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Exploring the Interaction of Probiotic Bacteria and Honey Bee Gut Metabolome

Master´s thesis

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Sustainable agriculture and food security

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Declaration

I hereby declare that I have authored this master's thesis carrying the name „ Exploring the Interaction of Probiotic Bacteria and Honey Bee Gut Metabolome“ independently under the guidance of my supervisor. Furthermore, I confirm that I have used only professional literature and other information sources that have been indicated in the thesis and listed in the bibliography at the end of the thesis. As the author of the master's thesis, I further state that I have not infringed the copyrights of third parties in connection with its creation.

In Prague on 19th of April 2024 ______________________________

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Exploring the Interaction of Probiotic Bacteria and Honey Bee Gut Metabolome

Summary:

This thesis aimed to investigate whether supplementation with specific bacteria would alter the metabolic composition of honey bee (*Apis mellifera*) gut and bodily tissue and whether this alteration would be influenced by the presence of a native gut microbiome obtained from older siblings. To achieve this, newly emerged bees were either supplemented with a mixture of bacterial strains (*Lactobacillus helsinborgensis*, *Bifidobacterium asteroides*, *Bombilactobacillus mellis* and *Lactobacillus apis*) or kept in sterile conditions, and were housed either with or without the presence of older bees. This results in four treatment combinations: Control ©, Probiotics (P), Control with older bees (COB) and Probiotics with older bees (POB).

Tissues of bees from each group were analysed by means of ^{1}H Nuclear Magnetic Resonance (NMR), generating metabolite spectra which allowed for identification of individual metabolites and comparison of their relative abundances. The results were interpreted by using hierarchical clustering, Principal Component Analysis (PCA), and Orthogonal partial least squares discriminant analysis (OPLS-DA). These methods were utilized to find differences between groups and to identify the most important compounds contributing to these differences.

Hierarchical clustering indicated an effect of the bacterial supplement, as treatments that received said supplement tended to cluster more closely with each other than with the control groups. PCA, however, shows that differences are rather subtle and inadequate to clearly distinguish control and supplemented groups.

In terms of specific compounds, the bacteria in the supplement mainly seem to influence the organic acid composition of the gut and tend to increase the acetate content. A phenomenon possibly caused by increased representation of *Bifidobacterium spp.* in the supplemented groups.

In the body, supplementation with the above-mentioned bacteria seems to slightly affect neuro-active compounds such as inosine and adenosine monophosphate (AMP).

Keywords:

Honey bee, Microbiota, Metabolomics, Probiotics, *Lactobacillus*, *Bifidobacterium*, Nuclear Magnetic Resonance

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1 Introduction

Pollinators are key to the functioning of our ecosystems and to the production of many of our food items. According to Ollerton et al. (2011), approximately 87.5% of all flowering plants are estimated to rely exclusively or partly on animals for pollination. Therefore, the loss of these pollinators inevitably leads to decline or loss of the associated plant species. A concept strongly supported by research such as Biesmeijer et al. (2006). Given that both pollinators and the plants they pollinate are an important food source for other organisms, the loss of these pollinating species could jeopardize the stability of entire ecosystem. Pollinating species are therefore often referred to as keystone species: A species without which the system crumbles.

When it comes to food production, 75% of plant species cultivated for human consumption benefit from or rely on biotic pollination (Klein et al., 2006). It is estimated that pollination services contribute 9.4% to the global agricultural gross domestic product (GDP), which amounted to between 190 to 467 billion Euros in 2009 (Lautenbach et al., 2012). The loss of pollinator communities could drastically reduce the abundance and the diversity of our food supply. Taken together with indirect effects due to ecosystem malfunction, such as increased incidence of pests, droughts, floods, and soil erosion, food security could be severely compromised. Therefore, maintaining healthy pollinator populations is of utmost importance, yet their numbers are declining globally (e.g. Kevan & Viana, 2003; Dicks et al., 2021)

A large share of the above-mentioned pollination services are provided by bees. It is estimated that pollination by honey bees, bumblebees and other wild types of bees contributes to about one third of the global food production (Khalifa et al., 2021). The western honeybee *Apis melifera*, is one of the most important species in pollination of commercial crops (Potts et al., 2016). Each year for example, it takes two million hives to sustain almond production in California alone (Champetier & Summer, 2019). More broadly, the species accounts for 75% of commercial pollination services in the US and 34% in the UK (Khalifa et al., 2021). Since honey bees are increasingly confronted with biotic and abiotic stressors such as foulbrood disease, *Varroa* mites, and pesticides, research into honey bee health (in addition to that of pollinators in general) is essential in the endeavour of guaranteeing food security and creating a sustainable future, both in ecology and agriculture.

This work will investigate the initial steps to determine whether supplementation with bacteria (probiotics) could be an effective strategy to counteract the threats this species is facing.

2 Scientific hypothesis and aims of the thesis

The aim of this thesis is to investigate if and how bacterial supplements alter the body and gut composition of honey bees (In this thesis, the term "honey bees" refers to the European honey bee, *Apis mellifera,* unless otherwise stated.), specifically in terms of metabolite abundances. Most studies regarding the gut microbiota of honey bees focus on the general effects of bacterial supplements, such as disease resistance, pesticide tolerance, and others. However, they often lack detailed information about the biochemical mechanisms underlying these effects. This study hence aims to provide insights into the biochemical changes induced by supplementation with specific gut bacteria and simultaneously seeks to investigate the interaction of such a supplement with the native gut microbiome.

This study hypothesised that bacterial supplementation and social interaction with older bees from the same hive can alter the metabolic profiles in the gut. Newly emerged young bees typically have few or no gut bacteria, but their gut microbiome develops as they mature (Martinson et al., 2012; Hroncova et al., 2015). The control group, which did not receive any treatment, is expected to exhibit minimally developed gut microbiome. Conversely, bees that received bacterial supplementation, artificially introduced older bees to develop their microbiome through social interaction, or both treatments are expected to develop different gut microbiomes. Consequently, these treatments are expected to significantly influence gut metabolome, which could then lead to changes in the body metabolome as well.

3 Current knowledge

As mentioned in the introductory part of this work, honey bees are a vital part of food production worldwide. However, their populations are struggling because their health is under serious threat. Here, I will start by exploring the gravity of the situation, what the main problems are and what we can do to solve them. The focus will then be shifted towards probiotics. A promising and relatively new addition to the arsenal of weapons to fight the demise of the honey bees.

3.1 The European honey bee, a species under pressure.

Honey bees, the European (*A. mellifera*) and eastern honey bees (*A. cerana*) in particular, are quite the extraordinary species since they are on the verge between domesticated and wild. The insect can and does survive in the wild, but its numbers have risen tremendously thanks to the ancient art of beekeeping. This unique relationship between honey bees and man, makes it a remarkably valuable tool in agricultural pollination and allows for close monitoring and managing of the insect's health. It is, therefore, a key species in ensuring stable food production for future generations and creating a sustainable economic system (Champetier & Summer, 2019; Khalifa et al., 2021).

Honey bees belong to the insect family 'Apidae' along with bumble bees, and to the genus 'Apis'. Currently, according to the US National Center for Biotechnology Information (NCBI) (Schoch et al., 2020), nine species of honey bee can be distinguished based on their genome: *Apis andreniformis* (black dwarf honey bee); *A. florea* (red dwarf honey bee); *A. dorsata* (giant honey bee); *A. koschevnikovi* (Koschevnikov's honey bee); *A. cerana* (eastern honey bee); *A. laboriosa* (Himalayan giant honey bee); *A. mellifera* (western/European honey bee); *A. nigrocincta* (Philippine honey bee); and *A. nuluensis*. Of these, only *A. mellifera* and *A. cerana* are managed by beekeepers and harvested for honey. This thesis will focus on the European honey bee (*A. mellifera*) since this is the main honey bee species important for pollination here in Europe. In the rest of this work, 'honey bee' will therefore refer to *Apis mellifera* unless stated otherwise.

3.1.1 Honey bee biology & lifecycle

Just like ants, wasps, termites and even naked mole rats, honey bees are so called eusocial animals (Burda et al., 2000; Cronin et al., 2013). Meaning that they live in colonies in which there's division of labour, cooperative brood care and overlap of generations within the colony

(Winston, 1987; Burda et al., 2000). A queen can build a colony consisting of several tens of thousands of workers at its peak (e.g. Loftus et al., 2016). Each of these workers lives around six weeks during the summer months which they fill with tasks such as tending for the brood, foraging, defending the nest, etc. (Winston, 1987).

Honey bees are holometabolic insects, which means that they are born as larvae and go through a pupae stage before emerging as adults. This process takes about three weeks during which the older workers carefully make sure the conditions are perfect for larval development (Winston, 1987). Once the adult bee emerges, it doesn't take long before they take up work themselves. The first three weeks are spent inside the hive as housekeepers. Feeding the brood, keeping the nest clean and at a comfortable temperature (around 35°C), building new honeycomb etc. Only when the worker gets old enough, it will start leaving the nest to forage increasingly frequently (Winston, 1987). By then, it will have mastered the famous bee dance and can show its sisters where to go. Thanks to this hard work and extraordinary cooperation, one hive can produce up to 27 kg of surplus honey a year. The average is estimated on 11kg per annum (British beekeeping association, n.d.). Other honey bee products commercially used are wax and propolis. The latter, due to its antimicrobial properties can be used as a natural medicine (Sforcin et al., 2016).

3.1.2 Trends in population density

The western honey bee has been managed by humans for centuries and from the 1600s onwards, it has been able to populate the entire globe largely thanks to man (van Engelsdorp & Meixner, 2010). Even to this day, there's more of *A. mellifera* than ever before. Data from the FAO shows that the number of managed honey bee colonies increased globally by 85% between 1961 and 2017 (Phiri et al., 2022). There are, however, tremendous regional differences. In Europe, the number of managed colonies has dropped by 11% with a steep decline at the end of last century and a slow recovery since (Fig. 1). Seen globally, the situation regarding honey bees is thus not as dire as it's sometimes made out to be. However, human population rises more quickly than that of honey bees (Phiri et al., 2022) and highly developed areas such as Europe and the U.S. where people's diets are packed with foods that need pollination, have low numbers of them. It is therefore entirely possible that pollination services could end up falling short for the rising food demand. Even though we might not be in a pollination crisis yet, it is vital to ensure stable, healthy pollinator populations for the future.

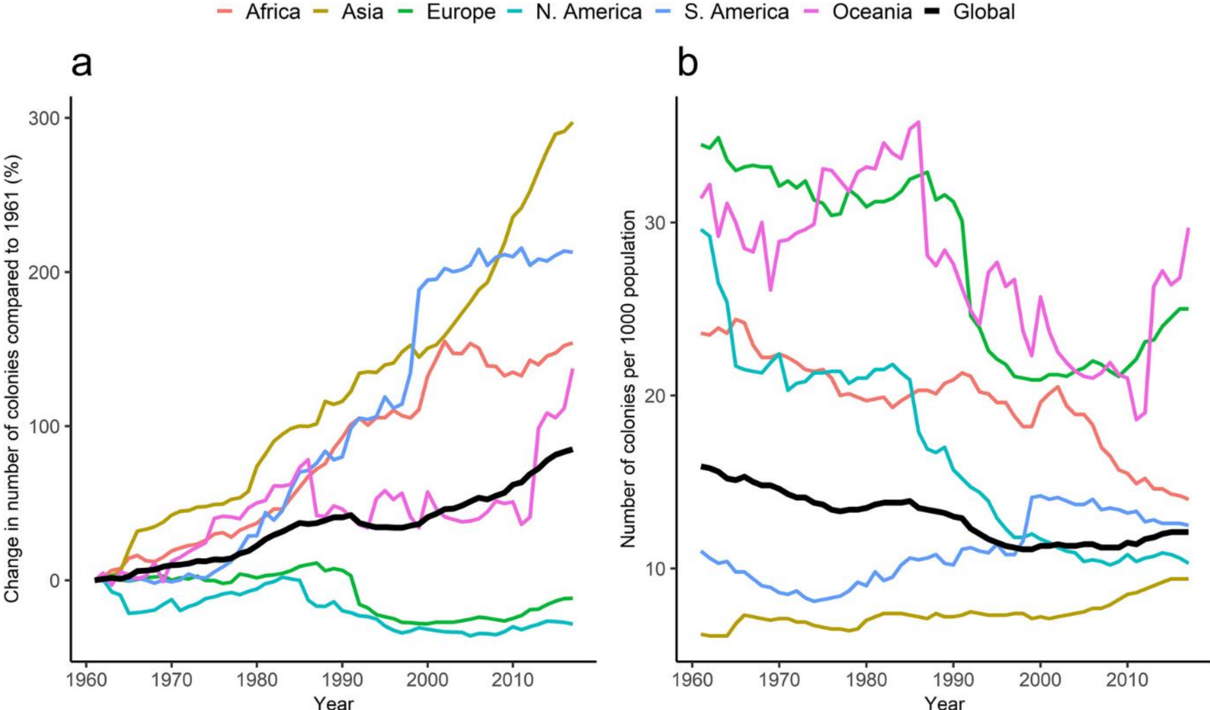


Figure 1: Overview of the global trends in number of managed honey bee colonies since 1961 (Phiri et al., 2022)

3.1.3 Threats to honey bee health

As Figure 1 depicts, the number of managed bee colonies is globally increasing. This, however, doesn't say much about how healthy each colony is. The global upward trend and the slight recovery in Europe is not so much the result of the absence of problems but more of the beekeeper's ability to overcome them. A wide array of threats, biotic and abiotic, are putting a constant strain on *A. mellifera*. The most important ones will be covered in this section.

3.1.3.1 Parasites

A very well-known organism within this category is an invasive ectoparasitic mite that carries the somewhat threatening, yet suiting name *Varroa destructor*. The mite can parasitise both adult bees and brood and therefore causes a broad range of symptoms which are jointly called varroosis (Traynor et al., 2020). A bee colony suffering from this infestation, can die within two years after the first mites found their way into the hive (Boecking & Genersch, 2008). Other than causing varroosis, and perhaps even more problematic, the mites can also be a vector to different kinds of viral pathogens (this subject is described in the following section). Consequently, controlling varroa infestation has had to become standard in beekeeping practices.

Such *Varroa* control is often executed by application of acaricides (substances that kill mites (Acariformes)) (Jack & Ellis, 2021). These substances range from synthetic to organic such as organic acids and essential oils (van der Steen & Vejsnæs, 2021). While these products deal quite effectively with *Varroa* infestation, timing and coordination between beekeepers is of the utmost importance to prevent reinfestation. This is often where the problem lies and why *V. destructor* still manages to cause the most significant economic losses of all honey bee diseases (Boecking & Genersch, 2008).

3.1.3.2 Viral pathogens

Another important cause of negative impacts on honey bee health is viruses. Some have coexisted with *A. mellifera* for a long time and do not cause any large scale problems, but those transferred by the exotic *V. destructor* mite, are reason for concern. One such virus has been inventively named 'deformed wing virus' (DWV) due to its tendency to cause irregular wing development in infected pupae. Adults with such deformations are soon expelled from the colony and die within 67 hours (Yang & Cox-Foster, 2007). These workers are therefore nothing but precious energy lost, resulting in weakening of the colony.

A second virus that poses a significant threat to honey bees is the acute bee paralysis virus (ABPV). Infected bees often remain symptom free, but when the virus finds its way into the haemolymph, it becomes highly lethal (Genersch, 2010). Since *V. destructor* accesses the haemolymph and fat bodies directly (Ramsey et al., 2019), any ABPV carried by this vector poses a serious threat (Chen & Siede, 2007). Both DWV and ABPV have been found to cause significant drops in winter survival, even when the infection is otherwise symptomless (Genersch, 2010).

Since *V. destructor* plays a critical role in spreading these viruses, a useful method of controlling them is by controlling their vector. Other than that, viruses are notoriously hard to kill without harming the host. Hence, the best final defence usually comes from the bee's immunity itself. More specifically, the gut microbiome seems to be an important factor in determining resistance to viral infection (Dosch et al., 2021; Svobodová et al., 2023) and therefore, probiotics might be an especially helpful tool in preventing viral diseases. However, data is still limited.

3.1.3.3 Bacterial pathogens

Regarding to bacterial pathogens, two well-known diseases in apiculture are American foulbrood (AFB) and European foulbrood (EFB) (Forsgren et al., 2018). Both of which are caused each by a single type of microbe. For EFB, *Melissococcus plutonis* is the culprit. Its American counterpart: *Paenibacillus larvae*. Both bacteria enter and infect larvae through contaminated food. Once established, the bacteria will grow uncontrollably and take away the nutrients from the larvae which then starves to death (Forsgren et al., 2018). The infection thus results in an unusually high larval mortality, hence the name 'foulbrood'.

When it comes to control of these diseases, symptomatic colonies usually have to be destroyed (Locke et al., 2019). Treatment with antibiotics is an option in the U.S. and some non-European countries (Locke et al., 2019), however, it can lead to increased susceptibility to other pathogens (Rayman et al., 2017). Therefore, new prevention and treatment methods are being developed. Very recently for example, world's first bee vaccine became available, giving honey bees enhanced resistance to American foulbrood (Dickel et al., 2022). Another viable option seems to be supplementation with lactic acid producing bacteria (Forsgren et al., 2010; Daisley et al. 2020).

3.1.3.4 Fungal pathogens

Nosema spp. is an obligatory intracellular fungal parasite that spreads amongst cells and individuals through spores (Formato et al., 2022). Two organisms belonging to this group (*N. apis* and *N. ceranae*) are known to infect the western and eastern honey bee respectively. *N. ceranae* can however, also infect *Apis mellifera* (Higes et al., 2008).

When spores are ingested by adult bees through contaminated faeces, the fungus can infect epithelial cells of the gut (Formato et al., 2022). There, it causes a potentially lethal disease that is generally called nosemosis and causes significant economic losses in some regions of the world (Genersch, 2010). Interestingly, nosemosis has also been found to change the behaviour of surviving workers and lead to precocious foraging (Wang & Moffler, 1970). Perhaps aiding in the spread of the pathogen amongst hives.

Nosemosis can be prevented or controlled by regularly feeding antifungal substances to the bees (Williams et al., 2008). Together with disinfecting all contaminated material, spreading the disease can largely be prevented. However, the efficacy is not infallible and more sustainable solutions are needed.

Another fungal pathogen in honey bees is *Ascophaera apis*. An ascomycete growing in the digestive tract and mainly affecting larvae causing so called chalkbrood disease (Aronstein & Murray, 2010). The gravity of this disease seems to largely depend on hygienic behaviour performed by worker bees. If they are quick to remove infested brood, spread of the disease can be largely prevented ('*Ascosphaera apis*', 2005). Other than that, good hygienic practices performed by the beekeeper are helpful to prevent outbreaks.

3.1.3.5 Abiotic stressors

A first important abiotic threat to honey bees is of course climate change (Le Conte & Navajas, 2008). Due to this phenomenon the bee's environment will undergo significant change and become more and more unpredictable. Temperature will increase along with the incidence of extreme weather events such as drought and heavy rainfall. Warmer, dryer conditions have been associated with lower winter survival in beehives (Switanek et al., 2017). Furthermore, changes in environmental conditions can cause a mismatch between fauna and flora since they might use different signals for timekeeping (e.g. temperature vs. length of day; Hegland et al., 2009) resulting in a potential shortage of foraging opportunities at certain times of year. Lastly, climate change opens opportunities for new pests to arise and new diseases to establish (Le Conte & Navajas, 2008). Therefore, it is important to consider these effects and of course mediate climate change.

The second major abiotic stressor is caused even more directly by us humans. In order to protect our crops from harmful insects, we use pesticides that are equally capable of killing the beneficial ones. Even though pollinators are usually exposed to lower doses, their individual and collective performance can still be significantly affected (Henry et al., 2012). Exposure to nonlethal doses of pesticides has been shown to reduce queen fertility (Gajger et al., 2017), lower individual immunity (Pettis et al., 2012) and negatively affect motor function, behaviour and cognition (Chmiel et al., 2020) in honey bees. All of which are evidently harmful to healthy colony functioning.

3.2 Probiotics and their role in honey bee health

As mentioned earlier, probiotics can be a viable solution to several of the problems described above. In order to understand the working mechanism however, it is helpful to have a basic knowledge of the honey bee digestive system. Therefore, before I elaborate on probiotics themselves, let me introduce you to the honey bee gut.

3.2.1 The honey bee gut

The intestines of honey bees essentially consist of three major compartments as depicted in Figure 2: foregut, midgut and hindgut. The first of which includes the oesophagus and crop, used for acquirement and storage of food and important in the social exchange of regurgitated food (trophallaxis) (Powell et al., 2014). Then there's the midgut which is the main part responsible for digestion and which connects to the hindgut through the pylorus. The hindgut, in turn, consists of the ileum followed by the rectum. Together, these two organs harbour the majority of the gut microbiota and the hindgut is therefore a major contributing factor to honey bee health (Rayman et al., 2018).

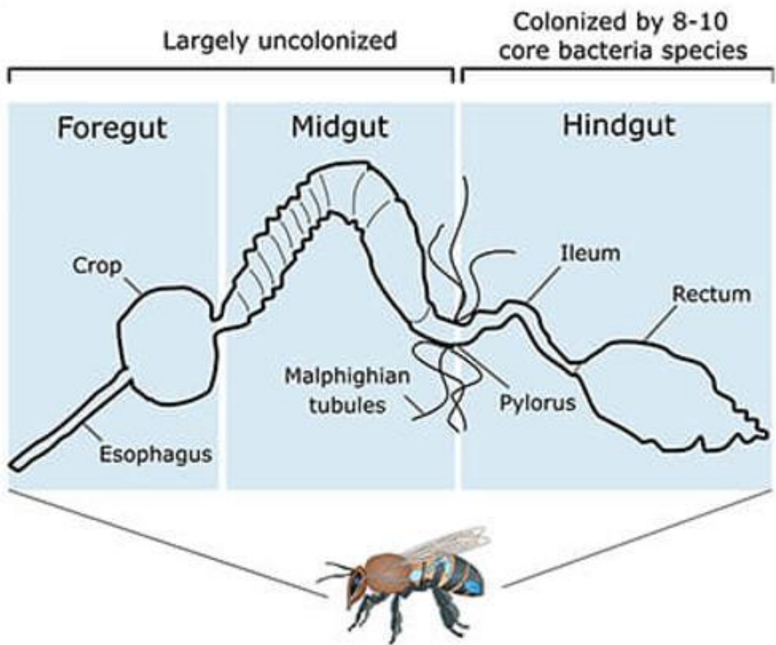


Figure 2: Schematic representation of the honey bee gut (McAfee, 2018)

3.2.2 The gut microbiome

A microbiome is a community of microorganisms. In this work more specifically one that lives in symbiosis with its host. This symbiosis is mutualistic in ideal circumstances but can just as well be communalistic or even antagonistic when conditions are suboptimal. Us humans have for example a skin microbiome, a bladder microbiome and perhaps most famously a gut microbiome. When these microbial communities are balanced, the individual is healthy. When disturbed however, (e.g. by unhealthy diet or antibiotics) all sorts of problems can arise, ranging

from diarrhoea, to depression and even auto-immune disease (Capuco et al., 2020, Xu et al., 2019).

3.2.2.1 Importance

In bees, much like in humans, the gut microbiome is essential to the health of the individual. Most likely it's even more important to the insects since bees have a notoriously low genetic immunity. The number of immune genes such as those coding for antimicrobial peptides (AMPs) is significantly lower in honey bees compared to other insects (Evans et al., 2006). A phenomenon at least partially explained by honey bee's reliance on social immunity, where the colony acts as a super organism and collective sanitising efforts reduce the average pathogen exposure in all members of the colony (Traynor et al., 2020).

This means however, that when a pathogen does find its way in, a bee has to rely on other mechanisms to protect itself and that is where the gut microbiome comes in. Studies have shown that gut microbiota can directly prevent pathogen invasion. Raymann et al. (2017) for example, demonstrated that disturbance of the microbiome with antibiotics increases susceptibility to opportunistic bacterial pathogens. Similarly, Kwong et al., (2017) showed that individuals with normal gut microbiota produce significantly more antimicrobial peptides and are more resistant to *E. coli* infection. Results published by Dosch and colleagues in 2021, furthermore, indicate that honey bees with a normal gut microbiome are less likely to die from deformed wing virus infection and honey bees that are able to survive varroosis appear to have a bacterial community which is less susceptible to multiple viruses (Svobodová et al., 2023).

Other than improved resistance against harmful organisms, a well-balanced gut microbiome is also very important in nutrient acquirement, with microorganisms being able to digest otherwise in- or less digestible substances (e.g. pollen cell walls) (Kwong & Moran, 2016; Bonilla-Rosso & Engel, 2018). Moreover, microbiota have another important protective effect which has developed quite recently. Namely, protection against manmade substances. Since we greatly influence our environment with our industry and agriculture, all organisms including honey bees are exposed to the chemicals and pollutants we release. It has been shown that a healthy microbiome can mediate the impact of at least some of them. Wu et al. (2020) found that a disturbed gut, lowers a bee's chance of surviving chronic exposure to thiacloprid (insecticide) and fluvalinate (acaricide) due to a diminished breakdown of these chemicals in the midgut. Another study by Rothman et al. in 2019, showed that some bee-associated bacteria can assimilate heavy metals, reducing the burden these elements place on their host. Lastly, a healthy gut also seems to ameliorate the effects of microplastics (Wang et al., 2021).

From the disquisition above, it is clear that the microbial community in the honey bee intestines is key to assuring good health and preventing colony loss. Yet several factors pose a significant threat to the balance of this community. Antibiotics evidently have profound effects by directly killing certain bacteria while leaving others unharmed, but also less conspicuous elements can disturb the honey bee gut. It is known for example that pesticides can alter the microbial composition (Kakumanu et al., 2016; Blot et al., 2019), just like microplastics (Wang et al., 2021) and heavy metals (Rothman et al., 2019). Hence, it seems that the very things that the microbiome protects against, also disturb it. To make sure that the microbial community keeps its ability to mediate harmful effects, it can therefore be advantageous to regularly reintroduce a mixture of bacteria to the gut. Before discussing the details of probiotic administration to honeybees, the next section will delve into the insect's intestinal inhabitants.

3.2.2.2 Community composition

The importance of the gut microbiome is clear, but what does a 'healthy' microbiome look like? In bees, nine species (also called phylotypes) of bacteria make up the bulk of the gut microbiome. Five of those, *Snodgrassella alvi*, *Gilliamella apicola*, two species of *Lactobacillus* (Firm-4 and Firm-5) and *Bifidobacterium asteroides* are abundant in all individuals and therefore make up the core microbiome (Nowak et al., 2021). This core is complemented by varying abundances of the four other, less universal bacterial clades: *Frischella perrarra*, *Bartonella apis*, *Commensalibacter* and *Bombella apis* (Engel et al. 2013; Corby-Harris et al. 2014b; Kešnerová et al. 2016).

3.2.2.2.1 Differences in community composition

The particular social strategy of honey bees causes some interesting patterns in the microbial composition of their gut. Solitary bees, for example, tend to have more diverse and variable microbial communities that largely depend on acquisition through the environment (Voulgari-Kokota et al., 2019), while social bees have more similar microbes that they acquire from one another (see 3.2.2.4).

Although the specific bacteria present in social bee's guts might be the same, there are notable differences in relative abundances between individuals within the same colony. When investigating the gut composition of nurse bees, foragers and food processors, Jones et al. (2018) found that bees taking on nest activities (nursing and food processing) harboured more *Lactobacillus mellis* (Firm-4) and *Bifidobacterium*. Furthermore, in these nest task performing bees, overall diversity of the microbiota was found to be higher as well. Possible explanations

according to the researchers are that bees spending a lot of time within the hive, perform mainly food related tasks (feeding larvae and food processing) and therefore have higher diversity and abundance of carbohydrate processing microbes (e.g. *Lactobacillus*). These individuals might also experience increased pressure of pathogens due to the hive conditions, possibly selecting for increased abundance of health promoting bacteria such as *Lactobacillus* and *Bifidobacterium*. Increased abundance of lactic acid bacteria in foragers compared to nurses has been found in other studies as well (Kapheim et al., 2015).

3.2.2.2.2 Non-bacterial inhabitants of the gut

Bacteria are not the only type of organisms populating honeybee intestines. Fungi are part of the community as well. The composition of the fungal community, however, is not as uniform as the bacterial one (Decker et al., 2023). What is present in the gut closely resembles what can be found in the hive environment and therefore greatly depends on location and circumstances (Decker et al., 2023). Consequently, it is much harder to pinpoint the function and possible benefits of the presence of certain fungi compared to that of the core bacteria. Since the latter are present in all bees regardless of location, it seems that they are vital to microbiome functioning. The focus of microbiome studies, probiotics and of this work is therefore mainly on the bacterial aspect.

3.2.2.3 Microbial distribution

The bacterial phylotypes described above are abundant in the honey bee gut, yet not equally distributed along all parts of it. The current understanding of this distribution is as follows: The crop contains few bacteria and those present are related to the environment or have some function in nectar metabolism. The midgut, because of its lining being renewed every so often, also does not harbour a significant bacterial community. Further down the digestive tract however, in the ileum and rectum respectively, approximately 10^8 and 10^9 bacterial cells can be found. While the ileum is mostly home to *Snodgrasella*, *Gilliamella* and *Frischella*, the rectum offers refuge to *Lactobacillus* and *Bifidobacterium* (Raymann & Moran, 2018). For clarity, the distribution is depicted in Figure 3.

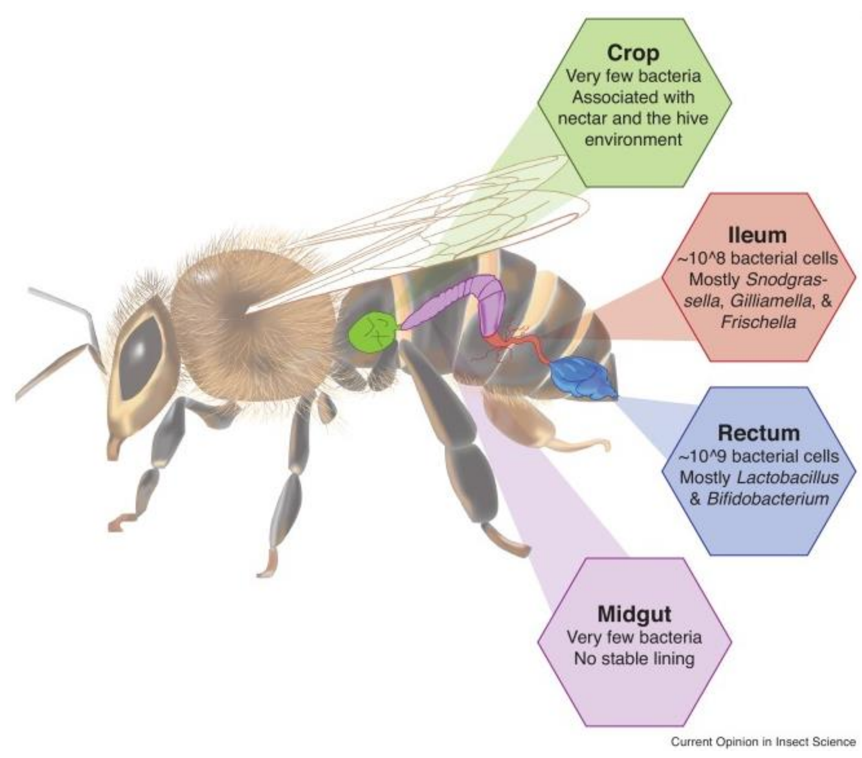


Figure 3: Overvieuw of the bacterial distribution in the honey bee gut (Raymann & Moran, 2018)

3.2.2.4 Acquisition of microbiota

It is now clear which bacteria are present and where they reside, but how do bees acquire them? Newly emerging adults tend to be free of microbes and are colonized within a few days (4-6) by the core microbiota (Martinson et al., 2012). This quick and uniform colonisation by the same bacterial species hint that they must be acquired from each other rather than from the environment. It is believed that one of the main transmission routes is trough so called proctodeal trophallaxis or anus-to-mouth feeding. Martinson et al. (2012) showed that a normal gut microbiome could be established by exposure of newly emerged bees to older workers or their excrements, while exposure to oral trophallaxis or hive components only, failed to do the same. Adult bees therefore most likely indeed acquire their core gut microbiota from older individuals, ensuring healthy, pathogen resistant bees throughout the colony and thus contributing to social immunity.

3.2.3 Probiotics: functions and benefits

Section 3.2.2.1 of this work explains the importance of the gut microbiome in the overall health of honey bees. Supporting the microbial composition of the gut by supplementation with important symbionts might therefore confer significant health benefits. Before I explain the science behind probiotics in honey bees, it is essential to define the term. According to the FAO/WHO, a probiotic is defined as:

"Live microorganisms which, when administered in adequate amounts, confer a health benefit to the host."

In honeybees, lactic acid bacteria (LAB) and *Bifidobacterium* are recognized as important symbionts (Vásquez et al., 2012) and are therefore thought to have beneficial effects on the host. Scientific enquiry into this topic seems to back up this statement indeed.

3.2.3.1 Resistance to pathogens

Forsgren et al. (2009) for example, found that bee specific LAB (*Lactobacillus*) and *Bifidobacterium* have in vitro and in vivo antagonistic effects against the American foulbrood causing bacteria *Paenibacillus larvae*. Supplementation of larval food with these bacteria, significantly reduced the number of infected larvae. Another study by Evans and Lopez in 2004 showed that administering *Lactobacillus* and *Bifidobacterium* to newly emerged bees induced an increase in the production of antimicrobial peptides. Possibly helping the insect fight pathogens by 'training' the immune system.

In both these studies, mixtures of LAB were provided, but in some cases, a single bacterial strain can provide protection against pathogens. *Lactobacillus kunkeei,* for example, has been found to reduce honey bee sensitivity to infection by the opportunistic pathogen *Serratia marcescens* (Chege et al., 2023).

Exposure to high quantities of a single organism should not, however, be done carelessly. A study by Ptaszyńska et al. in 2015 found that supplementation by *Lactobacillus rhamnosus* led to decreased survival rate in *Nosema ceranae* exposed bees. Additionally, Schwarz et al. (2016) showed inoculating newly emerged worker bees with *Snodgrassella alvi* increased susceptibility to a protozoan parasite *Lotmaria passim.* Remarkable since *S. alvi* is a member of the core gut bacteria in honey bees and is thought to have beneficial effects on the host. However, inoculation with the bacterium in such an early stage and with only this bacterium,

most likely results in an unbalanced development of the microbiome later in life[1] which could explain the heightened sensitivity to the parasite. Demonstrating the importance of species composition as well as the balance between them.

3.2.3.1.1 Mode of action

Beneficial gut bacteria prevent infection by pathogens in several distinct ways. A first mechanism is called colonisation resistance (Pickard et al., 2017) and relies on the 'first come, first serve' principle. Similar to the pioneer effect, micro-organisms that have already established a stable population within an habitat, will usually be able to outcompete newly introduced organisms simply by using up recourses and occupying space (competitive exclusion). In other words, a gut filled with neutral and beneficial bacteria leaves no space for pathogens and therefore results in a strong intestinal barrier against the latter.

Inhibition of such pathogens, however, also happens more directly. Probiotic bacteria are known to produce antimicrobial compounds with bacteriostatic or bactericidal effects such as bacteriocins (antimicrobial peptides; AMPs), lysozymes, hydrogen peroxide, siderophores, and proteases (Reis et al., 2012; Iorizzo et al., 2022). Furthermore, the pH reducing effect of volatile fatty acids and organic acids produced by some bacteria, can render the gut environment inhospitable to pathogens and several of these acidic compounds also have direct antimicrobial effects (Sorrentino et al., 2018). Lactic acid (LAB) and acetic acid (*Bifidobacterium*) are especially potent antimicrobial substances (Surve et al., 1991; Entani et al., 1998).

A last mechanism by which probiotics can inhibit pathogenic infection is by stimulation of the host's immune system. Honey bees produce several antimicrobial peptides such as defensin and apidaecin (Yi et al., 2014). As mentioned earlier, lactic acid bacteria and bifidobacteria have been found to upregulate the expression of such AMPs (Evans & Lopez, 2014).

3.2.3.2 Pesticide processing

As previously discussed, exposure to pesticides can put a significant impact on honey bee health. Mitigation of their effect is therefore one of the major assets probiotics might have to offer. LAB have been shown to be able to break down or sequestrate certain (organophosphorus) pesticides (Islam et al., 2010; Zhang et al., 2014), likely resulting in lower

[1] When a species is introduced first into an untouched environment, it will have the opportunity to reproduce without hindrance and dominate and/or supress other species that are introduced later, regardless of competitive abilities. In evolutionary biology, this phenomenon is referred to as 'pioneer effect'.

digestive uptake by the host. Such reduced uptake and thus pesticidal toxicity was for example demonstrated in *Drosophila melanogaster* upon applying a *Lactobacillus* probiotic (Trinder et al., 2016). Leska et al. (2022) found that LAB isolated from honey bees were able to reduce toxicity of the pesticides chlorpyriphos and coumaphos by adhering these chemicals to the bacterial cell wall. Results however, varied between bacterial strains and pesticides. Nonetheless, supplementing with the appropriate LAB may significantly reduce pesticide stress in honey bees.

3.2.3.3 Heavy metal detoxification

A similar beneficial effect of probiotics and LAB in particular is their ability to deal with heavy metals (HMs) so the host doesn't have to. Honey bees are mainly exposed to these toxic compounds through contaminated food sources and ingestion of such foods is known to have detrimental effect on adults and larvae (Di et al., 2016). The gut microbiome is therefore the first line of defence. Since HMs can't be broken down, the mode of action by which bacteria detoxify them is by adsorption or accumulation (Kinoshita, 2019; Bhakta et al., 2012). This, once again, lowers the host's exposure to these compounds and therefore reduces their toxicity.

3.2.3.4 Antioxidant production

Reactive oxygen species (ROS) are known to cause damage to DNA and other cell structures (Bertram & Hass, 2008). Although these molecules are normal by-products of the metabolism, and organisms have innate ways to deal with them, exogenous stressors such as pathogens and pesticides can disrupt this balance, causing ROS to wreak havoc.

When it comes to probiotics and oxidative stress in honey bees, there has been a specific emphasis on utilizing LAB for their role as natural antioxidants. Certain strains within this category exhibit both enzymatic and non-enzymatic antioxidant properties, possibly mitigating oxidative damage resulting from the accumulation of ROS during the digestive process (e.g. Mishra et al., 2015; Feng & Wang, 2020).

3.2.3.5 Digestive aid

Since symbiotic bacteria are vastly different from their host, they are often able to synthesise or break down compounds the host cannot. Comprising a vital addition to said host's metabolism. The insect gut microbiome is known to produce crucial nutrients such as essential amino acids and B vitamins (Douglas, 2015). Furthermore, honey bee specific microbes have been found to aid in the digestion of several compounds within the bee's diet. *Lactobacillus*

plantarum and *Gilliamella*, for example, provide support in the digestion of cellulose (Zheng et al., 2019). Other microbes, including LAB digest sugar compounds which are present in the honey bee diet, but not utilized as much by the bee or are even toxic to it (e.g. arabinose, lactose, xylose; Iorizzo et al., 2022). The above indicates that LAB might have a profound role in ameliorating digestion in honey bees.

3.2.4 Probiotics and their effect on body and gut metabolites

Since the scientific field regarding microbiota is relatively new, a lot remains to be discovered. It's been established that alteration of especially the gut microbiome can have significant effects on the host's overall health. Either positive or negative. We know probiotics can upregulate the expression of immunity related genes (Evans & Lopez, 2014) and increase the overall health of the host (Forsgren et al., 2009; Trinder et al., 2016). The exact mechanisms, however, and other effects on gene expression and metabolism are not yet fully understood. An interesting research line therefore is to compare the metabolic composition of bees that received probiotics to a non-probiotic control group. This will provide detailed information about which substances within the bee are influenced by the beneficial bacteria. Once such key compounds have been identified, further research can be performed regarding their function in the body.

3.2.5 Nuclear magnetic resonance spectroscopy

In order to get detailed and accurate information about metabolite abundances, honeybee tissues can be analysed by means of Nuclear Magnetic Resonance (NMR) spectroscopy (McDevitt et al., 2021) . This sophisticated technology is based on the differential behaviour of atomic nuclei upon exposure to magnetic pulses. The magnetic field generated by the machine, briefly disrupts the magnetic environment of the nuclei. When returning to the original state, each nucleus will generate a signal based on its unique magnetic environment within the molecule and each molecule will thus generate a distinct set of signals depending on its atomic composition and structure. Said molecules, in a next step, can be identified by comparison to standard NMR spectra. Their relative abundances can then be analysed by the relative height of the peaks in the generated spectrum (Hore et al., 2015). For examples of such NMR spectra, see section 5.1 of this work.

3.2.5.1 Human NMR applications

NMR technology is especially well suited for identifying organic compounds and is commonly used for medicinal applications, such as the analysis of biofluids (Vignoli et al.,

2019). NMR spectroscopy can provide detailed information about the biochemical composition of e.g. urine, blood, and faeces and thus, the biochemical state of an organism (Vignoli et al., 2019). Resulting in insights about disease, toxic insults etc. Given the non-invasive nature of biofluid collection, this method of diagnosis can be very valuable towards the future. Research into this subject mainly regards the detection of cancer (Carrola et al., 2011; Wang et al., 2013) but NMR might also be a feasible technique to diagnose other diseases such as Crohn's disease etc. (Fathi et al., 2014).

3.2.5.2 NMR in honey bees

In relation to (honey) bees, few studies using NMR have been performed and most of the ones that have been done, regard the composition of honey. For example, to identify adulteration (Yong et al., 2022) or the honey's botanical origin (Schievano et al., 2012). Research by McDevitt et al. (2021), shows that NMR is also a suitable technique to be used for analysis of a broad range of bodily tissues in honey bees. A finding that is supported by the quite remarkable demonstration of metabolic differences between summer and winter bees by Lee et al. (2022). Another study reported employed a multi-omics approach to unravel the metabolic changes induced by *Varroa destructor* (Kunc et al., 2023).

As is clear from the publishing dates of the studies above, the application of NMR to honeybee metabolomics is relatively new. The use of this technique can therefore shed a unique light on the bee's metabolic pathways under different conditions. Including exposure to bacterial supplements.

4 Methodology

4.1 Sample collection

On August 6, 2022, newly emerged honey bees were collected from two colonies, hive 6 and hive 7, located in an apiary at Kyvalka (49.1913056N, 16.4495556E). These bees were then organized into groups of 100 individuals each and placed into plastic cups. A mixture of bacterial strains - *Lactobacillus helsinborgensis*, *Bifidobacterium asteroides*, *Bombilactobacillus mellis,* and *Lactobacillus apis* - was adnimistered to designated treatment groups. This was carried out using a 3 ml aliquoat of sugar solution (3:2 w/w) with a bacterial concentration of 1×10^9 bacteria per group. The bacterial strains were applied to newly emerged bees in a single dose and were replaced by bacterial strain-free sugar solution after being consumed (the second day after the administration). Control groups were fed with probiotics-free sugar solution for the whole experiment. In some treatment groups (see below) five to ten day old honey bee workers collected from the respective colonies were included as well to ensure social interaction and transfer of native gut microbes, supporting the development of experimental bees.

The honey bees used in this study were kept in laboratory conditions and were divided into four groups as described below:

- C = Control: Newly emerged bees, administered with sucrose solution.
- COB = Control + older bees: Newly emerged bees administered with sucrose solution and kept together with older bees from the same hive.
- P = Probiotics: Newly emerged bees received a sucrose solution containing a mixture of bacterial strains.
- POB = Probiotics + older bees: Newly emerged bees received a sucrose solution containing a mixture of bacterial strains and were kept with older bees from the same hive.

These treatments were repeated on bees from two different hives, referred to as hive 6 and 7 in this thesis. Hive 7, however, did not include the control group, resulting in seven treatment groups instead of eight. Each of these groups contained 10 replicates and gut and body were analysed separately. Thus, in total 140 samples were processed (70 guts and 70 bodies).

4.2 Preparation of samples for NMR-analysis

Honey bee samples were dissected using sterile tweezers and forceps. Initially, the heads and venom sacs were discarded, and body segments such as the thoraces, abdomen, wings, and legs were gathered. The gastrointestinal tracts, except for the crops, were then retrieved using tweezers. Collected samples were homogenized with Ø 5 mm zirconium oxide grinding balls (Retsch 22.455.0009, Retsch GmbH, Haan, DE) in 1 ml of methanol at 25 Hz for three minutes (Retsch MM200, Retsch GmbH, Haan, DE). Thorough homogeneity was ensured by immerging the samples in an ultrasonic bath (5 minutes). Afterwards, the samples were centrifuged at 14 000 × g for 10 minutes at 4°C and the supernatant was collected and dried in a centrifugal vacuum concentrator at 40°C (MiVac Duo, Genevac, Ipswitch, UK).

To acquire the highest possible extraction rate, the procedure was repeated for the same homogenate samples with 1 ml of methanol. The supernatants were pooled and allowed to dry fully. Dried samples were stored at -80°C.

Prior to ^{1}H NMR analysis, the samples were resolubilised in 600 µl of D_2O by means of vortexing and an ultrasonic bath, followed by centrifugation (14 000 × g for five minutes). 540 µl of the supernatant was then mixed with 60 µl of NMR phosphate buffer (1.5 M K_2HPO_4 and NaH_2PO_4, pH 7.4, 5 mM 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid (TSP) and 0.2% NaN_3 in D_2O), and transferred into NMR tubes (5 mm, 7", High-Throughput, Willmad, NJ, US) for analysis. Figure 4 presents a simplified schematic overview of the process.

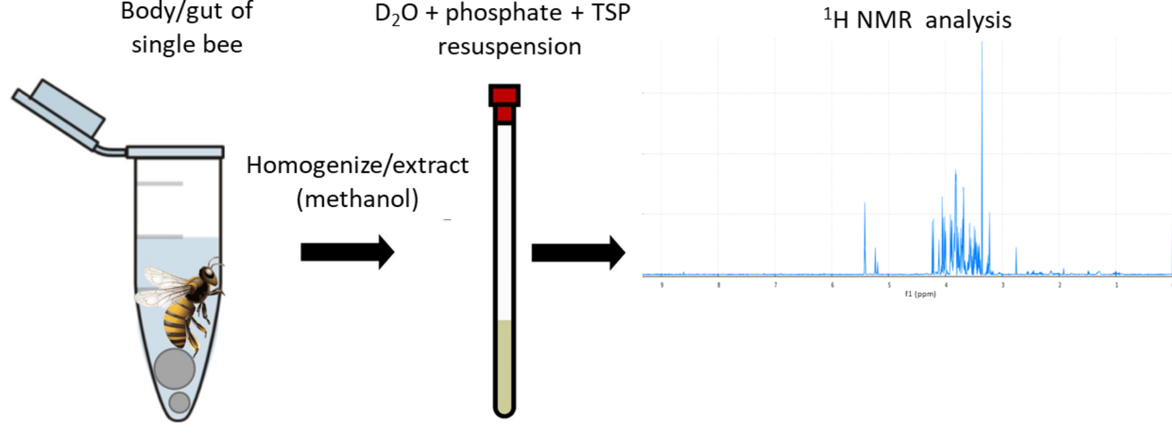


Figure 4: Graphic summary of the sample processing methodology (Adapted from McDevitt et al., 2021)

4.3 ^{1}H NMR analysis

^{1}H NMR spectra of honey bee methanol extracts, reconstituted in D_2O, were obtained using the 'noesypr1d' Bruker pulse program. The measurements were conducted on a Bruker Avance III spectrometer (operating at 500.18 MHz for ^{1}H observation) at 298 K, utilizing a 5 mm Broadband observe probe. The acquisition parameters included a 3.9 s acquisition time, 1 s relaxation delay (D1), 0.1 s mixing time, 8012.82 Hz spectral width, 64 K data points, and 128 scans.

4.4 Statistical analysis and data processing

The spectra were processed by zero-filling to 128 K, applying exponential multiplication with a line broadening of 0.3 Hz, manual phasing using Topspin 3.5 (Bruker, Billerica, MA, US). Metabolite identification was performed using Chenomx NMR Suite 8.6 (Chenomx, Edmonton, AB, Canada), which includes Reference Library 10 and an internally developed library containing approximately 80 compounds. A total of 58 metabolites were identified and fitted to the spectra.

Baseline correction was performed using NMRProcFlow 1.4. (Jacob et al., 2017), which was also utilised to select areas of interest within the spectra (buckets). Each of those ‘buckets’ was then manually allocated to the corresponding metabolite that was identified earlier. Out of the 316 selected areas however, 210 remain unknown or uncertain. Further investigation into their identity would only be required if their abundance proves to be altered between treatments and falls beyond the scope of this thesis.

For statistical analysis, the online software MetaboAnalist 6.0 (Pang et al., 2021) was used. Data were processed by means of probabilistic quotient normalization (PQN), log transformation and pareto scaling. Principal component analysis (PCA) was performed to examine the metabolic patterns and trends across the groups. Orthogonal partial least squares discriminant analysis (OPLS-DA) was utilized together with permutation test validation for 100 applications and the models underwent evaluation based on their goodness of fit (R^2Y) and predictive capability (Q^2). Variable importance in projection (VIP) were applied to determine the variables that contribute to the model's predictive ability.

Lastly, to visualize the average metabolite abundance for each group, hierarchical clustering heatmaps were created. In order to provide a clear overview of the general trends, only identified compounds were visualised and hives were analysed seperately as wel as data from gut and body. Clustering was based on a Euclidean distance measure and Ward clustering

method, and the data were processed beforehand in the same way as described above (PQN, log transformation, and pareto scaling). The resulting four figures are included in this work as appendices 9.1, 9.2, 9.3, and 9.4. Section 5.2.3 provides figures with a generalised overview of the results.

5 Results

5.1 NMR spectrum of honey bee tissue

As stated above, 58 compounds were identified in the honey bee extracts: 17 amino acids, 15 carboxylic acids, 4 phenolic compounds, 4 nucleosides, 2 nucleotides, 1 dinucleotide, 3 amines, 2 disaccharides, 2 monosaccharides, 3 choline containing compounds, 1 simple alcohol, 1 diol, 1 alkaloid, 1 sulphur containing compound, and 1 non-protein nitrogen compound. Figure 5 shows an NMR-spectrum resulting from analysis of the honey bee body while Figure 6 results from analysis of the gut. The range spanning from 0.5 to 3.5 ppm predominantly shows peaks originating from protons attached to aliphatic carbons or methyls of amino acids, organic acids, and lipids. Between 3.5 and 5.5 ppm, resonances primarily arise from protons of carbohydrates, while beyond 6.0 ppm, the spectrum mainly reflects protons attached to aromatic rings. Relative abundances vary between the gut and body. Especially in the carbohydrate, and amino and organic acid region.

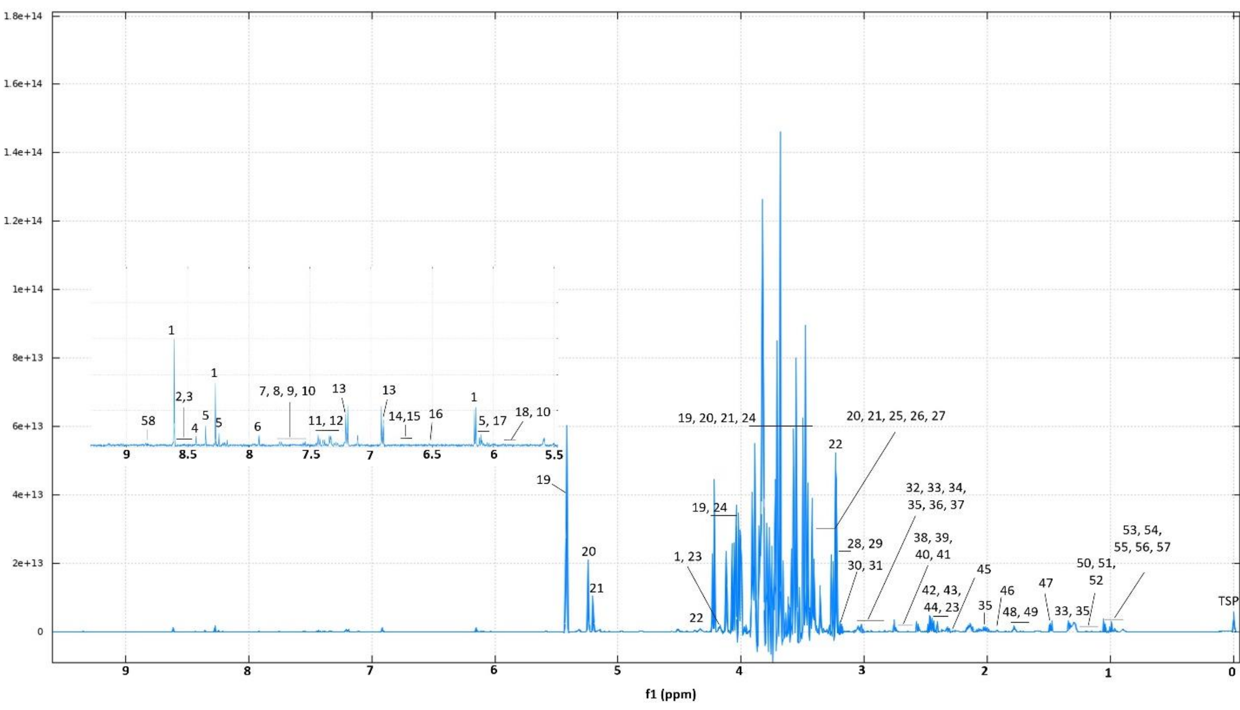


Figure 5: ^{1}H NMR spectrum of a honey bee body sample belonging to the COB group, which is most representative of the natural situation. Each number indicates an individual compound: 1. Adenosine monophosphate (AMP), 2. Inosine monophosphate (IMP), 3. Formic acid, 4. Oxidized nicotinamide adenine dinucleotide (NAD^+), 5. Inosine, 6. Histidine, 7. Thymidine, 8. Benzoate, 9. 4-Hydroxybenzoate, 10. Uracil, 11. Phenylalanine, 12. Caffeic acid, 13. Tyrosine, 14. Desamino-5,4 pentanoic acid, 15. 3,4 dihydroxy cinnamic acid, 16. Fumarate, 17. Adenosine, 18. Uridine, 19. Sucrose, 20. Glucose, 21. Trehalose, 22. Sn-glycero-3 phosphocholine, 23. Proline, 24. Fructose, 25.

O-phosphocholine, 26. Taurine, 27. Trimethylamine-N-oxide, 28. Choline, 29. β-alanine, 30. Dimethyl sulphone, 31. Malonate, 32. Ornithine, 33. Putrescine, 34. Creatine, 35. Lysine, 36. Asparagine, 37. Desaminotyrosine, 38. Sarcosine, 39. Dimethyl amine, 40. Aspartate, 41. Citrate, 42. Succinate, 43. 3-Hydroxy isovalerate, 44. Glutamate, 45. 2-Hydroxyglutarate, 46. Acetate, 47. Alanine, 48. Threonine, 49. Lactate, 50. 3-Amino isobutyrate, 51. Ethanol, 52. Propylene glycol, 53. Methyl succinate, 54. Isobutyrate, 55. Isoleucine, 56. Valine, 57. Leucine, and 58. Trigonelline

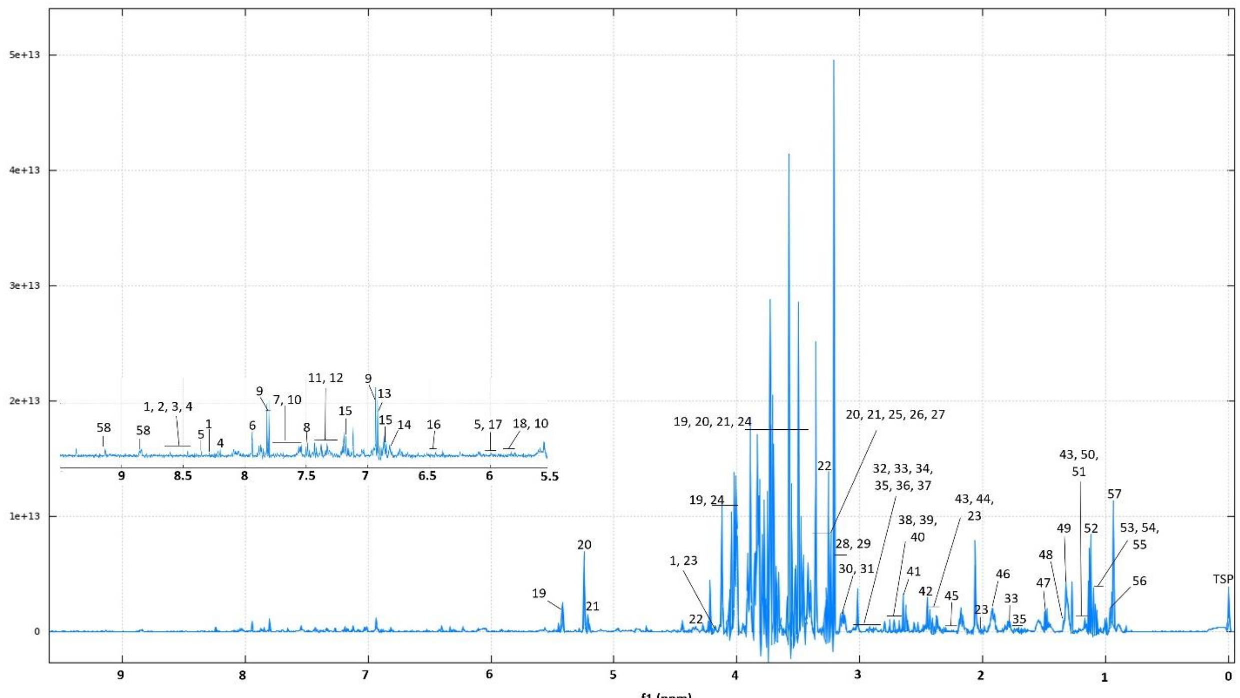


Figure 6: ¹H NMR spectrum of a honey bee gut sample belonging to the COB group (same individual as Fig. 5). Each number indicates an individual compound: 1. Adenosine monophosphate (AMP), 2. Inosine monophosphate (IMP), 3. Formic acid, 4. Oxidized nicotinamide adenine dinucleotide (NAD^+), 5. Inosine, 6. Histidine, 7. Thymidine, 8. Benzoate, 9. 4-Hydroxybenzoate, 10. Uracil, 11. Phenylalanine, 12. Caffeic acid, 13. Tyrosine, 14. Desamino-5,4 pentanoic acid, 15. 3,4 dihydroxy cinnamic acid, 16. Fumarate, 17. Adenosine, 18. Uridine, 19. Sucrose, 20. Glucose, 21. Trehalose, 22. Sn-glycero-3 phosphocholine, 23. Proline, 24. Fructose, 25. O-phosphocholine, 26. Taurine, 27. Trimethylamine-N-oxide, 28. Choline, 29. β-alanine, 30. Dimethyl sulphone, 31. Malonate, 32. Ornithine, 33. Putrescine, 34. Creatine, 35. Lysine, 36. Asparagine, 37. Desaminotyrosine, 38. Sarcosine, 39. Dimethyl amine, 40. Aspartate, 41. Citrate, 42. Succinate, 43. 3-Hydroxy isovalerate, 44. Glutamate, 45. 2-Hydroxyglutarate, 46. Acetate, 47. Alanine, 48. Threonine, 49. Lactate, 50. 3-Amino isobutyrate, 51. Ethanol, 52. Propylene glycol, 53. Methyl succinate, 54. Isobutyrate, 55. Isoleucine, 56. Valine, 57. Leucine, and 58. Trigonelline

5.2 Multivariate analysis

The aim of this study was to determine whether bees exposed to bacterial strains could be distinguished from the control group by their metabolites and whether the presence of older siblings influenced these potential differences in any way. This section will provide an overview of the PCA and OPLS-DA results, including VIP-scores of the 15 main contributing compounds for each treatment pair. Because of a notable hive effect (see 5.2.3), results from each hive will be reported separately.

5.2.1 Metabolic comparison in the body

5.2.1.1 Hive 6

5.2.1.1.1 Probiotics (P) vs. control (C)

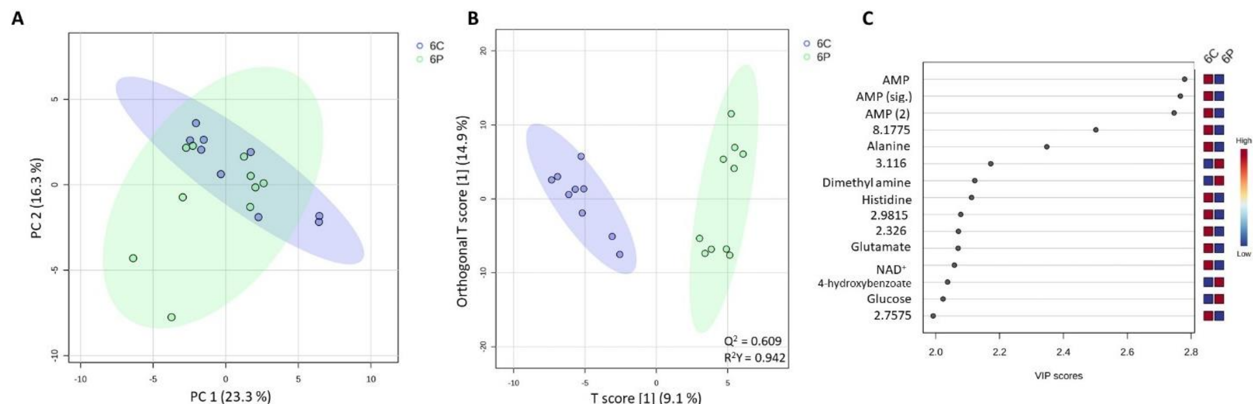


Figure 7: Metabolic comparison of body composition in the bacterial strain-supplemented group and the control group from hive 6. 6C, control group from hive 6; 6P, bacterial strain-supplemented group from hive 6. A) PCA, B) OPLSDA, C) VIP-scores of the top 15 compounds. The higher the score, the more its contribution to the difference between groups. Compounds that are present more than once are accompanied by a number signifying the distinct spectral position. (sig.) indicates the signature peak for a specific compound and unidentified compounds are labeled by a value representing their position on the NMR spectrum. Red or blue color indicates high or low abundance respectively.

The PCA results for comparison of the control (6C) and bacterial strain supplemented group (6P) (Fig. 7A), showed that 39.6 % of the total variation is explained by the two first principal components (PC 1 23.3%, PC 2 16.3%). The 6C group was more tightly clustered over PC 2 axis and more widely spread across PC 1 axis. The bacterial strain-supplemented group was more spread out overall, with the main variance over PC 1.

The OPLS-DA demonstrated good discriminant power and fit to the observed data, as indicated by an R^2Y value of 0.94. Q^2 measured the model's prediction accuracy at 0.61, which is a high level of predictive accuracy (Fig. 7B). Collectively, this suggests that the model fits

the training data effectively and possesses robust prediction capabilities. Subsequently, VIP-scores were applied to identify the top 15 metabolites. These metabolites are particularly noteworthy because they play a crucial role in distinguishing between the two groups, highlighting their importance in the analysis. Adenosine monophosphate (AMP) has the highest VIP-score and is therefore the most important compound that contributes to this difference. This compound tends to be more abundant in the control group. Alanine, dimethylamine, and histidine are the next known compounds in line. Of those, dimethylamine is less abundant in the control group while the other two compounds show the opposite trend (Fig. 7C).

5.2.1.1.2 Control (C) vs. control with older bees (COB).

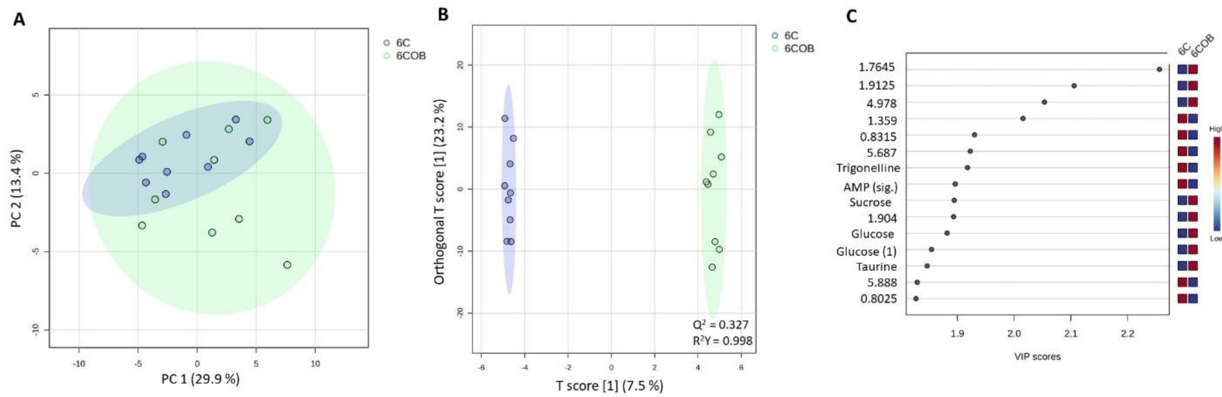


Figure 8: Comparison of body metabolites in control and COB group from hive 6. 6C, control group from hive 6; 6COB, control group exposed to older bees (hive 6). A) PCA, B) OPLSDA, C) VIP-scores of the top 15 compounds. The higher the score, the more its contribution to the difference between groups. Compounds that are present more than once are accompanied by a number signifying the distinct spectral position. (sig.) indicates the signature peak for a specific compound and unidentified compounds are labeled by a value representing their position on the NMR spectrum. Red or blue color indicates high or low abundance respectively. 6C, control group from hive 6; 6COB, control group exposed to older bees (hive 6).

When comparing the control group (6C) to the control with older bees group (6COB), PCA shows that PC 1 (29.9%) and PC 2 (13.4%) account for 43.3% of all variation (Fig. 8A). The control group was tightly clustered together along PC 2 with some variation across PC 1. The COB treatment resulted in an even spread for both principal components. These differences are mainly due to unknown compounds, however, of the ones that were identified, trigonelline, AMP and sucrose were the main contributors. The former two being upregulated in the C treatment and sucrose being more abundant when older siblings were present (Fig. 8C). With an R^2Y value of 0.998, the OPLS-DA-model effectively explains the variance in the observed

data. The model exhibits low to moderate predictive capabilities, as indicated by a Q^2 value of 0.327, which is below 0.5 (Fig. 8B). Meaning that the result applies to this dataset only.

5.2.1.1.3 Control with older bees (COB) vs. probiotics with older bees (POB)

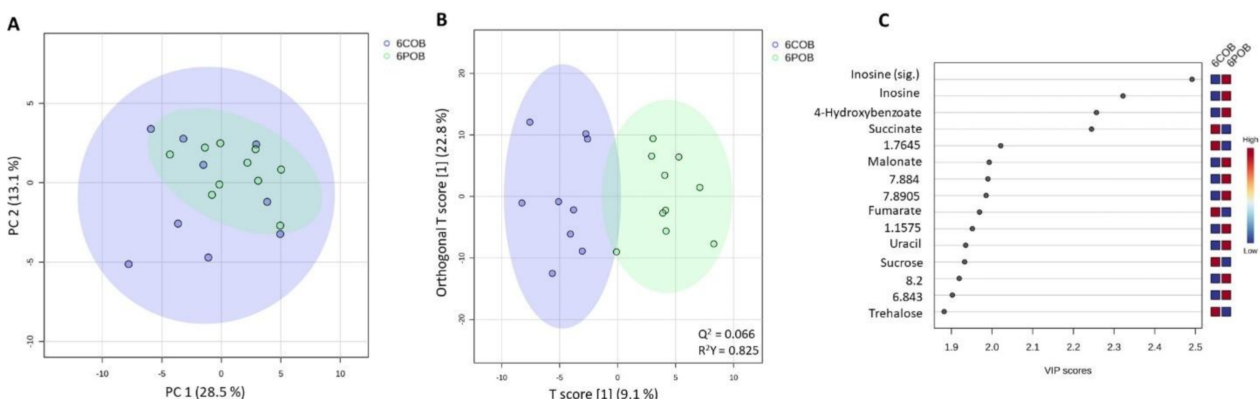


Figure 9: Comparison of body composition of COB and POB in hive 6 only. 6COB, control group with older bees from hive 6; 6POB, group that was bacterial strain supplemented and exposed to older bees (hive 6). A) PCA, B) OPLSDA, C) VIP-scores of the top 15 compounds. The higher the score, the more its contribution to the difference between groups. Compounds that are present more than once are accompanied by a number signifying the distinct spectral position. (sig.) indicates the signature peak for a specific compound and unidentified compounds are labeled by a value representing their position on the NMR spectrum. Red or blue color indicates high or low abundance respectively.

For the comparison of these treatments in hive 6, PCA shows a narrower clustering for the POB treatment, with the largest variation along PC 1. The COB data are spread widely and evenly across both principal components. Together, these PC's account for 41.6% (PC 1: 28.5%, PC 2: 23.1%) of total variation (Fig. 9A). OPLS-DA results in a well-fitting model (R^2Y: 0.285), but with a low predictive power (Q^2: 0.066) (Fig. 9B). The major contributor to the difference between the POB and COB group's result is inosine, followed by several organic acids such as 4-hydroxybenzoate, succinate, malonate, and fumarate. The first two of those together with malonate are more present in tissue of the POB treatment. The remaining compounds are less abundant in this treatment (Fig. 9C).

5.2.1.2 Hive 7

5.2.1.2.1 Control with older bees (COB) vs. probiotics with older bees (POB)

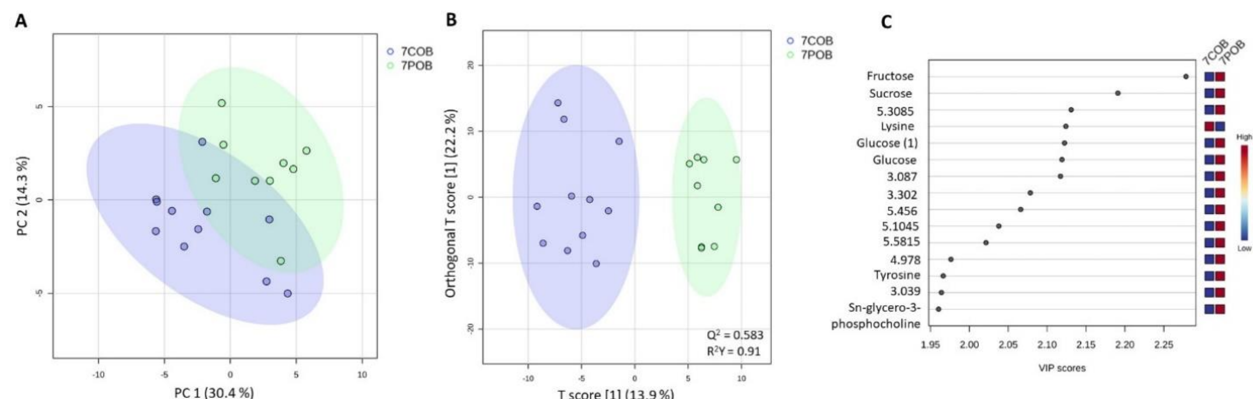


Figure 10: Comparison of body metabolites in the COB and POB treatments of hive 7. 7COB, control group exposed to older bees (hive 7); 7POB, supplemented with bacteria and exposed to older bees (hive 7). A) PCA, B) OPLSDA, C) VIP-scores of the top 15 compounds. The higher the score, the more its contribution to the difference between groups. Compounds that are present more than once are accompanied by a number signifying the distinct spectral position. Unidentified compounds are labeled by a value representing their position on the NMR spectrum. Red or blue color indicates high or low abundance respectively.

Compared to hive 6, the result for hive 7 is somewhat different, with the principal components accounting for 44.7% (PC 1: 30.4 %, PC 2: 14.3 %) of the variation and both treatments being relatively tightly clustered. The COB data is most widely spread along PC 1 while POB has more variation across PC 2 (Fig. 10A). The OPLS-DA model demonstrates strong predictive capability, indicated by its Q^2-value of 0.583, alongside a high capacity to explain the observed variance (R^2Y: 0.91) (Fig. 10B).

Carbohydrates seem to be important since fructose, sucrose, and glucose are among the top contributors. Accompanied by lysine, which is more abundant in the COB treatment, contrary to the sugar compounds (Fig. 10C).

5.2.2 Metabolic comparison in the gut

5.2.2.1 Hive 6

5.2.2.1.1 Probiotics (P) vs. control (C)

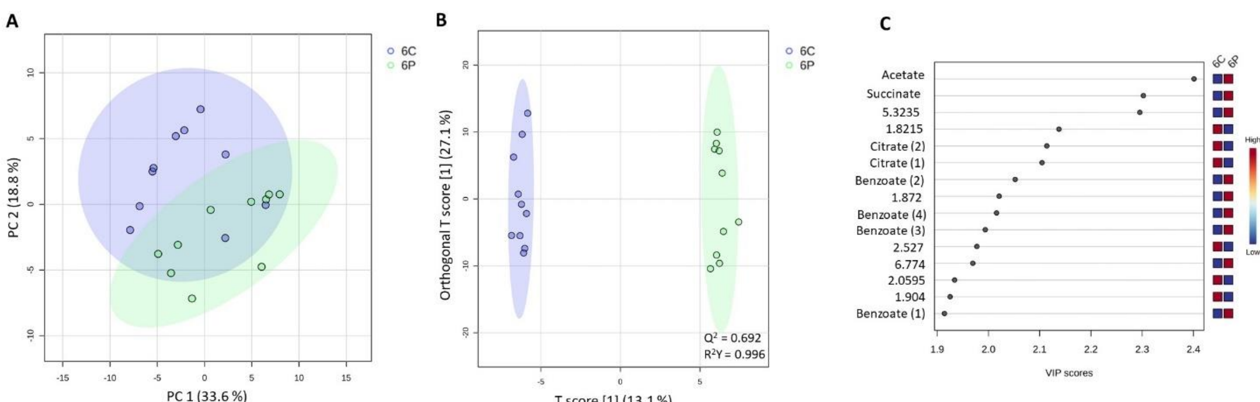


Figure 11: Comparison of gut metabolites in the probiotics and control treatments (hive 6). 6C, control group from hive 6; 6P, bacterial strain-supplemented group from hive 6. A) PCA, B) OPLSDA, C) VIP-scores of the top 15 compounds. The higher the score, the more its contribution to the difference between groups. Compounds that are present more than once are accompanied by a number signifying the distinct spectral position. Unidentified compounds are labeled by a value representing their position on the NMR spectrum. Red or blue color indicates high or low abundance respectively.

The PCA findings regarding the comparison of gut metabolites between the control and probiotics group (Fig. 11A) indicate that the combined influence of the first two principal components (PC 1: 33.6%, PC 2: 18.8%) accounts for 52.4% of the total variation observed. The control group was equally spread over both PC's and the probiotic group was more tightly clustered along PC 2, with notable variation for PC 1. The difference between treatments is mainly due to carboxylic acids such as acetate, succinate, citrate, and benzoate. Citrate is found in higher abundance in the control group, while the other compounds show the opposite trend (Fig. 11C). The OPLS-DA model is highly accurate in explaining the variance of the data (R^2Y: 0.996) and furthermore, has good predictive power (Q^2: 0.692) (Fig. 11B).

5.2.2.1.2 Control (C) vs. control with older bees (COB).

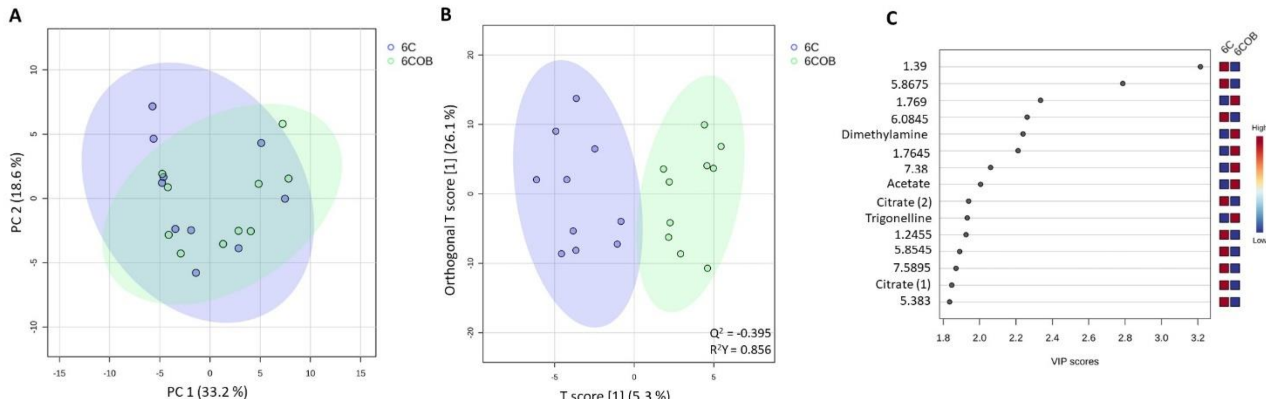


Figure 12: Comparison of gut metabolites in the control and COB groups (hive 6). 6C, control group from hive 6; 6COB, control group exposed to older bees (hive 6). A) PCA, B) OPLSDA, C) VIP-scores of the top 15 compounds. The higher the score, the more its contribution to the difference between groups. Compounds that are present more than once are accompanied by a number signifying the distinct spectral position. Unidentified compounds are labeled by a value representing their position on the NMR spectrum. Red or blue color indicates high or low abundance respectively.

When comparing the no-probiotics control group with the control and older bees group (COB), PCA analysis indicates that PC 1 (33.2 %) and PC 2 (18.6 %) collectively explain 51.8% of the total variance (Fig. 12A). The control group exhibits greater dispersion along PC 2, accompanied by noticeable variation along PC 1. In contrast, the COB treatment primarily varies along PC 1, with some variation along PC 2. These distinctions are primarily attributed to unidentified compounds. However, among those identified, dimethylamine, acetate, citrate, and trigonelline emerge as significant factors. Dimethylamine, acetate, and trigonelline are found more in the COB group, while citrate is more abundant without the presence of older bees (Fig. 12C). VIP-scores should however be interpreted with care since the model's predictive power is low (Q^2: -0.395). It does nonetheless have high explanative power regarding the variance of the data (R^2Y: 0.856) (Fig. 12B).

5.2.2.1.3 Control with older bees (COB) vs. probiotics with older bees (POB)

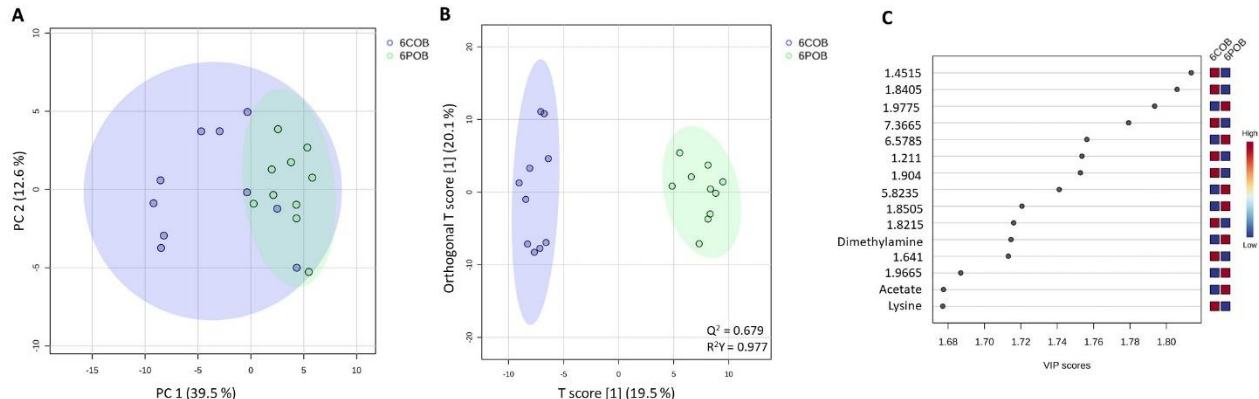


Figure 13: Comparison of gut metabolites in the COB and POB groups of hive 6. 6COB, control group with older bees from hive 6; 6POB, group that was bacterial strain supplemented and exposed to older bees (hive 6). A) PCA, B) OPLSDA, C) VIP-scores of the top 15 compounds. The higher the score, the more its contribution to the difference between groups. Unidentified compounds are labeled by a value representing their position on the NMR spectrum. Red or blue color indicates high or low abundance respectively.

For the comparison of the COB and POB treatments in hive 6, PCA shows a much narrower clustering for the POB treatment, with the largest variation along PC 2. The COB data are spread widely and evenly across both principal components. Together, these PC's account for 52.1% (PC 1: 39.5%, PC 2: 12.6%) of total variation (Fig. 13A). The model resulting from OPLS-DA is a good fit for the observed data (R^2Y: 0.977) and has good predictive power (Q^2: 0.679) (Fig. 13B). The observed difference between treatments is mainly due to unknown compounds. However, dimethylamine, acetate, and lysine have a role as well. The former two being more abundant in the POB treatment and the latter in the COB group (Fig. 13C).

5.2.2.2 Hive 7

5.2.2.2.1 Control with older bees (COB) vs. probiotics with older bees (POB)

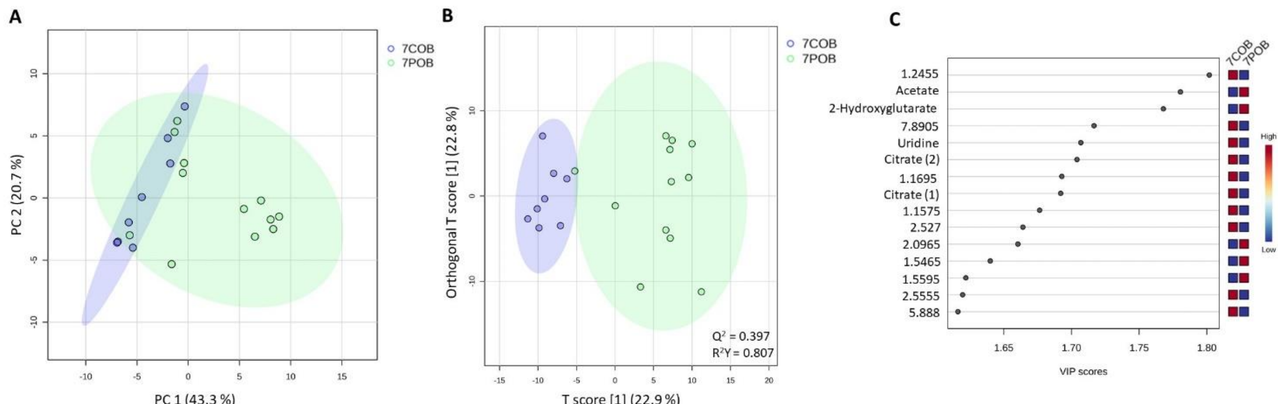


Figure 14: Comparison of gut metabolites in the COB and POB groups for hive 7. COB, control group exposed to older bees; POB, supplemented with bacteria and exposed to older bees A) PCA, B) OPLSDA, C) VIP-scores of the top 15 compounds. The higher the score, the more its contribution to the difference between groups. Compounds that are present more than once are accompanied by a number signifying the distinct spectral position. Unidentified compounds are labeled by a value representing their position on the NMR spectrum. Red or blue color indicates high or low abundance respectively.

The result for hive 7 indicates that the first two principal components account for 64 % of the variation (PC 1: 43.3%, PC 2: 20.7%). The POB data is most widely spread along PC 1 and also has notable variation along PC 2. COB on the other hand has most variation across PC 2 and is tightly clustered (Fig. 14A). Acetate, 2-hydroxyglutarate, uridine, and citrate are the most important known compounds contributing to this result. The former two being more abundant in the POB group while the other two are less abundant in this group (Fig. 14C). The OPLS-DA model has low predictive power (Q^2: 0.397), but explain the variance well (R^2Y: 0.807) (Fig. 14B).

5.2.3 Hierarchical clustering

5.2.3.1 Body metabolites

5.2.3.1.1 Hive 6

Carbohydrates	↓↓	↑↑	↑↑↑	↓↓↓
Amino acids & derivatives	↓↓↓	↑↑↑	↓↑↑	↓↑
Nucleotides & nucleosides	↓↓↑	↓↑	↓↑	↑
Organic acids of TCA-cycle	↑↑	↑↑	↑	↓↓↓
Other organic acids	↓↓↓	↑↑↑	↓↑	↓
Amines & amides	↑↑↑	↓↑	↑↑	↓↓↑

Figure 15: Generalised overview of the trends resulting from hierarchical clustering analysis of the body metabolites from hive 6. Results are shown from left to right for control (C), control and older bees (COB), bacterial strain supplemented group (P), and bacterial strain and older bees group (POB). Green arrows pointing up indicate high abundance of the metabolite group in the specific treatment. Blue downward pointing arrow means low abundance. Relative abundance is depicted by the number of arrows, with three being very high or very low. Combinations of up and downward arrows indicate that the trend varies within the compound group. The dominating trend is shown by the type of arrow that is most abundant.

According to the hierarchical clustering heatmap regarding body metabolites in hive 6 (appendix 9.1), control treatments and treatments involving bacterial supplementation are clustered together. Regarding specific compound groups, carbohydrates were present in low abundances in the bacterial strains with older bees treatment (POB) while high abundances were found in the bacterial supplement only group (P) (Fig. 15). Similarly, carbohydrate abundance was lower in the control treatment (C) and higher when older bees were present (COB).

Amino acids, mostly differed between C (low) and COB (high) while no uniform trend was found in the other treatments. When looking at specific compounds however, proline and alanine were more abundant in the bodies of both control groups, with the strongest difference between C and P (see appendix 9.1). Lysine, leucine, tyrosine, valine, and threonine were highly

abundant in the COB group while low in the other groups. Lastly, asparagine, was more abundant in the groups that were supplemented with bacteria.

For nucleosides, similar to amino acids, different compounds behaved differently across treatments. Adenosine monophosphate specifically however, was more abundant in both control groups, with the main difference between C and P. Inosine differed mainly between COB and POB, with the latter having the highest abundance of this compound.

Organic acids involved in the tricarboxylic acid cycle (TCA-cycle) were abundant overall, except in the POB group. Other organic acids mainly differed in abundance between the C and COB groups.

Amines and amides were found in higher abundances in the C and P treatments, while their behaviour was scattered in the other groups.

5.2.3.1.2 Hive 7

Carbohydrates	↓↓↓	↓↓	↑↑↑
Amino acids & derivatives	↓↑	↓↑	↓↑
Nucleotides & nucleosides	↑↑	↑↑	↓↓
Organic acids of TCA-cycle	↓↓	↑↑	↓↑
Other organic acids	↓	↓↓	↑↑
Amines & amides	↓↓	↓	↓↑

Figure 16: Generalised overview of the trends resulting from hierarchical clustering analysis of the body metabolites from hive 7. Results are shown from left to right for control and older bees, bacterial strain supplemented group, and bacterial strain and older bees group. Green arrows pointing up indicate high abundance of the metabolite group in the specific treatment. Blue downward pointing arrow means low abundance. Relative abundance is depicted by the number of arrows, with three being very high or very low. Combinations of up and downward arrows indicate that the trend varies within the compound group.

Carbohydrate abundances in the bodies of bees from hive 7, were mostly different between COB and POB treatments (appendix 9.2). With the most carbohydrates found in the POB group. In the other two treatments, carbohydrates were less abundant (Fig. 16).

No uniform trend was found for amino acids, while nucleosides/nucleotides were more abundant in the COB and P group. Organic acids from the Krebs-cycle (= TCA-cycle), were more present in the P group and less in the COB group. No clear trend was found in the POB group. Other organic acids mostly differed in abundance between P and POB treatments, while amines and amides leaned towards low abundance across treatments.

In this case, the clustering distance was largest between the POB and P treatment.

5.2.3.2 Gut metabolites

5.2.3.2.1 Hive 6

Carbohydrates	↑↑	↑↑↑	↓	↓↓↓
Amino acids & derivatives	↓↑↑	↓↑	↓↑	↓↑↑
Nucleotides & nucleosides	↑	↓↓	↑↑	↓↓↓
Organic acids of TCA-cycle	↑↑	↑↑	↑	↓
Other organic acids	↓	↓↓	↑↑	↑↑↑
Amines & amides	↓↓	↓	↑↑	↑↑

Figure 17: Generalised overview of the trends resulting from hierarchical clustering analysis of the gut metabolites from hive 6. Results are shown from left to right for control, control and older bees, bacterial strain supplemented group, and bacterial strain and older bees group. Green arrows pointing up indicate high abundance of the metabolite group in the specific treatment. Blue downward pointing arrow means low abundance. Relative abundance is depicted by the number of arrows, with three being very high or very low. Combinations of up and downward arrows indicate that the trend varies within the compound group. The dominating trend is shown by the type of arrow that is most abundant.

Gut metabolites are distributed similarly within the control treatments vs. the bacteria supplemented treatments given that C and COB are clustered together as well as P and POB

(appendix 9.3). Figure 17 shows that the differences between control and supplemented groups are mainly due to carbohydrates, organic acids (other than those involved in the TCA cycle), and amines and amides. Carbohydrates overall being more abundant in the guts of the control group, while the other two compound groups are more abundant in the treatments that received bacterial strains.

Once again, amino acids and their derivatives showed different responses to the treatments. Aspartate, leucine and lysine for example were highly abundant in the POB treatment, while low in all others. Alanine, phenylalanine, and threonine differed mostly between control and bacterial strain supplemented groups, with the latter containing less of these compounds.

Nucleoside and nucleotide abundance was generally low in treatments involving older bees, while higher in the other groups (Fig. 17).

5.2.3.2.2 Hive 7

Carbohydrates	↓↓	↓	↑↑
Amino acids & derivatives	↓↓	↑	↑↑
Nucleotides & nucleosides	↑↑↑	↑	↓↓↓
Organic acids of TCA-cycle	↑	↓↑	↓↓
Other organic acids	↓↓	↑↑	↓↑
Amines & amides	↓↑	↓	↓↑

Figure 18: Generalised overview of the trends resulting from hierarchical clustering analysis of the body metabolites from hive 7. Results are shown from left to right for control and older bees, bacterial strain supplemented group, and bacterial strain and older bees group. Green arrows pointing up indicate high abundance of the metabolite group in the specific treatment. Blue downward pointing arrow means low abundance. Relative abundance is depicted by the number of arrows, with three being very high or very low. Combinations of up and downward arrows indicate that the trend varies within the compound group.

Overall, in terms of clustering distance, COB and POB groups were furthest apart (appendix 9.4). This result is mostly due to differences in abundances of carbohydrates, amino acids, nucleotides/sides, and organic acids involved in the TCA-cycle (Fig 18). Especially nucleotides and nucleosides were highly different between COB and POB treatments, with abundances being low in the latter and high in the former.

In case of amino acids, especially valine and leucine are highly abundant in the POB group, while low in abundance in the COB group.

5.2.4 Hive variation study

Since results slightly differ between hives, a hive variation study was performed based on PCA-analysis of body and gut metabolites grouped by hive.

5.2.4.1 Body

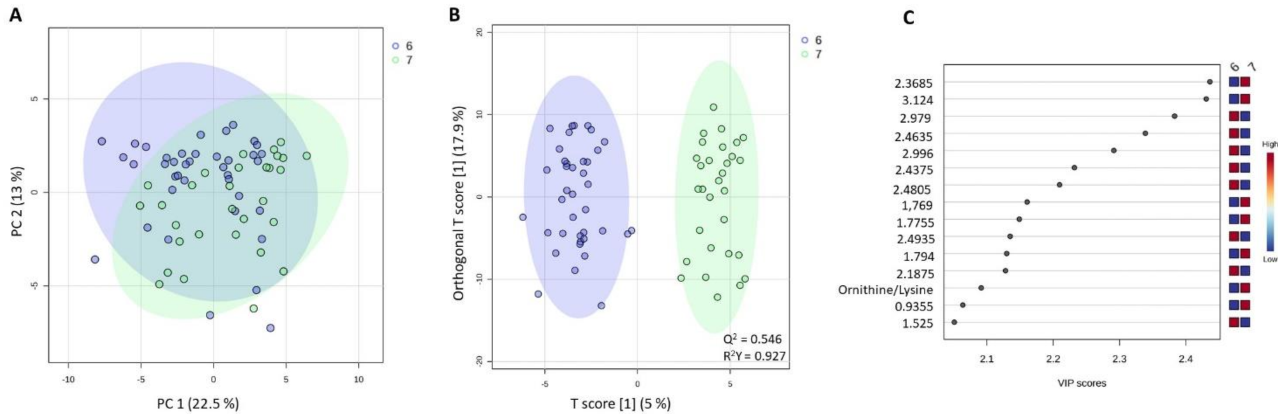


Figure 19: Comparison of body composition between hive 6 and 7. A) PCA, B) OPLSDA, C) VIP-scores of the top 15 compounds. The higher the score, the more its contribution to the difference between groups. Unidentified compounds are labeled by a value representing their position on the NMR spectrum. Red or blue color indicates high or low abundance respectively.

According to the PCA comparing body metabolites between the two separate hives, body composition is slightly different within each hive. Data from hive 6 is more scattered overall, with more variation across PC 2. Hive 7 is clustered somewhat more closely together and shows an opposite relationship with the principal components compared to hive 6. PC 1 (22.5 %) and PC 2 (13 %) account for 35.5 % of the total variation (Fig. 19A). Furthermore, the effect is mainly due to unknown compounds (Fig. 19C). OPLS-DA scores good for predictive power (Q^2: 0.546) and is a good fit for the observed data (R^2Y: 0.927) (Fig. 19B).

5.2.4.2 Gut

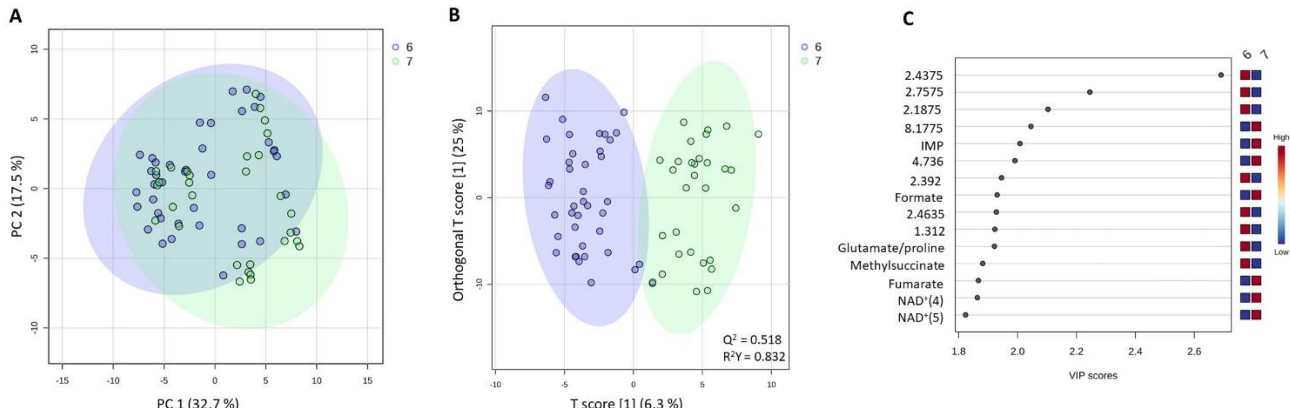


Figure 20: Comparison of gut metabolites between hive 6 and 7. A) PCA, B) OPLSDA, C) VIP-scores of the top 15 compounds. The higher the score, the more its contribution to the difference between groups. Compounds that are present more than once are accompanied by a number signifying the distinct spectral position. Unidentified compounds are labeled by a value representing their position on the NMR spectrum. Red or blue color indicates high or low abundance respectively.

Differences in gut metabolites between the two hives are somewhat more subtle, but similar to the previous section, both hives show opposite orientation along the PC's. PC 1 and PC 2 explain 32.7 % and 17.5 % of the total variation respectively (Fig. 20A). Together accounting for 50 %. The effect is mainly due to unknown compounds, but inosine monophosphate (IMP) and organic acids such as formate, glutamate and fumarate are among the most important known contributors (Fig. 20C). The OPLS-DA model fits the data well (R^2Y: 0.832) and has good predictive power (Q^2: 0518) (Fig. 20B).

6 Discussion

Based on the hierarchical clustering distances between treatment groups, supplementation with bacterial strains seems to influence the metabolic composition of honey bees since individuals exposed to bacterial strain supplements are more similar to each other than to the control treatments (with the exception of body metabolites in hive 7; section 5.2.3.1.2). The effect is more pronounced in the gut (Figs. 17 and 18), which can of course be explained by the fact that this is where the administered bacteria reside and thus will have the most direct influence.

In none of the comparisons however, principal component analysis resulted in a clear distinction between treatments. This indicates that the differences are rather subtle and should be regarded as such.

This section will explain the most important findings focussing mostly on the distinction between groups that received bacterial supplements and those that did not. Either in the presence of older bees or not.

6.1 Effect of bacterial strain supplementation

Comparison of the control group (C) to the group that received the bacterial supplement (P) allows for interpretation of the isolated effect of the specific bacterial strains that were administered to the bees.

6.1.1 Gut

The bacteria used in this experiment, seem to mainly influence the carboxylic acid composition of the intestines of previously sterile bees. Acetate, succinate, and benzoate leaned towards higher abundance in the gut of bees exposed to the bacterial supplement, while the citrate content in these guts was lower (Fig. 11).

Most likely, acetate, succinate, and benzoate are produced by the metabolism of the newly introduced bacteria. Acetate and succinate are typical metabolites in the microbial breakdown of dietary fibre found in pollen (Ricigliano & Anderson, 2020). While benzoate is specifically associated with the breakdown of aromatic compounds, including those derived from lignin (Michalska et al., 2012).

Acetate, succinate, and benzoate are important determinants in the bacterial composition of the gut microbiota. Acetate for example, is produced in high quantities by *Bifidobacterium sp.* and has been found to enhance the human gut's resistance to infection by increasing the

production of bacteriocins by lactic acid bacteria (Fukuda et al., 2011; Meng et al., 2021). Benzoate, in turn, has been demonstrated to ameliorate the intestinal flora of several livestock species such as chickens (Yousaf et al., 2017), pigs (Torrallardona et al., 2007), and even fish (Libanori et al., 2023), largely due to its antimicrobial properties. Succinate, lastly, is an important intermediate in the TCA-cycle but has also been associated with inflammation response (Tannahil et al., 2013), immune system functioning (Rubic et al., 2008) and glycemic control within the host organism (De Vadder et al., 2016).

Although the role of these compounds in insects is less studied than in mammals, it is reasonable to assume a similar function in these organisms. Even more so because they seem to be abundantly produced by the native honey bee microbiota. Quin and colleagues (2024) recently found that colonisation of the honey bee gut with *Snodgrasella alvi* and *Gilliamella spp.* resulted in a synergistic production of all three of the above mentioned compounds. Our results indicate a similar phenomenon. Overall, the increased abundance of these fermentation products are evidence for the importance of gut bacteria as a digestive aid for the host organism (as explained in section 3.2.3.5 of this work).

The remaining compound, citrate, was found to be less abundant in the presence of gut microbiota. This could be explained by the competition of bacterial strains for this substance. Citrate is an important compound in the TCA-cycle (also called citric acid cycle) and is therefore quickly used to produce energy to sustain the bacterial cells. Since the TCA-cycle is a fundamental metabolic pathway present in most organisms (Mailloux, 2015), competition for citric acid will inevitably be high. Depletion of citrate by the honey bee microbiota has been demonstrated in the case of colonisation with specific strains (e.g. *Lactobacillus spp.,* Brochet et al., 2021; Quinn et al., 2024) as well as for the full microbial community (Kešnerová et al., 2017). This finding can also partly explain the increased abundance of succinate since citrate is converted into this compound further down the line of the TCA-cycle (Alabduladhem & Bordoni, 2024).

6.1.2 Body

Regarding body metabolites, adenosine monophosphate (AMP) seems to be the most important compound and tends to be more abundant when gut bacteria were absent. AMP, mainly in its cyclic form (cAMP) is an important intracellular signalling molecule closely connected with dopamine receptors in insects (Verlinden, 2012). It is for example associated in honey bees with behavioural patterns including division of labour (Reim & Scheiner, 2014), and learning and memory (Raza & Su, 2020). Whether or not the differences in AMP found in

this study can alter the bee's behaviour in any meaningful way and whether or not the potential effect would be positive or negative, is beyond the scope of this work. It is however interesting to note that the gut microbiome seems to influence the expression of behaviour related compounds in the host's body.

6.2 Effect of the presence of older siblings

The effect of older bees can be isolated through the comparison of the C and COB treatments.

6.2.1 Gut

Similarly to supplementation with specific bacteria, the presence of older bees hints toward increased abundance of acetate and decreased abundance of citrate (Figure 12C). This would be an indication that older bees do indeed transfer at least in part, their gut microbiome to naïve siblings. Those gut microbes then influence organic acid abundances via the mechanisms described above. One could expect that the transfer of native gut microbes would lead to an increase of several other fermentation products as observed in the case of bacterial supplementation (see previous section). This, however, is not the case which can be explained by the phenomenon of cross feeding (Smith et al., 2019) where a more diverse microbial community uses metabolites produced by other strains more readily and efficiently resulting in a low nett abundance of these compounds.

Overall, the differences are only minor since PCA analysis shows a nearly complete overlap of both treatments and the OPLS-DA model's predictive ability was low ($Q^2 = -0.395$).

6.2.2 Body

VIP-scores appointed AMP once again among the important compounds contributing to the distinction between C and COB bees (Figure 8C). With this compound again being more present in the control group. Other influenced compounds were trigonelline and saccharides such as glucose and sucrose. PCA however showed strong overlap again and combined with a low predictive power of the OPLS-DA model, this indicates that the differences are small and might be produced merely by chance.

Overall, based on the PCA results (Figure 8A) variation within the COB group was quite large. Perhaps the haphazard nature of bacterial transfer via trophallaxis in the COB group (as explained in section 3.2.2.4), results in differential colonisation patterns depending on which of

the naturally present bacterial strains are introduced at what time during the experimental period. Differences in gut microbes could then translate to differences in body metabolite abundances. In mice for example, the gut microbiome is known to influence blood glucose levels (De Vadder et al., 2016) and a study by Lamichhane et al (2021) found microbiota dependent lipid levels in human blood. In honeybees in particular, the microbiome has been demonstrated to influence the abundance of several neuroactive metabolites in the haemolymph and brain (Cabirol et al., 2023a).

6.3 Effect of bacterial strain supplementation in the presence of older siblings

Lastly, the influence of the supplemented bacteria in the presence of older bees can be explored through the comparison of the COB and POB treatments.

6.3.1 Gut

Both for hive 6 and 7, acetate was among the top 15 most important compounds based on their VIP-scores (Fig. 13C & 14C). In both cases, acetate was more abundant in the POB treatment while citrate was less abundant in this treatment within hive 7. Thus, indicating that the strains used in the bacterial supplement increase the acetate abundance more than the native microbes. This phenomenon occurs likely due to *Bifidobacterium asteroides* since acetate is the main glucose fermentation product produced by *Bifidobacterium spp.* (de Souza Oliveira et al., 2012). Furthermore, citrate depletion was enhanced by the specific strains in the bacterial supplement as well. That is, in hive 7.

According to the hierarchical clustering heatmaps (appendix 9.3 & 9.4; figure 17 & 18), nucleotides and nucleosides become less abundant upon supplementation with bacteria in both hive 6 and 7. In the latter, uridine shows up as one of the five most important compounds according to VIP-Score (Fig. 14C). This nucleoside has been shown to alter the gut microbiome of mice on a high fat diet, assisting lipid metabolism (Liu et al., 2021) and to ameliorate epithelial cell renewal in the intestines of piglets (Li et al., 2019). The specific bacteria in the supplement seem to shift the balance of the natural microbiome away from uridine production, whether this result is reproducible is however uncertain since the predictive ability of the OPLS-DA model was relatively low ($Q^2 = 0.395$)

Interestingly, PCA for hive 6 showed once again increased variation within the COB group (Fig. 13A). This could mean that transfer of gut microbiota via the natural pathway (trophallaxis) lead to differential colonisation patterns and that these differences are reduced

through supplementation with specific bacterial strains. A logical finding since the supplementation regime was the exact same for all bees and would thus result in more similar colonisation of the gut. However, no such pattern was observed in hive 7. Quite the opposite in fact (Fig 14A).

Overall, gut metabolites within the COB and POB treatments were influenced differentially between hives (Figure 13 & 14). This indicates a dependency of the interaction between the natural microbiome and the bacterial supplement on the bees environment and/or genetics. Bees' food preferences (pollen/nectar ratio) have been found to depend on their genetic composition (Page et al., 1995) and differences in food sources, more than likely result in a different microbial composition and thus differential interaction with newly introduced strains.

6.3.2 Body

Body tissues of the COB and POB treatment showed different results in each hive. The differences for hive 6 were mainly due to nucleosides/tides such as inosine and uracil, and organic acids including malonate, succinate, fumarate, and 4-hydroxy benzoate (Fig. 9C) and similar to the gut metabolites, variation in the COB treatment is large (Fig. 9A). The OPLS-DA model has low predictive value ($Q^2 = 0.066$; Fig 9B) and the PCA shows a strong overlap between POB and COB data. Differences are therefore minor, but inosine seems to be of some importance, nonetheless. This nucleoside was overall more abundant when bees received a bacterial supplement. In mice, inosine is an intracellular signalling molecule with anti-depressant properties (Muto et al., 2014) and can improve neurological and behavioural recovery after stroke (Chen et al., 2002). In honey bees specifically, the phosphorylated form of inosine (inosine monophosphate; IMP) has been associated with increased sensitivity of taste receptors detecting the presence of amino acid in a food source (Lim et al., 2019). The effect of inosine is less studied in this insect, but in mammals, it has been found to have similar functionality as adenosine (Srinivasan et al., 2021) and adenosine is in fact a neuroactive compound in honeybees (Chen et al., 2021). The function of inosine is therefore likely similar in insects as well.

For hive 7, saccharides seem to be important determinants in the difference between COB and POB treatments since hierarchical clustering shows a strong distinction in carbohydrate abundance (Fig. 16; appendix 9.2) and fructose, sucrose and glucose are among the top five major contributors according to VIP-score (Fig. 10C). All those compounds are more abundant with bacterial strain supplementation. Since the bacterial strains within the supplement are

known to be involved in the sugar metabolism of honey bees, especially *Lactobacillus* and *Bifidobacterium spp.* (Engel & Moran, 2013), it is possible that the presence of these bacteria increases the uptake of saccharides into the haemolymph. A statement backed up by research of Lee et al. (2018) where the core honey bee microbes, including *Lactobacillus* and *Bifidobacterium* were found to be key determinants in digestion of carbohydrates and consequential weight gain in honey bee workers.

7 Conclusion

Although specific trends were overall subtle, hierarchical clustering indicates an effect of the bacterial supplement since groups that received this supplement tended to cluster together and thus were more similar to each other than to the control groups.

In more detail, supplementation with the specific bacterial strains in this experiment tends to mainly influence the organic acid content in the intestines of honey bees. When the bacterial supplement was the only source of gut colonising microbes, that is, when older bees were absent, a diverse selection of fermentation products was found to increase in abundance. This indicates a role of the supplemented bacteria as digestive aid, but also possibly hints towards a less efficient turnover of metabolites compared to the native and presumably more diverse microbial community.

Acetate is the main compound affected by the gut microbiome since it is more abundant when gut bacteria are present. Regardless of their origin (older bees or supplement). Furthermore, supplementation with the specific bacteria used here, increased acetate abundance in guts also colonised by natural bacteria. This is most likely due to the increased representation of *Bifidobacterium spp.* due to supplementation. *Bifidobacterium spp.* is known for its high acetate production. Since acetate can influence the microbial composition of the gut (see section 6.1.1), the specific supplement used in this experiment is likely to affect said composition through this mechanism.

Lastly, regarding bodily tissues, it is interesting to note that the presence of gut bacteria, natural or via supplementation, tends to influence compounds related to neurological functioning such as AMP and inosine. This might indicate the existence of a gut-brain axis, where the gut microbial community is capable of influencing neurology and behaviour in honey bees. A phenomenon backed up by previous scientific inquiry (Zhang et al., 2022; Cabirol et al., 2023b).

8 Bibliography

Alabduladhem, T. O., Bordoni, B. (2024) Physiology, Krebs Cycle. [Updated 2022 Nov 23]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; Available from: https://www.ncbi.nlm.nih.gov/books/NBK556032/

Aronstein, K. A., & Murray, K. D. (2010). Chalkbrood disease in honey bees. J*ournal of Invertebrate Pathology, 103, S20–S29*. https://doi.org/https://doi.org/10.1016/j.jip.2009.06.018

Ascosphaera Apis. (2005). In Encyclopedia of Entomology (p. 204). Dordrecht: Springer Netherlands. https://doi.org/10.1007/0-306-48380-7_319

Bertram, C. & Hass, R. (2008). Cellular responses to reactive oxygen species-induced DNA damage and aging. *Biological Chemistry*, *389*(3), 211-220. https://doi.org/10.1515/BC.2008.031

Bhakta, J. N., Ohnishi, K., Munekage, Y., Iwasaki, K., & Wei, M. Q. (2012). Characterization of lactic acid bacteria-based probiotics as potential heavy metal sorbents. *Journal of Applied Microbiology*, *112*(6), 1193–1206. https://doi.org/10.1111/j.1365-2672.2012.05284.x

Biesmeijer, J. C., Roberts, S. P. M., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., … Kunin, W. E. (2006). Parallel Declines in Pollinators and Insect-Pollinated Plants in Britain and the Netherlands. *Science*, *313*(5785), 351–354. https://doi.org/10.1126/science.1127863

Boecking, O., & Genersch, E. (2008). Varroosis – the Ongoing Crisis in Bee Keeping. *Journal Für Verbraucherschutz Und Lebensmittelsicherheit*, 3(2), 221–228. https://doi.org/10.1007/s00003-008-0331-y

Bonilla-Rosso, G., & Engel, P. (2018). Functional roles and metabolic niches in the honey bee gut microbiota. *Current Opinion in Microbiology*, *43*, 69–76. https://doi.org/https://doi.org/10.1016/j.mib.2017.12.009

British beekeepers association. (n.d.). Available online: https://www.bbka.org.uk/honey (Accessed on 25 September 2023)

Brochet, S., Quinn, A., Mars, R. A. T., Neuschwander, N., Sauer, U., & Engel, P. (2021). Niche partitioning facilitates coexistence of closely related honey bee gut bacteria. *ELife*, *10*, e68583. https://doi.org/10.7554/eLife.68583

Burda, H., Honeycutt, R. L., Begall, S., Locker-Grütjen, O., & Scharff, A. (2000). Are naked and common mole-rats eusocial and if so, why? *Behavioral Ecology and Sociobiology*, *47*(5), 293–303. https://doi.org/10.1007/s002650050669

Cabirol, A., Moriano-Gutierrez, S., & Engel, P. (2023a). Neuroactive metabolites modulated by the gut microbiota in honey bees. *Molecular microbiology*, 10.1111/mmi.15167. Advance online publication. https://doi.org/10.1111/mmi.15167

Cabirol, A., Schafer, J., Neuschwander, N., Kesner, L., Liberti, J., & Engel, P. (2023b). A defined community of core gut microbiota members promotes cognitive performance in honey bees. *BioRxiv*. https://doi.org/10.1101/2023.01.03.522593

Capuco, A., Urits, I., Hasoon, J., Chun, R., Gerald, B., Wang, J. K., … Viswanath, O. (2020). Current Perspectives on Gut Microbiome Dysbiosis and Depression. *Advances in Therapy*, *37*(4), 1328–1346. https://doi.org/10.1007/s12325-020-01272-7

Carrola, J., Rocha, C. M., Barros, A. S., Gil, A. M., Goodfellow, B. J., Carreira, I. M., … Duarte, I. F. (2011). Metabolic Signatures of Lung Cancer in Biofluids: NMR-Based Metabonomics of Urine. *Journal of Proteome Research*, *10*(1), 221–230. https://doi.org/10.1021/pr100899x

Chege, M., Kinyua, J., & Paredes, J. C. (2023). *Lactobacillus kunkeei* impacts the health of honey bees, *Apis mellifera scutellata*, and protects the bees against the opportunistic pathogen *Serratia*

marcescens. *International Journal of Tropical Insect Science*, *43*(6), 1947–1955. https://doi.org/10.1007/s42690-023-01103-6

Chen, P., Goldberg, D. E., Kolb, B., Lanser, M., & Benowitz, L. I. (2002). Inosine induces axonal rewiring and improves behavioral outcome after stroke. *Proceedings of the National Academy of Sciences*, *99*(13), 9031–9036. https://doi.org/10.1073/pnas.132076299

Chen, P., Lu, Y.-H., Lin, Y.-H., Wu, C.-P., Tang, C.-K., Wei, S.-C., & Wu, Y.-L. (2021). Deformed wing virus infection affects the neurological function of Apis mellifera by altering extracellular adenosine signaling. *Insect Biochemistry and Molecular Biology*, *139*, 103674. https://doi.org/https://doi.org/10.1016/j.ibmb.2021.103674

Chen, Y. P., & Siede, R. B. T.-A. in V. R. (2007). Honey Bee Viruses (Vol. 70, pp. 33–80). Academic Press. https://doi.org/https://doi.org/10.1016/S0065-3527(07)70002-7

Chmiel, J. A., Daisley, B. A., Pitek, A. P., Thompson, G. J., & Reid, G. (2020). Understanding the Effects of Sublethal Pesticide Exposure on Honey Bees: A Role for Probiotics as Mediators of Environmental Stress. *Frontiers in Ecology and Evolution*, *8*. https://doi.org/10.3389/fevo.2020.00022

Corby-Harris, V., Snyder, L. A., Schwan, M. R., Maes, P., McFrederick, Q. S., & Anderson, K. E. (2014). Origin and Effect of Alpha 2.2 Acetobacteraceae in Honey Bee Larvae and Description of Parasaccharibacter apium gen. nov., sp. nov. *Applied and Environmental Microbiology*, 80(24), 7460–7472. https://doi.org/10.1128/AEM.02043-14

Cronin, A. L., Molet, M., Doums, C., Monnin, T., & Peeters, C. (2013). Recurrent Evolution of Dependent Colony Foundation Across Eusocial Insects. *Annual Review of Entomology*, *58*(Volume 58, 2013), 37–55. https://doi.org/https://doi.org/10.1146/annurev-ento-120811-153643

Daisley, B. A., Pitek, A. P., Chmiel, J. A., Al, K. F., Chernyshova, A. M., Faragalla, K. M., … Reid, G. (2020). Novel probiotic approach to counter Paenibacillus larvae infection in honey bees. *The ISME Journal*, *14*(2), 476–491. https://doi.org/10.1038/s41396-019-0541-6

de Souza Oliveira, R. P., Perego, P., de Oliveira, M. N., & Converti, A. (2012). Growth, organic acids profile and sugar metabolism of Bifidobacterium lactis in co-culture with Streptococcus thermophilus: The inulin effect. *Food Research International*, *48*(1), 21–27

De Vadder, F., Kovatcheva-Datchary, P., Zitoun, C., Duchampt, A., Bäckhed, F., & Mithieux, G. (2016). Microbiota-Produced Succinate Improves Glucose Homeostasis via Intestinal Gluconeogenesis. *Cell Metabolism*, *24*(1), 151–157. https://doi.org/https://doi.org/10.1016/j.cmet.2016.06.013

Decker, L. E., San Juan, P. A., Warren, M. L., Duckworth, C. E., Gao, C., & Fukami, T. (2023). Higher Variability in Fungi Compared to Bacteria in the Foraging Honey Bee Gut. *Microbial Ecology*, 85(1), 330–334. https://doi.org/10.1007/s00248-021-01922-5

Di, N., Hladun, K. R., Zhang, K., Liu, T.-X., & Trumble, J. T. (2016). Laboratory bioassays on the impact of cadmium, copper and lead on the development and survival of honeybee (Apis mellifera L.) larvae and foragers. *Chemosphere*, *152*, 530–538. https://doi.org/https://doi.org/10.1016/j.chemosphere.2016.03.033

Dickel, F., Bos, N. M. P., Hughes, H., Martín-Hernández, R., Higes, M., Kleiser, A., & Freitak, D. (2022). The oral vaccination with Paenibacillus larvae bacterin can decrease susceptibility to American Foulbrood infection in honey bees—A safety and efficacy study. *Frontiers in Veterinary Science*, *9*. https://doi.org/10.3389/fvets.2022.946237

Dicks, L. V, Breeze, T. D., Ngo, H. T., Senapathi, D., An, J., Aizen, M. A., … Potts, S. G. (2021). A global-scale expert assessment of drivers and risks associated with pollinator decline. *Nature Ecology & Evolution*, 5(10), 1453–1461. https://doi.org/10.1038/s41559-021-01534-9

Dosch, C., Manigk, A., Streicher, T., Tehel, A., Paxton, R. J., & Tragust, S. (2021). The Gut Microbiota Can Provide Viral Tolerance in the Honey Bee. *Microorganisms*, *9*(4). https://doi.org/10.3390/microorganisms9040871

Engel, P., Bartlett, K. D., & Moran, N. A. (2015). The bacterium Frischella perrara causes scab formation in the gut of its honeybee host. *mBio* 6: e00193-15.

Engel, P., & Moran, N. A. (2013). Functional and evolutionary insights into the simple yet specific gut microbiota of the honey bee from metagenomic analysis. *Gut Microbes*, *4*(1), 60–65. https://doi.org/10.4161/gmic.22517

Entani, E., Asai, M., Tsujihata, S., Tsukamoto, Y., & Ohta, M. (1998). Antibacterial Action of Vinegar against Food-Borne Pathogenic Bacteria Including Escherichia coliO157:H7. *Journal of Food Protection*, *61*(8), 953–959. https://doi.org/https://doi.org/10.4315/0362-028X-61.8.953

Evans, J. D., Aronstein, K., Chen, Y. P., Hetru, C., Imler, J.-L., Jiang, H., … Hultmark, D. (2006). Immune pathways and defence mechanisms in honey bees Apis mellifera. *Insect Molecular Biology*, *15*(5), 645–656. https://doi.org/https://doi.org/10.1111/j.1365-2583.2006.00682.x

Fathi, F., Kasmaee, L. M., Sohrabzadeh, K., Nejad, M. R., Tafazzoli, M., & Oskouie, A. A. (2014). The Differential Diagnosis of Crohn’s Disease and Celiac Disease Using Nuclear Magnetic Resonance Spectroscopy. *Applied Magnetic Resonance*, *45*(5), 451–459. https://doi.org/10.1007/s00723-014-0530-x

Feng, T., & Wang, J. (2020). Oxidative stress tolerance and antioxidant capacity of lactic acid bacteria as probiotic: a systematic review. *Gut Microbes*, *12*(1), 1801944. https://doi.org/10.1080/19490976.2020.1801944

Formato, G., Rivera-Gomis, J., Bubnic, J., Martín-Hernández, R., Milito, M., Croppi, S., & Higes, M. (2022). Nosemosis Prevention and Control. *Applied Sciences*. https://doi.org/10.3390/app12020783

Forsgren, E., Locke, B., Sircoulomb, F., & Schäfer, M. O. (2018). Bacterial Diseases in Honeybees. *Current Clinical Microbiology Reports*, *5*(1), 18–25. https://doi.org/10.1007/s40588-018-0083-0

Forsgren, E., Olofsson, T. C., Váasquez, A., & Fries, I. (2010). Novel lactic acid bacteria inhibiting Paenibacillus larvae in honey bee larvae. *Apidologie*, *41*(1), 99–108. https://doi.org/10.1051/apido/2009065

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., … Ohno, H. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature*, *469*(7331), 543–547. https://doi.org/10.1038/nature09646

Gajger, I. T., Sakač, M., & Gregorc, A. (2017). Impact of Thiamethoxam on Honey Bee Queen (Apis mellifera carnica) Reproductive Morphology and Physiology. *Bulletin of Environmental Contamination and Toxicology*, *99*(3), 297–302. https://doi.org/10.1007/s00128-017-2144-0

Genersch, E. (2010). Honey bee pathology: current threats to honey bees and beekeeping. *Applied Microbiology and Biotechnology*, *87*(1), 87–97. https://doi.org/10.1007/s00253-010-2573-8

Hegland, S. J., Nielsen, A., Lázaro, A., Bjerknes, A.-L., & Totland, Ø. (2009). How does climate warming affect plant-pollinator interactions? *Ecology Letters*, *12*(2), 184–195. https://doi.org/https://doi.org/10.1111/j.1461-0248.2008.01269.x

Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., … Decourtye, A. (2012). A Common Pesticide Decreases Foraging Success and Survival in Honey Bees. *Science*, *336*(6079), 348–350. https://doi.org/10.1126/science.1215039

Higes, M., Martín-Hernández, R., Botías, C., Bailón, E. G., González-Porto, A. V, Barrios, L., …

Meana, A. (2008). How natural infection by Nosema ceranae causes honeybee colony collapse. *Environmental Microbiology*, *10*(10), 2659–2669. https://doi.org/https://doi.org/10.1111/j.1462-2920.2008.01687.x

Hore, P. J. (2015). Nuclear Magnetic Resonance (2nd ed). *Oxford chemistry primers.* ISSN 1367-109X. Oxford University Press.

Hroncova, Z., Havlik, J., Killer, J., Doskocil, I., Tyl, J., Kamler, M., … Rada, V. (2015). Variation in Honey Bee Gut Microbial Diversity Affected by Ontogenetic Stage, Age and Geographic Location. *PLOS ONE*, *10*(3), 1–17. https://doi.org/10.1371/journal.pone.0118707

Iorizzo, M., Letizia, F., Ganassi, S., Testa, B., Petrarca, S., Albanese, G., … De Cristofaro, A. (2022). Functional Properties and Antimicrobial Activity from Lactic Acid Bacteria as Resources to Improve the Health and Welfare of Honey Bees. *Insects*, *13*(3). https://doi.org/10.3390/insects13030308

Islam, S. M. A., Math, R. K., Cho, K. M., Lim, W. J., Hong, S. Y., Kim, J. M., … Yun, H. D. (2010). Organophosphorus Hydrolase (OpdB) of Lactobacillus brevis WCP902 from Kimchi Is Able To Degrade Organophosphorus Pesticides. *Journal of Agricultural and Food Chemistry*, *58*(9), 5380–5386. https://doi.org/10.1021/jf903878e

Jack, C. J., & Ellis, J. D. (2021). Integrated Pest Management Control of Varroa destructor (Acari: Varroidae), the Most Damaging Pest of (Apis mellifera L. (Hymenoptera: Apidae)) Colonies. *Journal of Insect Science*, *21*(5), 6. https://doi.org/10.1093/jisesa/ieab058

Jacob, D., Deborde, C., Lefebvre, M., Maucourt, M., & Moing, A. (2017). NMRProcFlow: a graphical and interactive tool dedicated to 1D spectra processing for NMR-based metabolomics. *Metabolomics*, *13*(4), 36. https://doi.org/10.1007/s11306-017-1178-y

Jones, J. C., Fruciano, C., Marchant, J., Hildebrand, F., Forslund, S., Bork, P., … Hughes, W. O. H. (2018). The gut microbiome is associated with behavioural task in honey bees. *Insectes Sociaux*, 65(3), 419–429. https://doi.org/10.1007/s00040-018-0624-9

Kapheim, K. M., Rao, V. D., Yeoman, C. J., Wilson, B. A., White, B. A., Goldenfeld, N., & Robinson, G. E. (2015). Caste-Specific Differences in Hindgut Microbial Communities of Honey Bees (Apis mellifera). *PLOS ONE*, 10(4), 1–14. https://doi.org/10.1371/journal.pone.0123911

Kešnerová, L., Mars, R. A. T., Ellegaard, K. M., Troilo, M., Sauer, U., & Engel, P. (2017). Disentangling metabolic functions of bacteria in the honey bee gut. *PLOS Biology*, *15*(12), 1–28. https://doi.org/10.1371/journal.pbio.2003467

Kevan, P. G., & Viana, B. F. (2003). The global decline of pollination services. *Biodiversity*, *4*(4), 3–8. https://doi.org/10.1080/14888386.2003.9712703

Khalifa, S. A. M., Elshafiey, E. H., Shetaia, A. A., El-Wahed, A. A. A., Algethami, A. F., Musharraf, S. G., … El-Seedi, H. R. (2021). Overview of Bee Pollination and Its Economic Value for Crop Production. *Insects*, *12*(8). https://doi.org/10.3390/insects12080688

Kinoshita, H. (2019). Biosorption of Heavy Metals by Lactic Acid Bacteria for Detoxification. In M. Kanauchi (Ed.), *Lactic Acid Bacteria: Methods and Protocols* (pp. 145–157). New York, NY: Springer New York. https://doi.org/10.1007/978-1-4939-8907-2_13

Klein, A. M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2006). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1608), 303–313. https://doi.org/10.1098/RSPB.2006.3721

Kunc, M., Dobeš, P., Ward, R., Lee, S., Čegan, R., Dostálková, S., … Hyršl, P. (2023). Omics-based analysis of honey bee (Apis mellifera) response to Varroa sp. parasitisation and associated factors reveals changes impairing winter bee generation. *Insect Biochemistry and Molecular*

Biology, *152*, 103877. https://doi.org/https://doi.org/10.1016/j.ibmb.2022.103877

Kwong, W. K., Mancenido, A. L., & Moran, N. A. (2017). Immune system stimulation by the native gut microbiota of honey bees. *Royal Society Open Science*, 4(2), 170003. https://doi.org/10.1098/rsos.170003

Kwong, W. K., & Moran, N. A. (2016). Gut microbial communities of social bees. *Nature Reviews Microbiology*, *14*(6), 374–384. https://doi.org/10.1038/nrmicro.2016.43

Lamichhane, S., Sen, P., Alves, M. A., Ribeiro, H. C., Raunioniemi, P., Hyötyläinen, T., & Orešič, M. (2021). Linking Gut Microbiome and Lipid Metabolism: Moving beyond Associations. *Metabolites*, *11*(1), 55. https://doi.org/10.3390/metabo11010055

Lautenbach, S., Seppelt, R., Liebscher, J., & Dormann, C. F. (2012). Spatial and Temporal Trends of Global Pollination Benefit. *PLOS ONE*, *7*(4), e35954. https://doi.org/10.1371/JOURNAL.PONE.0035954

Le Conte, Y., & Navajas, M. (2008). Changements climatiques : impact sur les populations d'abeilles et leurs maladies. *OIE Revue Scientifique et Technique*, *27*(2), 485–510. https://doi.org/10.20506/RST.27.2.1819

Lee, F. J., Miller, K. I., McKinlay, J. B., & Newton, I. L. G. (2018). Differential carbohydrate utilization and organic acid production by honey bee symbionts. *FEMS Microbiology Ecology*, *94*(8), fiy113. https://doi.org/10.1093/femsec/fiy113

Lee, S., Kalcic, F., Duarte, I. F., Titera, D., Kamler, M., Mrna, P., … Havlik, J. (2022). 1H NMR Profiling of Honey Bee Bodies Revealed Metabolic Differences between Summer and Winter Bees. *Insects*, *13*(2). https://doi.org/10.3390/insects13020193

Leska, A., Nowak, A., Miśkiewicz, K., & Rosicka-Kaczmarek, J. (2022). Binding and Detoxification of Insecticides by Potentially Probiotic Lactic Acid Bacteria Isolated from Honeybee (Apis mellifera L.) Environment—An In Vitro Study. *Cells*, *11*(23). https://doi.org/10.3390/cells11233743

Li, G., Xie, C., Wang, Q., Wan, D., Zhang, Y., Wu, X., & Yin, Y. (2019). Uridine/UMP metabolism and their function on the gut in segregated early weaned piglets. *Food Funct.*, *10*(7), 4081–4089. https://doi.org/10.1039/C9FO00360F

Libanori, M. C. M., Santos, G. G., Pereira, S. A., Ferrarezi, J. V. S., Ferreira, M. B., Cardoso, L., … Mouriño, J. L. P. (2023). Organic benzoic acid modulates health and gut microbiota of Oreochromis niloticus. *Aquaculture*, *570*, 739409. https://doi.org/https://doi.org/10.1016/j.aquaculture.2023.739409

Lim, S., Jung, J., Yunusbaev, U., Ilyasov, R., & Kwon, H. W. (2019). Characterization and its implication of a novel taste receptor detecting nutrients in the honey bee, Apis mellifera. *Scientific Reports*, *9*(1), 11620. https://doi.org/10.1038/s41598-019-46738-z

Liu, Y., Xie, C., Zhai, Z., Deng, Z., De Jonge, H. R., Wu, X., & Ruan, Z. (2021). Uridine attenuates obesity, ameliorates hepatic lipid accumulation and modifies the gut microbiota composition in mice fed with a high-fat diet. *Food Funct.*, *12*(4), 1829–1840. https://doi.org/10.1039/D0FO02533J

Locke, B., Low, M., & Forsgren, E. (2019). An integrated management strategy to prevent outbreaks and eliminate infection pressure of American foulbrood disease in a commercial beekeeping operation. *Preventive Veterinary Medicine*, *167*, 48–52. https://doi.org/https://doi.org/10.1016/j.prevetmed.2019.03.023

Loftus, J. C., Smith, M. L., & Seeley, T. D. (2016). How Honey Bee Colonies Survive in the Wild: Testing the Importance of Small Nests and Frequent Swarming. *PLOS ONE*, *11*(3), 1–11. https://doi.org/10.1371/journal.pone.0150362

Mailloux, R. (2015). Still at the Center of it All; Novel Functions of the Oxidative Krebs Cycle. *Bioenergetics Open Access*, *4*. https://doi.org/10.4172/2167-7662.1000122

Martinson, V. G., Moy, J., & Moran, N. A. (2012). Establishment of Characteristic Gut Bacteria during Development of the Honeybee Worker. *Applied and Environmental Microbiology*, 78(8), 2830–2840. https://doi.org/10.1128/AEM.07810-11

McAfee, A. (2018). Who's Got Guts: The Microbes Living in Bees - American Bee Journal. Retrieved September 18, 2023, from https://americanbeejournal.com/whos-got-guts-the-microbes-living-in-bees/

McDevitt, J. C., Gupta, R. A., Dickinson, S. G., Martin, P. L., Rieuthavorn, J., Freund, A., … Rovnyak, D. (2021). Methodology for Single Bee and Bee Brain 1H-NMR Metabolomics. *Metabolites*, *11*(12). https://doi.org/10.3390/metabo11120864

Mishra, V., Shah, C., Mokashe, N., Chavan, R., Yadav, H., & Prajapati, J. (2015). Probiotics as Potential Antioxidants: A Systematic Review. *Journal of Agricultural and Food Chemistry*, *63*(14), 3615–3626. https://doi.org/10.1021/jf506326t

Meng, F., Zhao, H., Nie, T., Lu, F., Zhang, C., Lu, Y., & Lu, Z. (2021). Acetate Activates *Lactobacillus* Bacteriocin Synthesis by Controlling Quorum Sensing. *Applied and environmental microbiology*, *87*(13), e0072021. https://doi.org/10.1128/AEM.00720-21

Muto, J., Lee, H., Lee, H., Uwaya, A., Park, J., Nakajima, S., … Mikami, T. (2014). Oral administration of inosine produces antidepressant-like effects in mice. *Scientific Reports*, *4*(1), 4199. https://doi.org/10.1038/srep04199

Nowak, A., Szczuka, D., Górczyńska, A., Motyl, I., & Kręgiel, D. (2021). Characterization of Apis mellifera Gastrointestinal Microbiota and Lactic Acid Bacteria for Honeybee Protection—A Review. *Cells*, *10*(3). https://doi.org/10.3390/cells10030701

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos*, *120*(3), 321–326. https://doi.org/https://doi.org/10.1111/j.1600-0706.2010.18644.x

Page, R. E., Waddington, K. D., Hunt, G. J., & Kim Fondrk, M. (1995). Genetic determinants of honey bee foraging behaviour. *Animal Behaviour*, *50*(6), 1617–1625. https://doi.org/https://doi.org/10.1016/0003-3472(95)80015-8

Pang, Z., Chong, J., Zhou, G., de Lima Morais, D. A., Chang, L., Barrette, M., … Xia, J. (2021). MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights. *Nucleic Acids Research*, *49*(W1), W388–W396. https://doi.org/10.1093/nar/gkab382

Pettis, J. S., vanEngelsdorp, D., Johnson, J., & Dively, G. (2012). Pesticide exposure in honey bees results in increased levels of the gut pathogen Nosema. *Naturwissenschaften*, *99*(2), 153–158. https://doi.org/10.1007/s00114-011-0881-1

Phiri, B. J., Fèvre, D., & Hidano, A. (2022). Uptrend in global managed honey bee colonies and production based on a six-decade viewpoint, 1961–2017. *Scientific Reports*, 12(1), 21298. https://doi.org/10.1038/s41598-022-25290-3

Pickard, J. M., Zeng, M. Y., Caruso, R., & Núñez, G. (2017). Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. *Immunological Reviews*, *279*(1), 70–89. https://doi.org/https://doi.org/10.1111/imr.12567

Potts, S. G., Imperatriz-Fonseca, V., Ngo, H. T., Aizen, M. A., Biesmeijer, J. C., Breeze, T. D., … Vanbergen, A. J. (2016). Safeguarding pollinators and their values to human well-being. *Nature*, *540*(7632), 220–229. https://doi.org/10.1038/nature20588

Powell, J. E., Martinson, V. G., Urban-Mead K., Moran, N. A. (2014). Routes of Acquisition of the Gut Microbiota of the Honey Bee *Apis mellifera*. *Appl. Environ. Microbiol.* 80. https://doi.org/10.1128/AEM.01861-14

Ptaszyńska, A. A., Borsuk, G., Zdybicka-Barabas, A., Cytryńska, M., & Małek, W. (2016). Are commercial probiotics and prebiotics effective in the treatment and prevention of honeybee nosemosis C? *Parasitology Research*, 115(1), 397–406. https://doi.org/10.1007/s00436-015-4761-z

Quinn, A., El Chazli, Y., Escrig, S., Daraspe, J., Neuschwander, N., McNally, A., … Engel, P. (2024). Host-derived organic acids enable gut colonization of the honey bee symbiont Snodgrassella alvi. *Nature Microbiology*, *9*(2), 477–489. https://doi.org/10.1038/s41564-023-01572-y

Raymann, K., & Moran, N. A. (2018). The role of the gut microbiome in health and disease of adult honey bee workers. *Current Opinion in Insect Science*, 26, 97–104. https://doi.org/https://doi.org/10.1016/j.cois.2018.02.012

Raymann, K., Shaffer, Z., & Moran, N. A. (2017). Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. *PLOS Biology*, *15*(3), e2001861. https://doi.org/10.1371/journal.pbio.2001861

Ramsey, S. D., Ochoa, R., Bauchan, G., Gulbronson, C., Mowery, J. D., Cohen, A., … vanEngelsdorp, D. (2019). Varroa destructor feeds primarily on honey bee fat body tissue and not hemolymph. *Proceedings of the National Academy of Sciences*, *116*(5), 1792–1801. https://doi.org/10.1073/pnas.1818371116

Raza, M., & Su, S. (2020). Differential roles for dopamine d1-like and d2-like receptors in learning and behavior of honeybee and other insects. *Applied Ecology and Environmental Research*, *18*. https://doi.org/10.15666/aeer/1801_13171327

Reim, T., & Scheiner, R. (2014). Division of labour in honey bees: age- and task-related changes in the expression of octopamine receptor genes. *Insect Molecular Biology*, *23*(6), 833–841. https://doi.org/https://doi.org/10.1111/imb.12130

Ricigliano, V. A., & Anderson, K. E. (2020). Probing the Honey Bee Diet-Microbiota-Host Axis Using Pollen Restriction and Organic Acid Feeding. *Insects*, *11*(5). https://doi.org/10.3390/insects11050291

Rothman, J. A., Leger, L., Kirkwood, J. S., & McFrederick, Q. S. (2019). Cadmium and Selenate Exposure Affects the Honey Bee Microbiome and Metabolome, and Bee-Associated Bacteria Show Potential for Bioaccumulation. *Applied and Environmental Microbiology*, *85*(21), e01411-19. https://doi.org/10.1128/AEM.01411-19

Rubic, T., Lametschwandtner, G., Jost, S., Hinteregger, S., Kund, J., Carballido-Perrig, N., … Carballido, J. M. (2008). Triggering the succinate receptor GPR91 on dendritic cells enhances immunity. *Nature Immunology*, *9*(11), 1261–1269. https://doi.org/10.1038/ni.1657

Schievano, E., Stocchero, M., Morelato, E., Facchin, C., & Mammi, S. (2012). An NMR-based metabolomic approach to identify the botanical origin of honey. *Metabolomics*, *8*(4), 679–690. https://doi.org/10.1007/s11306-011-0362-8

Schoch, C.L, et al. (2020) NCBI Taxonomy: a comprehensive update on curation, resources and tools. Databse (Oxford).

Schwarz, R. S., Moran, N. A., & Evans, J. D. (2016). Early gut colonizers shape parasite susceptibility and microbiota composition in honey bee workers. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(33), 9345–9350. https://doi.org/10.1073/pnas.1606631113

Sforcin, J. M. (2016). Biological Properties and Therapeutic Applications of Propolis. *Phytotherapy Research*, *30*(6), 894–905. https://doi.org/https://doi.org/10.1002/ptr.5605

Smith, N. W., Shorten, P. R., Altermann, E., Roy, N. C., & McNabb, W. C. (2019). The Classification and Evolution of Bacterial Cross-Feeding. *Frontiers in Ecology and Evolution*, *7*. https://doi.org/10.3389/fevo.2019.00153

Sorrentino, E., Tremonte, P., Succi, M., Iorizzo, M., Pannella, G., Lombardi, S. J., … Coppola, R. (2018). Detection of Antilisterial Activity of 3-Phenyllactic Acid Using Listeria innocua as a Model. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.01373

Srinivasan, S., Torres, A. G., & de Pouplana, L. (2021). Inosine in Biology and Disease. *Genes*, 12(4). https://doi.org/10.3390/genes12040600

Surve, A. N., Sherikar, A. T., Bhilegaonkar, K. N., & Karkare, U. D. (1991). Preservative effect of combinations of acetic acid with lactic or propionic acid on buffalo meat stored at refrigeration temperature. *Meat Science*, *29*(4), 309–322. https://doi.org/https://doi.org/10.1016/0309-1740(91)90010-N

Svobodová, K., Maitre, A., Obregón, D., Wu-Chuang, A., Thaduri, S., Locke, B., … Cabezas-Cruz, A. (2023). Gut microbiota assembly of Gotland varroa-surviving honey bees excludes major viral pathogens. *Microbiological Research*, *274*, 127418. https://doi.org/https://doi.org/10.1016/j.micres.2023.127418

Switanek, M., Crailsheim, K., Truhetz, H., & Brodschneider, R. (2017). Modelling seasonal effects of temperature and precipitation on honey bee winter mortality in a temperate climate. *Science of The Total Environment*, *579*, 1581–1587. https://doi.org/https://doi.org/10.1016/j.scitotenv.2016.11.178

Tannahill, G. M., Curtis, A. M., Adamik, J., Palsson-McDermott, E. M., McGettrick, A. F., Goel, G., … O'Neill, L. A. J. (2013). Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature*, *496*(7444), 238–242. https://doi.org/10.1038/nature11986

Torrallardona, D., Badiola, I., & Broz, J. (2007). Effects of benzoic acid on performance and ecology of gastrointestinal microbiota in weanling piglets. *Livestock Science*, *108*(1), 210–213. https://doi.org/https://doi.org/10.1016/j.livsci.2007.01.062

Traynor, K. S., Mondet, F., de Miranda, J. R., Techer, M., Kowallik, V., Oddie, M. A. Y., … McAfee, A. (2020). *Varroa destructor*: A Complex Parasite, Crippling Honey Bees Worldwide. *Trends in Parasitology*, *36*(7), 592–606. https://doi.org/10.1016/j.pt.2020.04.004

Trinder, M., McDowell, T. W., Daisley, B. A., Ali, S. N., Leong, H. S., Sumarah, M. W., & Reid, G. (2016). Probiotic Lactobacillus rhamnosus Reduces Organophosphate Pesticide Absorption and Toxicity to Drosophila melanogaster. *Applied and Environmental Microbiology*, *82*(20), 6204–6213. https://doi.org/10.1128/AEM.01510-16

van der Steen, J., & Vejsnæs, F. (2021). Varroa Control: A Brief Overview of Available Methods. *Bee World*, 98(2), 50–56. https://doi.org/10.1080/0005772X.2021.1896196

van Engelsdorp, D., & Meixner, M. D. (2010). A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *Journal of Invertebrate Pathology*, *103*, S80–S95. https://doi.org/https://doi.org/10.1016/j.jip.2009.06.011

Vásquez, A., Forsgren, E., Fries, I., Paxton, R. J., Flaberg, E., Szekely, L., & Olofsson, T. C. (2012). Symbionts as Major Modulators of Insect Health: Lactic Acid Bacteria and Honeybees. *PLOS ONE*, *7*(3), e33188. Retrieved from https://doi.org/10.1371/journal.pone.0033188

Verlinden, H. (2018). Dopamine signalling in locusts and other insects. *Insect Biochemistry and Molecular Biology*, *97*, 40–52. https://doi.org/https://doi.org/10.1016/j.ibmb.2018.04.005

Vignoli, A., Ghini, V., Meoni, G., Licari, C., Takis, P. G., Tenori, L., Turano, P., & Luchinat, C. (2019). High-Throughput Metabolomics by 1D NMR. *Angewandte Chemie (International ed. in English)*, *58*(4), 968–994. https://doi.org/10.1002/anie.201804736

Wang, D.-I., & Mofller, F. E. (1970). The Division of Labor and Queen Attendance Behavior of Nosema-Infected Worker Honey Bees12. *Journal of Economic Entomology*, *63*(5), 1539–1541. https://doi.org/10.1093/jee/63.5.1539

Wang, H., Wang, L., Zhang, H., Deng, P., Chen, J., Zhou, B., … Zhao, Y.-L. (2013). ^{1}H NMR-based metabolic profiling of human rectal cancer tissue. *Molecular Cancer*, *12*(1), 121. https://doi.org/10.1186/1476-4598-12-121

Wang, K., Li, J., Zhao, L., Mu, X., Wang, C., Wang, M., … Wu, L. (2021). Gut microbiota protects honey bees (Apis mellifera L.) against polystyrene microplastics exposure risks. *Journal of Hazardous Materials*, *402*, 123828. https://doi.org/https://doi.org/10.1016/j.jhazmat.2020.123828

Williams, G. R., Sampson, M. A., Shutler, D., & Rogers, R. E. L. (2008). Does fumagillin control the recently detected invasive parasite Nosema ceranae in western honey bees (Apis mellifera)? *Journal of Invertebrate Pathology,* *99*(3), 342–344. https://doi.org/https://doi.org/10.1016/j.jip.2008.04.005

Winston, M. (1987). The biology of the honey bee. Harvard University Press.

Wu, Y., Zheng, Y., Chen, Y., Wang, S., Chen, Y., Hu, F., & Zheng, H. (2020). Honey bee (Apis mellifera) gut microbiota promotes host endogenous detoxification capability via regulation of P450 gene expression in the digestive tract. *Microbial Biotechnology*, *13*(4), 1201–1212. https://doi.org/10.1111/1751-7915.13579

Xu, H., Liu, M., Cao, J., Li, X., Fan, D., Xia, Y., … Zhao, H. (2019). The Dynamic Interplay between the Gut Microbiota and Autoimmune Diseases. *Journal of Immunology Research*, *2019*, 7546047. https://doi.org/10.1155/2019/7546047

Yang, X., & Cox-Foster, D. (2007). Effects of parasitisation by Varroa destructor on survivorship and physiological traits of Apis mellifera in correlation with viral incidence and microbial challenge. *Parasitology*, 134(3), 405-412. doi:10.1017/S0031182006000710

Yi, H.-Y., Chowdhury, M., Huang, Y.-D., & Yu, X.-Q. (2014). Insect antimicrobial peptides and their applications. *Applied Microbiology and Biotechnology*, *98*(13), 5807–5822. https://doi.org/10.1007/s00253-014-5792-6

Yong, C.-H., Muhammad, S. A., Aziz, F. A., Nasir, F. I., Mustafa, M. Z., Ibrahim, B., … Seow, E.-K. (2022). Detecting adulteration of stingless bee honey using untargeted 1H NMR metabolomics with chemometrics. *Food Chemistry*, *368*, 130808. https://doi.org/https://doi.org/10.1016/j.foodchem.2021.130808

Yousaf, M.S., Goodarzi Boroojeni, F., Vahjen, W., Männer, K., Hafeez, A., Ur-Rehman, H., Keller, S., Peris, S., & Zentek, J. (2017) Encapsulated benzoic acid supplementation in broiler diets influences gut bacterial composition and activity, British Poultry Science, 58:2, 122-131, DOI: 10.1080/00071668.2016.1262000

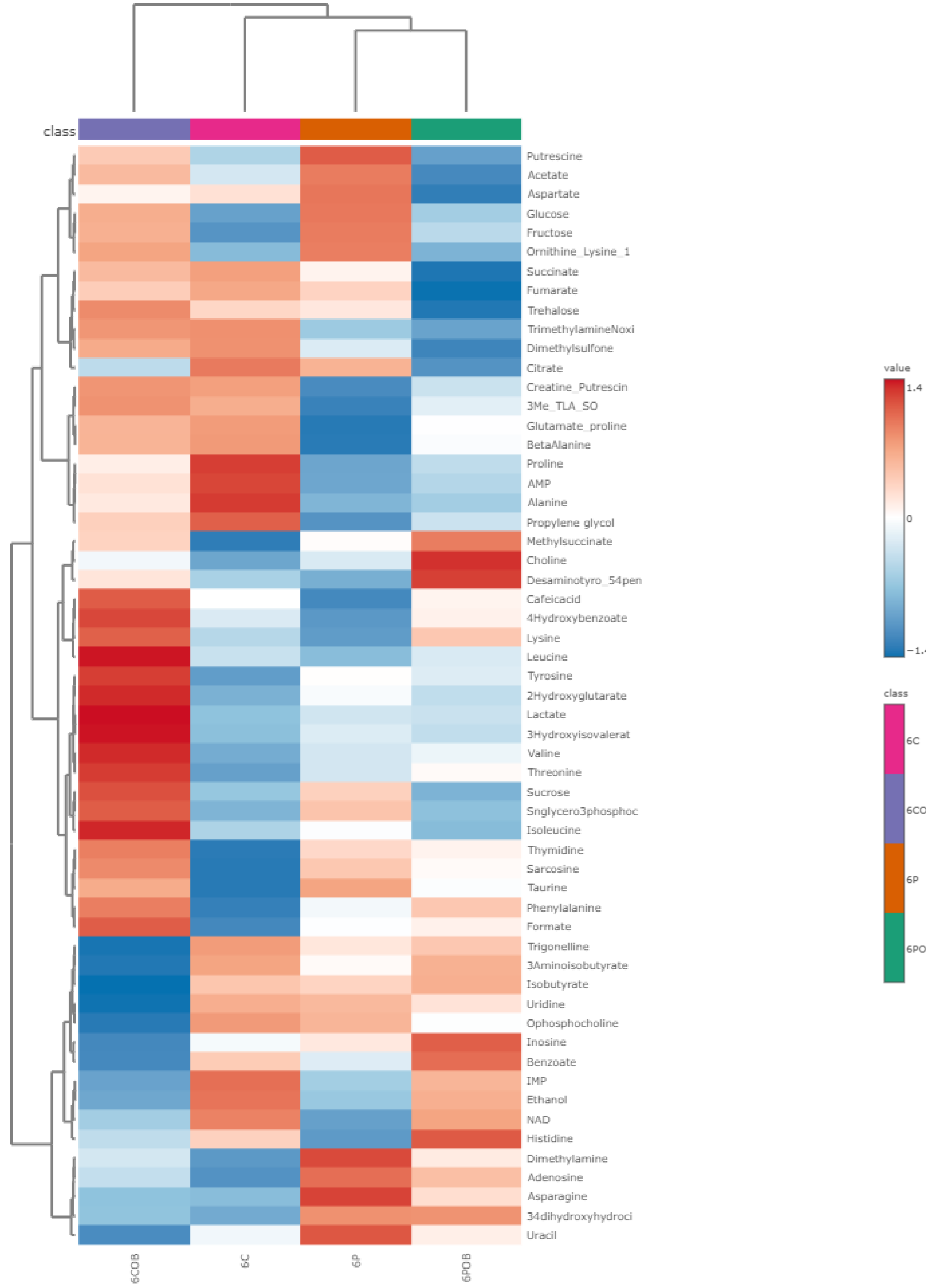
Zhang, Y.-H., Xu, D., Liu, J.-Q., & Zhao, X.-H. (2014). Enhanced degradation of five organophosphorus pesticides in skimmed milk by lactic acid bacteria and its potential relationship with phosphatase production. *Food Chemistry*, *164*, 173–178. https://doi.org/https://doi.org/10.1016/j.foodchem.2014.05.059

Zhang, Z., Mu, X., Cao, Q., Shi, Y., Hu, X., & Zheng, H. (2022). Honeybee gut Lactobacillus modulates host learning and memory behaviors via regulating tryptophan metabolism. *Nature Communications*, *13*(1), 2037. https://doi.org/10.1038/s41467-022-29760-0

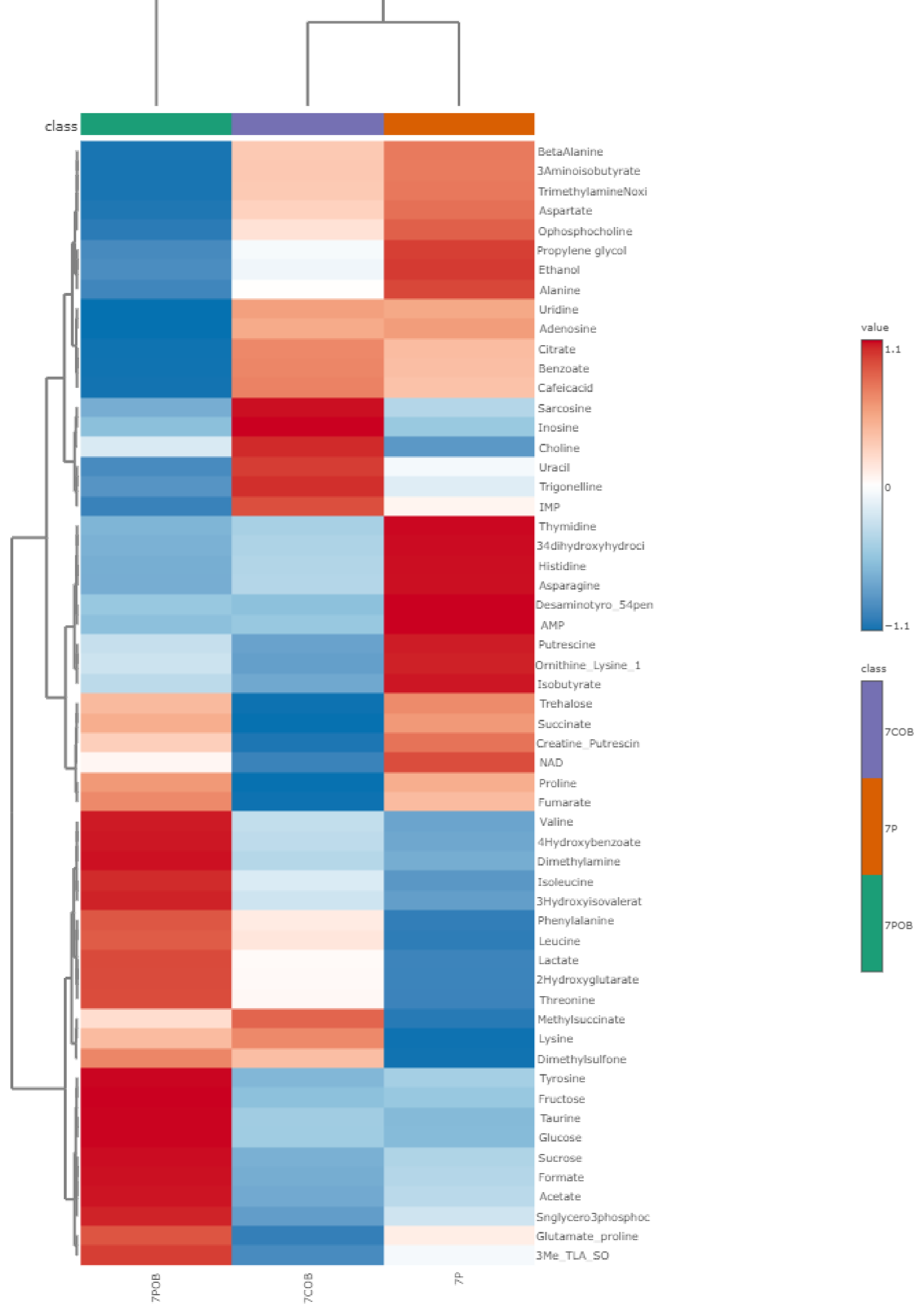
Zheng, H., Perreau, J., Powell, J. E., Han, B., Zhang, Z., Kwong, W. K., … Moran, N. A. (2019). Division of labor in honey bee gut microbiota for plant polysaccharide digestion. *Proceedings of the National Academy of Sciences*, *116*(51), 25909–25916. https://doi.org/10.1073/pnas.1916224116

9 Appendices

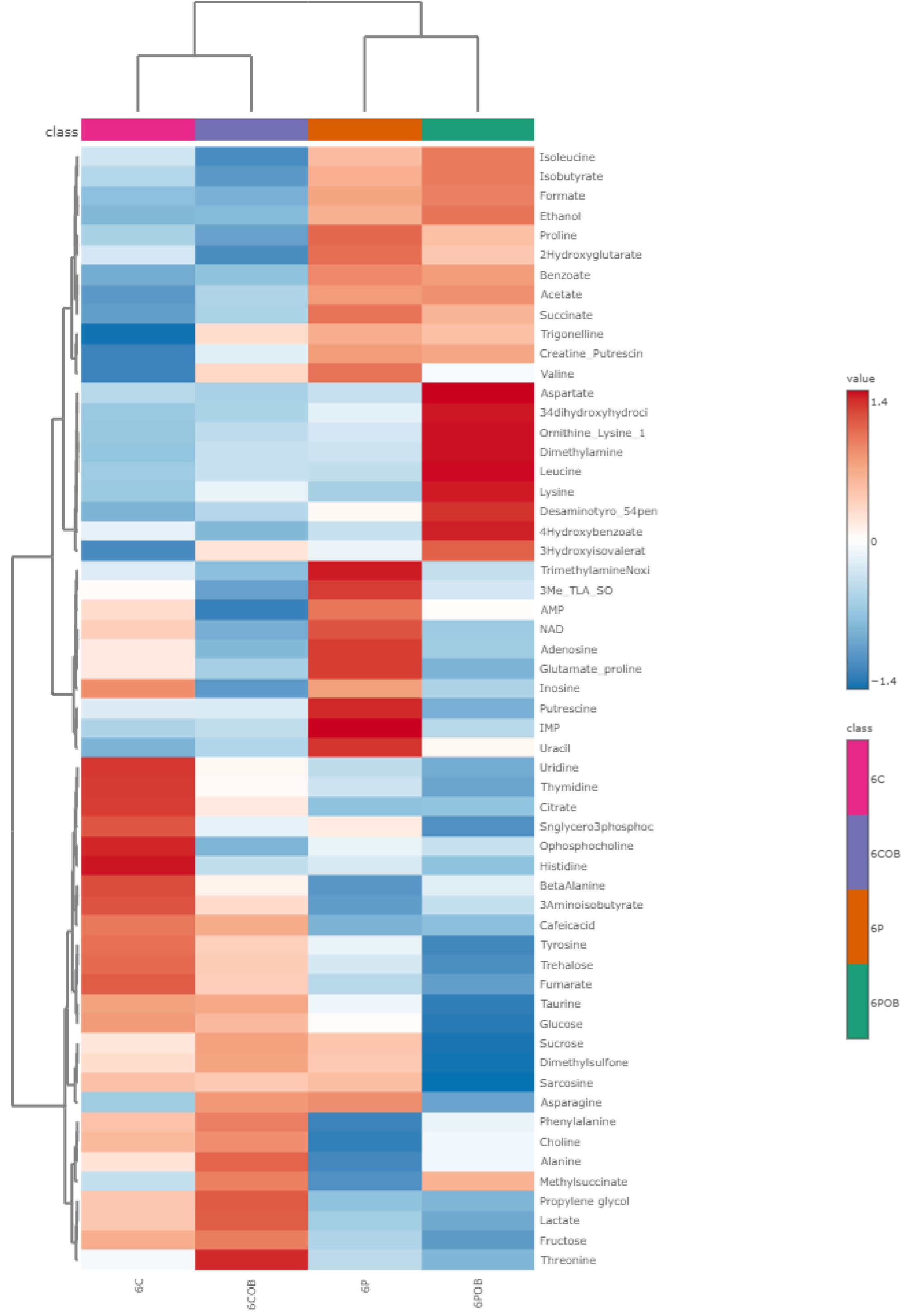
9.1 Heatmap of body metabolites in hive 6



9.2 Heatmap of body metabolites in hive 7



9.3 Heatmap of gut metabolites in hive 6



9.4 Heatmap of gut metabolites in hive 7