifespan, and egg production (Anagnostou, Dorsch, and Rohlfs 2010). Similar to the literature, the major yeast phyla in our samples were Ascomycota, Basidiomycota, and a much lower abundance of Mucoromycota. *P. manshurica* dominates all of the *Drosophila* samples in our samples at the species level. *P. manshurica* find in various fermented foods and enables to forming biofilm on abiotic surfaces (Perpetuini et al. 2021). Many members of the genus *Malassezia*, including *M. restricta*, form part of the gut microbiota. *M. restricta* can trigger immunological responses in the intestine by promoting the production of inflammatory cytokines (Spatz and Richard 2020; Wrighton 2019). Also, it is a lipophilic yeast and has also been shown to increase adiposity in mice (Guillot & Bond, 2020). *T. rufum*, a species with antibacterial, anti-inflammatory, and antioxidant qualities, was another prevalent yeast in the samples (Patel et al., 2017; Pattanayak et al., 2017). *P. ostreatus*, a member of the phylum Basidiomycota, contributes to decreasing blood sugar, and prior research on *D. melanogaster* also has demonstrated its anti-diabetic properties (Omale et al., 2020). *M. restricta* has been among the top ten species of *D. suzukii* fungal microbiota, with a rate of incidence 26% of samples after ITS amplicon sequencing (Jiménez Padilla et al. 2020). Overall, these observations highlight the diverse and essential roles of fungal microbiota in the *Drosophila* intestine.

The correlations between bacterial and fungal alpha diversity indices indicate no statistically significant relationships across the assessed diversity metrics. The Spearman correlation coefficients for the Shannon and Simpson indices showed p-values greater

than the significance level of 0.05. However, the observed taxa indices show a moderate positive association between bacterial and fungal taxa, but the relationship remains statistically insignificant. The absence of statistically significant evidence to support a consistent or significant association in alpha diversity analyses might be attributed to the small sample size that might reduce the statistical power needed to identify real associations.

2.5. Conclusion

This chapter findings highlight the prevalence of specific bacterial and fungal species in the Drosophila Genetic Reference Panel samples. They have an important impact on the host by influencing the immune system, assisting in nutrition metabolism, and protecting against infections. Bacterial species comprised a significant portion of the gut microbiota, but we also discovered stable fungal communities dominated by Ascomycota and Basidiomycota. Fungal studies on the *Drosophila* gut microbiota indicate a smaller but equally essential microbial population. While fungus is less abundant than bacteria, they have a substantial influence on host health and physiology. *Drosophila melanogaster*, as a model organism, provides valuable insights into host-microbe interactions, as well as a simplified system for investigating hypotheses that may be applied to other species, including humans. Understanding these interactions is critical for developing microbiota-based therapies to improve health, such as probiotics, prebiotics, and other microbiome-modulating methods.

CHAPTER 3

DETERMINATION OF METABOLIC POOLS *DROSOPHILA MELANOGASTER*

3.1. Introduction

3.1.1. Lipid metabolism

Lipids participate in a number of functions in *Drosophila*, including oogenesis, energy storage, determining the fluidity and structure of membranes, and embryo development. Neutral lipids such as triacylglycerol (TAG, known as also triglycerides (TG)) and diacylglycerides (DAG) are crucial in regulating the host lipid metabolism and energy homeostasis (Zhao and Karpac 2020). The most concentrated type of energy among the formed by chemically-bond, is known TAG. Moreover, almost ninety percent of stored lipids consist of triacylglycerols (Akhmetova, Balasov, and Chesnokov 2021). Similarly, adipocyte cells in mammals, triacylglycerols are the primary storage form of lipid droplets in *Drosophila* (Kühnlein 2012). Those reserves are a crucial component of the storage needed for survival when encountering hunger in the adult flies. Lipid metabolism of the fruit fly can be classified into two processes. The first is termed lipogenesis, and it is responsible for the formation of lipid in the form of TAG, whereas the second is lipolysis, which manages the breakdown of stored TAG into free fatty acids (Thanh et al. 2020). Lipases hydrolyze dietary TAG to produce free fatty acids, glycerol, or other acylglycerol precursors in the midgut after consumption. Linkage among insulin signaling and TAG metabolism is notably provided by Forkhead box subgroup O (Foxo). Foxo accelerates the enzymatically breakdown of triacylglycerol by increasing lipase activity. The main lipase activity is provided by Brummer lipase (Bmm), an ortholog of

mammalian adipocyte triglyceride lipase in DM and it converts TAG into free fatty acid and diacylglycerol. The other lipase called Magro (mag), by function as a homologue of mammalian gastric lipase, breaks down dietary triglycerides for fatty acid absorption (Sieber and Thummel 2012). Metabolites arise from TAG digestion are absorbed by enterocytes and they are transformed to diacylglycerol which is the primary transportation form of neutral lipids in the hemolymph. In addition to, excessive ones are held as lipid droplets, stored into fat body, or packed for carriage to other organs for catabolism. The fat body, which functions as a multifunctional organ analogous to the liver and adipose tissue of mammals, is the main nutrient reservoir (Musselman et al. 2013). In addition to the fat body, secretory cells known as oenocytes, which have functional similarity with the fundamental cells (hepatocytes) of the mammalian liver, absorb mobilized lipids during starvation in adult flies (Chatterjee and Perrimon 2021). During starvation, TAG stores are hydrolyzed in the enzymatic process called lipolysis and transported from the fat body to other tissues. Lipids are transported to organs through hemolymph by particular carriers known as lipoproteins. Lipoproteins transport dietary or newly generated lipids to the fat body for storage; but if encountered with hunger, they move lipids from fat body to peripheral tissues. In *Drosophila*, lipid transport is mediated by three lipoproteins: lipophorin, crossveinless d, and lipid transfer particle. Approximately ninety-five percent of lipids are transported by lipophorin in the hemolymph (Palm et al. 2012). Lipid transport proteins are made in enterocytes in mammals, while in *Drosophila*, they are manufactured in the fat body and then moved to the gut. Metabolic role of triacylglycerols includes more than just energy storage. Fatty acids utilized for TAG storage, can potentially be used in synthesis structural membrane lipids, or signaling molecules (Heier and Kühnlein 2018). Genes related to lipid metabolism, independently of energy source, also have a role in processes such as spermatogenesis (biological processes), oogenesis (eggshell construction), embryogenesis (cell migration), and late development (Figure 3.1).

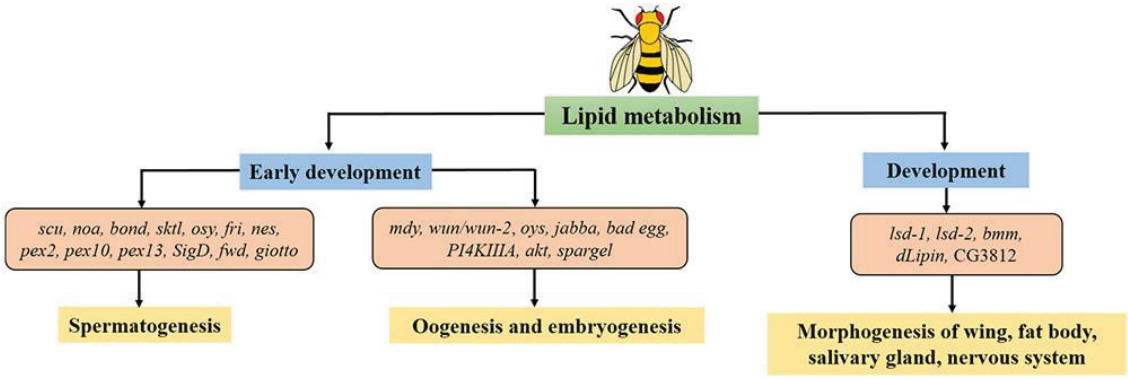

Figure 3. 1. Genes involved in lipid metabolism during various stages of development in fruit fly. (Source: Thanh et al. 2020).

3.1.2. Carbohydrate metabolism

In addition to lipid metabolism, host metabolism is also influenced by the metabolism of carbohydrates, the use of amino acids, and mitochondrial activity. In all animals, ability of usage and storage carbohydrates is crucial for preserving metabolic equilibrium. Both *Drosophila* and mammals release peptide hormones from specific cells in response to the digestion processing. These peptides are recognized by specialized receptors on muscle and adipose tissue, then trigger IIS and TOR signaling pathways so that enhance storage of glycogen and triglycerides. Peptide hormones present in *D. melanogaster* are essential for controlling lipid and glycogen stored in the fat body. *Drosophila* insulin-like peptides (DILPs, one to eight) are one of these hormones that functional homologues of insulin in vertebrates (Liu and Huang, 2013). Insulin is synthesized by pancreatic β cells in vertebrates and enhances the permeability of membranes for glucose. On the contrary the catabolism, insulin supports protein and lipid anabolism and stimulates the conversion of glucose to glycogen. Similar with insulin, DILPs regulate the carbohydrate and lipid metabolism, tissue growth, and lifespan of fruit fly. These hormones are secreted by insulin-producing cells (IPCs) that are analogous to the pancreatic β cells. It has been shown that, ablation of insulin-producing cells results in lower cell size in larvae and higher blood sugar levels (Rulifson, Kim, and Nusse 2002). Increased glycogen storage, a longer lifespan and higher level of circulating triglycerides

have been noted in insulin-producing cell knockdown flies. Knockdown studies conducted with each DILP type have indicated key points that refer different metabolism regulation. DILP2, which is primarily produced in the midgut, plays a key role in the metabolism of carbohydrates. For instance, DILP3 is in charge of the synthesis and release of trehalose, DILP2 and 5 control the deposition of glycogen, and the knockout of DILP7 resulted in decreased triglyceride levels and an increased in glycogen levels (Semaniuk et al. 2021). Another hormone, adipokinetic hormone, where produced in corpora cardiaca cells found in the larval ring gland, is functional homologue of glucagon in vertebrates. In *D. melanogaster*, AKH works antagonistically with insulin-like peptides to preserve metabolic homeostasis. In order to release stored glycogen in the fat body and raise blood glucose levels, AKH activates glycogen phosphorylase enzyme. If AKH is deficient in fruit flies, trehalose production from fat body glycogen cannot activated (Isabel et al. 2005). In addition to pancreatic cells that release glucagon and insulin hormones, our intestinal cells contribute to maintain overall energy balance. Enteroendocrine cells release a variety of hormones that affect metabolic homeostasis. Incretins consist of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), regulate metabolic responses in case of food consumption (Ezcurra et al. 2013). Incretins, an enteroendocrine hormone, enhance insulin production when stimulated by glucose. In *D. melanogaster*, neuropeptide F (NPF) hormone functions as an incretin-like hormone since it is sensitive to sugar; however, the primary structure of this protein varies from GIP and GLP-1. NPF is synthesized and secreted by midgut enteroendocrine cells (Figure 3.2). It then attaches to the NPF receptors located on insulin-producing cells or corpora cardiaca which is orthologues of the pancreatic α cells (Park et al. 2011). The disruption of the ligand-receptor signaling has been demonstrated to cause catabolic phenotypes related with AKH and insulin, as well as reduction of energy storage, hypoglycemia and hyperphagia (Yoshinari et al. 2021). As a result, incretin dysregulation can be linked to disorders including obesity and type 2 diabetes. In addition to the IIS, transforming growth factor beta (TGFβ) signaling pathway also a conserved mechanism regulates physiological functions, homeostasis, and immune responses (Bastin and Eleftherianos 2023). In high sugar intake, secretion of Dawdle (daw), the TGFβ ligand, increases. Daw is especially involved in the regulation of carbohydrate metabolism functions by stimulating the secretion of DILP2 and 5 (Chatterjee and Perrimon 2021; Upadhyay et al. 2017). Also, by suppressing many lipases, and carbohydrases like amylase, it adjusts the sugar

homeostasis. Mutations in the *daw* gene cause hyperglycemia with high levels of glucose and trehalose in the hemolymph (Bland 2023).

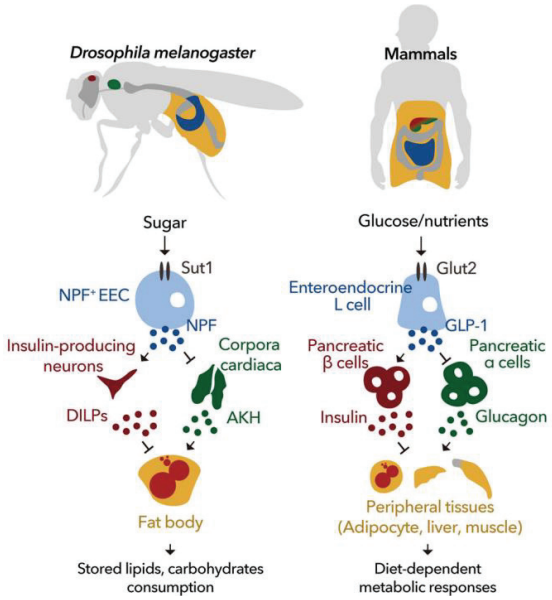

Figure 3. 2. Regulation of carbohydrate metabolism by AKH and DILPs in *Drosophila* (left); by insulin and glucagon in mammals (right). (Source: Yoshinari et al. 2021).

After enzymatically digestion of carbohydrates into monosaccharides, they are absorbed by intestinal cells and released into the hemolymph. Later, they are transformed and stored as glycogen in the fat body, or for metabolization in other tissues, they are concentrated into trehalose. The main blood sugar in *Drosophila* is trehalose which is a disaccharide (Na et al. 2013). Trehalose is produced by gathering two glucose molecules with trehalose-6-phosphate synthase (Tps1) enzyme in the fat body and is catabolized by trehalase (Treh) enzyme (Mattila and Hietakangas 2017). These sugars can be used as substrates in chemical reactions or stored as form of glycogen. While the most abundant sugar in circulation in mammals is glucose, in fruit fly it is trehalose. The majority of the fly carbohydrates are stored as glycogen, which serves as a primary source of energy during times of starvation or activity. In the hemolymph during the larval and adult flies, trehalose is nearly equal and plenty. Conversely, in larvae, circulatory glucose is minimal and increases as it matures (Tennessen et al. 2014). Trehalose is additionally needed in the fruit fly brain, because in glial cells it is utilized for lactate and alanine production in

order to supply energy to neurons (Volkenhoff et al. 2015). In the hemolymph, its level is a hundred-fold greater than glucose (Liguori, Mascolo, and Vernì 2021). Trehalose, unlike other reducing sugars like glucose or maltose, is not easily oxidized and reduced (Benaroudj and Goldberg 2001). Because of its non-reducing structure, trehalose is stable in hemolymph, resistant chemical degradation and enzymatic reactions that can occur with reducing sugars. This stability provides that trehalose remains a consistent energy supply for the fruit fly, even under changing physiological circumstances. Due to its difficult oxidized property, trehalose can function as an antioxidant, removing free radicals and protecting cellular components from oxidative damage inside the hemolymph (Paithankar et al. 2018).

3.1.3. Protein metabolism

Proteins, as well as carbohydrates and lipids, can be catabolized to obtain energy. Amino acids are the building blocks of proteins; therefore, the body does not often utilize them as an energy source due to their structural and functional responsibilities. Protein metabolism comprises an average of ten percent of the total metabolism in fruit fly (Marron et al. 2003). Long-term starvation makes proteins energy source due to the fact that enough energy requirement cannot be maintained. Firstly, proteins are broken down by a process known as proteolysis. After that, amino acids are transformed into acetyl-CoA, pyruvate, or tricarboxylic acid cycle intermediates to produce adenosine triphosphate (ATP). In *Drosophila melanogaster*, the fat body is an essential region for protein synthesis and secretion, including storage proteins known as hexamerin. Hexamerins are oligomeric storage proteins usually consisting of six polypeptides that are produced in the fat body before being released into the hemolymph. They act as a reservoir of amino acids that may be used in situations when resources are low, such as during metamorphosis or starvation. In addition to their role in food storage, it has been determined that hexamerins also play a role in processes such as clot formation (Vlisidou and Wood 2015). Amino acids have a crucial role in regulating metabolism. The TOR signaling pathway accompanies for in regulation protein synthesis and development in *Drosophila*. In the controlling of translation in mammals, eukaryotic translation initiation

factor 4E-binding protein (4E-BP) and ribosomal protein S6 kinase 1 (S6K1) proteins are regulated by mammalian target of rapamycin complex I (mTORC1) (Yang et al. 2014). Activation of S6K and inhibition of 4EBP by mTORC1 enhance protein synthesis (Jacobs, George, and Kemppainen 2020). 4E-BP is encoded by Thor in *Drosophila* and regulates the translation, response to stress, host immune defense, and cell growth (Bernal and Kimbrell 2000). The AMP-activated protein kinase (AMPK) controls cellular metabolism. AMPK suppresses mTORC1 activity, resulting in decreased protein synthesis (Su et al. 2019).

3.2. Materials and Methods

3.2.1. Measurement of metabolic pools

Metabolic pools of protein, glucose, trehalose (fly blood sugar), glycogen, and lipid (triglyceride) were measured in adult flies (5-7 days old) of each DGRP strain (Figure 3.3). First of all, 300 µl of NP40 Substitute Assay Reagent (Cayman Chemical, USA) buffer solution was used to homogenize two adult flies from each strain. The mixture was then centrifuged in a cooled centrifuge at maximum speed for 10 minutes in 4°C, and the supernatant was taken into a clean microtube. 20 µL of homogenate was separated for protein assays, and the remaining solution was denatured for 15 minutes at 70°C for quantification of triglyceride, glucose, trehalose, and glycogen measurements. Metabolite determinations were made by taking samples from this solution. In each metabolic pool, measurements were made in duplicates using Multiskan GO Microplate spectrophotometer (Thermo Scientific, USA) and the average of the measurements was utilized in further analyses. The metabolites in each sample were quantified using standard curves and linear regression formulas, which were created by graphing absorbance against increasing concentrations of each metabolite. All of the measurements were standardized by the amount of protein in order to minimize the effects of sampled flies' size and possible homogenization differences on metabolite levels; after that they were expressed as milligrams of metabolite per milligrams of protein.

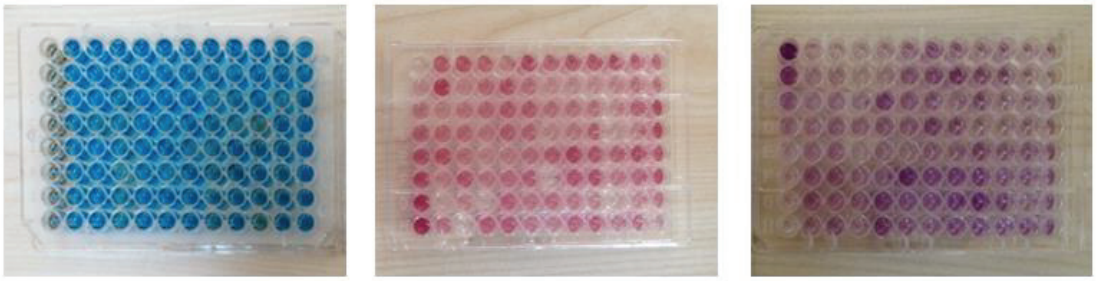

Figure 3. 3. Metabolic pool measurements with DGRP samples.

3.2.2. Protein Assay

Protein concentrations were measured based on the Bradford method as mentioned in the Protein Determination Kit (Cayman Chemical, USA) according to the manufacturer protocol. For the standard curve, 40 µg/ml intermediate stock was prepared by using 4 µl BSA standard (stock concentration of 10 mg/ml) diluting into 996 µl ddH_2O. By serial dilution from this intermediate stock, eight standards (range between 5.6 and 32.0 µg/ml) were prepared. 10 µl of fly homogenate was diluted into 450 µl ddH_20. 100 µl of each diluted fly homogenate and 100 µl of each prepared standard were loaded onto plate; they were incubated with 100 µl of Protein Determination Assay Reagent at room temperature for five minutes then measured in 595 nm.

3.2.3. Glucose, Glycogen and Trehalose Assays

Glucose Colorimetric Assay Kit (Cayman Chemical, USA) was used to determine glucose, trehalose, and glycogen levels. First of all, to identify glycogen, and trehalose levels in the samples, digestion into free glucose was done by utilizing enzymes. For glycogen determination, 15 µl of fly homogenate including 5 µl of 250 mM Na phosphate assay buffer (pH 7.2) was subjected to enzymatic digestion with 5 µl of amyloglucosidase (A1602 from Sigma, St. Louis) at 37°C for 1 hour. On the other hand, for the

determination of trehalose, 15 µl of fly homogenate samples including 5 µl of 250 mM Na phosphate assay buffer (pH 7.2) were enzymatically digested by 5 µl of trehalase (T8778 from Sigma, St. Louis) at 37°C for 24 hours. From each 100 mg/dl intermediate stock, glycogen (Cayman 700481) and trehalose (Cayman 20517) standards were prepared at different concentrations (obtained by eight serial dilutions), and they were subjected to enzymatic digestion process together with the samples. Glucose standard curve (range between 2.5 and 25.0 µg/ml) was utilized to determination of glucose levels in samples. To perform assay, 85 µl of Assay Buffer was added into 15 µl of sample and to initiate the reaction, 100 µl of enzyme mixture was used. After incubation at 37°C for 10 minutes, plates were measured in 500-520 nm. Afterwards, the absorbance of glucose in the untreated samples was subtracted from the absorbance of samples diluted with amyloglucosidase and trehalase, glycogen and trehalose levels were calculated respectively by utilizing glycogen and trehalose standard curves.

3.2.4. Triglyceride Assay

Triglyceride was measured with commercially available Triglyceride Colorimetric Assay Kit (Cayman Chemical, USA). 10 µl of fly homogenate denatured at 70°C for 15 minutes, was mixed with 150 µl of Triglyceride Enzyme Mixture, then incubated at 37°C for 30 min. Absorbance was measured in 530-550 nm. For triglyceride standard curve, firstly 200 mg/dl intermediate stock was prepared by adding 400 µl NP40 Standard Substitute Assay Reagent to 100 µl Triglyceride stock standard. By serial dilutions from this solution, standards were prepared at a range of concentration between 3.13 and 200 mg/dl.

3.3. Results

In adult flies (5-7 days old) of each DGRP strain, the metabolic pools of protein, glucose, trehalose, glycogen, and lipid (triglyceride) were measured. To reduce the

impact of flies' size and possible homogenization variations on metabolite levels, the amounts of triglycerides, glycogen, trehalose, and glucose in the samples were normalized to the amount of protein measured for each sample. As seen in the Figure 3.4, differences were observed in triglyceride, glycogen, trehalose, and glucose levels in DGRP samples. The mean (± SD) and median (25%, 75% quartiles) glucose measurements were 0.19 (± 0.12) and 0.18 (0.12, 0.23). For glycogen, trehalose, and triglycerides, these measurements were observed 0.36 (± 0.44) and 0.20 (0.09, 0.47), 0.03 (± 0.03) and 0.02 (0.01, 0.04), 1.03 (± 0.65) and 0.84 (0.59, 1.33), respectively.

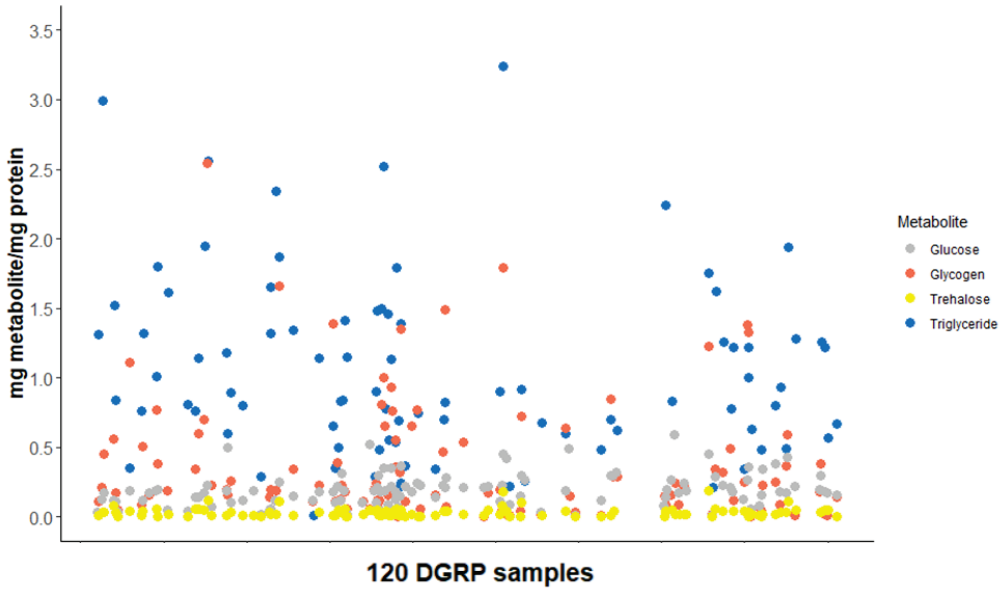

Figure 3. 4. Comparison of glucose, glycogen, trehalose, and triglyceride levels in the DGRP samples.

The largest metabolic pool of *Drosophila* was triglyceride, followed by glycogen (Figure 3.5). The Kruskal Wallis rank-sum test was utilized to evaluate host metabolism differences in 120 DGRP samples and statistically significant difference was observed among samples in accordance with metabolic pools (p-value < 2.2e-16). The pairwise comparisons conducted with Wilcoxon rank-sum test revealed three statistically significant results. First of all, glycogen, and triglyceride; then glycogen and trehalose;

and finally, trehalose and triglyceride were significantly different from each other (Wilcoxon rank-sum, Ps < 2.2e-16).

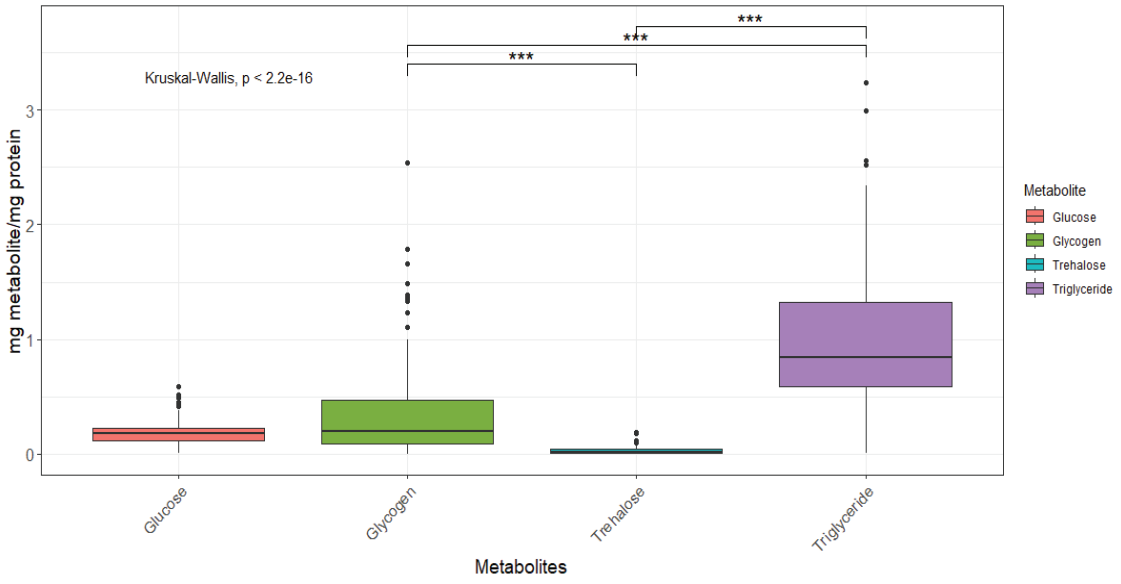

Figure 3. 5. Boxplot comparison of the triglyceride, trehalose, glycogen, and glucose metabolic pools between DGRP samples.

The Shapiro-Wilk test is a statistical method used to determine whether dataset follows a normal distribution. As shown in Figure 3.6, the results revealed significant deviations from the normal distribution for each metabolite (Shapiro–Wilk test Ps < 0.001). The bestNormalize is a R package that aims to determine the most appropriate normalization approach for non-normal data (Peterson 2021). It aids normalization of data distributions, which is required for many statistical analyses. Our data was transformed by automatically chosen appropriate data transformation methods to achieve normality utilizing bestNormalize package and for further analyses, normalized measurements were used.

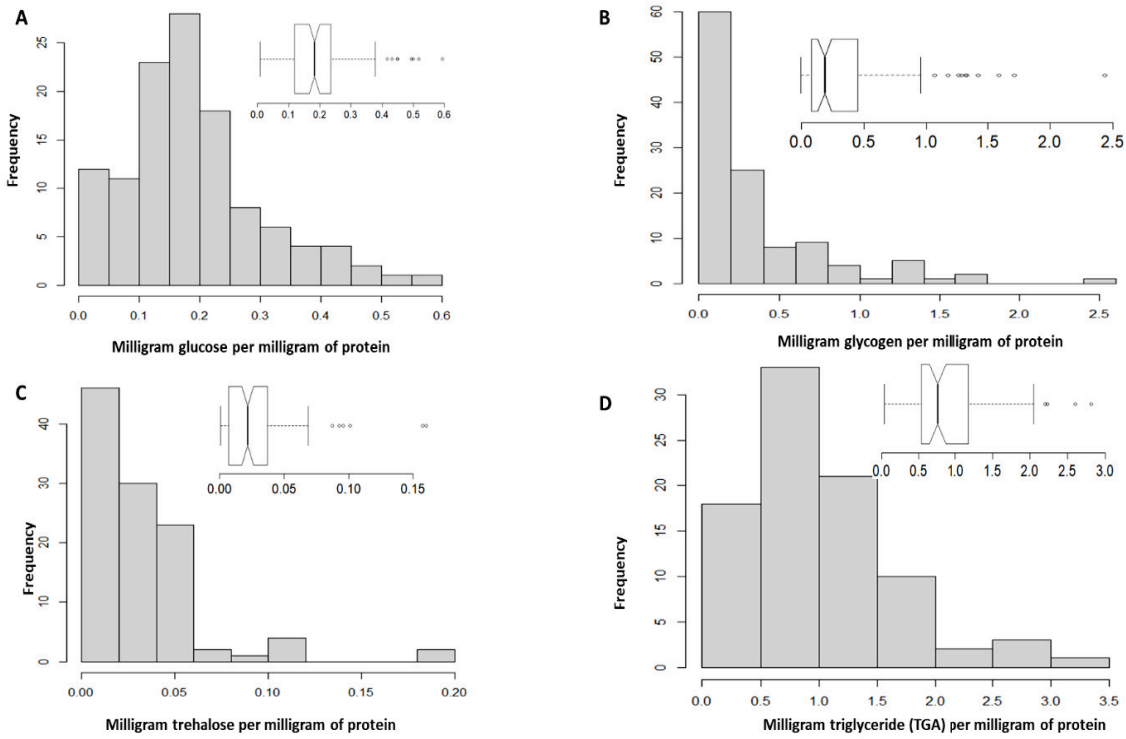

Figure 3. 6. Histograms and horizontal box plots of glucose (A), glycogen (B), trehalose (C), and triglyceride (D) measurements.

3.4. Conclusion

This chapter provided interesting revelations into the metabolic dynamics influenced by genetic variants come from each DGRP line. Studies have highlighted the impact of age, gender, and genotype on metabolic profiles in *Drosophila*, showing that genetic background can significantly affect metabolic characteristics such as lipid and carbohydrate metabolisms (Hoffman et al. 2014). Furthermore, using a systems biology approach, researchers discovered how nutrition interacts with genetic factors to influence lifespan in DGRP strains (Jin et al. 2020). Such findings imply that environmental factors, as well as genetic background, could have a role in the metabolic variations found in the samples. The observed differences in metabolic pool levels among our strains indicate not only the genetic diversity in the DGRP samples, but also the complex relationship between genetics and metabolism.

CHAPTER 4

ASSESSMENT OF THE RELATIONSHIPS BETWEEN INTESTINAL MICROBIOTA AND HOST METABOLISM

4.1. Introduction

Excessive immune response in both flies and mammals can cause dysregulation of metabolic storage, while a loss of metabolic homeostasis can impair the immune system. The primarily defense mechanism of fruit flies against pathogens is innate immune system, because they lack adaptive immunity. There is significant conservation of this innate immune response among DM and mammals. The components of *Drosophila*'s innate immunity include phagocytosis performed by hemocytes, melanization upon injury, and AMP synthesis (Hoffmann 2003). In response to immunological signals or infection, the host metabolism changes. During infection, the fruit fly's immunological and metabolic systems must work together harmoniously. Activating the immune system in response to infection triggers several high-energy processes, including phagocytosis, and cell proliferation (Bland 2023). Peptide hormones or cytokines secreted into the body play a dual role in immune response and nutritional balance management. The fly's reactivity to microbial threats is influenced by the important interplay between host metabolism and the immune system. Additionally, the metabolic function of *Drosophila* gut bacteria is dynamic and sensitive to dietary modulation. For example, *Wolbachia* infection in *Drosophila* has been shown to indicate higher lipid, triglyceride and glucose levels compared to control samples. Also, the expression level of the *Tps1* gene responsible for trehalose synthesis has been shown to be reduced in the infected samples (Karpova et al. 2023). When encountering PAMPs, Toll and Imd signaling pathways are triggered for neutralization and elimination in cellular and humoral immune processes. In DM, the immune response to gram-positive bacteria and fungi is mediated through the Toll system. Activation of the fat body Toll

signaling pathway in *D. melanogaster* causes the shift of fatty acids from nutrient storage to membrane phospholipid synthesis by decreasing the expression of diacylglycerol acyltransferase (DGAT), an enzyme that catalyzes the last step of triglyceride synthesis. This is disadvantageous as it leads to a reduction in stored energy required for metamorphosis and early adult life. It has been shown that flies with active Toll pathways enter the pupa stage with 50% lower triglyceride levels (Martínez et al. 2020). Since adequate triglyceride levels are not provided, it may lead to long-term deteriorations in the completion process of metamorphosis and stress resistance. Various AMPs are secreted simultaneously during infection, which implies that a large number of proteins are created during the immune response. However, some pathogens can weaken host defenses during infection by preventing the synthesis of essential proteins. Consequently, activated Toll and Imd signaling pathways also cause significant alterations in host lipid, carbohydrate, and protein metabolism. The immune response can also be regulated by dietary sugar alteration. It has been demonstrated that a high-glucose diet induces the expression of cell junction components that make up the intestinal epithelial barrier and increases basal AMP expression (Galenza and Foley 2021).

The first research to demonstrate the metabolic effects of infection in *Drosophila* indicated that *Mycobacterium marinum* infection led to decreased fat and glycogen storage as well as formation hyperglycemia in the fruit flies (Dionne et al. 2006). Stimulator of interferon genes (*STING*) is a kind of transmembrane protein involved in innate immunity by recognizing cytosolic DNA and triggering immune responses against pathogens. *STING* initiates a signaling cascade that leads to the production of type I interferons (IFNs) and other proinflammatory cytokines when activated by cytosolic DNA. *CG1667* (*Sting*) in *Drosophila* is the homologous of the mammalian *STING*. In the fruit fly, the fat body not only stores nutrients but also acts as an immunological regulator by producing AMPs during infection. Flies lacking *CG1667* have indicated a significant reduction in crucial storage metabolites such as TAG, trehalose, and glycogen, as well as a decrease in AMP expression (Akhmetova, Balasov, and Chesnokov 2021). Unlike pathogens, commensal bacteria in the gut promote nutritional symbiosis by utilizing nutrients in the lumen and assisting in nutrient uptake for the host. For instance, *Acetobacter pomorum* can induce the insulin signaling pathway throughout the host by releasing acetic acid from the lumen of the midgut, hereby it provides development and also causes reduction in lipid and sugar levels (Shin et al. 2011). Experimentally removing microbiota from the flies has resulted in hyperglycemia and hyperlipidemia (Wong et al.,

2014). The two main bacterial families in *Drosophila*, *Acetobacteraceae* and *Lactobacillales*, are able to utilize glucose. Thus, it may be assumed that lack of metabolic activity of these species is the cause of the high fat and carbohydrate levels seen in axenic flies. In fact, the triglyceride level in gnotobiotic flies generated with different microbiota contents, particularly harboring *Lactobacillus* and *Acetobacter* species together, has been observed to reach similar levels with flies in control group. When the individual impacts of the species were evaluated, it was also indicated a notable negative correlation among the abundance of *Acetobacter* and the triglyceride level, due to the biggest decrease seen in flies containing *Acetobacter* species (Newell and Douglas 2014). Myocyte enhancer factor 2 (Mef2), a transcription factor, mediates the transition between metabolism and the immune system in the fat body. S6K phosphorylates Mef2 to promote glycogen and lipid synthesis. However, during gram-negative bacterial infection, dephosphorylated Mef2 results in decreased triglyceride and glycogen production and a weakened immune system (Clark et al. 2013). Based on these findings, we can say that *D. melanogaster* is a perfect model organism for investigations aimed at maximizing metabolic health or reducing the likelihood of diseases like obesity by studying the impact of each microorganism on metabolism.

4.2. Results and Discussion

In this chapter, analyses were performed to assess the associations of intestinal microbiota members both within themselves and with the host metabolism. The first of these analyses was Principal Components Analysis (PCA). Principal Component Analysis is a widely used multivariate statistical technique. PCA allows researchers to understand the structure of data, detect outliers, and visualize complex datasets by identifying relationships among variables. By converting the data into principal components, it can reveal how different variables (specific microorganism or metabolic pools) contribute to similarities and differences between samples. This knowledge is important for understanding how certain microbial communities may interact with metabolic features. Moreover, clusters generated in PCA plots may indicate that different

metabolic profiles or microbiota members are related with particular species, thereby provide focus on hypotheses with respect to microbial impacts on host metabolism.

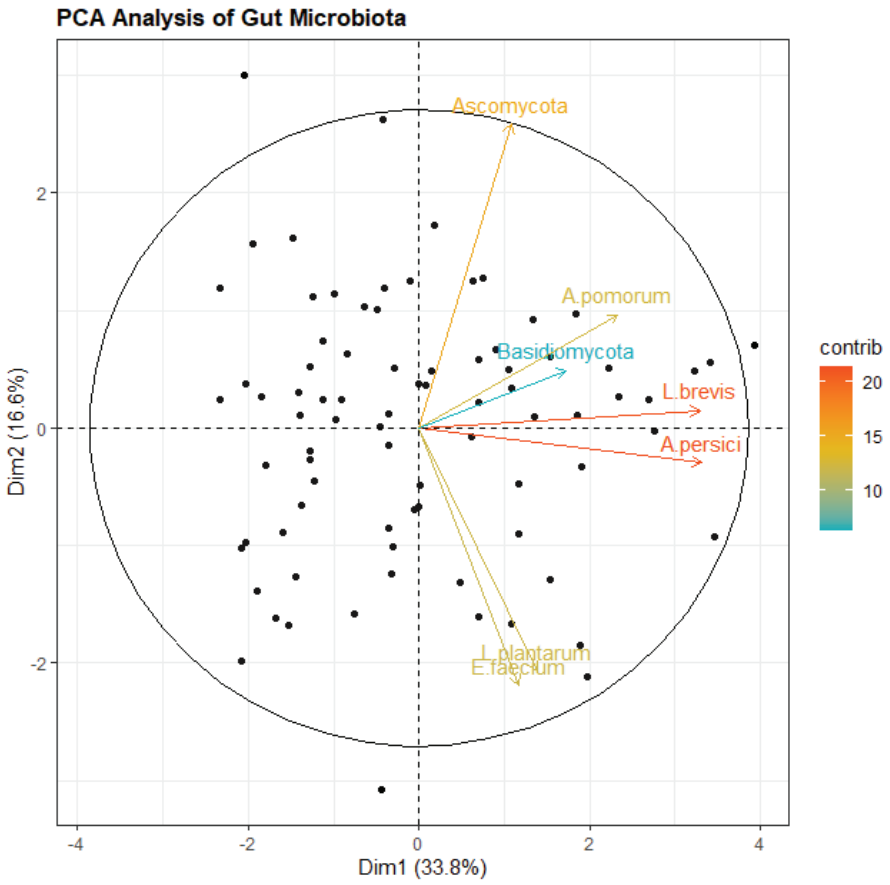

Figure 4. 1. Principal component analysis of the gut microbiota composition in DGRP samples.

To normalize and assumption of the normality of the data, bestNormalize package and Shapiro–Wilk test was utilized in R software, respectively. R packages named with FactoMineR and factoextra, were utilized to perform PCA analysis. The first PCA analysis was performed to investigate the relationships between gut microbiota composition (Figure 4.1). Dim1 (Component1) explained 33.8% of the data, whereas Dim2 (Component2) covered 16.6%. Dim1 appeared as the component that constitutes an important portion of the dataset variability with the largest contribution of the *A. persici* and *L. brevis* respectively. Since they point in almost the same direction, it is

possible that there may be an important positive association between these two microorganisms. The direction and length of the arrows on the figure, which represent different microbial taxa, show how much impact they have on the main components. Notably, the arrows of taxa like *A. pomorum*, *A. persici*, *L. brevis,* and Basidiomycota point in the same direction, suggesting a positive relationship between each of these taxa. This implies that if the abundance of Basidiomycota increases, the abundance of *A. pomorum*, *A. persici*, *L. brevis* may also increase. These results, depicted in Figure 4.1, highlight potential interactions within the gut microbiota, emphasizing that some fungal flora may positively affect bacterial populations or vice versa.

The subsequent PCA analysis was performed with metabolite data (Figure 4.2). According to the results of the PCA analysis, the first component (Dim1) explained the majority of the data (63.2%) so was the most contribution, while Dim2 explained 19.9% of the data. Because triglyceride, trehalose, and glycogen are all storage metabolites, their positioning according to Dim1 demonstrates positive correlations among them.

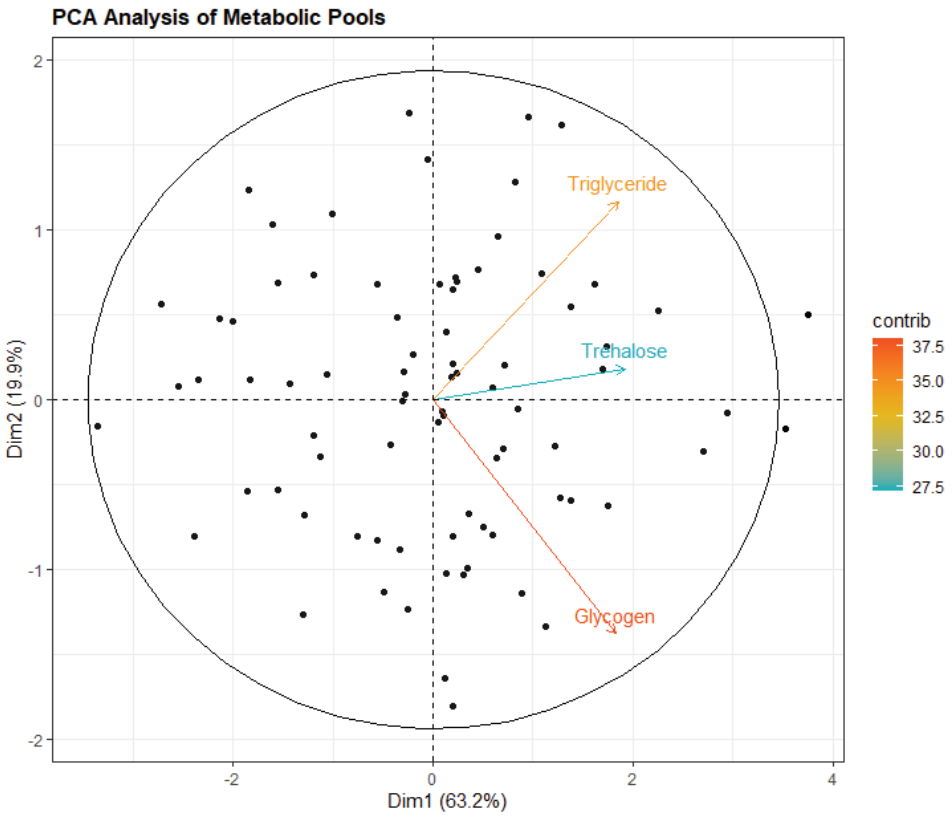

Figure 4. 2. Principal component analysis of the metabolic pools in DGRP samples.

The final PCA analysis was performed to investigate potential relationships and patterns that could be exist between microbiota and metabolism (Figure 4.3). The x-axis represents the Dim1, explained 31% of the data with the most contribution of the *A. pomorum* and *L. brevis* respectively. While Dim2 represented 17.9% of the data. Metabolites clearly separated from the microbiota and grouped closely. Thus, there is a negative association shown by the right-pointing arrows for the microbiota and the left-pointing arrows for the metabolic pools. One could say that a reduction in the amounts of storage metabolites is associated with an increase in the abundance of gut microbiota.

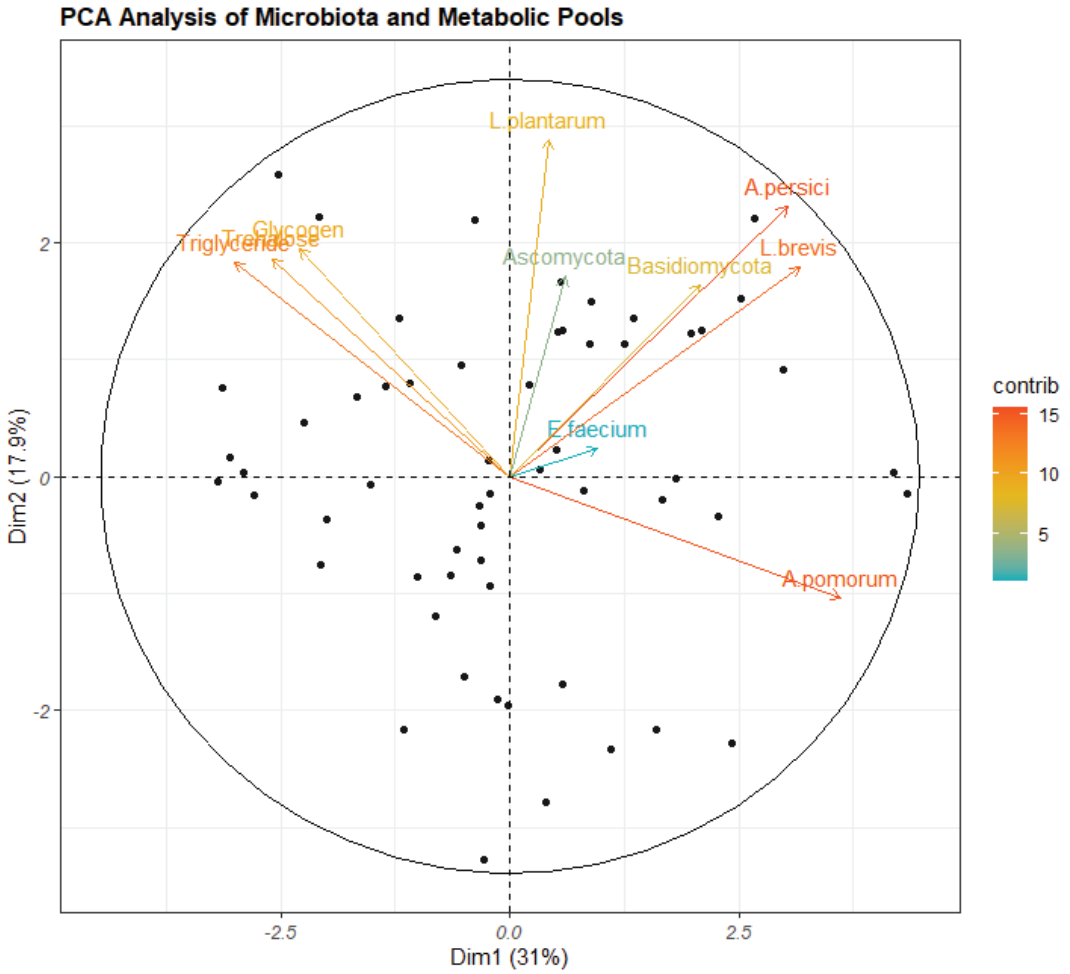

Figure 4. 3. Principal component analysis of between microbiota and metabolic pools in DGRP samples.

After discovering patterns through PCA, the next step was to assess the strength of the correlations between variables. Correlation analysis is useful to assess the correlations between different microbial members or microbiota abundance and host metabolism. It gives knowledge about the dynamics of dataset by assessing the degree of relationship among variables. Correlation analysis not only determines how strongly two variables are related, but it also examines the statistically significance of that association. This aids in separating significant relationships from coincidentally occur ones. There are several methods for performing correlation analysis. The Pearson correlation coefficient is often used to determine linear correlations between variables. On the other hand, Kendall tau and Spearman are utilized for non-parametric data or where normality assumptions do not fit.

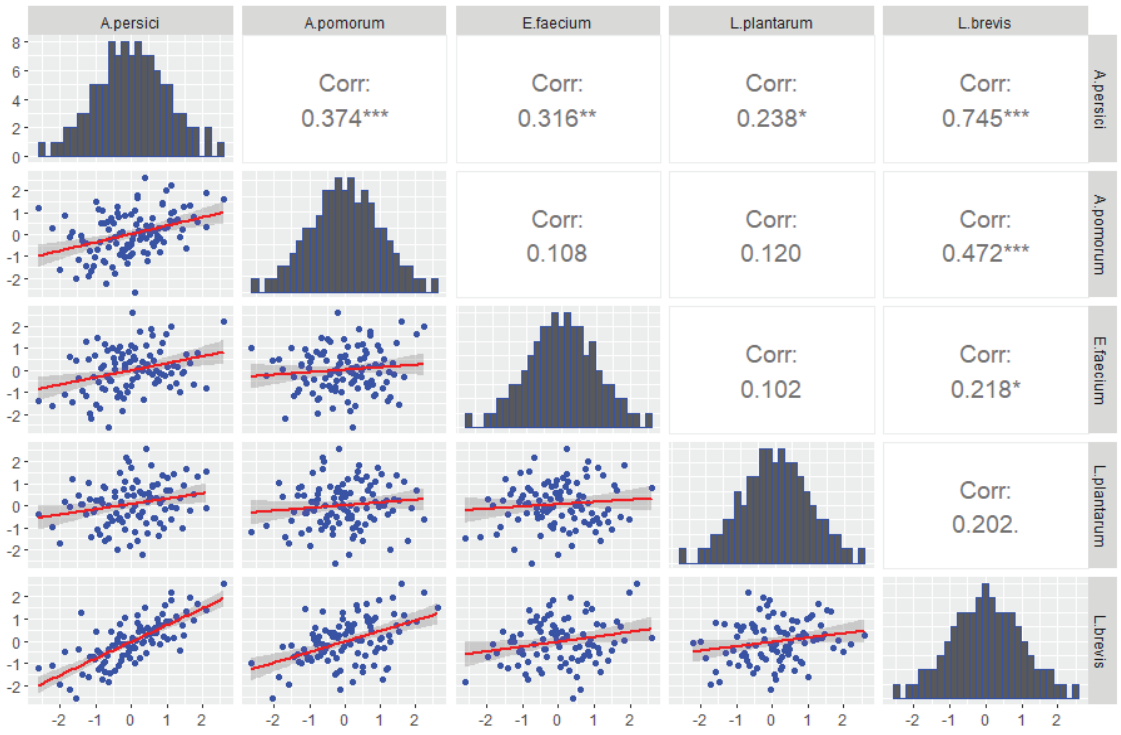

Figure 4. 4. Scatter plot of correlation matrix between bacterial species in *Drosophila.*

For investigation of the relationship among fungal and bacterial species, and metabolite variables, Pearson correlation coefficient was used. The Pearson correlation coefficient is a metric with a range of -1 to +1. A coefficient close to +1 or -1 indicates a

significant positive or negative association, respectively, whereas a value close to 0 implies no linear correlation. For each DGRP strain, firstly the presence of relationship between *L. plantarum*, *A. pomorum, E. faecium, L. brevis* and *A. persici*, which were screened with Real-Time PCR analysis, were determined by Pearson correlation coefficient. All statistical analyzes were performed utilizing R software (Version 4.3.1) (https://www.r-project.org). The GGally package in R was used to visualize multiple correlation matrices. Based on t-test statistics, representation of statistically significance was displayed as *** $p< 0.001$, ** $p< 0.01$, * $p< 0.05$, · $0.1<p< 0.05$. As seen in Figure 4.4, *A. persici* was positively correlated with all other bacterial species (r: 0.238-0.745, $Ps < 0.05$), while *L. brevis* displayed positive correlation with *A. pomorum* (r: 0.472, $p< 0.001$) and *E. faecium* (r: 0.218, $p< 0.05$). The strongest correlation was seen among *A. persici* and *L. brevis* species.

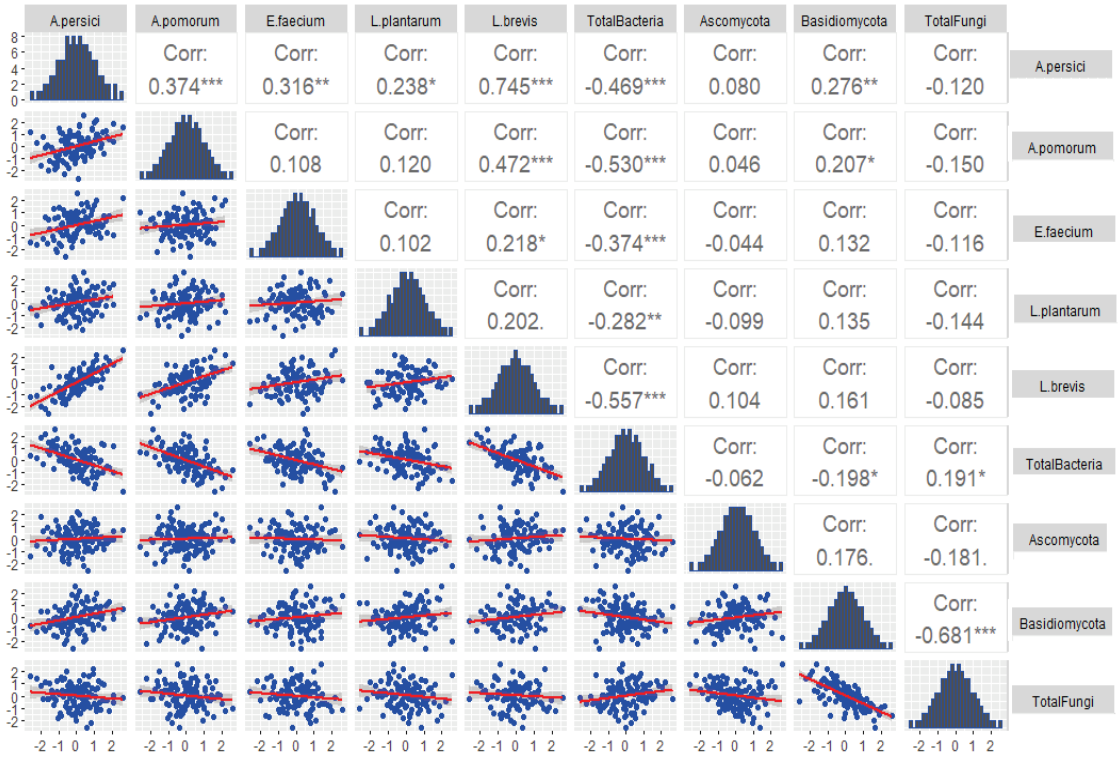

Figure 4. 5. Scatter plot of correlation matrix between bacterial and fungal taxa.

Next correlation analysis was performed among both the bacterial and fungal microbiota (Figure 4.5). Positive correlations were found among Basidiomycota and *Acetobacter persici* (r: 0.276, $p < 0.01$) and *Acetobacter pomorum* (r: 0.207, $p < 0.05$) when the relationships between fungal and bacterial taxa were examined. Additionally, there was a weak positive association found among the overall amounts of bacteria and fungus (r: 0.191, $p < 0.05$). Despite representing the most abundant phylum in mycobiota, Ascomycota did not show any statistically significant relationships with other microbiota members.

The combination of PCA and pairwise correlation analysis provides an effective basis for analyzing complicated interactions within the gut ecosystems. While PCA provides a general picture of the data structure and highlights taxa that contribute the most to variation throughout the dataset, the correlation matrix reveals detailed pairwise interactions as positive or negative that can be critical for identifying particular connections within the microbiota. The positive interaction between *Acetobacter* and *Lactobacillus* species might base on the providing of *Lactobacillus*-derived nutrients to *Acetobacter*. Specifically, the strong correlation seen between *A. persici,* and *L. brevis* might be attributable to their possible synergistic activities in the gut environment. In *Drosophila*, similar interactions like increased AMP expression, and enhanced ISC proliferation or improved nutrition absorption have been noted for *A. persici* and *L. brevis* respectively (Han et al., 2021; Onuma et al., 2023). Sommer and Newell have described a beneficial interaction between *Acetobacter fabarum* and *L. brevis*, facilitated by the exchange of metabolites in *Drosophila* (Sommer and Newell 2019). The *ppdK* gene in *A. fabarum* played an important role in the utilization of fermentation products of *L. brevis* as a carbon source. Moreover, mutations in this gene have been led to reduced bacterial density in co-colonizing flies, further emphasizing its importance. Actually, the interactions between bacteria can be species-specific, such as mutualistic relationships of *Acetobacter* with *L. brevis* or antagonistic with *L. fructivorans*, suggesting that species differences have a substantial influence on host. Additionally, positive relationships between Basidiomycota and bacteria such as *A. persici* and *A. pomorum* suggest symbiotic partnerships, which may contribute to resilience and stability of the gut microbiota. These interactions may help to break down complex carbohydrates while also influencing the host immune system (Sam, Chang, and Chai 2017).

Following the microbiota-based correlation analyses, correlations between the metabolic pools were examined (Figure 4.6). A positive association was revealed for triglyceride, trehalose, and glycogen metabolite levels. Flies with higher triglyceride levels also had higher glycogen and trehalose levels, and conversely (r: 0.412-0.480, Ps < 0.001).

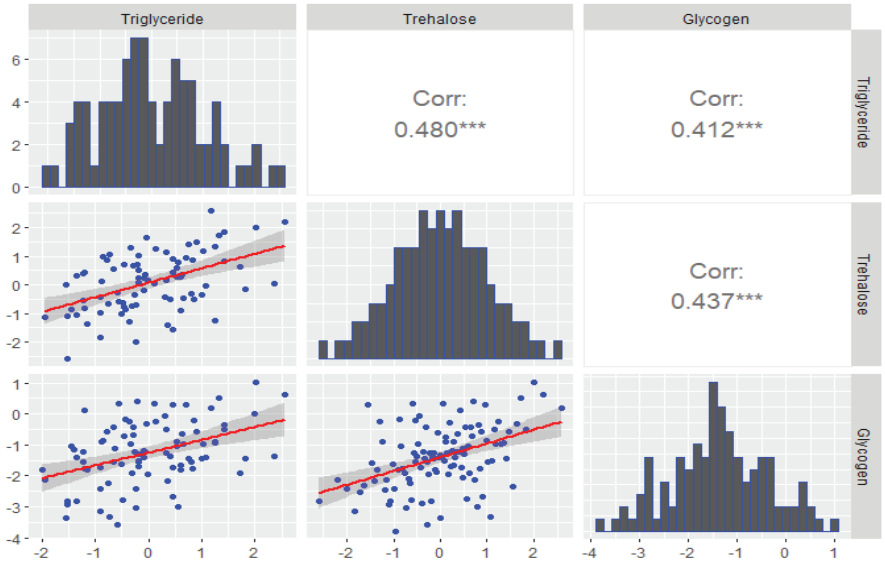

Figure 4. 6. Scatter plot of correlation matrix between metabolic pools.

The final correlation analyzes were done to reveal relationships among microbiota and metabolic pools. All of the microbial members indicated negative correlation with metabolites. Triglyceride level was negatively correlated with *A. persici* (r: −0.27, p< 0.05), *A. pomorum* (r: −0.72, p< 0.001), Basidiomycota (r: −0.22, p< 0.05), and *L. brevis* (r: −0.29, p< 0.01) abundance (Figure 4.7 A-D). Besides this, there was no notable association between Basidiomycota and other metabolites. In addition, trehalose negatively correlated with only *A. pomorum* (r: −0.33, p< 0.001) (Figure 4.7 E). The last metabolic pool, glycogen was shown a negative correlation with *A. pomorum* (r: −0.35, p< 0.001), and *E. faecium* (r: −0.33, p< 0.001) (Figure 4.7F and G). In addition, there was no significant association between Ascomycota abundance and any of the metabolites.

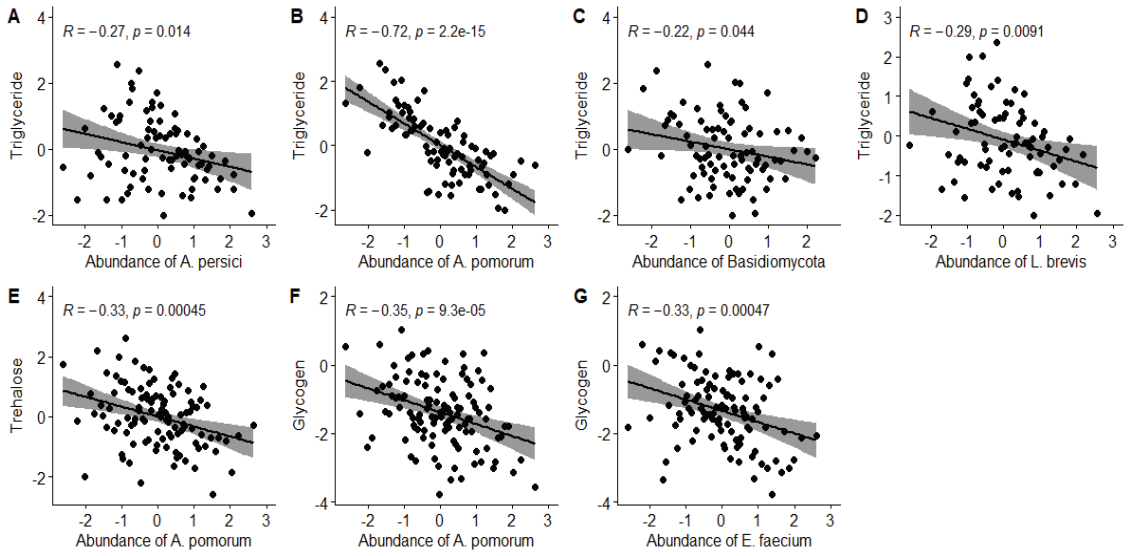

Figure 4. 7. Scatter plots of evaluated relationships between microbiota and metabolic pools.

Studies demonstrate that microbial interactions not only influence survival and proliferation but also have a significant impact on the host's metabolism, immune function, and overall health. Microbiota-host interactions contribute to key physiological functions including energy homeostasis, glucose regulation, and lipid metabolism. Especially members of *Acetobacteraceae*, *Lactobacillales*, and *Gammaproteobacteria* can contribute to the host's vitamin B synthesis and reduce energy storage as triglyceride and glycogen. In axenic flies or those experiencing dysbiosis, high glucose and triglyceride levels and reduced basal metabolism have been encountered. *Acetobacter* species (*A. pomorum*, *A. tropicalis*) alone are sufficient to regulate glucose levels. However, to control triglyceride metabolism, both *Acetobacter* and *Lactobacillus* species (*L. brevis*, *L. fructivorans*, *L. plantarum*) are required (Newell and Douglas 2014). Harmoniously with the literature, we observed a negative correlation between relative abundance of *Acetobacter* species (*A. persici, A. pomorum*) and triglyceride level. *L. brevis* symbiotically related with both *A. pomorum* and *A. persici*, also showed a negative correlation on triglyceride level. Acetate, a short-chain fatty acid produced by *A. pomorum*, stimulates the insulin pathway in *D. melanogaster*, thereby increases host growth and reduces sugar level (Shin et al. 2011). In a study investigating the probiotic and anti-diabetic effects of *E. faecium* on *Drosophila*, supplementation with *E. faecium*

has decreased the adverse effects of a high-fat diet in a *Drosophila* type-2 diabetes model. This was achieved by reducing the overexpression of insulin-like genes (Bhanja et al. 2022). The negative correlation of *E. faecium* with the glycogen level in our samples and the demonstration of its anti-diabetic effect on *Drosophila* in the literature provide a hypothesis for testing the effects of this bacteria on host metabolism in further studies. Even though we indicated significant positive relationships between Basidiomycota and the abundances of *A. pomorum* and *A. persici*, but not with *L. brevis*; all four had a significant reduction impact on triglyceride level.

4.3. Conclusion

Physical interactions, such as cell-cell attachment, and chemical interactions, such as the production of small molecules, between fungi and bacterial microbiota can affect intestinal homeostasis. The diversity of these interactions, which range from synergistic to antagonistic, is critical in determining the microbial community structure, regulating metabolic pathways, and altering the host immune response. Synergistic interactions among microorganisms often result in higher nutrient synthesis and absorption, thereby create a more efficient metabolic network. For example, certain bacteria may produce vitamins and other essential nutrients, therefore improve overall nutritional status and metabolic health. However, not all interactions in the gut microbiota are beneficial. Some relationships are antagonistic, in which one microorganism prevents the growth or activity of another. Such antagonistic interactions are essential for preventing pathogen overgrowth and ensuring a healthy microbial population. Furthermore, the dynamic interactions among intestinal microorganisms contribute to their durability and stability, allowing them to adapt efficiently to environmental changes. The gut microbiota is a highly adaptable ecosystem that may respond to changes in nutrition or other environmental variables. As an example, a high-fiber diet can encourage the growth of beneficial bacteria for production of short-chain fatty acids, which are essential for colon health and metabolic balance. Anomalies in the gut microbiota, known as dysbiosis, are associated with metabolic diseases, even gastrointestinal health conditions. Probiotics and prebiotics are frequently used to promote the growth of advantageous bacteria and restore

a healthy microbial balance. Therefore, understanding the complex relationships between fungus and bacterial microbiota is critical for designing targeted treatments to improve gut health.

CHAPTER 5

GENOME-WIDE ASSOCIATION (GWAS) AND GENE ONTOLOGY (GO) ENRICHMENT ANALYSES

5.1. Introduction

Genome-wide association studies (GWAS) are a research approach that aim to identify genetic variations associated with specific traits or diseases. It comprehensively scans the sample genome to detect single nucleotide polymorphisms (SNPs) that statistically correlated with a particular phenotypic characteristic (Begum et al. 2012). Genetic markers discovered by GWAS can be used to identify which genes are related with diseases and an individually risk of particular illnesses. The completion of the Human Genome Project in 2003, and the development of high-throughput genotyping technologies, provided key data for GWAS applications (Ku et al. 2010). The first notable achievement for GWAS was recorded in 2005 with the study of age-related macular degeneration, and then this technique swiftly applied to other fields of research (Klein et al. 2005). The International HapMap project, which was completed in two phases to verify the SNPs found by the Human Genome Project, expanded the usefulness of GWAS even further. The association test is the most commonly utilized statistical approach in GWAS. While performing multiple comparisons, methods like Bonferroni correction or false discovery rate (FDR) are used to adjust significance thresholds and minimize the probability of false positives (Kaler and Purcell 2019). Thus, the strongest ($p < 10^{-5}$) SNPs are obtained. The statistical model that was employed is as follows:

$$Y=\beta 0 + \beta 1X + (\beta 2Z) + \epsilon$$

Y is the dependent variable that has to be predicted or explained. Y represents the relative amount of each microbiota member. X is an independent variable that represents each genetic difference/variant (SNP) matrix on the genome. ϵ symbolizes model errors, while beta (β) symbols indicate regression coefficients for each variable. Z is another independent variable that can be included as an optional parameter, demonstrating variables such as age and gender. The main purpose of the model is to quantify the impact of each genetic variant in the X matrix on Y and determine whether this effect is statistically significant. This approach has also been effectively utilized to investigate the genetic basis of phenotypes with complicated genetic structure in the *Drosophila* model (Jumbo-Lucioni et al. 2010).

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	ID	MinorAllele	MajorAllele	RefAllele	MAF	MinorAlleleCount	MajorAlleleCount	SingleEff	SinglePval	SingleMixedPval	GeneAnnotation	RegulationAnnotation	
2	2R_6862081_	GTA	TAC	TAC	0,09434	10	96	9,561	3,84E-12	2,86E-13	SiteClass[|||],Tra	-	
3	3R_2413609	G	A	G	0,05172	6	110	10,42	2,57E-10	9,79E-10	SiteClass[|||],Tra	(TF_binding_site|mE1_	
4	2R_1389426_	A	G	G	0,06542	7	100	9,358	3,89E-10	5,01E-09	SiteClass[FBgn00(	(transposable_element	
5	2R_1908421	A	G	G	0,05357	6	106	9,637	1,06E-09	2,48E-08	SiteClass[FBgn00(	(TF_binding_site|BDTN	
6	2R_1908357	A	C	C	0,0885	10	103	7,549	1,44E-09	3,27E-08	SiteClass[FBgn00(	(TF_binding_site|BDTN	
7	2R_1383635_	G	C	C	0,1333	14	91	5,364	1,65E-09	9,40E-06	SiteClass[FBgn00(	-	
8	3R_6307438_	T	G	G	0,0531	6	107	10,44	2,32E-09	9,63E-10	SiteClass[|||],Tra	-	
9	3R_1230886	A	T	T	0,05357	6	106	10,44	2,77E-09	9,70E-10	SiteClass[FBgn00(	(TF_binding_site|mE1_	
10	3R_2091951	T	C	C	0,08929	10	102	7,508	2,93E-09	4,03E-08	SiteClass[|||],Tra	-	

Figure 5. 1. Contents of the Top Annot file obtained from GWAS analysis.

Following the GWAS, a Top Annot file is generated by selecting the SNP dataset with the highest significance (Figure 5.1). The “ID column” includes information about which chromosome the SNP is located on and its specific position on that chromosome. While “Minor and Major Allele” columns refer to the less and more prevalent variations found in the population, respectively. “Minor and Major Allele Counts” are the counts of how many times each minor and major allele appears in the sample population. “MAF (Minor Allele Frequency)” demonstrates how frequently the minor allele appears in the population. “SingleEff” is the estimated effect of a single SNP on the phenotype under study. “SinglePval” reflects the p-value associated with the SNP’s influence on the phenotype, whereas “SingleMixedPval” represents the p-value produced from a more comprehensive study using a mixed model. Mixed models are also account random effects (such as environmental variables). The “Gene Annotation” column lists the gene

names and codes associated with each SNP that have been listed in FlyBase database. The last column, titled “Regulation Annotation”, provides the regulatory region on the gene where the SNP is located and aids in understanding which regions SNP impacts.

Mackay and colleagues generated the Drosophila Genetic Reference Panel (DGRP), which consists of 192 inbred *Drosophila melanogaster* strains with completely sequenced genomes (Mackay et al. 2012). Nowadays, this panel consists of 205 strains. These strains with the same homozygous genotype make DGRP an ideal source for GWAS and other genetic analysis. DGRP is composed of inbred strains originating from a single population, so they are an excellent resource for GWAS due to their controlled genetic background, which allows for mapping of characteristics to genetic variants. One notable example is the identification of genetic modifiers of lifespan on a high sugar diet using DGRP strains. In this study, Patel and Talbert have conducted a GWAS for lifespan among 193 lines selected from DGRP fed a high sugar diet (Patel and Talbert, 2021). They have found significant lifespan-associated SNPs in regions of genes involved in behavior, development, etc. In another GWAS study using DGRP strains, resistance mechanisms to α-amanitin, a fungal toxin, were investigated and some genes associated with the TOR pathway were identified (Mitchell et al. 2017).

GWAS and candidate gene studies are two methodologies applied to identify genetic variants linked with specific characteristics or disorders. GWAS offers a comprehensive approach for investigating genetic impacts without assuming prior assumptions, whereas candidate gene studies give a more specific approach based on exist assumptions. While GWAS provides a greater possibility for finding unexpected genetic associations, candidate gene studies are limited to genes chosen based on prior information. Even though candidate gene studies can more specifically focused on essential biological pathways, they carry the risk of missing additional pathways that have not previously discussed.

5.2. Results and Discussion

GWAS analysis is a powerful approach for determining whether genetic variations are linked to particular situation. It can be used to find genetic variants linked to variations in the gut microbiota composition and host metabolism. For identification of genetic factors underlying the observed microbiota differences between DGRP strains, GWAS was performed by utilizing the relative abundance of each bacterial and fungal taxa that studied with qPCR. Drosophila Genetic Reference Panel (http://dgrp2.gnets.ncsu.edu/) and PLINK (Rentería et al., 2013) tools were preferred in order to performing GWAS.

First of all, genetic variants and their annotations correlated with the relative abundance of *A. persici, A. pomorum, E. faecium, L. brevis* and *L. plantarum* were determined by GWAS. More than 1.9 million variants in DGRP genomes were analyzed with GWAS analyses. In the Manhattan plot that P values were plotted throughout the chromosomes, the significant P values grouped together (Figure 5.2-5.6).

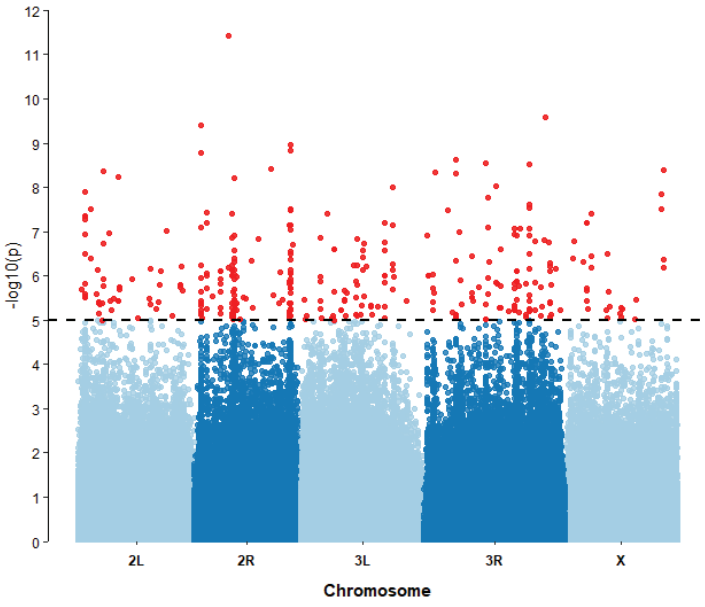

Figure 5. 2. Manhattan plot of the $\log_{10}$ inverse P-values from *A. persici* relative abundance. X-axis shows *Drosophila* chromosomes and its arms (L and R), Y-axis shows inverse $\log_{10}$ of the P-value.

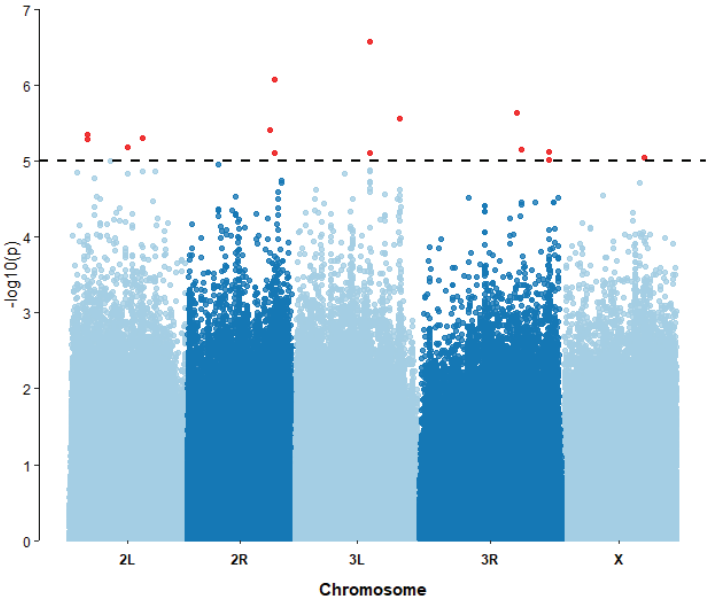

Figure 5. 3. Manhattan plot of the $\log_{10}$ inverse P-values from *A. pomorum* relative abundance.

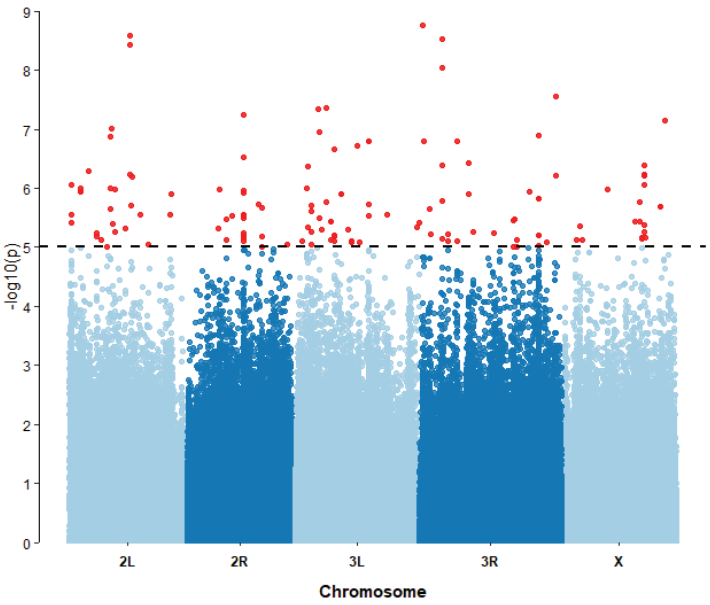

Figure 5. 4. Manhattan plot of the $\log_{10}$ inverse P-values from *E. faecium* relative abundance.

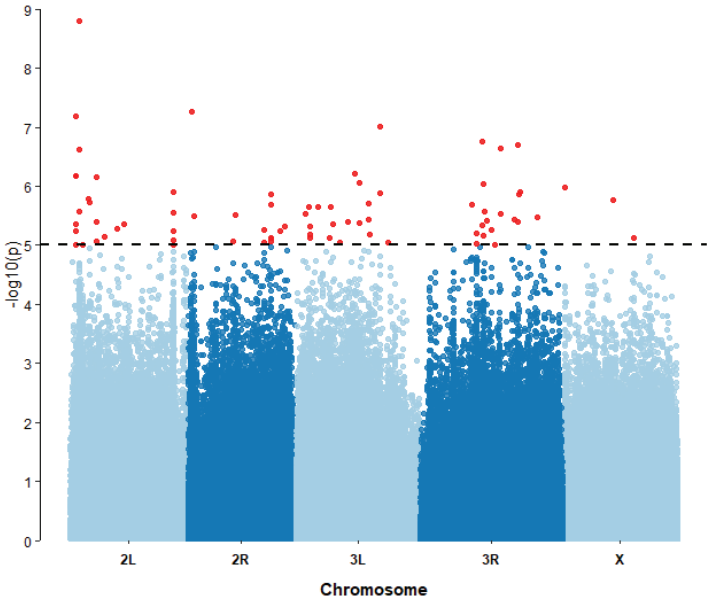

Figure 5. 5. Manhattan plot of the $\log_{10}$ inverse P-values from *L. brevis* relative abundance.

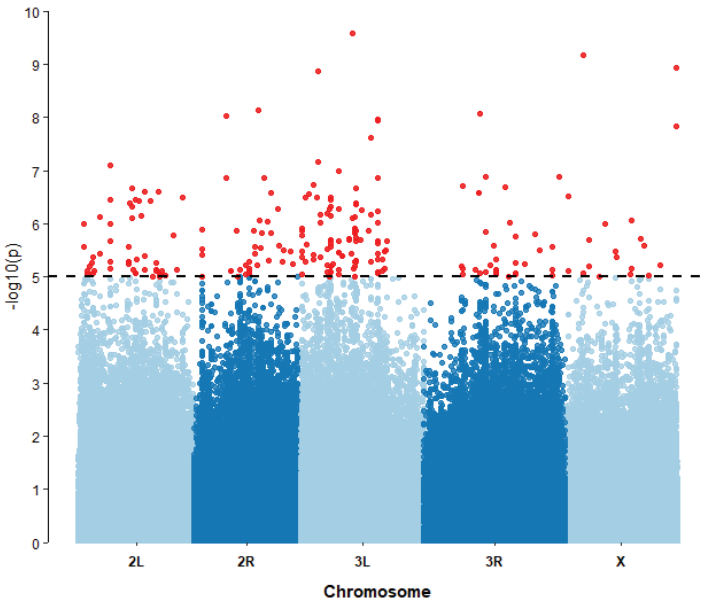

Figure 5. 6. Manhattan plot of the $\log_{10}$ inverse P-values from *L. plantarum* relative abundance.

Following the GWAS, all of the genes obtained from each bacterial species was searched in the FlyBase database (https://flybase.org/) (Larkin et al. 2021) and their functions were determined. Human orthologs of the genes were predicted by DRSC Integrative Ortholog Prediction Tool (DIOPT) tool (Hu et al. 2011). 391 statistically significant variants were obtained from 137 different genes for *A. persici*. From these genes, *Der-2, Tg, pyd, Uev1A, Myd88*, and *Pvf3* are related with gut homeostasis, defense, and immunity while *Pmp70* and *Pdk* are involve in host metabolism (Table 5.1). *Myd88* participates in the Toll pathway which responses to microbial infections in *Drosophila*, maintains intestinal homeostasis in humans. *MyD88*-mutant flies have been shown to be highly susceptible to infections of fungi and gram-positive bacteria (Tauszig-Delamasure et al. 2002). Alike *Drosophila*, *Myd88*-deficient individuals are responsive to pyogenic bacterial infections (Von Bernuth et al. 2008). Eighteen variants from nine genes obtained for *A. pomorum*. *RRAD* is human ortholog of *Rgk3* gene and it is related with type 2 diabetes mellitus in humans (Noreen et al. 2020). Another gene, *pes* is involved in mycobacterial infections, including *Mycobacterium fortuitum* and *Mycobacterium smegmatis* (Marshall and Dionne 2022). It was found 168 variants from 79 genes for the third bacteria, *E. faecium*. *bbg* and *Nost* genes are associated with immune system and epithelial cell differentiation. The *bbg* gene associated with *E. faecium* belongs to interleukin superfamily and it was shown a chronic inflammation of the midgut epithelium in *bbg*-mutant flies (Bonnay et al. 2013).

There were 301 variants obtained from 122 genes associated with *L. plantarum* in GWAS. The *CG33791* gene is involved in the tricarboxylic acid cycle (TCA). The *Drs* gene in the Toll pathway encodes antimicrobial peptides against fungi. In *Drosophila*, the response against gram-negative bacteria is generated by the Imd pathway. Although the *Drs* gene is participated in the Toll pathway, it has been observed that the *Drs* gene is also induced by Gram-negative peptidoglycan in Imd pathway genes mutant flies (Leulier et al. 2003). *Mesr4* gene has a role in innate immunity and regulates the fat storage of *Drosophila* by reducing the lipid accumulation. *CG17646* gene, human ortholog of *ABCG1*, is involved in regulation of triglyceride in *Drosophila*. The last gene associated with *L. plantarum* is *Hr96* and necessary to maintaining of lipid and cholesterol homeostasis.

Table 5. 1. Variants related with *Drosophila* gut microbiota and their mammalian orthologous.

Targeted bacteria	Chromosome	Base pair	Gene	P-value	Human ortholog
A. persici	3R	12308862	*Der-2*	9.70E-10	*DERL2*
	X	19647139	*Pmp70*	2.80E-09	*ABCD3*
	3R	4663467	*pyd*	1.99E-08	*TJP2*
	2L	8025865	*Tg*	2.09E-08	*TGM1*
	3L	5357819	*Uev1A*	2.75E-08	*UBE2V2*
	2R	5317318	*Pdk*	1.07E-06	*PDK3*
	2R	5193446	*Myd88*	1.13E-06	*MYD88*
	2L	7148467	*Pyf3*	9.79E-06	*PGF*
A. pomorum	3L	14681014	*ome*	2.52E-07	*FAP*
	2R	17153340	*Rgk3*	9.59E-07	*RRAD*
	2R	16390657	*Obp57c*	3.52E-06	-
	2L	14269190	*wb*	3.59E-06	*LAMA1*
	X	16163953	*kat80*	5.54E-06	*KATNB1*
	2L	7992403	*pes*	8,75E-06	*SCARB1*
E. faecium	3L	14478148	*bbg*	1.16E-07	*IL16*
	X	8945944	*Nost*	1.35E-06	*NOSTRIN*
	3R	19071557	*CG4467*	5.74E-06	*ERAP1*
L. plantarum	3L	1617211	*CG33791*	1.89E-07	*OGDH*
	3L	3369563	*Drs*	2.15E-07	-
	2R	13436026	*Mesr4*	3.32E-06	*ING4*
	2L	1733303	*CG17646*	6.09E-06	*ABCG1*
	3R	20852035	*Hr96*	9.96E-06	*NR1I2*
L. brevis	2L	1083181	*Atg4*	4.07E-08	*ATG4A*
	2R	16423159	*Bbd*	1.06E-06	-
	2L	3461783	*CG3246*	1.14E-06	-
	3R	19048115	*Nrf2*	4.77E-06	*NFE2L1*
	3L	8598338	*CG6282*	6.79E-06	-

The last GWAS analysis was performed for *L. brevis*. 93 variants associated with 50 genes were found related with *L. brevis*. The *Atg4* gene regulates autophagy by effecting autophagosome biogenesis. Like *Atg4*, *Bbd* gene contributes the host immunity by involved in antimicrobial humoral response. *CG3246* gene has a lipid binding activity. *CG6282* is an uncharacterized protein but its function is predicted to be related with lipid metabolic process. It has also been shown that the *Nrf2* gene creates an immune-independent response against lactobacilli (Jones et al. 2015).

Besides the GWAS, Gene Ontology (GO) enrichment analysis is a bioinformatic approach that involves the classification of genes under three categories according to their molecular function (defines basic activity of gene), cellular component (describe the active location of gene within the cell), and biological process (refers to multiple activities). Genetic variants (only P-value less than 10^{-5}) obtained GWAS were annotated by FlyBase database and PANTHER (http://pantherdb.org/) in terms of their molecular function, biological process, cellular component. The protein network interactions, GO annotations, biological pathway, and functional enrichment tests were evaluated using the STRING (https://string-db.org) web tool.

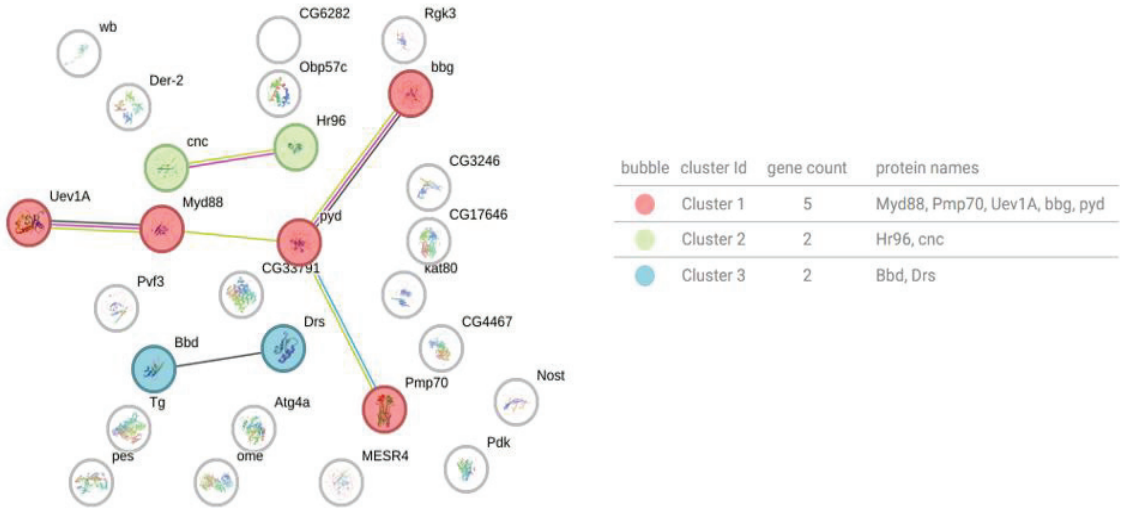

Figure 5. 7. Protein-protein interaction network of bacterial genes identified in GWAS.

According to the STRING analysis, three clusters were observed (Figure 5.7). In gene ontology analyses, utilizing all of the statistically significant genes in the GWAS, GO terms of the genes as molecular function, biological process, cellular component, and pathway were identified for five different bacteria by PANTHER and FlyBase databases (Figure 5.8). Binding (GO:0005488) and cellular process (GO:0009987) genes constituted the largest part in the molecular function and biological process respectively in all bacteria. In cellular component, membrane (GO:0016020) and intracellular anatomical structure (GO:0005622) shared most of the genes.

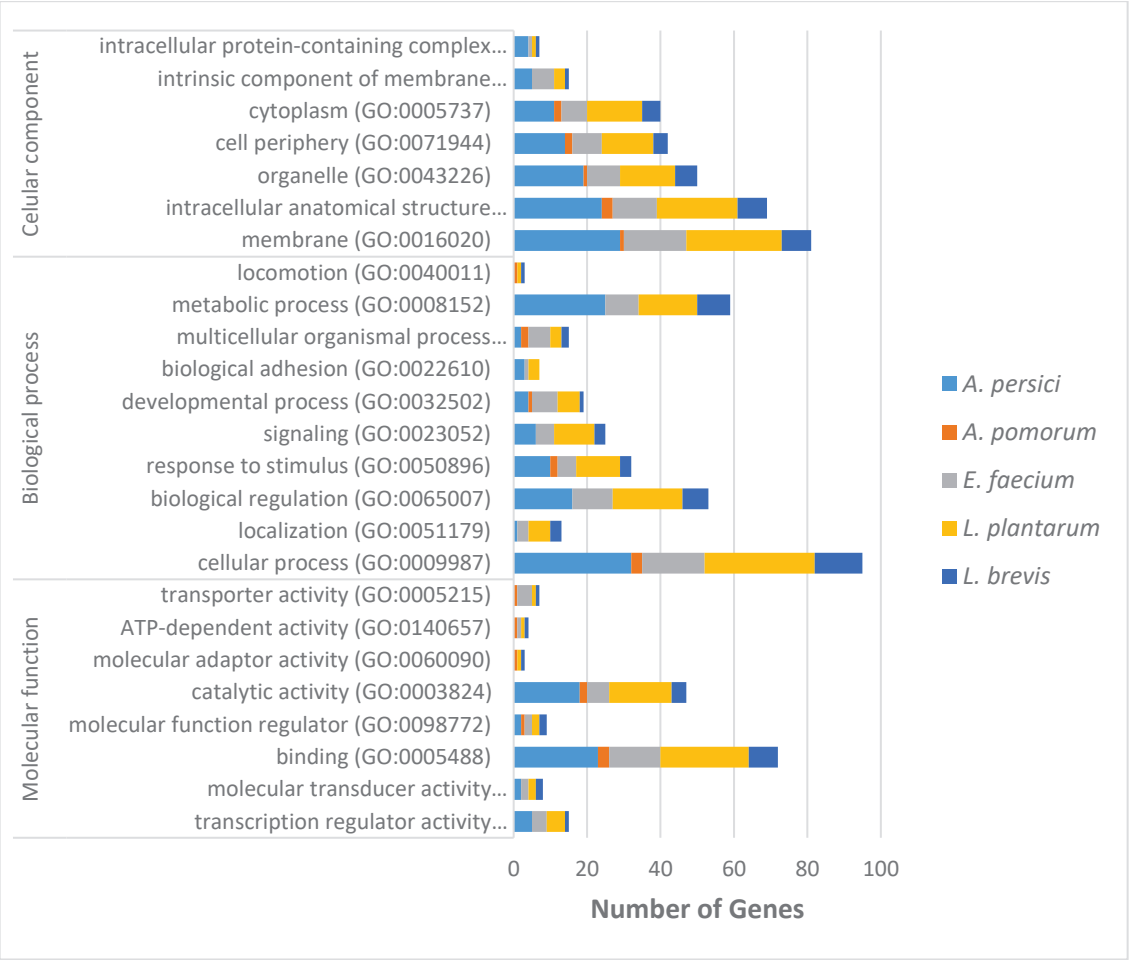

Figure 5. 8. Gene ontology analysis of bacterial species.

Following the investigation of bacteria's individual impacts, a STRING analysis was carried out using genes shown to be common among the five bacterial species (Figure 5.9). *A. persici* shared *CG32104*, *CG42339*, *SPR*, and *Octbeta1R* genes with *E. faecium*, but *cnc*, *CG42788*, *CG9899*, *mmp2*, and *rtnl1* genes with *L. brevis*. By regulating the immune system, particularly through immune-related genes like AMP, *cnc* can control lipid homeostasis (Karim et al., 2015). While the genes *CG42541*, *fru*, and *rols* were shared by *L. plantarum* and *A. persici*, *L. plantarum* and *E. faecium* shared the genes *kirre*, *lar*, *lola*, *msn*, *shi*, and *ship*. The *Drosophila* abdomen contains oenocytes secretory cells, which produce hydrocarbon molecules from long-chain fatty acids. These compounds are essential for courting behavior and resistance to desiccation. In these cells, *fru* helps to maintain lipid homeostasis (Sun et al. 2023). *L. plantarum* and *L. brevis* only shared the *CG4629* gene. *Lola* acts as a transcription factor that regulates the proliferation

of ISCs and the homeostasis of the midgut (Hao et al. 2020). *A. pomorum* did not have any similar genes with other bacterial species.

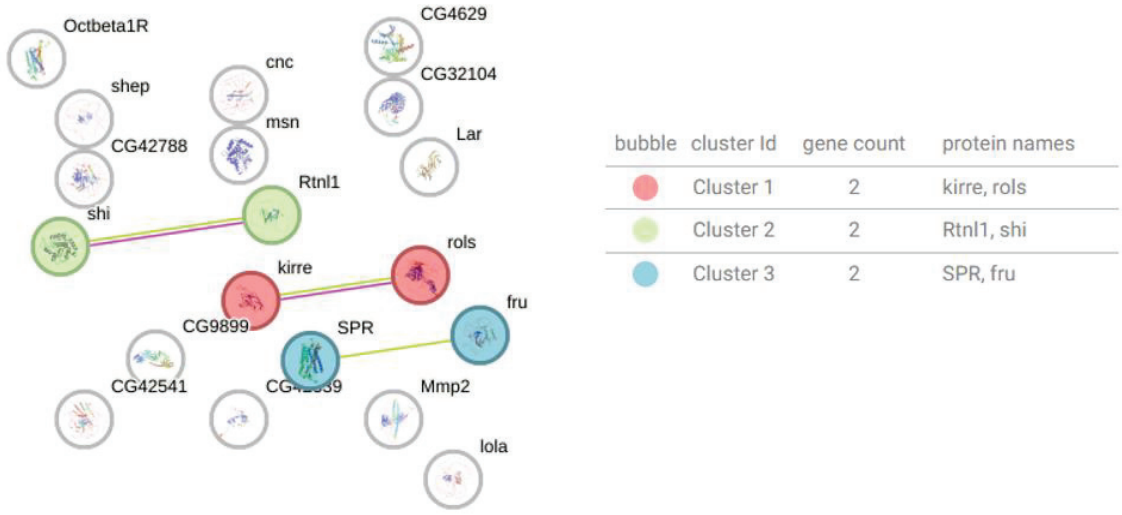

Figure 5. 9. Protein-protein interaction network of all bacteria intersection genes identified in GWAS.

Secondly, genetic variants and their annotations correlated with the abundance of *Ascomycota, Basidiomycota* and total fungi were determined by GWAS. Approximately 1.8 million variants in DGRP genomes were tested with GWAS analyses. In the Manhattan plots that p-values were plotted throughout the chromosomes, the significant p-values grouped together (Figure 5.10 and 5.11).

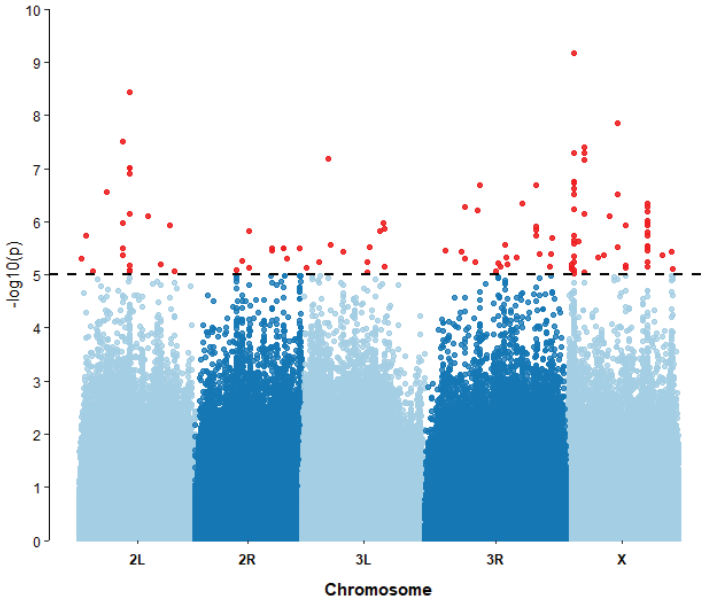

Figure 5. 10. Manhattan plot of the $\log_{10}$ inverse P-values from Ascomycota relative abundance.

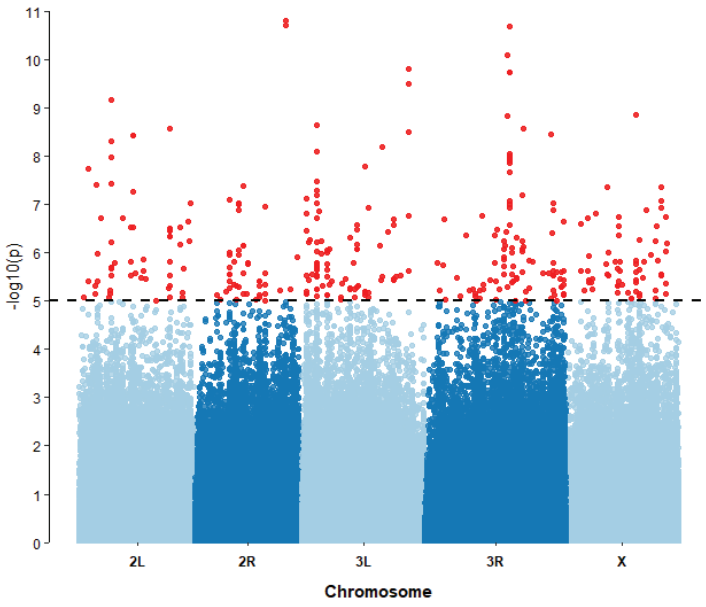

Figure 5. 11. Manhattan plot of the $\log_{10}$ inverse P-values from Basidiomycota relative abundance.

According to the GWAS results, 144 statistically significant variants (127 SNPs, 10 deletions, 7 insertions) were obtained from 70 genes for Ascomycota. Results for Basidiomycota was 430 variants (370 SNPs, 34 deletions, 24 insertions, 2 MNPs) from 194 genes and for overall fungi was 894 variants from 335 genes. In the Venn diagram presented in Figure 5.12, overlapping and unique genes associated with fungal groups identified through GWAS is illustrated. While the intersection of Ascomycota and Basidiomycota comprises *Nlg4*, *flw, grn*, *Gfrl*, *RhoGAP71E*, *trio* genes, the intersection of three groups has *bru-3* and *CG7985* genes (Figure 5.12).

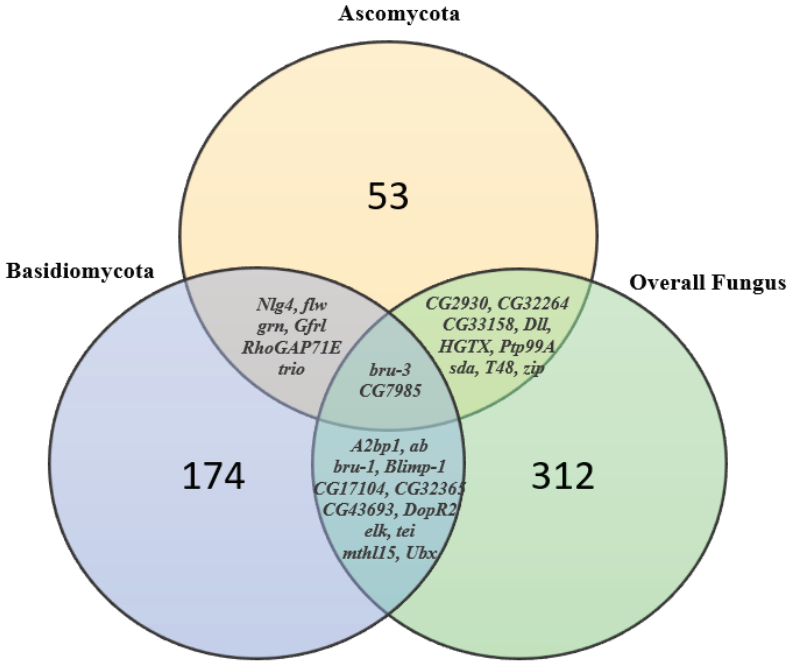

Figure 5. 12. Venn diagram illustration of the genes obtained by fungal GWAS analysis.

Protein-protein interactions were identified utilizing all intersection genes for fungi by STRING network analyses (Figure 5.13). The lines connecting genes indicate both physical interactions and functional relationships revealed by the STRING database. *Rbfox1* has been shown to contribute to generate adult muscle variety and development in *Drosophila* (Nikonova et al. 2022).

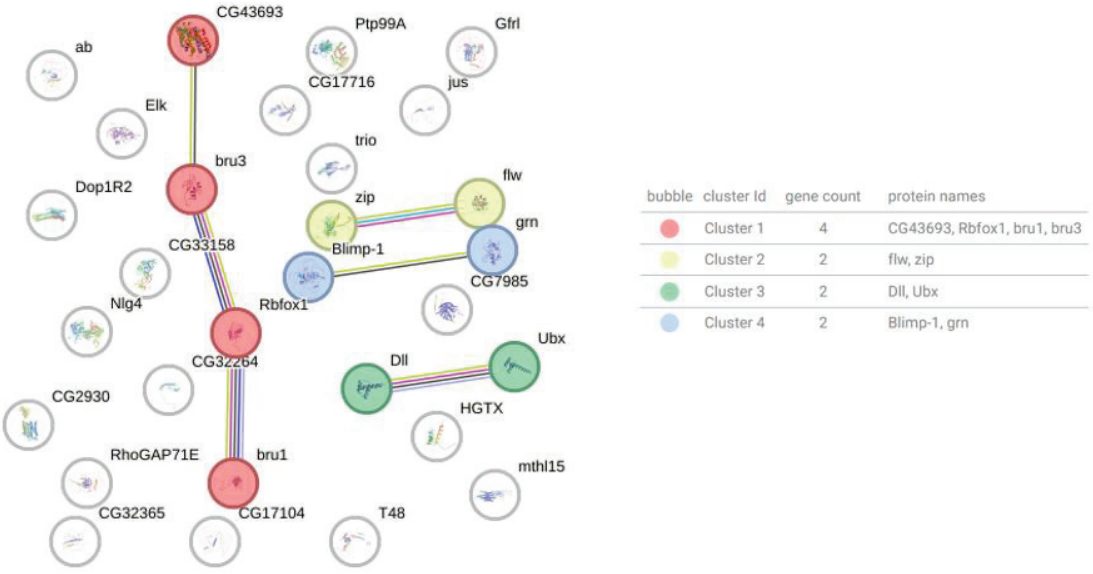

Figure 5. 13. Protein-protein interaction network of all fungal intersection genes identified in GWAS.

The functional enrichment analysis results give a perspective of the biological processes that are significantly connected with fungal microbiota-related genes. The Term ID and description columns represent gene ontology terms associated with a specific biological process. The observed gene count column shows the number of genes in the dataset that are associated with the specific GO term, out of the total number of genes that can potentially be linked to that term. The False Discovery Rate (FDR) represents the estimated percentage of false positives among the detected GO terms. An FDR < 0.05 is regarded strong proof that the enrichment is not the result of random chance. As shown in Table 5.2, mycobiota related genes have important roles in various developmental and regulatory processes, especially negative regulation of biosynthetic and metabolic processes, as well as organ development and differentiation. A small subset of genes (12 out of 1836) has a statistically significant association with phenotype of abnormal neuroanatomy in *Drosophila*.

Table 5. 2. Functional enrichments of the mycobiota.

Category	Term ID	Term description	Observed gene count	FDR	Proteins in the network
GO Process	GO:0010558	Negative regulation of macromolecule biosynthetic process	8 of 418	0.0121	*HGTX,Ubx,Rbfox1,grn,bru3,bru1,Blimp-1,ab*
GO Process	GO:0031327	Negative regulation of cellular biosynthetic process	8 of 434	0.0121	*HGTX,Ubx,Rbfox1,grn,bru3,bru1,Blimp-1,ab*
GO Process	GO:0007480	Imaginal disc-derived leg morphogenesis	4 of 70	0.0275	*trio,Ubx,RhoGAP71E,Dll*
GO Process	GO:0007275	Multicellular organism development	14 of 2125	0.0306	*flw,trio,HGTX,Ubx,RhoGAP71E,Dll,Nlg4,Ptp99A,Rbfox1,Gfrl,grn,Blimp-1,ab,T48*
GO Process	GO:0010605	Negative regulation of macromolecule metabolic process	9 of 828	0.0306	*HGTX,Ubx,Dll,Rbfox1,grn,bru3,bru1,Blimp-1,ab*
GO Process	GO:0000122	Negative regulation of transcription by RNA polymerase II	5 of 230	0.0338	*HGTX,Ubx,grn,Blimp-1,ab*
GO Process	GO:0002165	Instar larval or pupal development	7 of 544	0.0338	*flw,trio,Ubx,RhoGAP71E,Dll,Rbfox1,Blimp-1*
GO Process	GO:0007399	Nervous system development	9 of 980	0.0338	*trio,HGTX,Dll,Nlg4,Ptp99A,Rbfox1,Gfrl,grn,ab*
GO Process	GO:0007444	Imaginal disc development	7 of 545	0.0338	*flw,trio,Ubx,RhoGAP71E,Dll,Rbfox1,ab*
GO Process	GO:0009653	Anatomical structure morphogenesis	11 of 1464	0.0338	*flw,trio,Ubx,RhoGAP71E,Dll,Ptp99A,Rbfox1,grn,bru1,ab,T48*
GO Process	GO:0010092	Specification of animal organ identity	2 of 4	0.0338	*Ubx,Dll*
GO Process	GO:0032501	Multicellular organismal process	17 of 3342	0.0338	*flw,trio,HGTX,Ubx,jus,RhoGAP71E,Dll,Nlg4,Ptp99A,Rbfox1,Gfrl,grn,Dop1R2,bru1,Blimp-1,ab,T48*
GO Process	GO:0035114	Imaginal disc-derived appendage morphogenesis	6 of 325	0.0338	*flw,trio,Ubx,RhoGAP71E,Dll,Rbfox1*
GO Process	GO:0035120	Post-embryonic appendage morphogenesis	6 of 316	0.0338	*flw,trio,Ubx,RhoGAP71E,Dll,Rbfox1*
GO Process	GO:0048731	System development	11 of 1336	0.0338	*trio,HGTX,Ubx,Dll,Nlg4,Ptp99A,Rbfox1,Gfrl,grn,Blimp-1,ab*
GO Process	GO:0048856	Anatomical structure development	15 of 2531	0.0338	*flw,trio,HGTX,Ubx,RhoGAP71E,Dll,Nlg4,Ptp99A,Rbfox1,Gfrl,grn,bru1,Blimp-1,ab,T48*
GO Process	GO:0051172	Negative regulation of nitrogen compound metabolic process	8 of 662	0.0338	*HGTX,Ubx,Rbfox1,grn,bru3,bru1,Blimp-1,ab*
GO Process	GO:0007560	Imaginal disc morphogenesis	6 of 388	0.0347	*flw,trio,Ubx,RhoGAP71E,Dll,Rbfox1*
GO Process	GO:0030154	Cell differentiation	11 of 1558	0.0425	*flw,trio,HGTX,Ubx,RhoGAP71E,Ptp99A,Rbfox1,grn,bru1,Blimp-1,ab*
GO Process	GO:0048519	Negative regulation of biological process	11 of 1590	0.0476	*flw,HGTX,Ubx,Dll,Rbfox1,grn,Dop1R2,bru3,bru1,Blimp-1,ab*
Drosophila Phenotype (Monarch)	FBcv:0000435	Abnormal neuroanatomy	12 of 1836	0.0272	*zip,trio,HGTX,Ubx,CG17716,RhoGAP71E,Dll,Nlg4,Ptp99A,grn,Dop1R2,ab*

After examining the common effect of the mycobiota, STRING analysis was also performed with all genes obtained after Ascomycota and Basidiomycota GWAS analyzes with a P value less than 10^{-5} and 12 clusters were determined (Figure 5.14). The first three GO terms for biological process were nervous system development (GO:0007399), system development (GO:0048731), multicellular organism development (GO:0007275), respectively. For molecular function, just protein binding (GO:0005515) was observed. In cellular component, plasma membrane (GO:0005886), cell periphery (GO:0071944), and intrinsic component of plasma membrane (GO:0031226) shared most of the genes. Additionally, identification of protein families and domains were determined by InterPro and SMART databases. Immunoglobulin subtype 2 (IPR003598) and immunoglobulin C-2 Type (SM00408) was represented as the first results. Immunoglobulin domains are typically involved in immune responses; therefore, the results suggest that these genes may play a role in the recognition of fungal components.

bubble	cluster Id	gene count	protein names
	Cluster 1	125	Ac13E, AdamTS-A, Antp, Ars2, Atpalpha, Blimp-1, CG10749, CG1136, CG15117, CG17193, CG2930, CG30015, CG30461, CG32081, CG32264, CG32521, CG33158, CG34377, CG3632, CG42322, CG42324, CG42337, CG42524, CG43347, CG43693, CG45002, CG4972, CG7099, CG7985, CG8839, Cad99C, CadN2, Calx, Cbp53E, Clk, DAAM, DIP-epsilon, DIP-gamma, Dll, Elk, GC, Gfrl, Grip, HLH3B, HmgZ, Hs6st, Hsc70-2, Irk1, Irk2, KCNQ, Lsd-2, MED16, MED26, Mbs, Nlg4, Nrg, Pka-C1, Ptp61F, Ptp99A, Rap2l, Rbfox1, RhoGAP18B, RhoGAP71E, RpL7A, Rpb5, S-Lap5, SH3PX1, SK, SKIP, Sema1a, Sfmbt, Snoo, Strip, Task6, Ubx, Ugt304A1, Uros1, Wdr62, Xport-A, bab1, bip1, brm, bru1, bru3, caup, cdi, chb, dally, dlg1, dnc, dpr13, dpr17, dpr9, eEF1alpha2, eIF4EHP, eIF5B, exp, fand, feo, flw, for, foxo, frj, fru, grn, hpo, hth, kek5, me31B, mirr, neb, nolo, pdm3, rdgB, rg, shg, sosie, spri, sr, ste24b, tau, ttk, ttv, ush, zip
	Cluster 2	7	CG10175, CG1632, CG44153, Cht7, TyrRII, mthl15, obst-B
	Cluster 3	4	alphaTub67C, fs(1)N, stet, toc
	Cluster 4	3	CG42663, a, mnb
	Cluster 5	3	CG6191, CG6966, Kank
	Cluster 6	2	PVRAP, Spn31A
	Cluster 7	2	CG14100, CG4611
	Cluster 8	2	CG2187, salt
	Cluster 9	2	ADPS, euc
	Cluster 10	2	pinta, smal
	Cluster 11	2	Adar, mtt
	Cluster 12	2	Dop1R2, FMRFaR

Figure 5. 14. Clustering of Ascomycota and Basidiomycota related genes with p $<10^{-5}$ identified in the GWAS.

5.3. Conclusion

GWAS studies are essential for identifying specific genes that affect metabolic processes via interactions with the microbiota. In *Drosophila*, different genes are critical for metabolism, immune responses, and lipid homeostasis. Nutritional characteristics dependent on the microbiota composition can also strongly influenced by *Drosophila* host genetics (Dobson et al. 2015). The intestinal barrier constitutes one of the important defense mechanisms for the host as it physically separates the intestinal microbiota from the host. The *pyd* gene, which is included in this defense mechanism and found only in our *A. persici* GWAS results, regulates tight junctions that control the permeability of the intestinal barrier. The effect of the *pyd* gene was indicated among the genes statistically associated with the abundance of *A. tropicalis* in *Drosophila* (Chaston et al. 2016). The *Myd88* gene again related in only *A. persici*, is an adapter protein plays a central role in the innate and adaptive immunity and is involved in Toll-like receptors and interleukin-1 signaling pathways (Takeuchi and Akira 2001). *IL16*, the human ortholog of the *bbg* gene obtained from *E. faecium* GWAS analysis, is a pleiotropic cytokine that affects different phenotypic properties. Interleukins (IL), a member of cytokines, form an important part of our immune system by playing a role in defense against microbes. Polymorphisms in interleukin genes can cause to autoimmune diseases, inflammation, and diabetes mellitus. Additionally, polymorphisms in *ERAP1*, the human ortholog of *CG4467* gene, was reported to causative of both Ulcerative Colitis and Crohn's Disease (Küçükşahin et al. 2016; Pepelyayeva and Amalfitano 2019). The *Drs* gene, which is related with *L. plantarum* and a part of the Toll system, not only encodes antimicrobial peptides against fungi, but it is also stimulated by Gram-negative peptidoglycan even when the Imd pathway is not activated. These results suggest a crosstalk between immune signaling pathways. In addition to immune-related regulatory variations discovered in *L. plantarum*, *Mesr4* regulates fat storage by decreasing lipid accumulation. Similarly, the *CG17646* gene regulates triglyceride levels, so highlighting the genetic basis of lipid regulation. Another gene of *Hr96* is required for maintaining lipid and cholesterol homeostasis, demonstrating the importance of genetic influences in metabolic processes. Additionally, genetic abnormalities in mice cause significant changes in gut microbiota, which are associated with metabolic disorders such as increased body fat and reduced

glucose tolerance, especially when the mice are fed a high-fat diet (Ussar et al. 2015). Utilizing GWAS analyses, individual genetic variants may be analyzed and correlated with variances in microbial composition to uncover genes that impact digestion, immune responses, and metabolic health. This chapter provides a knowledge of how host genetics might predispose host to specific health disorders by modulating gut biodiversity. Finally, such research provides development of customized therapies that take into account both genetic and microbial profiles, resulting in more effective preventative and treatment approaches for metabolic and gastrointestinal illnesses.

CHAPTER 6

MICROBIOTA INVESTIGATION BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

6.1. Introduction

Loop-mediated isothermal amplification (LAMP), that has been first developed in 2000, is one of the DNA amplification methodologies used in molecular biology that is similar to PCR (Notomi et al. 2000). It is especially utilized to detect a specific DNA sequence within a sample (such as plant, food, blood, saliva, environmental samples). This technique is preferable due to simplicity and efficiency since it operates under isothermal conditions. In this method, basically a set of primers, an enzyme mix (*Bst* DNA polymerase), and a template DNA are mixed. Therefore, primers are managed a major part of the amplification. Either four or six primer sets are utilized in the reaction for detection of target DNA as possible as quickly (Wong et al. 2018). The primer sets are categorized as three main parts: inner primers (FIP and BIP), outer primers (F3 and B3), and loop primers. First of all, after amplification of DNA with BIP primer (with B1C-B2 sequences linked by a poly-T linker), B3 is bound the synthesized strand and amplified it. Then this step is repeated using FIP (with F1C-F2 sequences linked by a poly-T linker) and F3 primers. Afterward, on both arms, complementary regions (F1-F1C and B1-B1C) are folded and constituted a dumbbell structure (Parida et al. 2008). In the dumbbell structure, there are many regions that provide binding site to primers within the reaction mixture. Finally, binding of primers through many sites result in exponential amplification, and the final products, concatemers are formed (Figure 6.1). The optional part here is the usage of loop primers. Because loop primers initiate the reaction more quickly, they can prefer as a speed enhancer so that reduce the reaction time (Nagamine, Hase, and Notomi 2002).

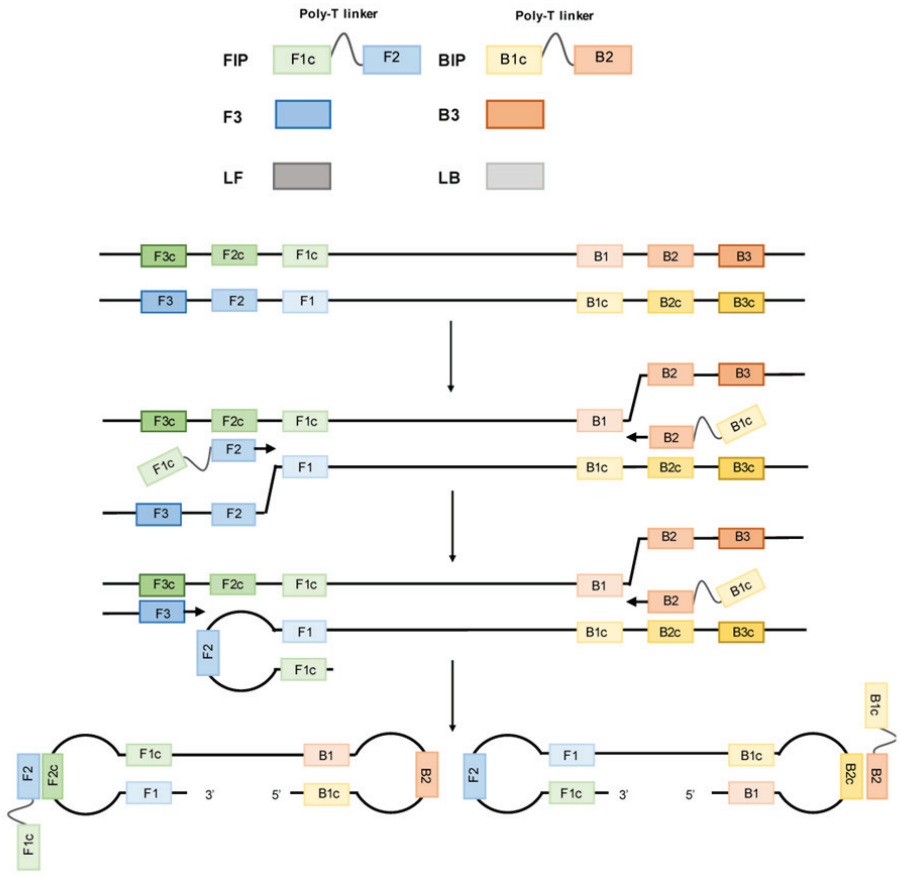

Figure 6. 1. Overview of LAMP reaction. (Source: García-Bernalt Diego et al. 2021).

To visualize and interpret the LAMP reaction, different approaches can be utilized. The first one is colorimetric assay. When using phenol red as an indicator, color change happens during the amplification because of the shifting pH. Reaction begins with a red color, as the amplification progresses correctly color changes to yellow. Color change is a visual confirmation of the accomplished amplification of the targeted DNA sequence. In another naked eye scanning, formation of magnesium pyrophosphate can be utilized like colorimetric assay. Pyrophosphate ions come from the reaction, are reacted with magnesium ions, and formed a white, insoluble precipitate called magnesium pyrophosphate. LAMP reaction can be also performed with an intercalating dye, such as SYBR Green, similar to that of qPCR (Fischbach et al. 2015).

6.2. Methods

6.2.1. Designing of LAMP primers

Primer sets for *A. pomorum*, *L. brevis* and *M. restricta* were designed by NEB LAMP Primer Design Tool (https://lamp.neb.com/#!/). Then, properties of primers were identified by IDT SciTools OligoAnalyzer 3.1 software and specificity of primers were checked with BLAST. Primers are listed in Table 6.1. Primers were synthesized by Macrogen (Korea).

Table 6. 1. Primers utilized in LAMP reactions.

Targeted species	DNA sequences of the primers (5′–3′)		References
Acetobacter pomorum	F3	ACGGTACCCGTAGAAGAAGC	This study
	B3	ACAACCCTCTCTCACACTCT	
	FIP	CCAGTCATTCCGAGCAACGCTATTTTCGGCTAACTTCGTGCCAG	
	BIP	GCGTAAAGGGCGTGTAGGCGTTTTCGTATCAAATGCAGCTCCCA	
	LoopF	CCTTCGTATTACCGCGGCTG	
	LoopB	CAGTCAGATGTGAAATCCCCGG	
Lactobacillus brevis	F3	GGCGGGAAAAAGTATGCCAT	(Tsuchiya et al. 2007)
	B3	AGAAGGCCAGTTCCCGAAT	
	FIP	GCCGTGCTGATCTTGTGGTAGAGCCTTTGACCATGGTCATGT	
	BIP	CACGATCGTGCACTTCTTGCCACCGCGTCGTCAATGTCTT	
	LoopF	CAATCACGTGCATTGGCGTC	
	LoopB	CCGAGAAACGACCGTCTTTG	
Malassezia restricta	F3	TGCGTAGGTGGTAGTCAGC	This study
	B3	TGCGCACTTACACTTGTTCA	
	FIP	ACCCGGCCTCTCTCTCTCTCTACGGATTCATGTTCCCATGT	
	BIP	CATCTGCTTCGCACGGACCCACTCCATCCACTGTCGGC	
	LoopF	CTCCCCTGTACACATCAAAGGAA	
	LoopB	AGAAAGAAGTGAAGGGACTCACTCA	

6.2.2. Performing the LAMP reactions

LAMP experiments were conducted using the colorimetric method. For a single reaction, 9 µl of dH_2O, 2.5 µl of LAMP primer mix, 12.5 µl of WarmStart Colorimetric LAMP 2X Master Mix with UDG (New England Biolab's, USA) were mixed and total volume was adjusted to 24 µl without the DNA sample. With 1 µl of DGRP DNA sample, the final volume was completed to 25 µl (Table 6.2). The LAMP reaction was carried out by incubating at 65°C for 30-45 minutes within SimpliAmp Thermal Cycler (Applied Biosystems (ABI), USA). Because the LAMP reaction has been done preferring colorimetric assay, data analysis has completed by naked eye.

Table 6. 2. Reaction components of LAMP.

Components (Per reaction)	**Volume**
dH_2O	9 µl
WarmStart Colorimetric LAMP 2X Master Mix with UDG	12.5 µl
LAMP Primer Mix (10X)	2.5 µl
DNA sample	1 µl
Total Volume	**25 µl**

6.3. Results and Discussion

In order to identify three specific microbial species in the gut samples of DGRP lines, loop-mediated isothermal amplification analysis was carried out in this chapter. Prior to performing the LAMP analysis, qPCR was used to confirm the existence of these target species in the gut microbiota. *A. pomorum* and *L. brevis* were chosen based on earlier results showing their significant lowering effect on *Drosophila* triglyceride metabolism (see Figure 4.7 in Chapter 4). Furthermore, we reported a negative correlation (r: -0.22, $p< 0.05$) between the triglyceride level and Basidiomycota in Chapter 4. Hence, we concentrated on *M. restricta* and *P. ostreatus*, two of the most prevalent

Basidiomycota species, and quantified their abundance in all samples using qPCR. *M. restricta* was found to be more abundant than *P. ostreatus*, as shown in Figure 6.2A (Wilcoxon rank-sum, p-value = 0.321). Regression analysis was then carried out to evaluate their association with triglyceride metabolism. We observed a significant negative relationship (r: -0.54, p-value = 9.4e-05) between *M. restricta* abundance and triglyceride levels, as illustrated in Figure 6.2B. In contrast, *P. ostreatus* and triglyceride levels exhibited a positive but non-statistically significant correlation (r: 0.33, p-value = 0.2) shown in Figure 6.2C. The significant negative relationship seen between *M. restricta,* and triglyceride levels suggests its potential impact on *Drosophila* lipid metabolism. Given these results, we continued with the LAMP analyses, focused on *M. restricta*.

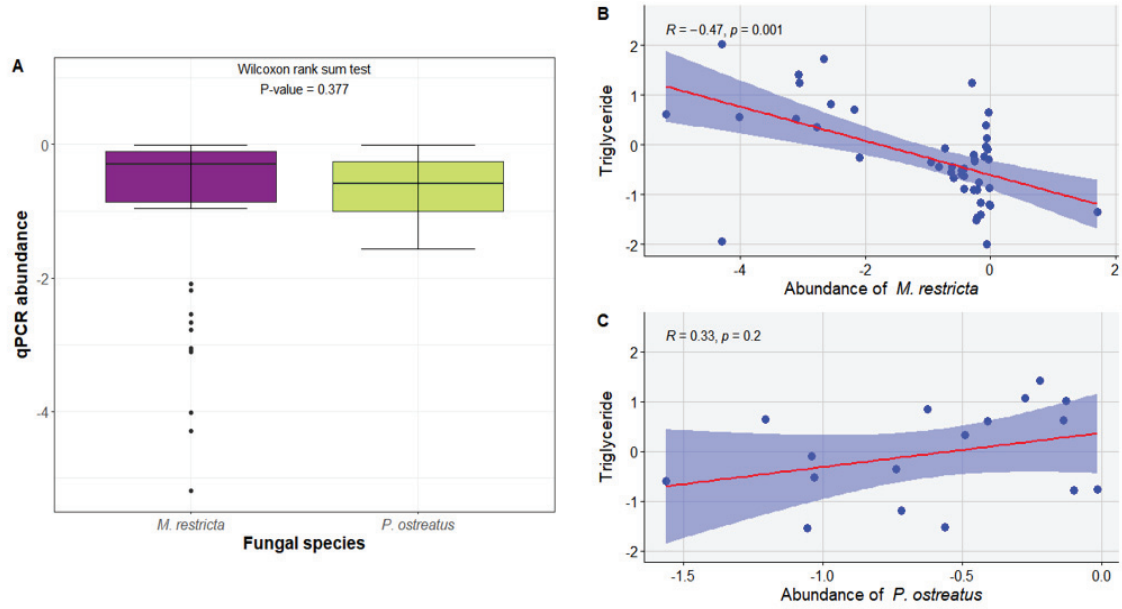

Figure 6. 2. Relative abundance of *M. restricta* and *P. ostreatus* within gut samples (A), their relationships with triglyceride levels respectively (B and C).

LAMP reactions were carried out for *A. pomorum*, *L. brevis*, and *M. restricta* by utilizing the WarmStart Colorimetric LAMP 2X Master Mix with UDG Kit which provides a colorimetric readout. The colorimetric methods eliminate the need for equipment, make the process more accessible, especially in low-sample situations. Since

the pH is reducing due to production of protons during reaction, the pH sensitive dye turns from pink to yellow as the reaction progresses showing the presence of the target DNA. That's why, LAMP results were interpreted with the naked eye. While the original color pink was considered negative; the yellow color obtained as a result of amplification was reported as positive. According to the findings for *A. pomorum*, 84.62% of the samples positive, while 15.38% negative (Figure 6.3A). For *L. brevis*, 61.54% of the samples were positive and 38.46% were negative (Figure 6.3B). Concerning the last target microorganism, *M. restricta*, 21.37% of the samples were positive, and 78.63% negative (Figure 6.3C).

Figure 6. 3. LAMP reaction results of *A. pomorum* (A), *L. brevis* (B) and *M. restricta* (C) in DGRP gut samples.

The comparison between qPCR and LAMP for detecting *A. pomorum*, *L. brevis*, and *M. restricta* in DGRP gut samples was performed by utilizing boxplot analyses. The box plots in Figure 6.4 show the qPCR abundances of these species and the LAMP results corresponding to these values. The boxplots for *A. pomorum* and *L. brevis* are indicating clear distinction in relative abundance of microbiota between LAMP positive and negative results. For samples with higher abundance, the LAMP results were consistently positive for *A. pomorum*, particularly below Ct values of 32.01. These results indicate a strong correlation between high bacterial load and LAMP positivity. The significant difference in *A. pomorum* abundance between LAMP positive and negative samples was confirmed by the Wilcoxon rank-sum test result (p-value = 2.7e-09) (Figure 6.4A). Likewise with *A. pomorum*, the abundance of *L. brevis* demonstrated a continuous positive result below the Ct of 31.76, with a remarkable distinction (Wilcoxon rank-sum,

p-value = 8.3e-07) between LAMP positive and negative results (Figure 6.4B). So, LAMP has indicated a high sensitivity to detection *A. pomorum* and *L. brevis* when the bacterial load is high (values less than almost 32 Ct). For *M. restricta*, the LAMP results were not as clear-cut for distinction (Wilcoxon ran-sum, p-value = 0.12) (Figure 6.4 C). The fact that the fungi abundance is lower than the bacterial species suggests that higher Ct values can provide both positive and negative LAMP findings.

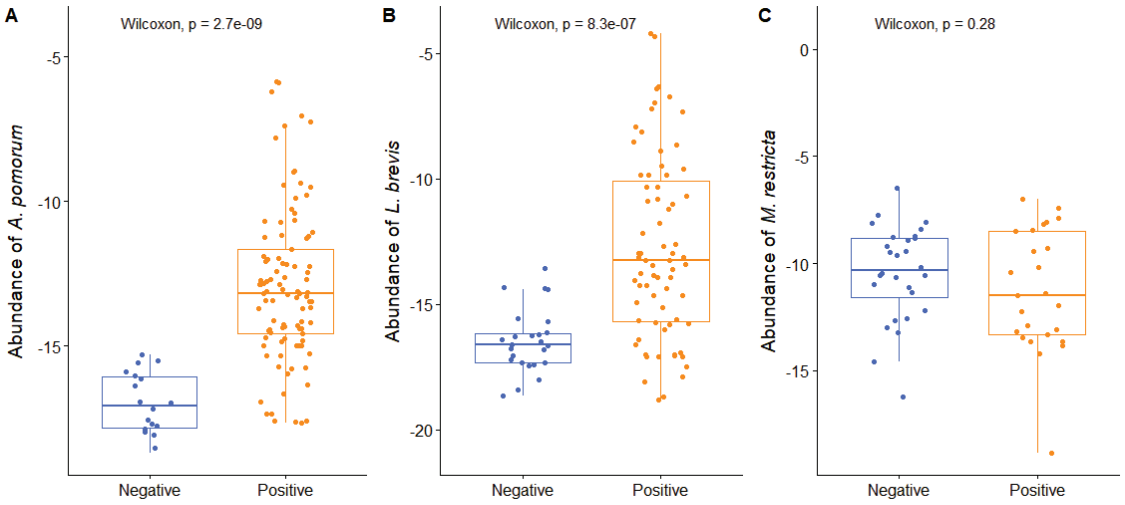

Figure 6. 4. Boxplots that indicate comparison between LAMP results and relative abundance of *A. pomorum* (A), *L. brevis* (B) and *M. restricta* (C) in DGRP gut samples.

There is very limited number of LAMP studies conducted in *Drosophila*, and within the larger taxonomic order Diptera, specifically the Drosophilidae family. LAMP assays have been developed for detecting *Drosophila suzukii*, which is closely related to *Drosophila melanogaster* and the effectiveness of LAMP for field applications has been successfully demonstrated (Kim et al., 2016). While this study mainly focused on ensuring high sensitivity and accuracy to identify *D. suzukii* alone; another study aimed to differentiate *D. suzukii* from other drosophilids such as D*rosophila affinis* and *Drosophila simulans* (Hong, Michel, and Long 2023). On the other hand, LAMP has been utilized to rapidly detection of fruit flies such as *Dacus ciliatus* (cucumber fruit fly), *Ceratitis capitata* (Mediterranean fruit fly), and *Bactrocera trivialis* (New Guinea fruit

fly), which are relatives of Drosophilidae within the Diptera order (Huang et al. 2009; Sabahi et al. 2018; Starkie et al. 2022).

Microbiota analysis is generally performed via fecal samples in human due to its ease of collection and the richness of microbial content. Also, durability of LAMP against inhibitors found in fecal samples enhances its utility in microbiota research (Francois et al. 2011). In human studies, LAMP has been successfully employed to detect gut microbiota, hereby its high sensitivity and specificity even in complicated sample types has been proven (Fernández-Soto et al. 2016; Rahman et al. 2017). This link among *Drosophila* and human investigations demonstrates adaptability and efficacy of LAMP in identifying gut microbiota, emphasizing its importance in basic and practical microbiological research.

6.4. Conclusion

In this chapter, we assessed the efficacy of LAMP and qPCR for identifying targeted microbial species in DGRP gut samples. We performed these analyses using optimized protocols to target three specific microbial species. The qPCR findings confirmed the existence of the targeted species in the intestinal samples and utilized as a reference for comparison. Following the qPCR analysis, we performed LAMP on the same samples without serial dilutions of the DNA to determine whether LAMP could achieve equivalent sensitivity and specificity. During the comparison, findings in samples reveals that while LAMP is effective for higher bacterial loads, its sensitivity decreases with lower bacterial concentrations. The significant association between qPCR and LAMP results supports that LAMP may be used for rapid and cost-effective screening. The statistically significant differences for *A. pomorum* and *L. brevis* support the reliability of the LAMP approach. Percentage of the LAMP positive results for *A. pomorum, L. brevis*, and *M. restricta* were to be 84.62%, 61.54%, and 21.37%, respectively. Fungal microbiota abundance was approximately three to four times lower than that of bacterial species. So, we concluded the posing challenges for achieving statistically significant results come from this lower abundance. Nonetheless, the LAMP technique effectively detected fungal microbiota despite its lower abundance and smaller

size of sample. To achieve statistically significant outcomes, future studies should study with larger sample sizes.

CHAPTER 7

CONCLUSION

Due to its simpler and more easily controllable microbiota compared to mammals, *Drosophila melanogaster* serves as an effective model for studying the interactions between host metabolism and microbiota. While the distribution of commensal and pathogenic microorganisms in *Drosophila* and mammalian guts differs, research indicates that the pathways through which these microorganisms interact with the host gut are highly conserved. In both organisms, the intestinal structure keeps bacteria away from the epithelium, provides a surface for bacterial attachment, and serves as a nutritional source for intestinal bacteria. Although the cell types and intestinal signaling pathways regulating host metabolism are similar in both *Drosophila* and mammals, there are some differences. The flat intestinal epithelium in flies allows more complete access of the microbiota to the epithelial surface, unlike in mammals. Despite these structural differences, *Drosophila* remains a valuable model for exploring fundamental principles of gut microbiota interactions and their impact on host health.

The gut microbiome affects our health by ensuring healthy development, enough nutrition, and appropriate carbohydrate and lipid metabolism throughout life. Therefore, maintaining a microbiota ideally matched to the host is of great importance in improving human health. In this study firstly (Chapter 2), intestinal microbiota composition was revealed by 16S rRNA and ITS amplicon sequencing in DGRP samples. The sequencing data provided a comprehensive overview of the microbial ecosystem found in the fruit fly gut, demonstrating the diversity and abundance of different bacterial and fungal species. These results highlight the necessity of taking into account the composition and abundance of fungal species in gut-based studies; since differences between these groups of fungi may have implications for interactions within the gut microbiota studied. By understanding these microbial compositions, we can acquire insights into the relationships between host and microbiota, and how these interactions influence metabolic processes and overall health. Further studies should aim to distinguish the roles of especially live yeasts under various nutritional conditions. This approach can enhance

our knowledge of yeasts, both as integral members of the microbiota and as an important source of protein.

In the second section (Chapter 3), focused on the measurement of several metabolic pools in DGRP samples, including protein, glucose, glycogen, trehalose, and triglycerides. This part is critical for understanding the metabolic profiles of different genetic backgrounds and their interactions with the microbiota. By measuring these metabolic pools, it can be learned about how genetic variants affect metabolic pathways and energy balance in *Drosophila melanogaster*. Lipids, particularly triacylglycerols, are essential reserves of energy and have important roles in many biological processes. Carbohydrate metabolism in *Drosophila*, primarily regulated by insulin-like peptides and hormones such as adipokinetic hormone, reflects mammalian systems in controlling glucose metabolism, hence maintains metabolic equilibrium. The unique stability and resistance to oxidation of trehalose, make it a reliable energy source for *Drosophila*, even under varying physiological circumstances. Furthermore, its antioxidant properties protect cellular components from oxidative damage, indicating its critical function in the metabolic and physiological resilience of the fruit fly. These metabolic pathways highlight the complexity and interdependent nature of nutrient consumption in *Drosophila*, providing important insights into metabolic regulation and its consequences for health and disease in more complex organisms. Future research aiming at distinguishing the particular roles of microbial interactions and genetic differences in these metabolic pathways might improve our understanding and offer potential applications for managing metabolic health and disease.

The following chapter (Chapter 4) highlights the impact of microbial interactions both with each other and on host metabolism. The diverse relationships between fungi and bacterial microbiota play crucial roles in maintaining intestinal homeostasis and regulating key physiological functions such as energy homeostasis, glucose regulation, and lipid metabolism. Specific microbial species, such as *Acetobacter* and *Lactobacillus*, contribute significantly to energy storage regulation. Studies demonstrating negative correlations between *Acetobacter* species and triglyceride levels, as well as the beneficial effects of *E. faecium* in reducing insulin-like gene overexpression, emphasize the importance of these microorganisms in host health. The dynamic interactions among gut microbiota members, ranging from synergistic to antagonistic, are essential for metabolite synthesis, pathogen defense, and overall microbial stability. Dysbiosis, or microbial imbalance, is linked to metabolic and gastrointestinal diseases, emphasizing the necessary

for targeted probiotic and prebiotic therapies to restore healthy microbiota. Understanding these microbial interactions is critical for developing effective strategies to enhance gut health and prevent metabolic disorders.

The next chapter (Chapter 5) emphasized the significant role of GWAS in uncovering the relationships between host genetics and gut microbiota. GWAS is utilized for identifying specific genes that influence metabolic processes through interactions with the microbiota. In *Drosophila*, important genes have been discovered that regulate metabolism, immune responses, and lipid homeostasis. For instance, the *pyd* gene, identified in the *A. persici* GWAS results, is crucial for maintaining intestinal barrier integrity, while the *Myd88* gene, also associated with *A. persici*, plays a fundamental role in immune signaling pathways. Other genes, such as the *bbg* and *CG4467*, highlight the genetic links to immune system function and susceptibility to diseases like diabetes and inflammatory bowel disease. Furthermore, genes associated with lipid regulation, such as *Mesr4*, *CG17646*, and *Hr96*, emphasize the genetic basis of metabolic processes. The findings presented in this chapter can be important for developing personalized therapies that consider both genetic and microbial profiles, leading to more effective prevention and treatment strategies for metabolic and gastrointestinal diseases.

Loop-mediated isothermal amplification is a basic technique highlighted in the final chapter (Chapter 6) for its powerful ability to amplify DNA with high specificity and efficiency, particularly from very small samples like *Drosophila* gut samples. The importance and reason for preference of LAMP is its simplicity and rapidity, allowing for the detection of target genes without the need for sophisticated equipment or extensive sample preparation. This technique is especially advantageous for studying the microbiota in *Drosophila melanogaster*, as it enables precise amplification of microbial DNA from tiny amounts of gut tissue. The successful use of LAMP in this research shows its potential to reveal detailed microbial dynamics within the gut, contributes to our understanding of host-microbiota relationships. By utilizing the LAMP technique, reliable results can be obtained even from the smallest biological samples, thereby advances can be made in genetic and microbiota studies that will have important consequences for health and disease.

In conclusion, this thesis has demonstrated the beneficial application of *Drosophila melanogaster* as a model organism for investigating the detailed relationships between host metabolism, microbiota, and genetics. This study can also serve as a basis for several other research that discover the interaction between host microbiota,

metabolism, and genetics in a variety of ways. For example, the effects of *Acetobacter persici*, *Acetobacter pomorum*, *Lactobacillus brevis*, and *Malassezia restricta* on lipid metabolism can be investigated directly by administering a high-fat diet to *Drosophila melanogaster*. Axenic flies can be used in another investigation to understand whether reducing effects of these species on triglyceride metabolism are independent or not. This approach will allow us to reveal for potential synergistic interaction between fungal (*M. restricta*) and bacterial (*A. persici, A. pomorum, L. brevis*) species. Additionally, mutant *Drosophila* strains in terms of immune system and metabolism related genes that were identified by GWAS, can be utilized to assess the genetic influences on microbiota-metabolism interactions.

As a different perspective, customized microfluidic devices and chip technologies can be preferable for *Drosophila* studies because of their automated manipulation, injection, and other treatment procedures. To monitor the cardiac activity of *Drosophila* larvae exposed to various chemicals, microchips have been developed (Ardeshiri et al. 2016). Also, these devices have been utilized to monitor foraging behaviors by feeding *Drosophila* adult flies at regular intervals (Navawongse et al. 2016). This system can be modified by constructing chambers that include different microbiota compositions and automated feeding systems. Hereby, it can allow the study of how changes in gut microbiota affect feeding behavior. On the other hand, different gut-on-a-chip systems have been developed to examine the effect of host-microbiota interactions on intestinal physiology on a species basis for our complex intestinal system (Beck et al. 2016). Burmeister and colleagues have developed a platform to investigate commensalistic interactions among bacteria that have a role in industrial production (Burmeister et al. 2018). In future studies, microfluidic systems can be used to load specific bacterial and fungal species into the gut of *Drosophila*, and to control the impact of different microbiota compositions on host metabolism. Additionally, microchip-based platforms designed with sensors can enable real-time monitoring of metabolic pools such as trehalose, triglycerides, glycogen providing dynamic insights into metabolic regulations.

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APPENDIX A

OTU TABLES

Table A. 1. ITS OTU Table of DGRP samples in family level.

#Otu Table (Relative Abundance)	S1	S2	S3	S4	S5	S6	S7	S8	S9
Fungi;Ascomycota;Dothideomycetes;Botryosphaeriales;Botryosphaeriaceae	0.00	0.30	0.00	0.27	0.29	0.01	0.00	0.00	0.35
Fungi;Ascomycota;Dothideomycetes;Capnodiales;Cladosporiaceae	0.00	0.55	0.00	1.04	0.00	0.00	0.00	0.00	1.05
Fungi;Ascomycota;Dothideomycetes;Capnodiales;Mycosphaerellaceae	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Dothideomycetes;Dothideales;Aureobasidiaceae	0.00	0.14	0.00	0.33	0.12	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Dothideomycetes;Dothideales;Dothioraceae	0.00	0.06	0.00	0.21	0.09	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Dothideomycetes;Pleosporales;Didymellaceae	0.00	2.37	0.00	0.86	1.67	0.01	0.00	0.00	0.00
Fungi;Ascomycota;Dothideomycetes;Pleosporales;Melanommataceae	0.00	0.28	0.00	0.25	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Dothideomycetes;Pleosporales;Phaeosphaeriaceae	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Dothideomycetes;Pleosporales;Pleosporaceae	1.14	1.74	0.00	0.23	0.28	0.00	0.00	0.02	0.00
Fungi;Ascomycota;Dothideomycetes;Venturiales;Sympoventuriaceae	0.00	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Eurotiomycetes;Chaetothyriales;Herpotrichiellaceae	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Eurotiomycetes;Eurotiales;Aspergillaceae	0.07	0.87	0.00	16.18	0.55	0.01	0.00	0.00	0.84
Fungi;Ascomycota;Eurotiomycetes;Onygenales;Ascosphaeraceae	0.00	0.02	0.00	0.00	0.07	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Eurotiomycetes;Phaeomoniellales;Phaeomoniellaceae	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Laboulbeniomycetes;Pyxidiophorales;Pyxidiophoraceae	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Lecanoromycetes;Acarosporales;Acarosporaceae	0.00	0.30	0.00	0.00	0.27	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Lecanoromycetes;Caliciales;Caliciaceae	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Lecanoromycetes;Caliciales;Physciaceae	0.00	0.22	0.00	0.18	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Lecanoromycetes;Lecanorales;Lecanoraceae	0.00	0.12	0.00	0.19	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Lecanoromycetes;Lecanorales;Parmeliaceae	0.00	0.27	0.00	0.55	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Lecanoromycetes;Peltigerales;Collemataceae	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Lecanoromycetes;Pertusariales;Megasporaceae	0.00	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Lecanoromycetes;Teloschistales;Teloschistaceae	0.00	0.16	0.00	0.00	0.06	0.00	0.00	0.00	0.14
Fungi;Ascomycota;Leotiomycetes;Helotiales;Cenangiaceae	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00

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Table A.1. (cont.)

#Otu Table (Relative Abundance)	S1	S2	S3	S4	S5	S6	S7	S8	S9
Fungi;Ascomycota;Leotiomycetes;Helotiales;Helotiaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Leotiomycetes;Helotiales;Helotiales_fam_Incertae_sedis	0.00	2.78	0.00	0.24	0.52	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Leotiomycetes;Helotiales;Sclerotiniaceae	0.00	0.67	0.00	0.00	0.09	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Leotiomycetes;Phacidiales;Phacidiaceae	0.00	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Leotiomycetes;Rhytismatales;Rhytismataceae	0.00	0.04	0.00	0.21	0.00	0.00	0.00	0.00	0.07
Fungi;Ascomycota;Leotiomycetes;Thelebolales;Pseudeurotiaceae	0.00	0.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Pezizomycetes;Pezizales;Pezizaceae	0.00	0.03	0.00	0.00	0.03	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Pezizomycetes;Pezizales;Sarcoscyphaceae	0.00	0.11	0.00	0.00	0.06	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Pezizomycetes;Pezizales;Tuberaceae	0.00	0.95	0.00	3.36	1.52	0.00	0.01	0.01	0.07
Fungi;Ascomycota;Saccharomycetes;Saccharomycetales;Debaryomycetaceae	0.00	0.42	0.00	0.00	0.11	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Saccharomycetes;Saccharomycetales;Phaffomycetaceae	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.07
Fungi;Ascomycota;Saccharomycetes;Saccharomycetales;Pichiaceae	97.50	61.39	99.45	0.25	69.71	0.09	99.67	97.74	0.00
Fungi;Ascomycota;Saccharomycetes;Saccharomycetales;Saccharomycetaceae	0.65	9.01	0.51	32.77	9.88	95.08	0.01	2.03	4.92
Fungi;Ascomycota;Saccharomycetes;Saccharomycetales;Saccharomycetales_fam_Incertae_sedis	0.00	0.30	0.00	0.59	0.22	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Sordariomycetes;Diaporthales;Diaporthaceae	0.00	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Sordariomycetes;Glomerellales;Glomerellaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.28
Fungi;Ascomycota;Sordariomycetes;Hypocreales;Bionectriaceae	0.00	0.12	0.00	0.00	0.18	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Sordariomycetes;Hypocreales;Clavicipitaceae	0.00	0.40	0.00	0.32	0.19	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Sordariomycetes;Hypocreales;Cordycipitaceae	0.00	1.23	0.00	1.89	0.86	0.01	0.00	0.00	1.05
Fungi;Ascomycota;Sordariomycetes;Hypocreales;Hypocreaceae	0.00	0.03	0.00	0.00	0.08	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Sordariomycetes;Hypocreales;Hypocreales_fam_Incertae_sedis	0.00	0.75	0.00	0.30	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Sordariomycetes;Hypocreales;Nectriaceae	0.00	2.79	0.00	0.00	1.60	0.00	0.00	0.00	0.07
Fungi;Ascomycota;Sordariomycetes;Hypocreales;Stachybotryaceae	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Sordariomycetes;Sordariales;Chaetomiaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07

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Table A.1. (cont.)

#Otu Table (Relative Abundance)	S1	S2	S3	S4	S5	S6	S7	S8	S9
Fungi;Ascomycota;Sordariomycetes;Xylariales;Amphisphaeriaceae	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Ascomycota;Sordariomycetes;Xylariales;Sporocadaceae	0.00	0.00	0.00	0.00	0.18	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Agaricomycetes;Agaricales;Agaricaceae	0.00	0.00	0.00	0.38	0.00	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Agaricomycetes;Agaricales;Agaricales_fam_Incertae_sedis	0.00	0.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Agaricomycetes;Agaricales;Amanitaceae	0.00	0.18	0.00	0.00	0.05	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Agaricomycetes;Agaricales;Bolbitiaceae	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Agaricomycetes;Agaricales;Cortinariaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Agaricomycetes;Agaricales;Hymenogastraceae	0.00	0.21	0.00	0.00	0.18	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Agaricomycetes;Agaricales;Omphalotaceae	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.01	0.00
Fungi;Basidiomycota;Agaricomycetes;Agaricales;Pleurotaceae	0.00	0.00	0.00	1.14	0.00	0.01	0.00	0.00	0.63
Fungi;Basidiomycota;Agaricomycetes;Agaricales;Pluteaceae	0.00	0.00	0.00	0.41	0.00	0.00	0.00	0.00	0.07
Fungi;Basidiomycota;Agaricomycetes;Agaricales;Psathyrellaceae	0.00	0.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Agaricomycetes;Agaricales;Schizophyllaceae	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Agaricomycetes;Agaricales;Tricholomataceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14
Fungi;Basidiomycota;Agaricomycetes;Cantharellales;Cantharellales_fam_Incertae_sedis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.14
Fungi;Basidiomycota;Agaricomycetes;Cantharellales;Ceratobasidiaceae	0.00	0.01	0.00	0.00	0.05	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Agaricomycetes;Hymenochaetales;Tubulicrinaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07
Fungi;Basidiomycota;Agaricomycetes;Polyporales;Fomitopsidaceae	0.00	0.00	0.00	0.54	0.00	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Agaricomycetes;Polyporales;Phanerochaetaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14
Fungi;Basidiomycota;Agaricomycetes;Polyporales;Steccherinaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.70
Fungi;Basidiomycota;Agaricomycetes;Russulales;Albatrellaceae	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Agaricomycetes;Russulales;Peniophoraceae	0.00	0.00	0.00	2.20	0.00	0.00	0.00	0.00	0.14
Fungi;Basidiomycota;Agaricomycetes;Thelephorales;Thelephoraceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.35

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Table A.1. (cont.)

#Otu Table (Relative Abundance)	S1	S2	S3	S4	S5	S6	S7	S8	S9
Fungi;Basidiomycota;Agaricomycetes;Trechisporales;Hydnodontaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.21
Fungi;Basidiomycota;Cystobasidiomycetes;Cystobasidiomycetes_ord_Incertae_sedis;Buckleyzymaceae	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Cystobasidiomycetes;Erythrobasidiales;Erythrobasidiaceae	0.00	0.00	0.00	0.03	0.01	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Malasseziomycetes;Malasseziales;Malasseziaceae	0.01	0.30	0.00	3.02	0.35	0.06	0.22	0.00	8.22
Fungi;Basidiomycota;Microbotryomycetes;Microbotryomycetes_ord_Incertae_sedis; Microbotryomycetes_fam_Incertae_sedis	0.00	0.00	0.00	0.58	0.00	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Microbotryomycetes;Sporidiobolales;Sporidiobolaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07
Fungi;Basidiomycota;Tremellomycetes;Cystofilobasidiales;Cystofilobasidiaceae	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Tremellomycetes;Filobasidiales;Filobasidiaceae	0.09	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Tremellomycetes;Holtermanniales;Holtermanniales_fam_Incertae_sedis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07
Fungi;Basidiomycota;Tremellomycetes;Tremellales;Bulleribasidiaceae	0.07	0.05	0.00	0.00	0.08	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Tremellomycetes;Tremellales;Tremellaceae	0.00	0.25	0.00	0.08	0.00	0.00	0.00	0.00	0.00
Fungi;Basidiomycota;Tremellomycetes;Trichosporonales;Trichosporonaceae	0.00	0.79	0.00	0.00	0.81	0.01	0.00	0.00	0.00
Fungi;Basidiomycota;Wallemiomycetes;Wallemiales;Wallemiaceae	0.00	0.98	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Mucoromycota;Mucoromycetes;Mucorales;Cunninghamellaceae	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Mucoromycota;Mucoromycetes;Mucorales;Mucoraceae	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungi;Mucoromycota;Mucoromycetes;Mucorales;Rhizopodaceae	0.00	0.05	0.00	0.29	0.02	0.00	0.00	0.00	0.00

Table A. 2. 16S rRNA OTU Table of DGRP samples in family.

#Otu Table (Relative Abundance)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Actinomycetaceae	0.00	0.00	0.00	0.00	0.01	0.01	0.86	0.00	0.04	0.60
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.01	0.25
Bacteria;Actinobacteria;Actinobacteria;Corynebacteriales;Corynebacteriaceae	0.00	0.20	0.00	0.13	0.11	0.12	2.33	0.00	0.46	5.06
Bacteria;Actinobacteria;Actinobacteria;Corynebacteriales;Mycobacteriaceae	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.00
Bacteria;Actinobacteria;Actinobacteria;Geodermatophilales;Geodermatophilaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00
Bacteria;Actinobacteria;Actinobacteria;Micrococcales;Beutenbergiaceae	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteria;Actinobacteria;Actinobacteria;Micrococcales;Brevibacteriaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17
Bacteria;Actinobacteria;Actinobacteria;Micrococcales;Dermabacteraceae	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.09
Bacteria;Actinobacteria;Actinobacteria;Micrococcales;Microbacteriaceae	0.00	0.00	0.00	0.00	0.12	0.09	0.00	0.00	0.09	0.09
Bacteria;Actinobacteria;Actinobacteria;Micrococcales;Micrococcaceae	0.00	0.09	0.00	0.08	0.13	0.13	0.48	0.00	0.01	2.50
Bacteria;Actinobacteria;Actinobacteria;Micrococcales;Promicromonosporaceae	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
Bacteria;Actinobacteria;Actinobacteria;Micrococcales;Ruaniaceae	0.00	0.01	0.00	0.00	0.01	0.04	0.00	0.00	0.00	0.00
Bacteria;Actinobacteria;Actinobacteria;Nakamurellales;Nakamurellaceae	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Bacteria;Actinobacteria;Actinobacteria;Propionibacteriales;Propionibacteriaceae	0.00	0.03	0.00	0.40	0.03	0.12	9.65	0.00	0.81	7.90
Bacteria;Actinobacteria;Coriobacteriia;Coriobacteriales;Atopobiaceae	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Bacteria;Actinobacteria;Coriobacteriia;Coriobacteriales;Coriobacteriaceae	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.08
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae	0.00	0.24	0.01	0.36	0.35	1.69	6.13	0.01	0.62	2.51
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Barnesiellaceae	0.00	0.00	0.00	0.00	0.02	0.05	0.61	0.00	0.17	0.00
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Dysgonamonadaceae	0.01	0.97	0.00	0.04	0.73	0.76	0.00	0.00	0.00	0.00
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Odoribacteraceae	0.00	0.00	0.01	0.01	0.00	0.12	0.00	0.00	0.12	0.05
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Paludibacteraceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae	0.00	0.04	0.00	0.02	0.02	0.10	0.99	0.00	0.00	0.00
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae	0.00	0.30	1.62	0.50	0.35	1.21	5.62	0.00	1.11	1.45
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Rikenellaceae	0.00	0.06	0.01	0.01	0.10	0.14	0.58	0.00	0.13	0.78

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Table A.2. (cont.)

#Otu Table (Relative Abundance)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Tannerellaceae	0.00	0.08	0.00	0.02	0.07	0.07	0.06	0.00	0.10	0.29
Bacteria;Bacteroidetes;Chitinophagia;Chitinophagales;Chitinophagaceae	0.00	0.04	0.00	0.00	0.02	0.05	0.10	0.00	0.00	0.00
Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cytophagaceae	0.00	0.00	0.00	0.06	0.01	0.00	0.29	0.00	0.00	0.14
Bacteria;Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae	0.03	3.22	0.02	0.12	2.61	2.67	1.12	0.02	0.13	1.30
Bacteria;Bacteroidetes;Sphingobacteriia;Sphingobacteriales;Sphingobacteriaceae	0.01	1.63	0.01	0.07	1.79	1.55	0.61	0.02	0.12	0.12
Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae	98.61	80.13	96.20	6.07	76.72	70.93	9.90	99.15	6.25	32.76
Bacteria;Firmicutes;Bacilli;Bacillales;Paenibacillaceae	0.00	0.06	0.00	0.00	0.17	0.14	0.00	0.00	0.00	0.03
Bacteria;Firmicutes;Bacilli;Bacillales;Planococcaceae	0.08	0.31	0.00	0.04	0.41	0.28	0.00	0.08	0.00	0.12
Bacteria;Firmicutes;Bacilli;Bacillales;Sporolactobacillaceae	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae	0.00	0.00	0.05	0.14	0.00	0.00	5.59	0.00	4.07	8.40
Bacteria;Firmicutes;Bacilli;Lactobacillales;Aerococcaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.93	0.00	0.00	0.38
Bacteria;Firmicutes;Bacilli;Lactobacillales;Carnobacteriaceae	0.00	0.00	0.00	0.02	0.00	0.00	1.50	0.00	0.00	0.12
Bacteria;Firmicutes;Bacilli;Lactobacillales;Enterococcaceae	0.00	0.61	0.00	0.11	0.50	0.48	1.25	0.01	0.29	0.36
Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae	0.00	0.02	0.01	0.41	0.00	0.14	16.87	0.00	3.94	0.76
Bacteria;Firmicutes;Bacilli;Lactobacillales;Leuconostocaceae	0.00	0.04	0.00	0.00	0.03	0.05	0.13	0.00	0.00	0.00
Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae	0.05	2.25	0.05	0.40	1.69	1.83	5.18	0.08	0.27	2.76
Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae	0.00	0.01	0.00	0.00	0.02	0.04	0.00	0.00	0.15	0.16
Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiales incertae sedis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00
Bacteria;Firmicutes;Clostridia;Clostridiales;Eubacteriaceae	0.00	0.00	0.01	0.02	0.00	0.14	0.32	0.00	0.10	0.06
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae	0.00	0.09	0.07	0.15	0.09	0.57	2.04	0.00	0.62	0.44
Bacteria;Firmicutes;Clostridia;Clostridiales;Oscillospiraceae	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00
Bacteria;Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae	0.00	0.03	0.00	0.00	0.03	0.02	0.00	0.00	0.00	0.08
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae	0.00	0.06	0.57	0.22	0.13	0.68	4.54	0.00	0.75	1.77
Bacteria;Firmicutes;Erysipelotrichia;Erysipelotrichales;Erysipelotrichaceae	0.00	0.00	0.03	0.03	0.00	0.07	0.32	0.00	0.29	0.10

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Table A.2. (cont.)

#Otu Table (Relative Abundance)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Bacteria;Firmicutes;Negativicutes;Acidaminococcales;Acidaminococcaceae	0.00	0.02	0.80	0.00	0.02	0.09	0.42	0.00	0.04	0.20
Bacteria;Firmicutes;Negativicutes;Selenomonadales;Selenomonadaceae	0.00	0.01	0.00	0.08	0.00	0.05	2.84	0.00	0.23	0.02
Bacteria;Firmicutes;Negativicutes;Veillonellales;Veillonellaceae	0.00	0.00	0.00	0.04	0.02	0.06	1.05	0.00	0.28	0.72
Bacteria;Firmicutes;Tissierellia;Tissierellales;Peptoniphilaceae	0.00	0.00	0.00	0.04	0.00	0.06	0.42	0.00	0.42	1.12
Bacteria;Fusobacteria;Fusobacteriia;Fusobacteriales;Fusobacteriaceae	0.00	0.08	0.00	0.01	0.11	0.07	0.22	0.00	0.11	0.07
Bacteria;Proteobacteria;Alphaproteobacteria;Caulobacterales;Caulobacteraceae	0.00	0.36	0.01	0.03	0.66	0.84	0.00	0.00	0.21	0.11
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Bradyrhizobiaceae	0.00	0.14	0.00	0.01	0.21	0.25	0.22	0.00	0.05	0.00
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Brucellaceae	0.00	0.57	0.00	0.00	0.53	0.58	0.00	0.01	0.05	0.00
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Hyphomicrobiaceae	0.00	0.00	0.00	0.00	0.03	0.10	0.00	0.00	0.00	0.29
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Methylobacteriaceae	0.00	0.05	0.01	0.09	0.03	0.06	0.00	0.00	0.22	0.62
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae	0.00	0.27	0.00	0.00	0.26	0.44	0.00	0.00	0.06	0.00
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae	0.00	0.06	0.00	0.00	0.10	0.05	0.00	0.00	0.00	0.00
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Xanthobacteraceae	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae	0.00	0.01	0.00	0.01	0.06	0.08	0.00	0.00	0.00	0.21
Bacteria;Proteobacteria;Alphaproteobacteria;Rhodospirillales;Acetobacteraceae	0.85	0.18	0.14	89.71	3.56	3.92	1.76	0.23	76.04	17.53
Bacteria;Proteobacteria;Alphaproteobacteria;Rhodospirillales;Rhodospirillaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00
Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae	0.00	0.22	0.00	0.20	0.34	0.47	1.50	0.00	0.55	1.19
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Alcaligenaceae	0.00	0.17	0.00	0.00	0.26	0.26	0.00	0.00	0.04	0.00
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiaceae	0.00	0.00	0.00	0.00	0.00	0.00	4.28	0.00	0.00	1.04
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae	0.01	1.03	0.02	0.01	0.84	0.96	0.32	0.01	0.04	0.21
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae	0.00	0.00	0.00	0.02	0.00	0.01	0.00	0.00	0.03	0.00
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Sutterellaceae	0.00	0.00	0.12	0.00	0.03	0.09	0.45	0.00	0.04	0.07
Bacteria;Proteobacteria;Betaproteobacteria;Neisseriales;Chromobacteriaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

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Table A.2. (cont.)

#Otu Table (Relative Abundance)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Bacteria;Proteobacteria;Betaproteobacteria;Neisseriales;Neisseriaceae	0.00	0.00	0.00	0.01	0.01	0.02	3.26	0.00	0.00	0.22
Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Azonexaceae	0.00	0.00	0.00	0.00	0.01	0.03	0.00	0.00	0.00	0.00
Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00
Bacteria;Proteobacteria;Gammaproteobacteria;Aeromonadales;Aeromonadaceae	0.01	0.12	0.00	0.01	0.14	0.15	0.00	0.01	0.00	0.20
Bacteria;Proteobacteria;Gammaproteobacteria;Aeromonadales;Succinivibrionaceae	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.21
Bacteria;Proteobacteria;Gammaproteobacteria;Alteromonadales;Shewanellaceae	0.00	0.13	0.00	0.00	0.12	0.08	0.00	0.00	0.00	0.08
Bacteria;Proteobacteria;Gammaproteobacteria;Chromatiales;Chromatiaceae	0.00	0.04	0.00	0.00	0.05	0.04	0.00	0.00	0.00	0.02
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae	0.28	3.77	0.17	0.13	3.91	3.92	0.38	0.28	0.30	0.55
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Erwiniaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.19
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Hafniaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Morganellaceae	0.01	0.22	0.01	0.00	0.19	0.27	0.00	0.02	0.01	0.00
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Yersiniaceae	0.00	0.02	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.02
Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales;Halomonadaceae	0.00	0.02	0.00	0.04	0.01	0.02	0.42	0.00	0.12	0.24
Bacteria;Proteobacteria;Gammaproteobacteria;Pasteurellales;Pasteurellaceae	0.00	0.00	0.00	0.01	0.00	0.00	2.59	0.00	0.00	1.46
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae	0.04	1.34	0.01	0.10	1.34	1.50	0.00	0.06	0.21	1.21
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae	0.00	0.01	0.00	0.00	0.05	0.08	0.29	0.00	0.00	0.19
Bacteria;Proteobacteria;Gammaproteobacteria;Vibrionales;Vibrionaceae	0.00	0.09	0.00	0.00	0.13	0.15	0.00	0.00	0.00	0.00
Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae	0.01	0.51	0.00	0.03	0.51	0.71	0.13	0.00	0.00	0.16
Bacteria;Proteobacteria;Oligoflexia;Bdellovibrionales;Bdellovibrionaceae	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
Bacteria;Verrucomicrobia;Opitutae;Opitutales;Opitutaceae	0.00	0.00	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00
Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Akkermansiaceae	0.00	0.00	0.00	0.00	0.02	0.06	0.58	0.00	0.17	0.01
Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Verrucomicrobiaceae	0.00	0.07	0.00	0.00	0.07	0.02	0.00	0.00	0.00	0.00

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- Izmir Institute of Technology, Scientific Research Project, 2020IYTE0076 (2020-2021)

Selected Publications:

- Bozkurt, B., Terlemez, G., & Sezgin, E. (2023). Basidiomycota species in Drosophila gut are associated with host fat metabolism. *Scientific Reports*, 13(1), 13807.

- Sezgin, E., Terlemez, G., **Bozkurt, B.**, Bengi, G., Akpinar, H., & Büyüktorun, İ. (2022). Quantitative real-time PCR analysis of bacterial biomarkers enable fast and accurate monitoring in inflammatory bowel disease. *PeerJ*, 10, e14217.

- İrkin, R., **Bozkurt, B.**, & Tümen, G. (2021). Determination of the prevalence of Salmonella spp. and S. aureus in meat products by Real-Time PCR and testing their antibiotic susceptibility. *Med. Weter,* 77(7).