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Effects of gut microbiota composition on vitellogenin expression in honeybees

Elisa Cervetti
Ecology

Abstract

The relationship between animals and their gut microbiota is one of the most important symbiotic relationships in nature. The western honeybee, *Apis mellifera*, has received particular attention in microbiota research because of both its utility as a model organism and its economic importance. Honeybees have a specific gut microbiota which consists of few core members and contributes to the normal metabolism, immunity, behavior and endocrine signaling of the host. Among other genes, the honeybee microbiota affects the expression of vitellogenin, a multifunctional lipid-carrier protein that affects aging, immunity and behavior. The link between vitellogenin and the microbiota and the overlap between their functions suggest that vitellogenin could be the mediator of the microbiota effects on bee physiology.

In this thesis, we explored how microbiota composition affects vitellogenin expression in honeybees, both locally and systemically. To do so, we used quantitative real-time PCR to study vitellogenin expression in the gut and fat body of honeybees experimentally inoculated with different members of the normal gut microbiota.

Our results support the hypothesis that the various members of the microbiota contribute differently to the regulation of vitellogenin expression. We found that the gram-negative members of the honeybee microbiota, *Snodograssella alvi* and *Gilliamella apicola,* might be responsible for a systemic suppression of vitellogenin expression. We also found the gram-positive members of the microbiota to stimulate vitellogenin expression in the gut tissue, but we believe this might be an artifact of the experimental design. Nonetheless, the low efficiency of vitellogenin amplification and our inability to verify the presence and composition of the experimental microbiota in our bees reduce the quality of our data and prevent us from drawing any definitive conclusions.

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Abbreviations

Act	Actin
bp	Base pair
cDNA	Complementary DeoxyriboNucleic Acid
Ct	Threshold cycle
DNA	DeoxyriboNucleic Acid
F	Treatment with lab-cultured *F. perrara*
G+	Treatment with lab-cultured *B. asteroides*, *L. mellis* and *L. kullabergensis*
G-	Treatment with lab-cultured *G. apicola* and *S. alvi*
mRNA	Messenger RiboNucleic Acid
MRS	De Man, Rogosa and Sharpe agar
NG	Treatment with homogenized gut of hive bees
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
qPCR	Quantitative real-time Polymerase Chain Reaction
RNA	RiboNucleic Acid
SC	Treatment with no bacteria source
SG	Treatment with homogenized gut of sterile bees
T_a	Annealing temperature
TOT	Treatment with all the lab-cultured bacteria strains
TSA	Tryptic soy agar
Vg	Vitellogenin

1 Introduction

The relationship between animals and the complex community of microorganisms living in their digestive tract, i.e. their gut microbiota, is arguably amongst the most important symbiotic relationships in nature. Bacterial gut symbionts have been observed in many phylogenetically distant animals including nematodes (Clark & Hodgkin, 2016; Dirksen et al., 2016; Jiang & Wang, 2018), mollusks (Dudek et al., 2014), echinoderms (Hakim et al., 2019; Pagan-Jimenez et al., 2019), annelids (Murakami et al., 2015; Siddall et al., 2011), crustaceans (Li et al., 2018; Nougue et al., 2015), insects (Engel & Moran, 2013), tunicates (Dishaw et al., 2014), and several vertebrate groups, including fish (Clements et al., 2014; Givens et al., 2015), birds (Waite & Taylor, 2015) and mammals, most notably humans (Ley et al., 2008). While interaction stability and effects on the host vary greatly in different species and contexts (Douglas, 2014; Hammer et al., 2019), the host and its microbiota often form a mutualistic relationship. The animal's gut provides a stable, nutrient-rich, and relatively competition-free ecological niche for the bacteria, which in return benefit the animal. Bacteria can do this by performing novel functions, such as aiding metabolism or defense against pathogens, or, when the interaction is stable enough to act as an evolutionary pressure, by modulating host's traits (Douglas, 2014). Some of these interactions can be so intimate as to become obligated when the bacteria are unable to survive outside of the host's gut and the host depends on the microbiota to develop and maintain the normal phenotype and health. Because of their ubiquity and great impact on both human and animal health, over the past few decades, an increasing effort has been devoted to studying host-microbiota interactions in various species. One species that has received particular attention in this field, because of both its utility as a model organism and its inherent importance, is the western honeybee (*Apis mellifera*).

Honeybees possess several characteristics that make them suitable as a model organism for gut microbiota research. Firstly, it is both easy and inexpensive to obtain a great number of individuals for research. Beehives last for years, are mostly self-sustaining once established, and contain 30000-80000 adult workers each, with the queen laying 1000-2000 eggs per day under favorable conditions (Bodenheimer, 1937). Secondly, the anatomy and biology of honeybees are both well understood. In fact, honeybees have long been used in research, particularly as model organisms to study behavior, developmental plasticity, and aging (Zheng et al., 2018). This is also due to research on invertebrates being subject to fewer ethical concerns and more relaxed regulations compared to other animals. Lastly, the honeybee gut microbiota,

which will be described in detail later, is well characterized and presents several parallels with the microbiota of mammals, particularly humans, while being simpler in composition and easier to manipulate for research. For example, in both humans and bees, the microbiota is acquired through social interactions, the bacteria occupy similar positions and ecological niches within the gut (Kwong & Moran, 2016), and disturbances of the microbiota have been shown to contribute to similar health problems (see Wang et al. (2018) for examples). For these reasons, research on the honeybee gut microbiota is feasible and can lead to useful insights that could be applied to other organisms.

Nonetheless, the factors influencing honeybee health are worth studying even when they do not apply to other organisms. Firstly, honeybees are among the insects most important to humankind. Since the Neolithic, humans have used bees to produce honey and beeswax (Roffet-Salque et al., 2015). To this day, honey arguably remains the best-known use of this insect, with a growing global market estimated to be worth over 8 billion dollars in 2018 (Grand View Research, 2019). Nonetheless, the most important human use of honeybees is in agriculture as a provider of pollination services. Depending on the region, bee pollination is estimated to be worth from 30 to 100 times the value of honey and wax production (Bradbear, 2009). In North America alone, honeybees pollinate over 100 commercially grown crops and the contribution of this service to the economy is estimated to be as high as $19 billion annually (National Research Council, 2007). Secondly, honeybee populations have been declining over the past few decades. This has been in line with the decline experienced by almost half of the insect species worldwide (Sanchez-Bayo & Wyckhuys, 2019), but concerns for honeybees specifically have increased after the rise in 2006 of colony collapse disorder, a syndrome characterized by the sudden loss of worker bees from the hives and whose causes remain largely unclear (VanEngelsdorp et al., 2009). The vulnerability of this species and its economic importance offer compelling reasons to explore the drivers of honeybee health.

1.1 The honeybee gut microbiota

The gut microbiota changes dramatically through the development of honeybees. When the egg hatches, the larval gut is mostly devoid of bacteria (Martinson et al., 2012). The little bacteria that has been observed at this stage, appears to be transient rather than a stable community (Kwong & Moran, 2016; Zheng et al., 2018). At the end of the larval stage, nurse bees seal the cell containing the larva with a wax cap so that pupation can begin. At the beginning and end of the metamorphosis, the exoskeleton is shed, in a process called ecdysis. This process also removes the gut lining, effectively eliminating any bacteria that might have been present at the

larval stage (Zheng et al., 2018). If pupae are removed from their cells and raised under sterile conditions, they will emerge as microbiota-free adults and never acquire the characteristic gut biota. Under normal conditions, once the metamorphosis is completed, the adult bee emerges from its cell by chewing through the wax cap. While some bacteria may be acquired during the process (Schwarz et al., 2016), the newly emerged worker remains largely bacteria-free (Martinson et al., 2012). In the following days, as the bee interacts with other individuals and with the hive surfaces, the number of bacteria in the gut increases. Around 5 days after emergence, before it ever leaves the hive, the young bee has acquired all the characteristic members of the adult microbiota, which it will maintain over the rest of its life, and the number of bacteria in the gut stabilizes at 10^8-10^9 bacterial cells (Powell et al., 2014).

The gut microbiota of adult worker honeybees is remarkably consistent and made up of few bacterial taxa, all of which can be cultured in the lab and used to inoculate microbiota-free bees (Kwong & Moran, 2016). Each taxon corresponds to a species or a cluster of closely related species and, similarly to the members of the human microbiota (Schloissnig et al., 2013), often include genetically diverse strains even within individual bees (Engel et al., 2012; Moran et al., 2012). The most abundant taxa, which are present in virtually all honeybees, are two clusters of gram-positive species, *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5, and the two gram-negative bacteria *Snodgrassella alvi* and *Gilliamella apicola*. The gram-positive *Bifidobacterium asteroides* and other bacteria of the same genus are also ubiquitous, although less abundant. In addition, *Frischella perrara*, *Bartonella apis*, *Parasaccharibacter apium,* and a cluster referred to as Alpha2.1 are also commonly present in lower numbers. The relative abundance of each taxon can vary through a worker's life and between workers, even between individuals of similar age from the same hive (Moran et al., 2012). Nonetheless, these nine taxa account for over 95% of the gut microbiota of individual honeybees worldwide (Martinson et al., 2012)(figure 1a).

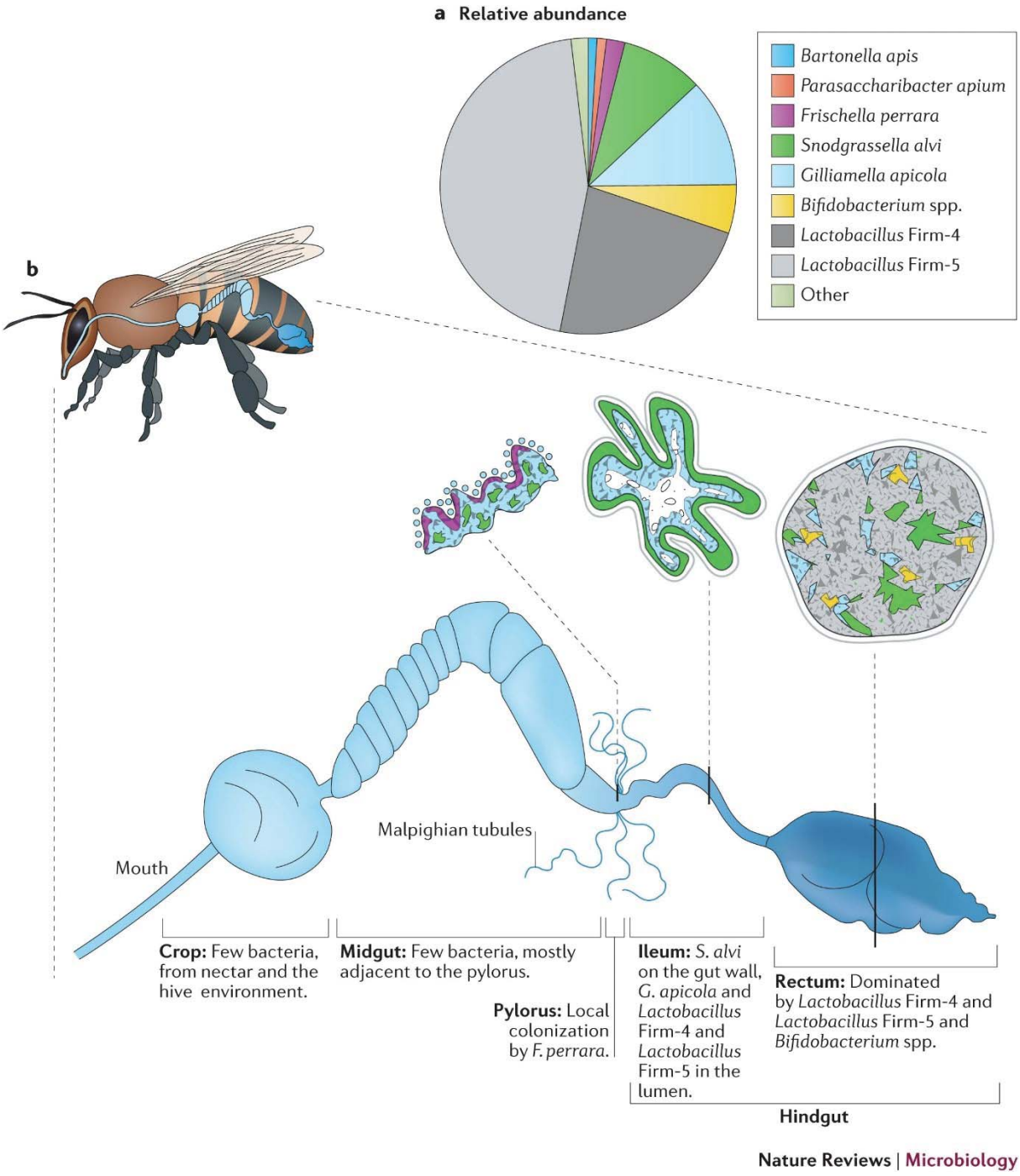

Figure 1 – Typical composition and spatial distribution of the bacterial gut microbiota of honeybees

a – Average composition of the gut microbiota of and adult worker honeybee. **b –** Spatial distribution of bacteria in the compartments of the honeybee gut. Figure from Kwong and Moran (2016)

This bacterial community has a characteristic, unequal distribution in the honeybee gut (figure 1b). The most anterior part of the gut is the crop, an organ used to store nectar, which contains very little bacteria despite the availability of nutrients (Martinson et al., 2012) and mostly species that inhabit nectar and hive materials (Kwong & Moran, 2016). The crop is followed by the midgut, the main site of digestion and absorption. The constant shedding of the midgut lining makes this compartment unsuitable for bacteria, which are present in very low numbers

(Martinson et al., 2012). Almost all the microbiota resides in the most posterior part of the gut, the hindgut. The ileum, the first section of the hindgut, is a small, tubular organ with deep longitudinal folds that absorbs nutrient not assimilated in the midgut. The bacterial community in the ileum is dominated by the gram-negative *S. alvi* and *G. apicola*, which form a stratified biofilm on the gut wall, while *Lactobacillus* species are less abundant and can be found in the lumen (Martinson et al., 2012). The small region connecting the ileum to the midgut, the pylorus, presents the same bacterial community, but its epithelium is also specifically colonized by *F. perrara*, which is most abundant here (Engel et al., 2015). The most posterior part of the hindgut, where waste is retained until defecation, is the rectum. The bacterial community hosted in the lumen of this region accounts for most of the microbiota and is dominated by the gram-positive *Lactobacillus* and *Bifidobacterium* species (Kwong & Moran, 2016; Martinson et al., 2012).The bacterial microbiota is well adapted to thrive in the bee hindgut, as suggested by its genetic and metabolic characteristics. Although it lacks certain core metabolic functions (Bonilla-Rosso & Engel, 2018), the microbiota possesses an unusually high number of genes for the metabolism of carbohydrates, an adaptation to the carbohydrate-rich diet of bees, mostly consisting of pollen and nectar (Engel et al., 2012). Furthermore, since most of the readily available nutrients are absorbed in the midgut, the bacteria must rely on substrates that the host is not able to utilize. In fact, different members of the microbiota can metabolize a great variety of such substrates, especially recalcitrant pollen-derived compounds, as shown by both metagenomic and metabolomics studies (Kesnerova et al., 2017; Zheng et al., 2016). The host potentially benefits from this microbial activity because the breakdown products are more readily available as nutrients and have antimicrobial properties (Kesnerova et al., 2017). Evidence also shows that different members of the microbiota engage in cross-feeding interactions through complementary metabolic pathways (Kesnerova et al., 2017), suggesting that the different bacterial species have coevolved to occupy specialized niches within the gut community. Coevolution between honeybees and their microbiota is also suggested by the fact that native bacteria strains colonize the bee gut more effectively than non-native strains and supported by the comparison of host and microbe phylogenies (Zheng et al., 2018). In addition to potentially aiding host metabolism, evidence suggests that the microbiota also affects honeybee immunity. Bees whose microbiota is altered display increased mortality associated with higher susceptibility to several parasites and pathogens (Maes et al., 2016; Raymann et al., 2017; Schwarz et al., 2016). The microbiota could strengthen the host's immunity by producing antimicrobial substances. This is supported by the fact that some members of the microbiota can inhibit the growth of pathogenic microorganisms *in vitro* (Forsgren et al., 2010; Killer et

al., 2014). But the microbiota also plays a role in regulating the host's own immune response. When compared to bees with the normal microbiota, bees lacking gut microbes or treated with antibiotics show significant downregulation of genes coding for crucial components of the honeybee innate immune response normally released upon infection (Kwong et al., 2017; Li et al., 2017). The fact that Kwong et al. (2017) observed this regulation effect in the hemolymph indicates that the microbiota stimulates the honeybee immune response at a systemic level, rather than locally in the gut. They also observed that inoculation with *S. alvi* alone induces the normal upregulation in the expression of one but interestingly not all families of antimicrobial peptides, suggesting that different members of the microbiota contribute differently to the regulation of the host's immunity.

The idea that different bacteria affect host immunity differently is also supported by the unique response elicited by *F. perrara*. This bacterium causes a localized activation of several parts of the host immune system and is the only member of the microbiota to induce melanization of the area of the gut epithelium it colonizes (Emery et al., 2017). Melanization is a part of the bee innate immune response typically observed upon pathogen infection and tissue damage. It remains unclear whether this strong immune reaction indicates a pathogenic effect of *F. perrara* or whether the host befits from the immune activation, for example through enhanced resistance to subsequent encounters with pathogens (Emery et al., 2017).

The microbiota also has a broader effect on honeybee gene expression and endocrine signaling, which are connected to behavior. Kesnerova et al. (2017) found that *B. asteroides* stimulates the production of host-derived prostaglandins and juvenile hormone derivatives involved in bee development, including the shift from nurse bee to forager bee. Another study by Zheng et al. (2017) found that the bees lacking the normal gut microbiota had reduced expression of several genes involved in the insulin/insulin-like signaling pathway. This shift inhibited the response to sugar in microbiota-free bees and consequently caused them to have lower body and gut weights compared to bees with the normal gut bacteria (Zheng et al., 2017). One gene along the insulin/insulin-like signaling pathway that contributed to the effects found by the authors codes for a protein called vitellogenin, which was found to be downregulated in the abdomen of bees without a microbiota. Intriguingly, vitellogenin has the potential to be involved in several other changes induced by the microbiota.

1.2 Vitellogenin

Vitellogenin is an ancient lipid-carrier protein. It is present in most egg-laying animals, where it provides lipids necessary for egg-yolk formation (Havukainen et al., 2013). In insects it is mainly produced in the fat body, an organ lining the abdomen wall and functionally homologous to the vertebrate liver and adipose tissue, and then secreted into the hemolymph (Amdam et al., 2012; Tufail & Takeda, 2008). In social insects, including honeybees, vitellogenin also serves several functions other than yolk formation.

Firstly, in honeybees vitellogenin regulates aging and inflammation. Bee castes characterized by a longer lifespan also have higher levels of vitellogenin in their hemolymph (Salmela & Sundström, 2017). This is true both in queens, which typically live 1-3 years, and in winter workers, which have a lifespan of 6-8 months, in contrast to summer workers, whose lifespan is usually 3-8 weeks (Corona et al., 2007; Fluri et al., 1977). The anti-aging effect of vitellogenin is probably related to its anti-inflammatory properties. One way in which this protein reduces inflammation is by acting as an antioxidant, as vitellogenin has been shown to neutralize free radicals both *in vivo* and in cell culture (Salmela & Sundström, 2017). Furthermore, vitellogenin has been shown to identify and bind to dead and damaged cells, a behavior typical of anti-inflammatory blood proteins in mammals and that could reduce inflammation by promoting cell clearance (Havukainen et al., 2013). These antioxidant and anti-inflammatory actions are believed to be the basic mechanism through which vitellogenin delays senescence and extends longevity.

Secondly, vitellogenin has a role in immunity. Immunological functions of vitellogenin are possibly as ancient as its role in egg formation and are observed in many distant animal groups (Du et al., 2017). Fish vitellogenin has many antibacterial, antiviral, and antifungal effects, most of which are probably shared by insect vitellogenin (Salmela & Sundström, 2017). A study by Salmela et al. (2015) showed that honeybee vitellogenin recognizes and binds to pathogen-associated molecular patterns on the bacterial surface, which probably shields against infection by facilitating the phagocytosis of the pathogen. Thanks to its role in egg yolk formation, vitellogenin is then able to carry these pathogenic pattern molecules to the eggs in the queen ovaries, allowing the transfer of immunological information to the offspring even without antibodies, which insects lack (Salmela et al., 2015). Vitellogenin also contributes to immunity by interacting directly with the immune cells. In particular, in honeybees vitellogenin is the main carrier of zinc, which is necessary for immune cells to maintain their function (Amdam et al., 2004b).

Finally, vitellogenin is involved in regulating behavior, in particular food-related worker behavior. As previously mentioned, vitellogenin interacts with the insulin/insulin-like signaling pathway to regulate nutritional status and determine the normal sugar response (Zheng et al., 2017). But vitellogenin also determines the shift from nursing to foraging behavior in workers. Young nurse bees have high levels of vitellogenin which suddenly drop immediately preceding the onset of foraging (Salmela & Sundström, 2017). Experimentally silencing the vitellogenin gene anticipates foraging onset, demonstrating that vitellogenin level determines the timing of the behavioral shift (Nelson et al., 2007). Interestingly, the change in worker task is also determined by a rise in juvenile hormone which inhibits vitellogenin expression (Amdam et al., 2012), and accompanied by a rearrangement of the gut microbial community (Miller et al., 2019).

There is a significant overlap between the functions of vitellogenin and the effect of the gut microbiota in honeybees. As mentioned, both vitellogenin and the microbiota affect food-related behavior, such as sugar response and foraging, immunity, and potentially caste-related behavior, although the role of the microbiota in this last area remains under-explored. Furthermore, the microbiota has been shown to cause changes in vitellogenin expression (Zheng et al., 2017). These facts suggest that the effects of the microbiota on bee physiology could be mediated by an effect on vitellogenin expression.

1.3 qPCR

Real-time quantitative polymerase chain reaction (qPCR) is a powerful technique for the quantification of nucleic acids. To do so, it combines the principle of qualitative polymerase chain reaction (PCR) and fluorometry.

PCR is a technique for the amplification of a specific DNA sequence, first proposed in 1985 (Saiki et al., 1985). It uses a cyclical reaction in the presence of the template DNA sequence, a heat-resistant DNA-polymerase, DNA primers specific to the sequence of interest, and nucleotide triphosphates. A PCR cycle consists of three temperature steps: first, the temperature is raised to denature the DNA double-helix, separating the two DNA strands; then, the temperature is lowered to allow the primers to bind to the template DNA; finally, the temperature is raised to the optimal temperature for the polymerase to copy the template by adding new nucleotides to the primers. This cycle is repeated several times, with the number of copies of the sequence of interest increasing exponentially with each cycle. This method allows to detect and study even small amounts of DNA and has found several applications in research,

medical sciences, and forensics. Furthermore, the technique can easily be extended to study RNA by adding a preliminary step, during which the sample of interest is reverse-transcribed into its complementary DNA sequence (cDNA). Nonetheless, there are limitations to traditional PCR. For instance, the efficiency of the reaction decreases as reagents become scarcer after repeated cycles, meaning that the amount of PCR product is essentially independent of the initial number of template copies and quantification virtually impossible.

This limitation was overcome in 1992 with the development of qPCR (Higuchi et al., 1992). In qPCR the target is amplified and detected at the same time by using a fluorescent detector. The detector can be non-specific fluorescent dyes, activated by binding to any double-stranded DNA, or sequence-specific fluorescent probes, activated by binding specifically to the target DNA sequence (Ståhlberg et al., 2005). The fluorescence increases as the PCR product accumulates and can be measured through a fluorometer in real time. Before the availability of reagents become limiting, the fluorescence is proportional to the initial number of target sequences, the number of PCR cycles, and the efficiency of the amplification reaction. The reaction efficiency varies between 1 when there is no amplification and 2 when every target molecule is successfully copied, and depends exclusively on the reagents and temperature used. Therefore, differences in the initial amount of DNA will be reflected in the number of PCR cycles necessary for fluorescence to reach a threshold level, with higher initial amounts giving lower threshold cycle (Ct) values. Thanks to its accuracy, sensitivity, and reproducibility, this technique is now one of the most common methods for the quantification of nucleic acids.

A field in which qPCR has found one of its widest application is gene expression analysis. In this context, reverse transcription is used in combination with qPCR to quantify the mRNA of the gene of interest present in a sample. Although it is possible to determine the absolute number of copies present at the beginning of the qPCR reaction by relating the Ct value to a standard curve (absolute quantification), it is normally not necessary. Most commonly, the expression level of a gene is determined in comparison with a reference sample (relative quantification), such as an untreated control. Usually, the expression of a second gene expected to have a similar expression in all samples, such as a housekeeping gene, is also measured. The expression of this second gene is then used to normalize the expression of the gene of interest, to account for differences in baseline protein expression and amount of biological material in different samples (Kubista et al., 2006). The two samples can then be compared with statistical methods of different complexity and the results are presented as the change in expression in the sample compared to the control.

1.4 Aim of this thesis

This thesis aimed to explore how gut microbiota composition influences vitellogenin expression in honeybees. To do so, we used qPCR to quantify vitellogenin expression in bees experimentally inoculated with different bacteria. Newly emerged, microbe-free bees were inoculated with either no microbiota, the complete microbiota obtained from hive bees, or different groupings of lab-cultured members of the microbiota. We focused on the core microbiota members, which we grouped based on their spatial and ecological distribution within the gut to reduce the number of experimental groups and increase the statistical power of our experiment. Nonetheless, we examined the role of *F. perrara* in isolation. This was done to avoid the confounding effect of the unique immune response this bacterium elicits in the host, which could be reflected in a peculiar effect on vitellogenin expression. We also included a group of bees inoculated with all the lab-cultured bacteria strains used in this experiment: if our experiment included all relevant bacterial groups this group would show similar vitellogenin expression as bees inoculated with the microbiota obtained from hive bees, supporting the biological relevance of our findings.

We measured vitellogenin expression separately in the bee gut and fat body. This was done because changes in vitellogenin expression in the gut could reflect a local immune response elicited by direct contact with the bacteria. On the other hand, changes in vitellogenin expression in the fat body would reflect a systemic impact of the microbiota on the host endocrine signaling.

2 Materials and methods

2.1 Experimental procedures

2.1.1 Experimental design

The bees involved in this study were collected during August 2019 from colonies kept at NMBU in Ås, Norway. Pupae with lightly pigmented eyes but lacking body pigmentation (Powell et al., 2014) were aseptically removed from capped brood cells, placed in sterile plastic cages equipped with a feeder modified from a plastic syringe (figure 2)(Huang et al., 2014; Williams et al., 2013), and incubated at approx. 35°C. Each cage contained a total of approximately 21 pupae of similar age, which were collected from 3 different hives to ensure genetic diversity. Under these conditions, the pupae emerged as adult bees within 5-8 days after collection.

Figure 2 – Example of cages and feeders used in this experiment

During the first 2 days following the emergence of the first bee in each cage, the newly emerged bees were provided with filter-sterilized 1:1 v:v sugar water (figure 3). From the 3rd day after the emergence of the first bee in the cage, the sugar solution was mixed with an equal amount of a protein source to obtain the base feeding solution. The protein source consisted of the supernatant of a 20% w:v pollen suspension during the first 2 days, and of a 40% w:v royal jelly solution for the following 3 days; to ensure their sterility and to inhibit the antimicrobial properties of royal jelly, the pollen suspension and the royal jelly solution were autoclaved for 15 minutes at 121°C and 115°C respectively.

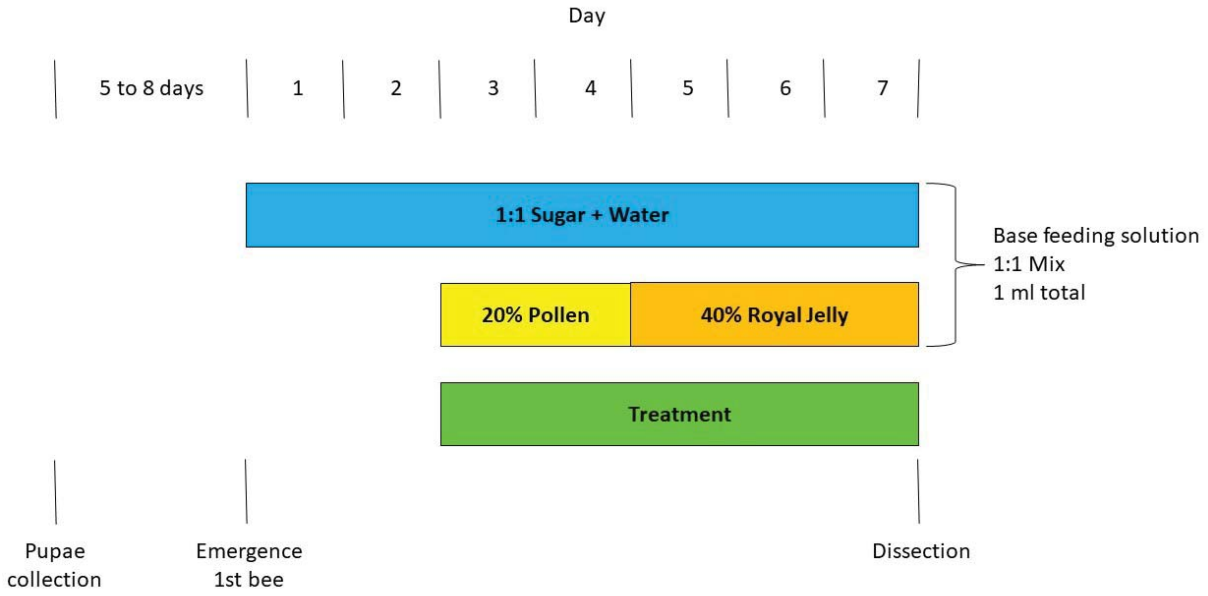

Figure 3 – Overview of the diet administered to honeybees each day of the experiment

On the 3rd day after the emergence of the first bee, the cages were also randomly assigned to one of 7 treatments:

1) Normal gut treatment (NG): meant to represent bees in natural conditions, these bees were fed a complete gut biota obtained by crushing the gut of adult hive bees;
2) Sterile gut treatment (SG): these bees were fed the crushed gut of adult bees lacking the normal gut biota, which allows us to test for effects of the gut and its content;
3) Sugar control treatment (SC): the baseline control group, these bees were only given the feeding solution, without any additional bacteria source;
4) Gram+ treatment (G+): these bees were fed cultured *B. asteroides*, *Lactobacillus mellis* (a Firm-4 representative), and *Lactobacillus kullabergensis* (a Firm-5 representative);
5) Gram- treatment (G-): these bees were fed cultured *G. apicola* and *S. alvi*;
6) Fischella treatment (F): these bees were fed cultured *F. perrara*;

7) Total treatment (TOT): these bees were fed all the six cultured bacteria strains fed to the other groups.

Each treatment was run in parallel on 2 different cages, except for the sterile gut treatment, which was administered to only 1 cage due to the unforeseen die-off of the second cage.

A complete overview of the bacterial strains used in this thesis can be found in table 1. *B. asteroides*, *L. mellis*, and *L. kullabergensis* were cultured on MRS gel supplemented with 2% fructose and 0.1% L-cysteine in anaerobic conditions (*AnaeroGen™ 3.5L*, *Thermo scientific*)(Ellegaard et al., 2019). *G. apicola* and *S. alvi* were cultured on tryptic soy agar supplemented with 5% horse blood in CO_2-enriched atmosphere (*CO_2Gen™ 2.5L*, *Thermo scientific*)(Engel et al., 2015). *F. perrara* was cultured on tryptic soy agar supplemented with 5% horse blood in anaerobic conditions (*AnaeroGen™ 3.5L*, *Thermo scientific*). All bacteria were incubated for 2 days at 36°C before use, and fresh cultures were used every day of treatment.

Table 1 – Details of the bacteria used in this thesis. TSA = tryptic soy agar; MRS = De Man, Rogosa and Sharpe agar.

Bacterial strain	Treatment group	Culturing condition	Reference	Catalogue number
Gilliamella apicola wkB1	G- and TOT	TSA, 36°C, microaerophilic	(Kwong & Moran, 2013)	ATCC BAA-2448
Snodgrassella alvi wkB2	G- and TOT	TSA, 36°C, microaerophilic	(Kwong & Moran, 2013)	NCIMB 14803
Frischella perrara PEB0191	F and TOT	TSA, 36°C, anaerobic	(Engel et al., 2013)	NCIMB 14821
Bifidobacterium asteroides C51	G+ and TOT	MRS, 36°C, anaerobic	(Scardovi & Trovatelli, 1969)	DSM 20089
Lactobacillus mellis Hon2N	G+ and TOT	MRS, 36°C, anaerobic	(Olofsson et al., 2014)	DSM 26255
Lactobacillus kullabergensis Biut2N	G+ and TOT	MRS, 36°C, anaerobic	(Olofsson et al., 2014)	DSM 26262

Every day, the SG group was fed 1 mL of feed containing 1 gut from an uninoculated bee. Uninoculated bees were obtained similarly to experimental bees and fed the base feeding solution containing pollen from the third day after their emergence until their use in the experiment. The gut was put directly into the feed and homogenized for a few seconds with an electric pestle and was assumed to contain approx. 10^5-10^6 bacterial cells (Powell et al., 2014).

For the NG group, the gut of 1 hive-bee was homogenized in PBS and diluted to the same bacterial concentration, assuming it originally contained around 10^9 bacterial cells (Kwong & Moran, 2016; Zheng et al., 2018), before adding it to the feeding solution. To eliminate any effect from the gut content, the hive bees were isolated and fed the base feeding solution containing pollen for at least 3 days before their gut was used in the NG treatment. For the remaining groups, the cultured bacteria were suspended in PBS and quantified by measuring the suspension's optical density at 600nm (*UV-1800* spectrophotometer, *Shimadzu*) before adding them to the feeding solution in a concentration of 10^6 bacterial cells per 1mL. This procedure ensured that all treatment groups received a similar number of bacteria and that each strand was equally represented in treatments involving more than one bacteria group.

2.1.2 Preliminary bacteria survival tests

To ensure treatment continuity, two tests were conducted to confirm that the bacteria administered could survive in the feeding solution. The first test meant to verify that all bacteria strands could survive in the sugar solution. For each of the bacterial strands involved in the experiment, approximately 10^6 bacterial cells were suspended in 1mL of filtered sterilized 1:1 v:v sugar water and incubated at room temperature (ca. 23°C). After 1 and 2 days, 20µL of the solution were cultured as described above. The second test meant to verify that the antibiotic properties of royal jelly (Fratini et al., 2016) were inactivated by autoclaving. *L. kullabergensis* and *G. apicola* were plated on their respective growth mediums. Then, a 40% w:v royal jelly solution which had previously been autoclaved at 115°C for 15 minutes was dripped on different spots of the plate. The plates were then incubated for 2 days as described above.

2.1.3 Gut and fat body collection

After 5 days of treatment, the bees were dissected to collect an intestine sample consisting of midgut and ileum, and the fat body. First, bees were put into a chill coma by cooling them at 4°C (Free & Spencer-Booth, 1960). Then, the gut was extracted by pulling the stinger apart from the body and the rectum was cut off. Finally, the abdomen was pulled apart from the thorax. The intestine sample and the abdomen were then immersed in 300µL and 500µL (respectively) of *RNAlater*™ solution (*Ambion*)(*RNAlater*™ *user manual*, 2014) and stored at -20° C. To avoid RNA degradation, the handling of each sample took no longer than 1 minute, from the initial extraction of the gut to the immersion of the samples in the preservative solution.

2.2 Sample analysis

To measure protein expression in the samples, RNA was isolated. Samples were homogenized and lysed using *TRIzol™ reagent* by *Thermo scientific* (*TRIzol™ User Guide*, 2016) and RNA was isolated using the *RNeasy Mini Kit* in combination with the *RNase-Free DNase Set*, both by *Qiagen* (*DNase Set Product Sheet*, 2018; *RNeasy Mini Handbook*, 2013). The complete protocol used for RNA extraction and isolation can be found in Appendix 1.

The isolated RNA was then reverse-transcribed into DNA. The *FIREScript® RT cDNA synthesis Mix with Oligo (dT) and Random primers* (*Solis Biodyne*) was used. 2µL of RNA were mixed with the kit components following the kit instructions (*FIREScript® data sheet*, 2017), and cDNA synthesis was conducted with an *Applied Biosystems 2720 Thermal Cycler*. The program used for cDNA synthesis consisted of 7 min at 25°C (primer annealing), 20 min at 45°C (reverse transcription) and 5 min at 85°C (enzyme inactivation), before samples were cooled down at 4°C and stored at -20°C.

The cDNA was then quantified using a *Qubit™ dsDNA HS Assay Kit* and a *Qubit™ 2.0 fluorometer* (*Thermo Scientific*) following the manufacturer's recommendations (*Qubit® user manual*, 2015) and diluted with PCR grade water to a concentration of approximately 2 µg/ml before qPCR.

Vitellogenin and actin, the gene chosen for normalization (Lourenço et al., 2008), were then quantified through qPCR. For each gene examined, the qPCR mix consisted of:

- 2 µl or approximately 4 ng of template DNA,
- 4 µl of *5x HOT FIREPol® EvaGreen® qPCR Supermix* (*Solis BioDyne*)(*EvaGreen® data sheet*, 2017),
- 0,4 µl of forward primer (table 2), for a final concentration of 0.2 µM
- 0,4 µl of reverse primer, for a final concentration of 0.2 µM
- 13,2 µl of PCR-grade water.

The qPCR protocol used consisted of an initial activation step at 95°C for 15 minutes followed by 40 amplification cycles (30s at 95°C for denaturation; 30s for annealing at 60°C for actin and at 54°C for vitellogenin, during which fluorescence was measured; 30s at 72°C for elongation). Melting curves for the qPCR products were also recorded, to ensure the specificity of the PCR reaction. The qPCR was performed using a *CFX96 Touch Real-Time PCR Detection System* (*Bio-Rad*).

Table 2 – Details of primers used for qPCR in this experiment. T_a = annealing temperature; bp = base pair

Gene	Primer	Sequence 5'-->3'	T_a (°C)	Reference	Amplicon length (bp)
Actin	Forward	TGCCAACACTGTCCTTTCTG	60	(Lourenço et al., 2008)	155
	Reverse	AGAATTGACCCACCAATCCA	60	(Lourenço et al., 2008)	
Vitellogenin	Forward	GTTGGAGAGCAACATGCAGA	54	(Amdam et al., 2004a)	150
	Reverse	TCGATCCATTCCTTGATGGT	54	(Amdam et al., 2004a)	

2.3 Data analysis

2.3.1 Statistical analysis

The PCR data was processed separately for the guts and the fat bodies. Individual samples were excluded from the analysis if the melting curve suggested that the PCR reaction had not been specific, i.e. if the melting curve showed more than one melting peak or a single melting peak at a temperature different from the one expected for the intended amplicon.

The raw data was compiled into excel and imported into the program *LineRegPCR* (Ruijter et al., 2009) to calculate the PCR efficiency (E) for each amplicon in each sample type. The calculated efficiencies and the Ct values were then imported into R (R Core Team, 2018) and analyzed.

The relationship between the Ct value and the initial number of copies (n) in the qPCR reaction is expressed by the relationship

$$Ct = k \times \log n$$

where k is the slope of a standard curve obtained through a serial dilution of a standard (Kubista et al., 2006). Since said slope is related to the efficiency (E) of the reaction through the equation

$$k = -\frac{1}{\log E}$$

(Kubista et al., 2006; Ståhlberg et al., 2005), the logarithm of the initial amount of DNA in the reaction can be calculated as

$$\log n = -Ct \times \log E.$$

Based on this relationship, a quantification of the expression of vitellogenin (Vg) relative to that of actin (Act) on which to perform the statistical analysis was calculated for each sample as:

$$\log {}^{Vg}/_{Act} = \log Vg - \log Act = (log(E_{Act}) \times Ct_{Act}) - \left(log\left(E_{Vg}\right) \times Ct_{Vg}\right).$$

Within each treatment group, outliers were identified and excluded from further analysis. Samples were considered outliers when their Vg/Act ratio fell more than 1,5 interquartile range below the first quartile or above the third quartile of the distribution for their treatment group.

The Vg/Act ratio values which were not considered outliers were then analyzed to find statistically significant differences among groups. Non-parametric statistical tests were favored, as the data did not always meet the assumption for parametric tests. First, Wilcoxon tests and, when possible, t-tests were performed to verify there were no significant differences between cages assigned to the same treatment. Then, independently of the results of this test, data from the two cages assigned to the same treatment were pooled together and a Kruskal-Wallis test and a posthoc pairwise Mann-Whitney U test with a Bonferroni correction were performed to detect differences between treatments.

2.3.2 Fold change estimate

The average Ct was calculated for both actin and vitellogenin in each experimental group. For all groups, the fold change in vitellogenin expression, relative to the expression in the SC group, was estimated from these averages using two relative quantification methods. In the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) it was assumed that the PCR reaction had perfect efficiency for both the target and calibrator gene, and the change in the expression of vitellogenin was calculated as:

$$Fold\ change = 2^{-\left(\Delta Ct_{sample} - \Delta Ct_{SC}\right)},$$

where

$$\Delta Ct = Ct_{Vg} - Ct_{Act}.$$

In the efficiency correction method (Pfaffl, 2001) differences in the efficiencies of the amplification reactions were instead kept into account, and the fold change was calculated as:

$$Fold\ change = \frac{\left(E_{Vg}\right)^{\Delta Ct_{Vg}(SC-sample)}}{\left(E_{Act}\right)^{\Delta Ct_{Act}(SC-sample)}}.$$

If a significant difference was found between two treatments other than the SC group, the change in Vg expression was also calculated between those two treatments with the same methods, by setting one of the 2 groups as the baseline instead of the SC group.

3 Results

3.1 Preliminary bacteria survival tests

In our first preliminary test, all experimental bacteria were able to grow after having been incubated in the sugar solution for 1 day. After 2 days, all bacteria apart from *F. perrara* were able to grow. These results confirmed that all the strands could survive in the base feeding solution between 24 and 48 hours. In the second test, both *L. kullabergensis* and *G. apicola* were able to grow on the entire plate, including the areas where the royal jelly solution had been dripped (figure 4), suggesting that the royal jelly did not interfere with bacterial survival.

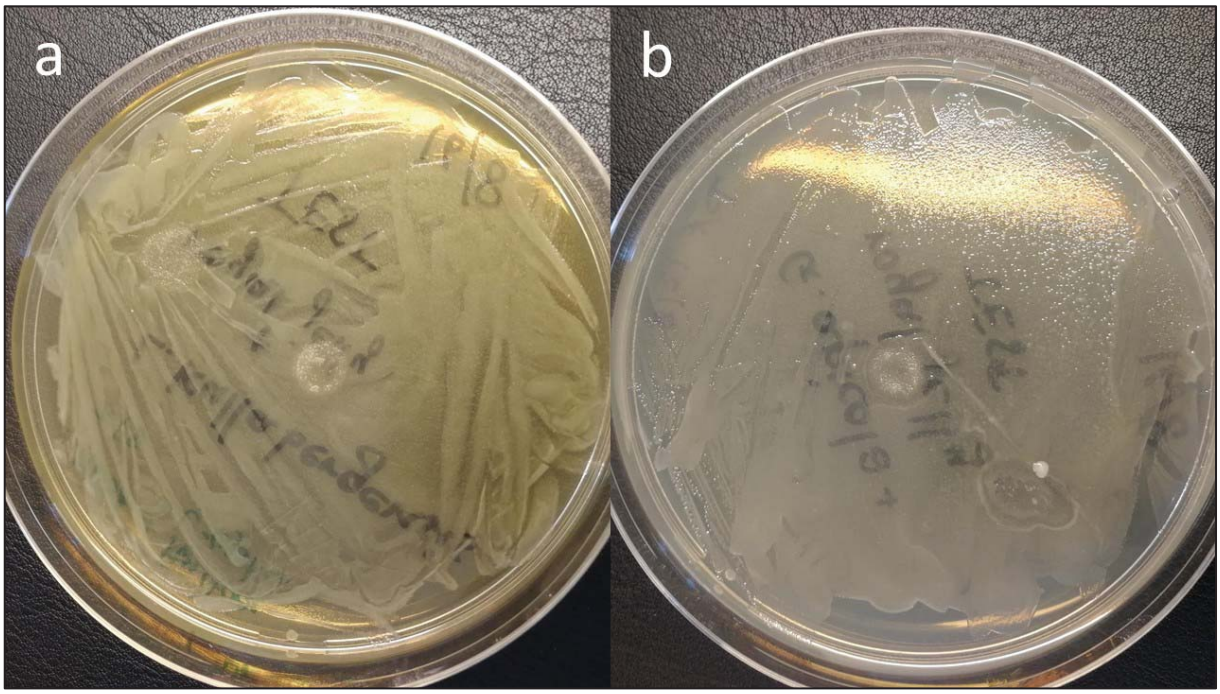

Figure 4 – Bacterial growth in the presence of autoclaved royal jelly

a – *Lactobacillus kullabergensis*. **b** – *Gilliamella apicola*.

3.2 Main results

A total of 180 bees were dissected. mRNA extraction and reverse transcription were successful in all the 360 samples collected, with cDNA yield ranging from 1,5µg/mL to 9,5µg/mL in fat body samples and from 4,1µg/mL to 12,4µg/mL in gut samples. The mean amplification efficiency calculated through *LineRegPCR* was above 2 for actin in both fat body and gut samples (2,049 and 2,078 respectively). The efficiency was lower for vitellogenin, with a mean value of 1,72 in fat bodies and 1,672 in guts. Because of this difference in efficiency, we consider the efficiency correction method the most appropriate to calculate the changes in gene expression, and present the results of the $2^{-\Delta\Delta Ct}$ calculations in Appendix 2. In gut samples,

vitellogenin amplification was also less specific: half of the samples showed a melting peak consistent with the formation of primer-dimers in addition to or instead of the expected peak for vitellogenin and were therefore excluded from the analysis. The number of samples included in the analysis after outlier removal and their repartition between treatments and cages can be found in table 3.

Table 3 – Number of samples (n) from each treatment and cage included in the analysis

	Fat body		Gut	
Treatment	n	n by cage	n	n by cage
F	25	11	8	7
		14		1
G-	22	13	10	5
		9		5
G+	25	14	21	11
		11		10
NG	27	13	15	9
		14		6
SC	26	12	12	7
		14		5
SG	16	16	14	14
TOT	32	16	8	3
		16		5
	173		88	

3.2.1 Fat body

The distribution of the logVg/Act ratio in fat body samples from each cage, after outliers removal, can be seen in figure 5. The average logVg/Act value across treatments was 2,23. Results and exact p-values for all statistical tests conducted can be found in Appendix 2. The Wilcoxon test revealed significant differences between the cages that received the G- treatment and between those that received the TOT treatment (in both cases, $p < 0,001$), while there were no significant differences between the replicates of the other treatments. The t-test had similar results.

The Kruskal-Wallis and posthoc Mann-Whitney U tests showed that Vg expression in the G- and NG treatments was significantly different than in the SC treatment ($p = 0,0049$ and $p = 0,0228$ respectively). In both cases, the expression of vitellogenin decreased in the treated samples compared to the sugar control group. When treatments were compared with the

efficiency correction method, Vg expression decreased by 46% in the NG treatment, and by 60% in the G- treatment (figure 6). The magnitude of the change was similar when calculated with the $2^{-\Delta\Delta Ct}$ method (figure S1a).

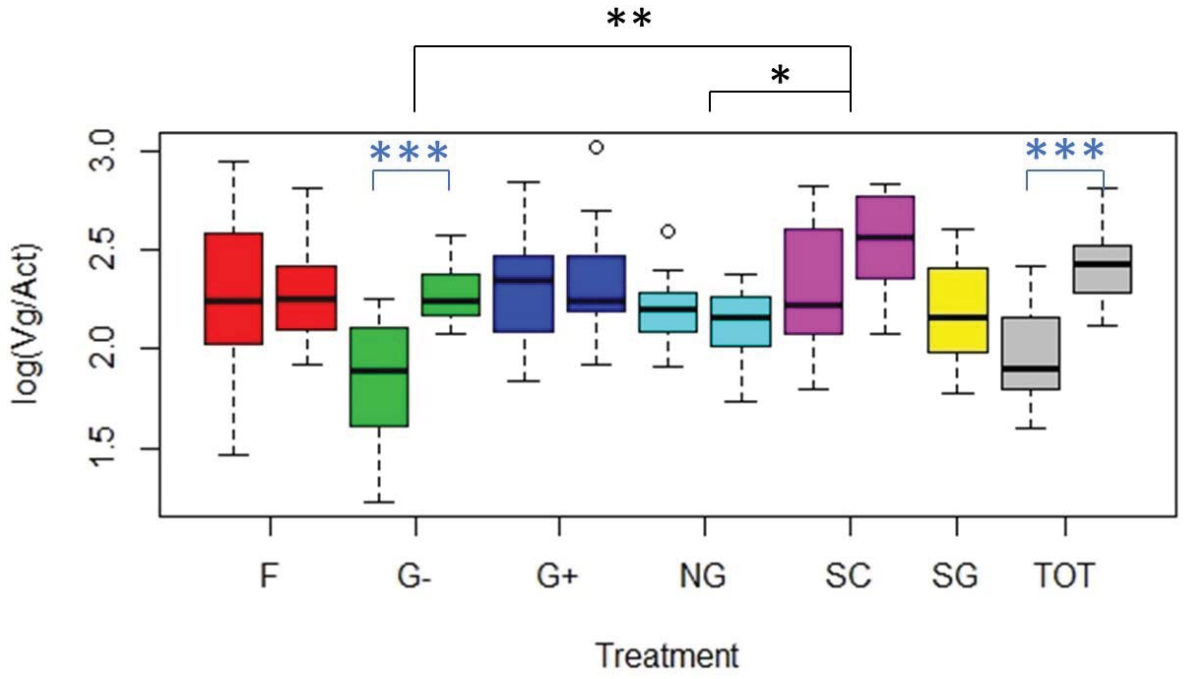

Figure 5 – Vitellogenin expression in the fat body of bees with different gut bacterial communities

Asterisks indicate significant differences between treatments (black) and between replicates of the same treatment (blue). * p < 0,05. ** p < 0,01. *** p < 0,001.

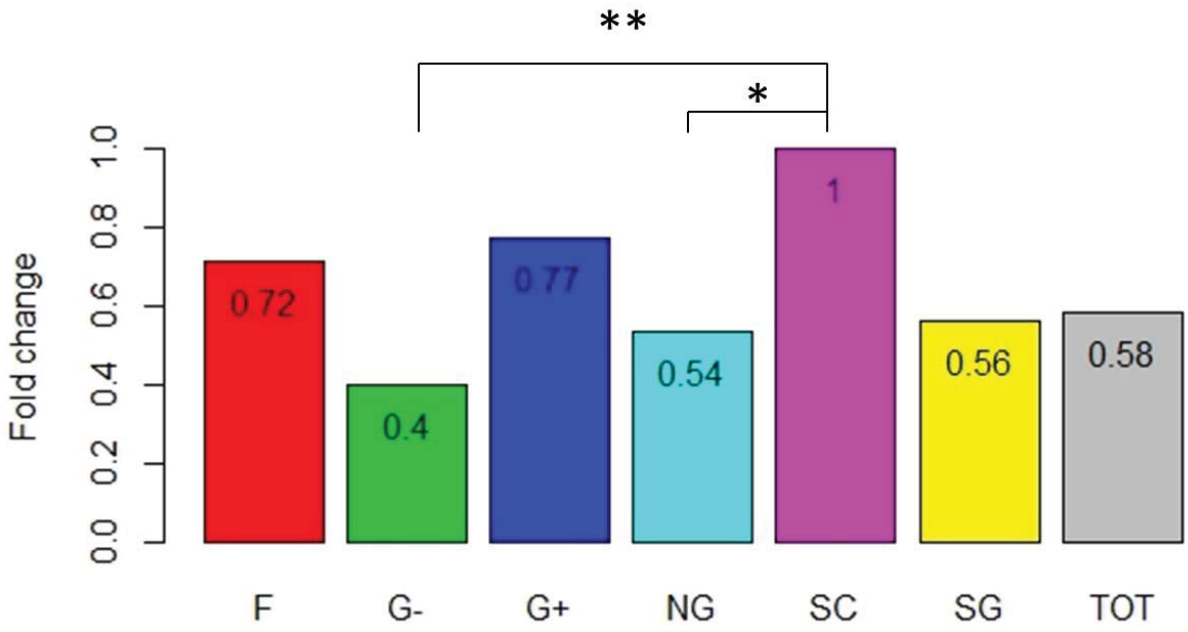

Figure 6 – Change in vitellogenin expression in the fat body of bees with different gut bacterial communities, relative to the SC group

Lines with asterisks above bars indicate statistically significant changes. * p < 0,05. ** p < 0,01. *** p < 0,001.

3.2.2 Gut

The distribution of the log relative Vg/Act ratio in gut samples, after outliers removal, can be seen in figure 7. The average logVg/Act value across treatments was 1,50. The Wilcoxon test revealed significant differences between the cages that received the NG treatment (p = 0.029), while there were no significant differences between the replicates of the other treatments. For those treatments that met the conditions to perform t-tests, the results of the 2 tests were similar.

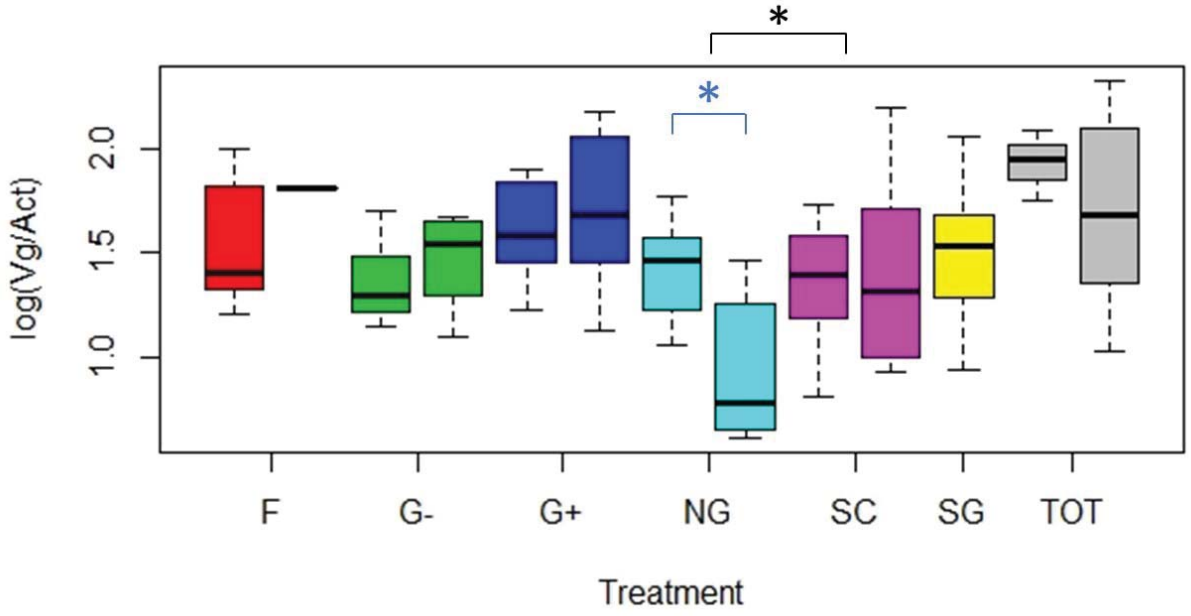

Figure 7 – Vitellogenin expression in the gut of bees with different gut bacterial communities

Asterisks indicate significant differences between treatments (black) and between replicates of the same treatment (blue). * p < 0,05. ** p < 0,01. *** p < 0,001.

The Kruskal-Wallis and posthoc Mann-Whitney U tests showed a significant difference in Vg expression between the G+ and NG treatment (p = 0,034), while all other comparisons were not significant. Vg expression increased almost 3-fold in the G+ treatment compared to the NG treatment, when the treatments were compared with the efficiency correction method (figure 8). The magnitude of the change was even greater when calculated with the $2^{-\Delta\Delta Ct}$ method (figure S2). It is also worth noting that, compared to the SC treatment, Vg expression seemed to increase in the G+ treatment and decrease in the NG treatment, although these differences were not statistically significant (figure 9 and figure S1b in Appendix 2).

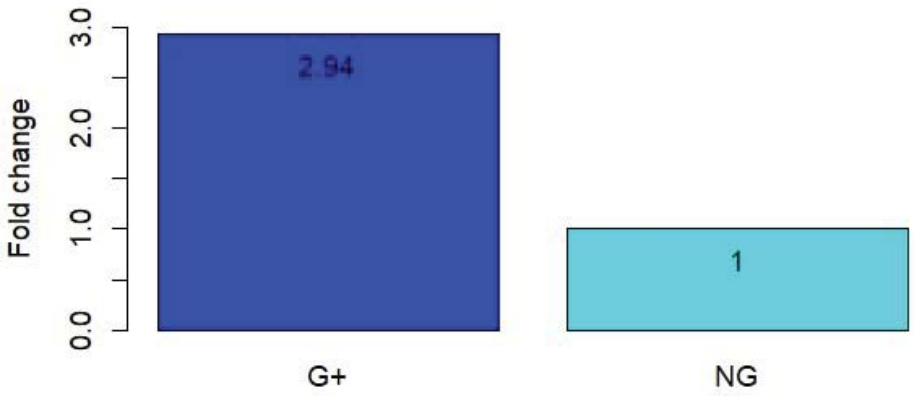

Figure 8 – Change in vitellogenin expression in the gut of bees inoculated with *Lactobacillus mellis*, *Lactobacillus kullabergensis* and *Bifidobacterium asteroides* relative to bees with a normal gut microbiota

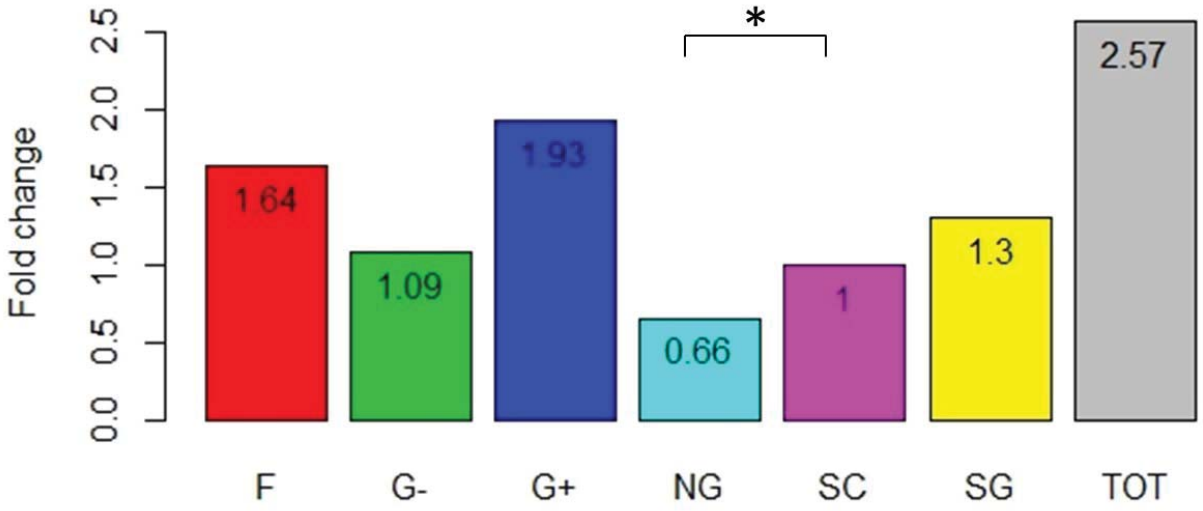

Figure 9 – Change in vitellogenin expression in the gut of bees with different gut bacterial communities, relative to the SC group

Lines with asterisks above bars indicate statistically significant changes. * $p < 0,05$. ** $p < 0,01$. *** $p < 0,001$.

4 Discussion

Overall, we found that vitellogenin expression, as reflected by the log Vg/Act ratio, was higher in the fat body than in the gut. This is consistent with the fat body being the main site of vitellogenin synthesis.

We found that vitellogenin expression in the fat body of bees in the NG group decreased by 46% compared to the SC group. A similar decrease was observed in the gut, although this difference was not statistically significant. These trends seem to suggest that the presence of the normal microbiota suppresses vitellogenin expression both locally and systemically. In stark contrast, Zheng et al. (2017) found gut vitellogenin expression to increase almost 5-fold in bees with the normal microbiota compared to germ-free bees. This discrepancy could be due to differences in methods, such as differences in bee diet or inoculation method, or reflect ecological differences between different honeybee populations, such as different degrees of dependence on the microbiota for endocrine regulation.

Vitellogenin expression in the fat body also decreased significantly in bees in the G- group. This result suggests that the presence of either *S. alvi* or *G. apicola* could be responsible for the systemic-level suppression of vitellogenin also observed in the NG group. Nonetheless, we did not find a significant decrease in the TOT group, which also included these bacteria. This contradicting result is possibly caused by part of the data in the G- and TOT groups being unreliable. In both these groups, the two cages to which the treatment was administered showed significantly different levels of fat body vitellogenin. Differences between cages suggest that one or both cages might not be representative of the treatment because of external effects, and our data does not allow us to identify if and where this is the case. Therefore, since we cannot exclude that part of the data might be driving an artificially significant result (or lack thereof) in these treatments, these results should be interpreted with caution.

In the gut, we found that vitellogenin expression was almost 3 times higher in the G+ group than in the NG group. Although we are not aware of any studies directly linking the gram-positive members of the microbiota and vitellogenin expression, Kesnerova et al. (2017) found that *B. asteroides* stimulates the production of juvenile hormone. Since high levels of juvenile hormone inhibit vitellogenin synthesis (Amdam & Omholt, 2003), our finding that gram-positive bacteria increase vitellogenin expression is particularly surprising. Interestingly, we only found this effect in the gut, and more specifically in the midgut and ileum. Under normal conditions, the midgut contains very little bacteria and the ileum is dominated by the gram-

negative members of the microbiota, while the gram-positive bacteria colonize the rectum. If, in the absence of the gram-negative bacteria, the ileum of bees in the G+ group was colonized by gram-positive bacteria, this abnormal distribution could have elicited a localized immune response and an increase in vitellogenin expression in this area of the gut. This dynamic would also explain why vitellogenin expression would be promoted in bees in the G+ group but not in those in the NG group, which would presumably have a normal distribution of bacteria and no immune response. Unfortunately, we are not able to confirm or deny this hypothesis, as we did not monitor spatial distribution of the bacteria in the different treatments.

We found no effect of *F. perrara* on vitellogenin expression in either the gut or fat body. This suggests that, although this bacterium activates the host's immune system and induces a melanization reaction (Emery et al., 2017), it does not do so by influencing vitellogenin expression. This is in line with the current understanding of the insect melanization response, as the mechanisms that generate it do not involve vitellogenin (Nakhleh et al., 2017).

4.1 Dataset limitations

Some limitations of our datasets emerge from both our results and methodology, and should be considered when drawing conclusions from the data we presented.

Firstly, the low amplification efficiency for vitellogenin suggests that our qPCR protocol was not adequately optimized for this protein. This could result from sub-optimal primer sequences or reaction conditions, i.e. temperature, pH, etc. While the efficiency correction method accounts for sub-optimal amplification when calculating changes in gene expression, the inadequate reaction optimization caused additional problems in gut samples. Here the melting curve of the qPCR products suggested an extensive formation of unintended amplification products, probably mostly consisting of primer dimers. We are not able to establish whether this resulted from particularly low vitellogenin expression in the gut or from the gut content interfering with the reaction. All samples where amplification had not been specific to vitellogenin had to be excluded from our analysis. This halved the sample size of our gut dataset and potentially introduced a systematic bias in our data. Overall, this reduced the statistical power of our experiment and left our analysis of gut samples more susceptible to false negatives and to positive results being driven by potential outliers.

Secondly, we could not verify the presence and composition of the experimental microbiota in our bees. To this end, we originally collected the complete gut of 1 bee from each cage and preserved it in 300µL of DNA preservative solution (*S.T.A.R. buffer*, *Roche*) after removing the

stinger. With the same procedure, we also collected the gut of 5 bees, each from a different cage, immediately before the beginning of treatment to verify that cages had not been contaminated before the experiment. Unfortunately, these samples could not be analyzed due to the unforeseen time constraint imposed by the 2020 COVID-19 pandemic. Without genetic data, we cannot confirm that all the bacteria included in our treatments successfully colonized the bee gut. We also cannot exclude colonization by unintended bacteria species, either from the environment or through cross-contamination of different treatments. Hence, the levels of vitellogenin expression observed in part of our data could result from experimental failure or contamination rather than from treatment. This occurrence is particularly likely in those treatments where replicate cages showed significantly different results.

For these reasons, our results should be interpreted cautiously, especially those regarding vitellogenin expression in the gut tissue.

5 Conclusion

This thesis explored how different members of the honeybee gut microbiota influence the expression of an important multifunctional protein, vitellogenin. Although evidence that the microbiota affects vitellogenin expression exists in the literature (Schwarz et al., 2016; Zheng et al., 2017) this is, to our knowledge, the first attempt to identify which members of the microbiota are responsible for this effect and to differentiate between local and systemic impacts.

We found some evidence to support our hypothesis that the various members of the microbiota contribute differently to the regulation of vitellogenin expression. Our results suggest, in contrast with previous studies, that the *S. alvi* and *G. apicola* might be responsible for a systemic suppression of vitellogenin expression. We also found the gram-positive members of the microbiota to stimulate vitellogenin expression in the gut tissue, but we believe this might be an artifact of the experimental design. We also found no impact of *F. perrara* on vitellogenin expression. Although these results seem promising, the limitations of our data prevent us from drawing any definitive conclusions.

We propose that this experiment should be replicated focusing on overcoming its limitations. Firstly, the qPCR protocol for vitellogenin amplification should be optimized, especially for the analysis of gut samples. Secondly, the presence and composition of the microbiota in each experimental group should be confirmed through genetic analysis. Lastly, the number of replicates in each treatment should be increased to limit the risk of contamination and increase statistical power.

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Appendix 1

RNA purification protocol

The first 7 steps in this protocol are derived from the TRIzol reagent protocol (*TRIzol™ User Guide*, 2016). The remaining steps follow the RNeasy mini handbook (*RNeasy Mini Handbook*, 2013), including steps 11 to 14 referring to the use of the RNase-free DNAse set.

All centrifugation steps were conducted at room temperature unless otherwise specified, using an *Eppendorf Centrifuge 5804 R*. All incubation steps were conducted at room temperature (20–30°C).

1. Thaw tissue, extract from RNA later (be sure to remove any crystals that may have formed), transfer to a new tube and add 3 sterile, RNase-free 3mm ceramic beads.
2. Add 1 mL of TRIzol™ Reagent to each fat body sample and 0,5 mL of TRIzol™ Reagent to each gut sample. Homogenize for 1 minute at 20 oscillations per second using a mixer mill (*RETSCH MM 400*).
3. Incubate for 5 minutes to permit complete dissociation of the nucleoproteins complex.
4. Add 0.2 mL of chloroform per 1 mL of TRIzol™ Reagent used for lysis, then securely cap the tube and mix by inversion.
5. Incubate for 2–3 minutes.
6. Centrifuge the sample for 15 minutes at 12,000 × g at 4°C. The mixture separates into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase.
7. Transfer the aqueous phase containing the RNA (300 µl for fat body samples and 200 µl for gut samples) to a new tube by angling the tube at 45° and pipetting the solution out.
8. Add 1 volume of 70% ethanol to the solution and mix immediately by pipetting. Do not centrifuge. Proceed immediately to the next step.
9. Transfer the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.
10. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
11. Before using the RNase-Free DNase Set for the first time, prepare DNase I stock solution. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNasefree water provided. To avoid loss of DNase I, do not open the vial. Inject RNasefree water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex.

12. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
13. Add the DNase I incubation mix (80 µl) directly to the RNeasy spin column membrane, and incubate for 15 min.
14. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.
15. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
16. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane.
    After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through.
17. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA.

Store purified RNA at -80°C.

Appendix 2

Comparison of log ratio by cage in fat body samples of the same treatment		
	Wilcoxon rank sum test with continuity correction alternative hypothesis: true location shift is not equal to 0	Welch Two Sample t-test alternative hypothesis: true difference in means is not equal to 0
F	W = 82, p-value = 0.8054	t = 0.15174, df = 14.507, p-value = 0.8815 95 percent confidence interval: -0.3072997 0.3542564 sample estimates: mean in group A mean in group B 2.298417 2.274939
G-	W = 8, p-value = 0.0008411	t = -4.4672, df = 19.098, p-value = 0.0002612 95 percent confidence interval: -0.6651019 -0.2408024 sample estimates: mean in group A mean in group B 1.846102 2.299054
G+	W = 72, p-value = 0.8054	t = -0.36432, df = 20.919, p-value = 0.7193 95 percent confidence interval: -0.2988858 0.2097923 sample estimates: mean in group A mean in group B 2.298623 2.343169
NG	W = 99, p-value = 0.7159	t = 0.60184, df = 24.455, p-value = 0.5528 95 percent confidence interval: -0.1049230 0.1914234 sample estimates: mean in group A mean in group B 2.183091 2.139841
SC	W = 52, p-value = 0.1052	t = -1.8468, df = 20.718, p-value = 0.07911 95 percent confidence interval: -0.45624540 0.02723786 sample estimates: mean in group A mean in group B 2.315363 2.529867
TOT	W = 23, p-value = 8.2e-05	t = -5.6954, df = 28.169, p-value = 4.079e-06 95 percent confidence interval: -0.6079211 -0.2863682 sample estimates: mean in group A mean in group B 1.974189 2.421333

Comparison of log ratio by cage in gut samples of the same treatment		
	Wilcoxon rank sum test with continuity correction alternative hypothesis: true location shift is not equal to 0	Welch Two Sample t-test alternative hypothesis: true difference in means is not equal to 0
F	W = 2, p-value = 0.6625	NA
G-	W = 10, p-value = 0.6761	t = -0.54461, df = 7.9262, p-value = 0.601 95 percent confidence interval: -0.4263770 0.2636708 sample estimates: mean in group A mean in group B 1.368678 1.450031
G+	W = 45, p-value = 0.5035	t = -0.80311, df = 15.16, p-value = 0.4343 95 percent confidence interval: -0.3880011 0.1754887 sample estimates: mean in group A mean in group B 1.602532 1.708788
NG	W = 46, p-value = 0.02924	t = 2.9505, df = 7.9393, p-value = 0.01857 95 percent confidence interval: 0.1060355 0.8694289 sample estimates: mean in group A mean in group B 1.4105738 0.9228416
SC	W = 17, p-value = 1	t = -0.29984, df = 6.1122, p-value = 0.7742 95 percent confidence interval: -0.7269016 0.5675733 sample estimates: mean in group A mean in group B 1.349519 1.429183
TOT	W = 9, p-value = 0.7656	t = 0.91932, df = 5.1565, p-value = 0.3989 95 percent confidence interval: -0.4153468 0.8844416 sample estimates: mean in group A mean in group B 1.927767 1.693220

Fat body log ratio by treatment

Kruskal-Wallis rank sum test

chi-squared = 20.172, df = 6, p-value = 0.002581

P values for pairwise comparisons using Wilcoxon rank sum test. Adjustment method: bonferroni

	F	G-	G+	NG	SC	SG
G-	0.7317	-	-	-	-	-
G+	1.0000	0.1823	-	-	-	-
NG	1.0000	1.0000	0.9936	-	-	-
SC	1.0000	0.0049	1.0000	0.0228	-	-
SG	1.0000	1.0000	1.0000	1.0000	0.2185	-
TOT	1.0000	1.0000	1.0000	1.0000	0.1621	1.0000

Gut log ratio by treatment

Kruskal-Wallis rank sum test

chi-squared = 16.771, df = 6, p-value = 0.01016

P values for pairwise comparisons using Wilcoxon rank sum test. Adjustment method: bonferroni

	F	G-	G+	NG	SC	SG
G-	1.000	-	-	-	-	-
G+	1.000	1.000	-	-	-	-
NG	1.000	1.000	0.034	-	-	-
SC	1.000	1.000	1.000	1.000	-	-
SG	1.000	1.000	1.000	0.772	1.000	-
TOT	1.000	0.720	1.000	0.178	0.994	1.000

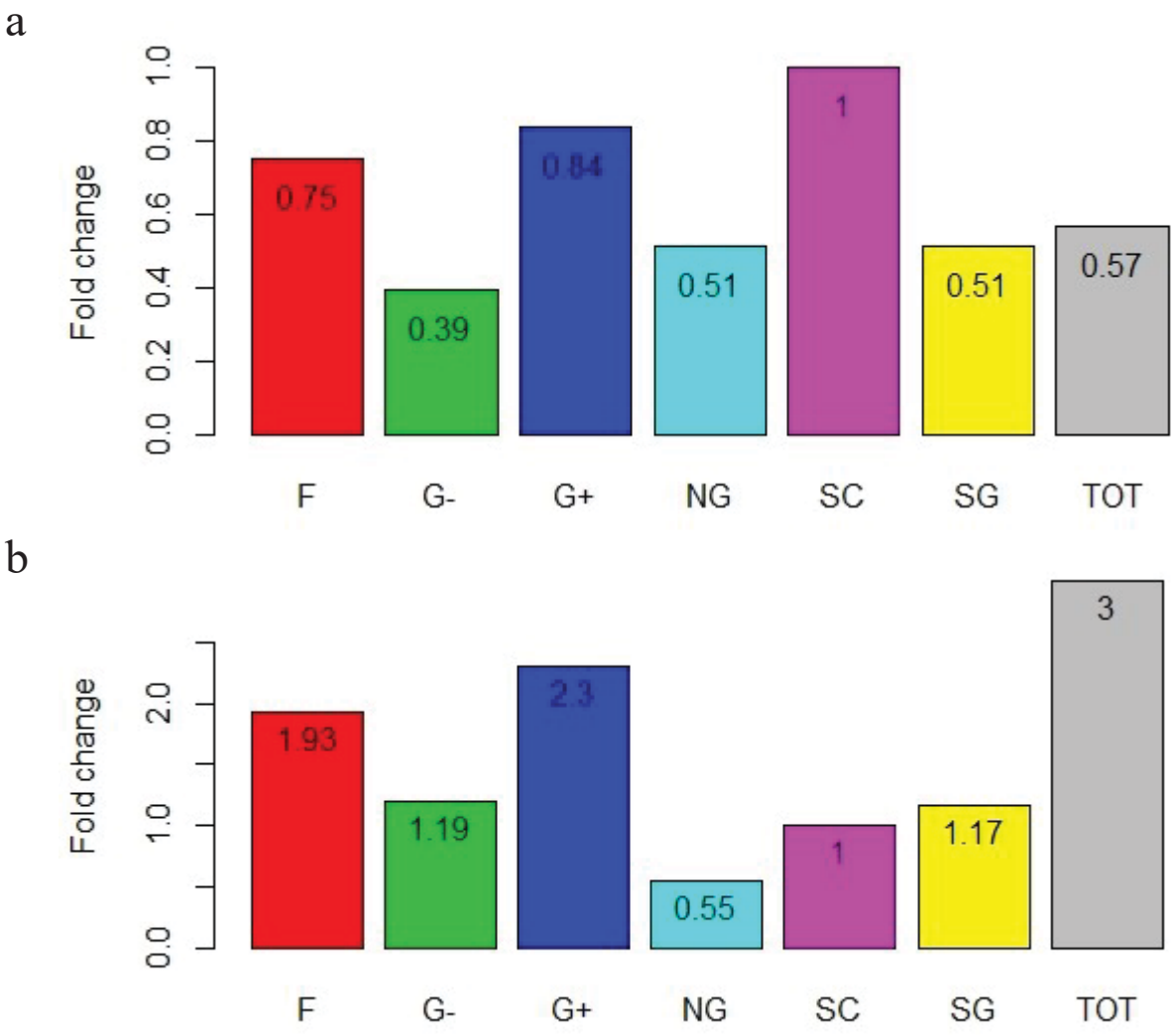

Figure S1 –Vitellogenin expression in fat body (a) and gut (b) samples in *Apis mellifera* with different gut biota composition relative to a group with no gut biota (SC), calculated with the $2^{-\Delta\Delta Ct}$ method.

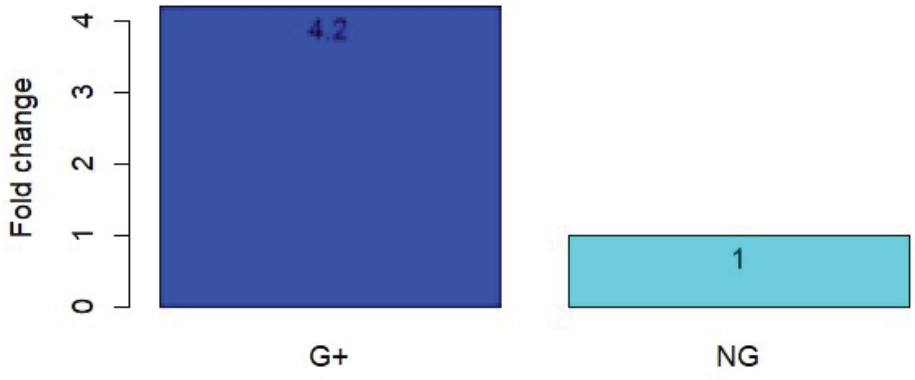

Figure S 2 – Relative vitellogenin expression in the gut *Apis mellifera* in the G+ and NG treatment, calculated with the $2^{-\Delta\Delta Ct}$ method.

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway