Year : 2019

Interactions between the gut symbiont Frischella perrara and its host the honey bee [Apis mellifera)

Emery Olivier

Emery Olivier, 2019, Interactions between the gut symbiont Frischella perrara and its host the honey bee [Apis mellifera)

Originally published at : Thesis, University of Lausanne

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Document URN : urn:nbn:ch:serval-BIB_06E4E8E84D5E2

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Faculté de biologie
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Département de Microbiologie Fondamentale

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Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Olivier EMERY

Master en Génomique et Biologie Expérimentale de l'Université de Lausanne
Master en Bioinformatique et Analyse de Données en Biologie de l'Université de Genève

Jury

Prof. Michel Chapuisat, Président
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UNIL | Université de Lausanne
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Ecole Doctorale
Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président·e	Monsieur	Prof.	Michel **Chapuisat**
Directeur·trice de thèse	Monsieur	Prof.	Philipp **Engel**
Co-directeur·trice	Monsieur	Prof.	Stephan **Gruber**
Expert·e·s	Monsieur	Prof.	Bruno **Lemaître**
	Monsieur	Prof.	Jan Roelof **van der Meer**

le Conseil de Faculté autorise l'impression de la thèse de

Monsieur Olivier Emery

Master en génomique et biologie expérimentale* / Master en biologie**, Université de Lausanne * Université de Genève, Suisse **

intitulée

Interactions between the gut symbiont *Frischella perrara* and its host the honey bee *(Apis mellifera)*

Lausanne, le 27 mai 2019

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Michel Chapuisat

“The bee’s life is like a magic well: the more
you draw from it, the more it fills with water”

Karl von Frisch

Acknowledgements

I would first like to thank Prof. Philipp Engel for allowing me to join his laboratory to perform this PhD work and for his guidance and support all along these years. I am also thankful to the members of my PhD commitee Prof. Bruno Lemaitre, Prof. Jan Roelof van der Meer, Prof. Michel Chapuisat and Prof. Stephan Gruber for taking the time to evaluate this work.

I would like to thank all colleagues from the Engel lab, in particular Konstantin Schmidt and Lucie Kešnerová who contributed to chapters 2 and 3 of this thesis, respectively. I am thankful to Berra Erkosar from the Department of Ecology and Evolution for her collaboration in chapter 3 and with the help she has provided me with statistics.

I want to thank the genomic technologies facility (GTF) staff in particular Johann Weber and Keith Harshman for their help to sequence RNA sequencing. I also thank Jean Daraspe and Bruno Humbel from the electron microscopy facility (EMF) for their help to take microscopy pictures from honey bee guts.

I would like to thank Catherine Berney and Jason Buser from the Department of Ecology and Evolution (DEE) Benjamin Dainat (Agroscope) for their help and counseling on beekeeping practices.

I would also like to thank the DMF staff including Vladimir Sentchilo, Nazife Beqa and the secretary office for the work that they do daily which contributes to make the DMF a confortable place to work. I thank the interns I have supervised over the years who have contributed to this work one way or another: Maya Makaric, Laurine Pelissier, Katherine Lane. I also thank organizers and assistants of the « sequence a genome » course.

Last but not least, I would like to thank my family and Magali Züger, my beloved one, for their constant support over the years.

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Résumé

Tous les symbiotes intestinaux ne sont pas forcément bénéfiques pour l'hôte. Certains peuvent être avoir un effet neutre alors que d'autres peuvent être nuisibles. Déterminer l'effet d'un symbiote intestinal donné est compliqué étant donné que les frontières entre être bénéfique ou nuisible sont souvent floues, et les bactéries intestinales font généralement partie d'une communauté plus complexe et hautement variable composée de nombreuses espèces. L'abeille mellifère possède un microbiote intestinal relativement simple qui permet d'étudier les effets de chaque espèce. Parmi les quelques membres du microbiote intestinal de l'abeille, *Frischella perrara* est une gammaproteobactérie qui colonise une region spécifique de l'intestin des abeilles où elle cause le phénotype dit "scab", une bande de couleur foncée qui apparaît à la surface de l'épithelium intestinal du côté du lumen. Il a été proposé que le scab est dû à de la mélanisation, une réponse courante des insectes provoquée par des blessures ou par l'exposition à des pathogènes. Malgré cette réponse immunitaire putative, il n'y a actuellement pas de données montrant que *F. perrara* soit pathogène pour les abeilles. De fait, *F. perrara* est fréquente parmi les abeilles adultes de colonies en bonne santé tout autour du monde. Ceci soulève de nombreuses questions intéressantes au sujet de la symbiose entre *F. perrara* et l'hôte. Est-ce que le scab correspond bien à de la mélanisation? Est-ce *F. perrara* affecte la santé de l'hôte? Quels gènes de *F. perrara* sont responsables de la colonisation et de la formation du phénotype scab? La fréquence de *F. perrara* ou ses intéractions avec d'autres membres du microbiote ou avec des pathogènes, varient-elles en function des saisons? La présente thèse aborde ces questions en étudiant la symbiose entre *F. perrara* et l'abeille mellifère à partir de trois perspectives: du point de vue de l'hôte (chapitre 1), du point de vue du symbiote (chapitre 2) et dans le contexte de la ruche au fil des saisons (chapitre 3).

Afin de comprendre comment *F. perrara* influence l'homéostasie de l'intestin et l'état immunitaire de l'hôte, j'ai utilisé la technique du RNA-Seq dans le but de déterminer les changements de l'expression des gènes de l'hôte dans l'intestin en réponse à une colonisation expérimentale avec *F. perrara*. Ceci a démontré que la colonisation avec *F. perrara* conduit à la surexpression spécifique de nombreux gènes de l'hôte impliqués dans la réponse immunitaire et dans le transport de molécules. En particulier, de nombreux gènes de la cascade de la mélanisation ont été surexprimés par la colonisation avec *F. perrara*, renforçant l'hypothèse que le scab est en effet le résultat de mélanisation de l'hôte. Malgré cette forte réponse immunitaire, la colonisation avec *F. perrara* n'a pas réduit l'espérance de vie des abeilles par rapport à des abeilles non-colonisées, ou par rapport à des abeilles colonisées avec un autre symbiote ne causant pas le phénotype scab en conditions de laboratoire.
Dans le but d'identifier les gènes de *F. perrara* impliqués dans la colonisation de l'hôte, la persistance dans l'intestin ou la formation du scab, en collaboration avec

un autre doctorant, nous avons étudié les changements de l'expression des gènes du symbiote *F. perrara* cultivé en laboratoire (in vitro) par rapport à des cellules de *F. perrara* récupérées à partir d'intestins d'abeilles (in vivo). Nous avons trouvé un certain nombre de gènes exprimés différemment, dont plusieurs gènes surexprimés in vivo impliqués dans la biosynthèse du tryptophane, dans le transport de sucres ou d'ions et quelques gènes impliqués dans la tolérance au stress oxidatif. Parmi les gènes sous-exprimés, nous avons trouvé des gènes impliqués dans la mobilité de la cellule et d'autres gènes participant au métabolisme du soufre.
Finalement, afin d'identifier les conditions spécifiques dans l'intestin des abeilles influençant la colonisation de *F. perrara*, nous avons déterminé la composition du microbiote d'abeilles individuelles d'une ruche au fil du temps. Alors que n'avons pas trouvé de correlations significatives entre *F. perrara* et d'autres membres du microbiote ou pathogènes, nous avons découvert que les abeilles d'hiver possédaient une différente structure de microbiote par rapport aux butineuses et que le régime alimentaire pouvait contribuer à changer la structure du microbiote. En particulier, nous avons observé que *F. perrara* était la seule espèce ayant des niveaux significativement plus bas dans les abeilles d'hiver par rapport au butineuses.

Globalement, cette thèse nous permet de conclure que le phénotype scab est très probablement le résultat de mélanisation en réponse à la colonisation avec *F. perrara*. L'absence d'effets négatifs de *F. perrara* sur l'hôte est cohérente avec sa distribution étendue au travers du temps et de l'espace. Cependant, d'autres pathogènes sont également fréquents parmi les colonies en bonne santé. Il est ainsi possible que l'effet négatif de *F. perrara* soit suffisamment petit pour que ce symbiote soit toléré dans l'intestin de l'abeille. La réponse immunitaire déclenchée par l'hôte pourrait jouer un rôle sur la tolérance de la part de l'hôte. Plutôt que d'éliminer *F. perrara,* la réponse immunitaire spécifique pourrait servir à garder la bactérie sous contrôle. Cependant, d'autres expériences seront nécessaires pour tester cette hypothèse. Au contraire, nous ne pouvons pas exclure non plus que *F. perrara* aie un role bénéfique pour l'hôte. En particulier, l'activation immunitaire de l'hôte par *F. perrara* pourrait protéger l'hôte de futures attaques de pathogènes et la biosynthèse de l'acide amine essentiel tryptophane ou d'autres composés chimiques par *F. perrara* pourraient être utilisés par l'hôte.

En résumé, *F. perrara* est un exemple evident d'un symbiote intestinal qui ne peut pas facilement être classifié parmi les trois catégories classiques qui englobent les mutualistes, les pathogènes et les commensaux. Ceci fait ressortir le besoin de considérer la symbiose comme un continuum entre pathogénicité et mutualisme, et de trouver des mesures précises pour quantifier les coûts et les bénéfices pour les différents partenaires impliqués.

Summary

Not all bacterial gut symbionts are necessarily beneficial to the host. Some of them may be neutral while others can even have detrimental effects. Determining the impact of individual gut symbionts can be challenging because the borders between being beneficial and detrimental are often fuzzy, and gut bacteria typically live in complex and highly variable multispecies communities. The honey bee possesses a relatively simple gut microbiota, providing a trackable model to study the effects of individual species. Among the few members of the honey bee gut microbiota, *Frischella perrara* is a gammaproteobacterium that colonizes a specific gut region where it causes the so-called "scab" phenotype, a dark colored band that appears on the luminal side of the epithelial surface. The scab has been hypothesized to result from melanization, a common insect immune response typically elicited after wounding or pathogen exposure. Despite inducing this putative immune response, there is currently no evidence that *F. perrara* is pathogenic for bees. In fact, *F. perrara* is highly prevalent among adult worker bees in healthy colonies across the world. This raises a number of interesting questions about the symbiosis between *F. perrara* and the host. Is the scab really a melanization response? Does *F. perrara* impact bee health? What genes from *F. perrara* are responsible for gut colonization and scab formation? Are there seasonal patterns of *F. perrara* prevalence along the year or interactions with other microbiota members or pathogens? The present thesis tackles these questions while investigating the symbiosis between *F. perrara* and the honey bee from three perspectives: the host side (chapter 1), the symbiont side (chapter 2) and in the context of the hive along seasons (chapter 3).

In order to understand how *F. perrara* affects the gut homeostasis and immune status of the host, I used RNA-Seq to determine changes in host gene expression in the gut in response to experimental colonization with *F. perrara*. This showed that colonization with *F. perrara* led to the specific upregulation of many genes involved in the host immune response. In particular, multiple genes of the melanization cascade were upregulated by *F. perrara*, supporting the idea that the scab corresponds to a host melanization response. Despite this strong immune response, experimental colonization with *F. perrara* did not reduce the lifespan of bees relative to non-colonized bees or bees colonized with another symbiont not causing the scab.
To identify *F. perrara* genes involved in colonization, persistence or scab formation, I investigated gene expression changes with RNA-Seq in *F. perrara* during host colonization relative to growth on agar plates, in collaboration with another PhD student. We found a number of interesting differentially expressed genes, with many genes upregulated in vivo involved in tryptophan biosynthesis, carbohydrate or ion transport, and some genes involved in tolerance to oxidative stress. Downregulated genes included genes coding for cell motility and sulfur metabolism.

Finally, to identify specific conditions in the bee gut that impact colonization by *F. perrara*, we monitored the microbiota of individual bees from a hive through time. While we did not find significant correlations between *F. perrara* and other gut microbiota members or pathogens, we found that winter bees had a distinct microbiota structure than foragers that may be dictated at least in part by diet. In particular, *F. perrara* was the only species to be at significantly lower levels in winter bees relative to foragers.

Overall, we can conclude from this PhD thesis that the scab phenotype is very likely the result of a melanization response upon *F. perrara* colonization. The absence of any detectable detrimental effect of *F. perrara* on the host is in line with its wide distribution across space and time. However, other pathogens are also highly prevalent in thriving honey bee colonies. Hence it is possible that the negative effect of *F. perrara* is small enough so that this gut symbiont is tolerated in the bee gut. The immune response mounted by the host may play an important role for the tolerance of the host. Rather than eliminating *F. perrara,* the specific immune response may keep the bacterium in check. However, further experiments need to be performed to test this hypothesis. On the contrary, we cannot exclude either that *F. perrara* has a beneficial role for the host. In particular, host immune activation by *F. perrara* may protect against subsequent pathogen assaults and the biosynthesis of the essential amino acid tryptophan or other chemical compounds by *F. perrara* may be used by the host.

In summary, *F. perrara* is a clear example of a gut symbiont that cannot be easily classified according to the three classical categories encompassing mutualists, pathogens and commensals. This highlights the need to think about symbiosis as a continuum between pathogenicity and mutualism, and to find precise measures to quantify the costs and benefits for the involved partners.

General introduction

Host-microbe interactions

Microorganisms, or microbes, were the first form of life on Earth, are ubiquitous, participate to the planet biochemical cycles and account for most of the diversity of life (Falkowski et al., 2008; Cavalier-Smith, 2006; Pace, 1997). By contrast, multicellular macroorganisms (i.e. animals and plants) appeared much later during evolution (Valentine, 1978) and, since then, have co-evolved with microbes (McFall-Ngai, 2015; McFall-Ngai et al., 2013; Erwin et al., 2011; Hickman, 2005). The study of host-microbe associations has historically focused on host-pathogen interactions as these play a central role in diseases of humans, as well as of plants and animals of agricultural importance (McGuire and Coelho, 2011; Herman and Williams, 2012; Fleming, 1871). Pathogens typically produce obvious negative effects on their hosts which can impose a severe burden to human society (Scholthof, 2007). Conversely, interactions between hosts and non-pathogenic or beneficial microorganisms do not exhibit such striking features and hence have received relatively little attention from scientists until recently despite being omnipresent and highly abundant on our planet and influencing almost all ecosystems in manifold ways (Bar-On et al., 2018; Pommerville, 2013).

Interactions between different species constitute a symbiosis and the interacting partners are called symbionts, with the one forming most of the biomass usually referred to as the "host". The definition of symbiosis is not fully agreed upon due to historical debates on i) whether it should include all forms of interactions between species or be restricted to mutually beneficial interactions only; and ii) whether prolonged duration of the interaction is required to define a symbiosis (Martin and Schwab, 2012a, 2012b). Here, De Bary's definition of symbiosis, i.e. "the living together of differently named species" (De Bary, 1879), will be used and includes all possible interactions between symbionts without making explicit assumptions on the duration of the interaction. The outcomes of host-microbe symbioses range from beneficial for one member of the interaction at the expense of the other (pathogenic) to reciprocally beneficial for both members of the interaction (mutualistic), including beneficial for one member but providing neither harm nor benefit to the other (commensal).

These outcomes are based on the relative costs and benefits for each of the interacting partners in terms of their ability to survive and produce offspring (i.e. fitness). For instance, human pathogenic bacteria such as *Mycobacterium tuberculosis* can thrive and multiply at the expense of the host which may develop tuberculosis, and eventually die if untreated (Tiemersma et al., 2011). By contrast, the mutualistic interaction that takes place between

aphids and their intracellular bacteria *Buchnera aphidicola* benefits both partners: the bacteria profit from nutrients from the host diet and, in return, produce and provide essential amino acids to the host which are absent from the phloem-based diet of aphids (Braendle et al., 2003; Sasaki and Ishikawa, 1995). As previously noticed (Douglas, 2018), the negative part of the definition of commensalism in host-microbe interactions (i.e "providing neither harm nor benefit") implies that proving a case of commensalism is impossible in practice: one would need to prove that a microorganism has no effect on its host under all possible conditions while measuring all possible effects. For these reasons, commensalism in host-microbe interactions cannot be strictly proven but the term tends to be used to describe symbioses in which microorganisms do not cause obvious effects to their host.

The pathogenicity-commensalism-mutualism continuum

The costs and benefits between interacting members of a symbiosis can be dynamic and vary in response to a multitude of factors. The latter include the health state of the host, genetic background of either partner, and environmental factors (Nishiguchi et al., 2008). Changes in symbiotic outcomes in response to host health state can be illustrated by the case of opportunistic pathogens: while they normally do not harm their host and can thus be considered commensals, they can suddenly turn into pathogens and

cause disease if the host is immunocompromised (Sepkowitz, 2002). Host-microbe symbiotic costs and benefits can also shift in response to microbial genetic backgrounds: bacterial strains harboring different virulence genes (e.g. toxin-producing or antibiotic-resistance genes) have varying degrees of pathogenicity and hence incur more or less severe costs to the host (Kadioglu et al., 2008; Wu et al., 2008; Bray Speth et al., 2007). Host genetics can also impact the symbiotic outcome, for example plant genotypes have been shown to play a significant role in the disease-suppressing interaction of plants with a microbial biocontrol agent (Smith et al., 1999). Symbiotic costs and benefits can also be affected by environmental factors: in the symbiosis between a fungal endophyte and its grass host, the benefits of the symbiosis for the host were strongest in water limiting conditions (Davitt et al., 2011). Hence the outcomes of host-microbe interactions form a continuum from pathogenicity to commensalism to mutualism, which can shift gradually or abruptly form one type towards another for a given pair of interacting partners (**Fig. 1**, Dimijian, 2000).

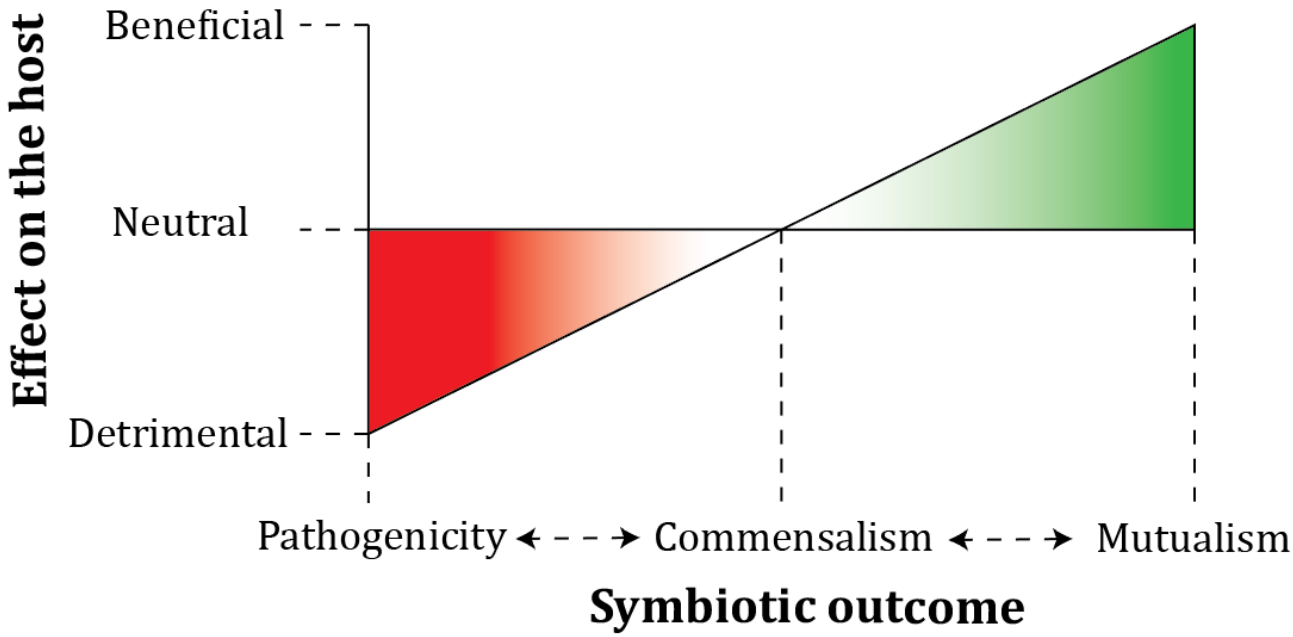


Fig. 1 *The pathogenicity-commensalism-mutualism continuum in host-microbe interactions. The different symbiotic outcomes form a gradient from pathogenicity to mutualism, rather than clearly separated categories.*

The gut microbiota

While the examples of symbioses presented so far correspond to one host – one symbiont species, many symbioses that can be found in nature take place between one host species and a consortium of different microbial species called the microbiota (Engel and Moran, 2013; Kohl, 2012; Lozupone et al., 2012; Cantas et al., 2012; Ley et al., 2008). Such complex symbioses imply not only diverse host-microbe interactions but also microbe-microbe interactions that influence each other and the host (Fraune et al., 2015; Charlier et al., 2009; Michel Fons, 2000). For example, the gut microbiota can protect the host from opportunistic pathogens through colonization

resistance (Buffie and Pamer, 2013; Kamada et al., 2013a; Stecher and Hardt, 2011; Michel Fons, 2000). In addition, microbes already established in the host can indirectly affect the presence of other microbes in the host by modulating the host immune response (Rooks and Garrett, 2016; Round and Mazmanian, 2009). Furthermore, the composition of most microbial communities is not static, but highly dynamic, changing over space and time during host development but also in response to changes in host diet or lifestyle (Bäckhed et al., 2015; Baldo et al., 2015; Korem et al., 2015; David et al., 2014a). This makes it extremely challenging to determine how a given microbiota affects host health and which community member engages in which type of symbiotic interaction with whom.

The gut microbiota of animals is of particular interest for studying symbiotic interactions between the host and different community members. First, the gut microbiota of an individual host animal is typically composed of tens to hundreds of different bacterial species that coexist at a given time point (Hird et al., 2015; Romero et al., 2014; Engel and Moran, 2013a; Ley et al., 2008). Second, the gut microbiota is in direct contact with the host via the epithelial cell surface and hence can exchange signals or metabolites (Jakobsson et al., 2015; Ashida et al., 2012; Wells et al., 2011). Third, it is composed of a taxonomically diverse set of bacteria that are supposed to be mostly beneficial for the host, but which also includes opportunistic pathogens, and

many bacteria for which the symbiotic relationships with the host have remained elusive (Nagao-Kitamoto et al., 2016; Zhu et al., 2013; Ghoshal et al., 2012; Zhu et al., 2013; Delzenne and Cani, 2011; Chow et al., 2011; Chow and Mazmanian, 2010). Fourth, the gut microbiota is an 'open' ecosystem that is influenced by the environment, i.e. it changes with the lifestyle of the host (e.g. dietary habits) and can be invaded by bacteria from the 'outside' or from communities of other body sites (Bäumler and Sperandio, 2016; David et al., 2014b, 2014a; Arimatsu et al., 2014).

Finally, selection can act at different levels in host-associated microbial communities. At the level of the bacterial community, selection will favor the most competitive bacteria, which may come at an expense for the host, because beneficial services of bacteria for the host are believed to be expensive. This can favor the evolution of cheaters, i.e. bacteria that have a growth advantage over their beneficial ancestors, because they do not invest in the host services anymore. In contrast, at the level of the host, selection will favor the community that is most beneficial to the host, i.e. the community which enhances the fitness of the host. Therefore, host-associated microbiomes have been proposed to evolve as 'an ecosystem on a leash' (Foster et al, 2017). But how is the evolutionary conflict between host and bacterial evolution solved? It has been predicted that the host can somehow exert control on its gut microbiota to select and tolerate beneficial

microorganisms while at the same time exclude pathogens (Schluter and Foster, 2012).

The study of complex multispecies microbial communities such as the gut microbiota has been greatly facilitated in recent years by the development of culture-independent, high throughput and affordable DNA sequencing and analysis techniques (Shendure and Ji, 2008; DeLong, 2005; Tringe and Rubin, 2005; Tringe et al., 2005; Torsvik and Øvreås, 2002). In particular, these developments have allowed to comprehensively profile the microbiota of the gastrointestinal tract of mammals, revealing its complexity with a high diversity of species within and between individuals (The Human Microbiome Project Consortium et al., 2012; Andersson et al., 2008). Moreover, experiments with germ-free animals, especially mice, have become routine and permit controlled experiments to probe the impact of entire microbial communities or individual species on host phenotypes (Rooks and Garrett, 2016). Using experiments with gnotobiotic animals, the gut microbiota has been shown to play important roles for the host in nutrition (Flint et al., 2012; Turnbaugh et al., 2006), immune system development (Sommer and Bäckhed, 2013; Round and Mazmanian, 2009) and protection from invading pathogens (Buffie and Pamer, 2013). Moreover, imbalance in the gut microbiota composition (i.e. dysbiosis) has been associated to a myriad of human diseases. These include, but are not limited to, inflammatory bowel

disease, celiac disease, asthma, allergies, metabolic syndrome, cardiovascular disease or neurological diseases like depression and Parkinson's disease (Sampson et al., 2016; Kelly et al., 2016; Shen et al., 2013; Clemente et al., 2012; Cryan and Dinan, 2012; Ley et al., 2006).

Despite the great advances made in the field, determining the underlying interactions in the gut of mammals between the host and individual microbiota members, or interactions between microbiota species, remains a challenging task. One of the difficulties in studying mammalian gut microbiotas is their complexity: they can be composed of hundreds to thousands of microbial species which corresponds to even more possible interactions (The Human Microbiome Project Consortium et al., 2012; Andersson et al., 2008; Ley et al., 2008). In addition, their species abundance and composition are variable between individual hosts, but also within each host in function of many factors such as diet, age or health status (Rampelli et al., 2015; Amato et al., 2015; Claesson et al., 2012; Yatsunenko et al., 2012; Filippo et al., 2010). Hence, simpler and more tractable models of the gut microbiota can be helpful in order to study the basic processes involved in the interactions between microbial communities and their hosts, and between microbiota members. In this regard, the gut microbiota of some insects present attractive study systems as they possess relatively fewer microbial species than that of mammals (Engel and Moran, 2013a).

The insect gut microbiota

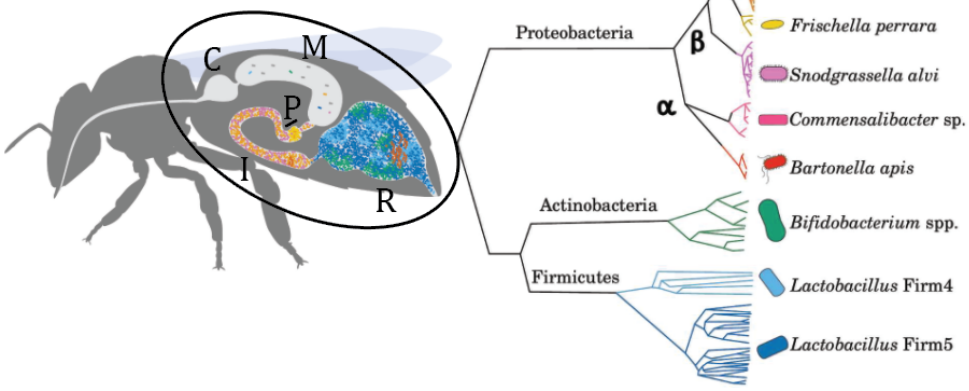
With over a million species, insects form the largest group in the animal kingdom accounting for ~66% of all species (Zhang, 2011). They are essential in the maintenance of natural and agricultural ecosystems through their role in the food-web and pollination. However, insects can also be detrimental by consuming and damaging large amounts of plants, or by acting as disease vectors (Robinson et al., 2011). Despite the enormous diversity of existing insect species, the global structure of the insect gut is rather conserved and can be divided in three main sections (i.e. the foregut, the midgut and the hindgut) which exhibit various morphological and physiochemical adaptations to specific diets across species (Chapman and Chapman, 1998). The gut microbiota of many insects contains few microbial species compared to mammalian guts and, depending on the host species, consists of either transient microorganisms that are acquired from the environment and simply pass through the gut without establishment of a stable colonization, or resident microorganisms that colonize, replicate, and persist in the gut environment (Engel and Moran, 2013a). Examples of transient insect gut microbiota include that of the fruit fly *Drosophila melanogaster* which is highly influenced by horizontally-acquired free-living bacteria from diet (Wong et al., 2013; Staubach et al., 2013) and the mosquito gut microbiota which corresponds to a low diversity bacterial community

acquired from the environment (Coon et al., 2014). By contrast, the core gut microbiota of the omnivorous cockroach *Periplaneta americana* is resilient to dietary shifts (Tinker and Ottesen, 2016) and broad-headed bugs and stinkbugs possess gut microbiotas which are highly specific and are dominated by a single bacterial species (Ohbayashi et al., 2015; Kikuchi et al., 2005).

Although the number of species in most insect gut microbiotas are usually lower than those of mammals and may range from loose to highly specialized associations, they nevertheless play key roles in host nutrition, development and immune response (Broderick et al., 2014; Wong et al., 2014; Coon et al., 2014; Rosengaus et al., 2011; Buchon et al., 2009).

The honey bee gut microbiota

The honey bee *Apis mellifera* is a social insect and a key pollinator which lives in large colonies consisting of tens of thousands of non-reproductive female workers and a single reproductive queen (Winston, 1991). Recently, high-throughput DNA sequencing methods have allowed to show that the honey bee gut bacterial community consists mostly of eight phylotypes (**Fig. 2**). Phylotypes refer here to clusters of bacterial strains that share >97% sequence identity in the 16S rRNA gene and thus can be taken as an equivalent to a bacterial species. These phylotypes are consistently found in



Core species	Gut region
Yes	Ileum
No	Pylorus
Yes	Ileum
No	n.d.
No	n.d.
Yes	Rectum
Yes	Rectum
Yes	Rectum

Fig. 2 *The honey bee gut microbiota members and their localization in the gut. The honey bee gut consists of five main regions : crop (C), midgut (M), pylorus (P), ileum (I) and rectum (R). The highest honey bee gut bacterial densities are found in the rectum (~10^9 cells), followed by the ileum (~10^8 cells) while few bacteria are present in the midgut and the crop. Core species are species consistently found in all adult worker bees of a given colony, while the three non-core species shown here are typically present in a given colony, but only found in a subset of the bees. The gut region indicated for each species corresponds to where the species is most abundant. n.d : non determined*
Adapted from Bonilla-Rosso and Engel, 2018.

adult workers irrespective of geographical location, life stage or season (Zheng et al., 2018; K wong and Moran, 2016; Kwong et al., 2014; Engel and Moran, 2013b; Engel et al., 2012; Moran et al., 2012; Martinson et al., 2011). Moreover, divergent strains of most of these phylotypes can also be found in other social corbiculate bees including bumble bee and other species of the genus *Apis*. The similarity of the gut community among honey bees from

different continents and the presence of related communities in other social bee genera indicates that these species share a prolonged co-evolution with their host (Kwong and Moran, 2016). These phylotypes include gram-negative proteobacteria such as alphaproteobacteria (*Bartonella apis, Commensalibacter sp.*), betaproteobacteria (*Snodgrassella alvi*), and gammaproteobacteria (*Gilliamella apicola* and *Frischella perrara*, Moran, 2015). Gram-positive bacteria are also part of the honey bee gut microbiota and include two species clusters in the Firmicutes phylum designated *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5, and the actinobacterium *Bifidobacterium asteroides*. While the honey bee gut microbiota phylotypes are consistently found within a honey bee colony, not all phylotypes are present in each individual bee. *S. alvi*, *G. apicola*, *Lactobacillus* Firm-4, *Lactobacillus* Firm5 and *B. asteroides* are found in the vast majority of honey bees. Therefore, these five phylotypes are designated as the core microbiota to distinguish them from the other gut microbiota phylotypes that are less prevalent and in general less abundant. The non-core bacteria include *F. perrara*, *B. apis*, and, for some researchers, also *Commensalibacter sp.* (Alpha2.1).

An important property of honey bee gut microbiota formation is that newly emerged bees are mostly devoid of bacteria and acquire them through contacts with nest mates and hive materials (Powell et al., 2014; Martinson

et al., 2012). This allows to collect and isolate bee pupae before emergence of imagos and raise them in an incubator so as to obtain microbiota-free bees without the need of antibiotics or an expensive germ-free facility. In addition, each of the bacterial phylotypes can be cultured in vitro (Kešnerová et al., 2017; Kwong and Moran, 2013; Engel et al., 2013c; Bottacini et al., 2012) which enables to selectively colonize microbiota-free bees with defined bacterial assemblages so as to produce gnotobiotic bees to test the effect of different bacterial communities. With its fast reproduction rate, relatively simple diet and gut microbiota amenable to manipulation, the honey bee is thus a promising model for host-microbiota studies.

The honey bee gut symbiont F. perrara causes a putative melanization response

F. perrara is a particularly interesting honey bee gut microbiota member. It colonizes a restricted area in the gut, the pylorus (located between midgut and the ileum) where it causes the so-called scab phenotype. This phenotype can be observed from outside the dissected gut and appears as a dark-colored band that partially surrounds the pylorus (**Fig. 3**, Engel et al., 2015a, 2013c). The coloring corresponds to a material that is deposited on top of the cuticle lining of the pylorus and that is reminiscent of melanin. In insects, the formation and deposition of melanin (i.e. melanization) corresponds to an

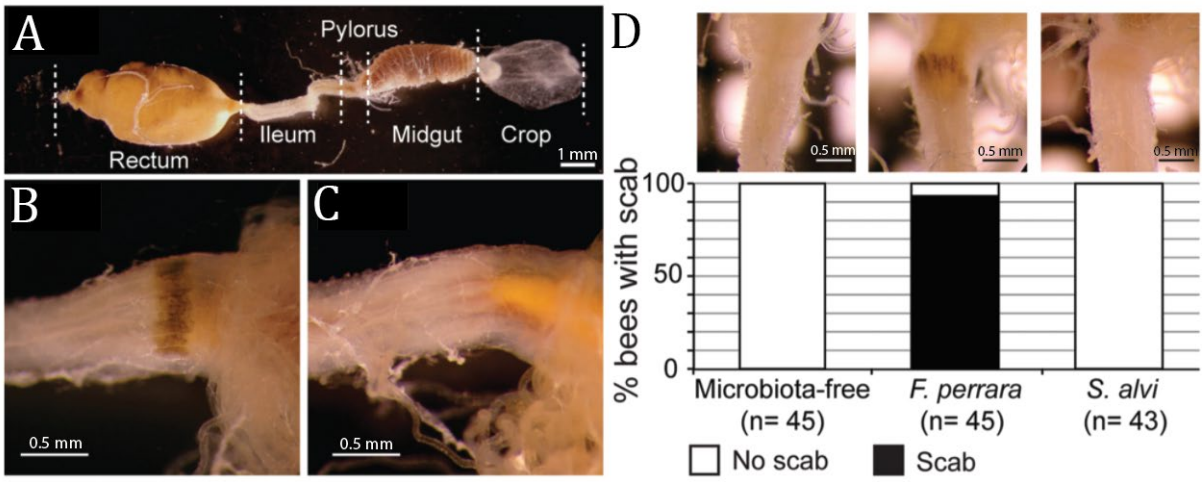


Fig. 3 *Scab phenotype in the pylorus of honeybees. (A) Dissected gut of an adult honeybee. Different gut regions, including the region defined as the pylorus, are outlined with dashed lines. The rectum is filled with pollen. (B and C) Pylorus region with scab and without scab, respectively. (D) Experimental colonization of microbiota-free (MF) bees with F. perrara causes scab development. Data are the percentage of 10-day-old bees with scab phenotypes after exposure to F. perrara or S. alvi or when left microbiota-free. n, number of animals analyzed. Only bees that were successfully colonized were included in the analysis. Adapted from Engel et al. 2015a*

immune response and wound healing mechanism (Nappi and Christensen, 2005). Melanization is triggered rapidly in response to microbial invasion or physical injury and is regulated by a complex proteolytic cascade known as the melanization cascade or prophenoloxidase–activating system (proPO-activating system, Cerenius et al., 2010). The melanization cascade is initiated by the recognition of microbes by pattern recognition proteins (PRPs) or by damaged tissues. This is followed by a multi-step chain of reactions which ultimately leads to the activation of the enzyme proPO to its

active form (i.e. phenoloxidase or PO) which catalyzes the oxidation of mono- and diphenols to orthoquinones. The orthoquinones are then polymerized by non-enzymatic reactions to form melanin (Sugumaran, 2002). Melanization occurs in insects in response to invading pathogens or parasites and can lead to melanotic encapsulation and death of the invaders (Götz, 1986). The melanization response also leads to the formation of many highly reactive intermediate species that participate in neutralizing pathogens; but that the host also needs to control in order to avoid self-damage (Zhao et al., 2011; Nappi and Vass, 2001; Saul and Sugumaran, 1989). However, in contrast to other examples where melanization plays a role as an immune response in insects, the putative melanization response in the symbiosis of *F. perrara* and the honey bee seems to occur in the gut lumen. Therefore, an alternative explanation for the production of the scab phenotype could be that *F. perrara* is able to produce melanin, as some microorganisms including bacteria have previously been shown to have this ability (Castro-Sowinski et al., 2002; Cubo et al., 1988; Ivins and Holmes, 1980; Turick et al., 2002).

It is intriguing that *F. perrara* is the only honey bee gut microbiota member which elicits the scab phenotype and, possibly, melanization in its host. The nature and the consequences of the symbiosis between the honey bee and *F. perrara* are yet to be determined. What are the effects on the host? Which

genes are involved in colonization/scab formation by *F. perrara*? While *F. perrara* seems to elicit a phenotype reminiscent of a pathogen, it is not cleared out from the gut following scab formation. In contrast to known pathogens, the prevalence or abundance of *F. perrara* have not been linked to detrimental effects on the host so far. Hence *F. perrara* is an interesting symbiont to study in order to further understand host-bacteria symbiotic interactions.

Aims of the PhD

The main goal of this PhD thesis is to better understand the interactions between *F. perrara* and its host, by assessing the effects of *F. perrara* on the honey bee, and by characterizing different aspects of *F. perrara* colonization and the scab phenotype. **Chapter 1** focused on the impact of *F. perrara* on the host. Therefore I conducted an RNA-Seq experiment to identify the host response to *F. perrara* colonization. In addition, I performed survival experiments under different dietary regimes to assess if *F. perrara* colonization would affect host lifespan and tested the applicability of a chemical inhibitor of melanization to block the scab phenotype.

In **chapter 2**, I turned the attention towards the symbiont and investigated how *F. perrara* gene expression was affected in response to colonization in the honey bee gut. To do so, I used RNA-Seq to compare the whole-genome gene expression of *F. perrara* in vivo upon colonization of the pylorus relative to *F. perrara* cells grown in vitro on agar plates. In order to provide candidate bacterial mechanisms important for symbiosis establishment and persistence, I further assessed the gene functions overrepresented among differentially expressed genes.

In **chapter 3**, I investigated if *F. perrara* abundance showed seasonal patterns and whether it correlated with the abundance of other microbiota members under in-hive conditions. The abundance of seven gut microbiota

phylotypes including *F. perrara* as well as two pathogens were monitored monthly in a hive for a period of two years, and the presence and intensity of the scab were recorded. Correlations between microbiota species and with pathogens were determined in order to assess possible interactions between microbial species pairs. In addition, I explored the relationship between scab intensity and *F. perrara* levels from this dataset and monitored the percentage of bees with the scab phenotype monthly from multiple hives along the year.

Together these three chapters investigate the interaction between *F. perrara* and its host from different perspectives and provide novel insights into this symbiosis.

1) Effects of *F. perrara* on *A. mellifera*

Summary

Single gut microbiota species can interact in multiple ways with their host ranging from mutualism to pathogenicity. In honey bees, the gammaproteobacterium *Frischella perrara* causes the scab phenotype which is reminiscent of melanization. In order to determine the effects of *F. perrara* colonization on the host, we investigated host transcriptome changes in the pylorus in response to bacterial colonization using RNA-seq. Mono-colonization of bees with *F. perrara*, but not with *S. alvi*, led to a strong activation of the immune system as measured by the upregulation of immune related genes including genes coding for antimicrobial peptides and pattern recognition receptors. In addition, the melanization cascade was upregulated by *F. perrara* colonization, suggesting that the scab results from melanization of the host. A transcriptome analysis of in-hive bees with versus without the scab phenotype further showed that *F. perrara* is also able to stimulate the host immune system in the presence of other gut microbiota species.

This chapter consists of the aforementioned results previously published in the form of an article in the journal *Molecular Ecology*, as well as two additional subchapters: one on honey bee survival in response to bacterial colonization with either *S. alvi* or *F. perrara*, and in non-colonized bees and the other on melanization inhibition using phenyltiourea (PTU). We found that *F. perrara* did not have a deleterious effect on honey bee lifespan under laboratory conditions, whether bees were fed a diet with or without proteins in the form of pollen. The melanization inhibition using PTU was not effective at blocking melanization and scab formation and we determined that PTU was toxic for the bees.

MOLECULAR ECOLOGY

Molecular Ecology (2017) **26**, 2576–2590 doi: 10.1111/mec.14058

Immune system stimulation by the gut symbiont *Frischella perrara* in the honey bee (*Apis mellifera*)

OLIVIER EMERY, KONSTANTIN SCHMIDT and PHILIPP ENGEL

Department of Fundamental Microbiology, University of Lausanne, CH-1015, Lausanne, Switzerland

Abstract

Gut bacteria engage in various symbiotic interactions with their host and impact gut immunity and homeostasis in different ways. In honey bees, the gut microbiota is composed of a relatively simple, but highly specialized bacterial community. One of its members, the gammaproteobacterium *Frischella perrara* induces the so-called scab phenotype, a dark-coloured band that develops on the epithelial surface of the pylorus. To understand the underlying host response, we analysed transcriptome changes in the pylorus in response to bacterial colonization. We find that, in contrast to the gut bacterium *Snodgrassella alvi*, *F. perrara* causes strong activation of the host immune system. Besides pattern recognition receptors, antimicrobial peptides and transporter genes, the melanization cascade was upregulated by *F. perrara*, suggesting that the scab phenotype corresponds to a melanization response of the host. In addition, transcriptome analysis of hive bees with and without the scab phenotype showed that *F. perrara* also stimulates the immune system under in-hive conditions in the presence of other gut bacterial species. Collectively, our study demonstrates that the presence of *F. perrara* influences gut immunity and homeostasis in the pylorus. This may have implications for bee health, because *F. perrara* prevalence differs between colonies and increased abundance of this bacterium has been shown to correlate with dietary alteration and impaired host development. Our transcriptome analysis sets the groundwork for investigating the interplay of bee gut symbionts with the host immune system.

Keywords: antimicrobial peptides, insect, melanization, microbiota, RNAseq, *Snodgrassella alvi*, transcriptome

Received 17 October 2016; revision received 1 February 2017; accepted 2 February 2017

Introduction

The honey bee (*Apis mellifera*) is a keystone pollinator species for agricultural and natural ecosystems, and recent colony losses have highlighted the necessity to intensify research on bee health (vanEngelsdorp *et al.* 2009). Bacterial communities inhabiting the gastrointestinal tract are known to be key players in health and disease for a wide range of animals (Engel & Moran 2013; Kostic *et al.* 2013). However, their potential symbiotic role for honey bees has long been neglected. This is surprising, because honey bees possess a relatively simple and remarkably conserved gut microbiota. It is composed of 8–10 species typically comprising more than 95% of all bacteria present in the adult gut (Martinson *et al.* 2011; Engel *et al.* 2012; Moran *et al.* 2012; Sabree *et al.* 2012). In parallel to the mammalian gut microbiota, gut bacteria of the honey bee are host-specific, they are spatially organized, have the highest abundance in the hindgut and thrive under anaerobic or microaerophilic conditions (Kwong & Moran 2016). Cultures of all major community members have recently been established (Kwong & Moran 2012; Engel *et al.* 2013; Corby-Harris *et al.* 2014a; Olofsson *et al.* 2014; Kešnerová *et al.* 2016), and newly emerged bees can be colonized with cultured strains under laboratory conditions (Engel *et al.* 2015a). This enables experimental investigations of the bee gut microbiota generating new leads directly relevant to bee health. Several findings, including evidence for pathogen protection or host nutrition, have indicated that the bee gut microbiota

Correspondence: Philipp Engel, Fax: +41 21 692 56 05;
E-mail: philipp.engel@unil.ch

may provide important beneficial functions for the host (Audisio & Benítez-Ahrendts 2011; Koch & Schmid-Hempel 2011; Engel *et al.* 2012; Lee *et al.* 2014). However, the precise roles of different community members and their influence on the host's immune system, metabolism or physiology have so far remained elusive.

We have recently shown that *Frischella perrara*, one of two gammaproteobacteria in the honey bee gut, is responsible for causing the 'scab' phenotype (Engel *et al.* 2015a). This phenotype is characterized by a dark brown to black deposit forming a localized thin band in the pylorus at the midgut–hindgut boundary, in close proximity to the malpighian tubules (Fig. 1A). The phenotype develops 5–7 days after adult worker bees have emerged. The proportion of bees with scab varies between colonies (typically 40–90%), but strongly correlates with the abundance of *F. perrara* in the gut (Engel *et al.* 2015a). Experiments with laboratory emerged bees have shown that colonization with a cultured strain of *F. perrara* is sufficient to induce the scab (Fig. 1A). In contrast, bees colonized with *Snodgrassella alvi*, another symbiont colonizing a similar region of the gut as *F. perrara* (Fig. 1D–F), or noncolonized bees, did not develop the phenotype (Fig. 1B and C) (Engel *et al.* 2015a).

While the presence of a scab can be observed from the outside of a dissected gut, the actual phenotype is located within the gut, on the cuticle layer lining the gut epithelium and separating the host from the bacteria in the lumen (Fig. 1G). Fluorescence *in situ* hybridization (FISH) shows that *F. perrara* specifically colonizes the epithelial regions where the scab is formed (Fig. 1D) (Engel *et al.* 2015a), suggesting a direct link between the association of *F. perrara* with the host

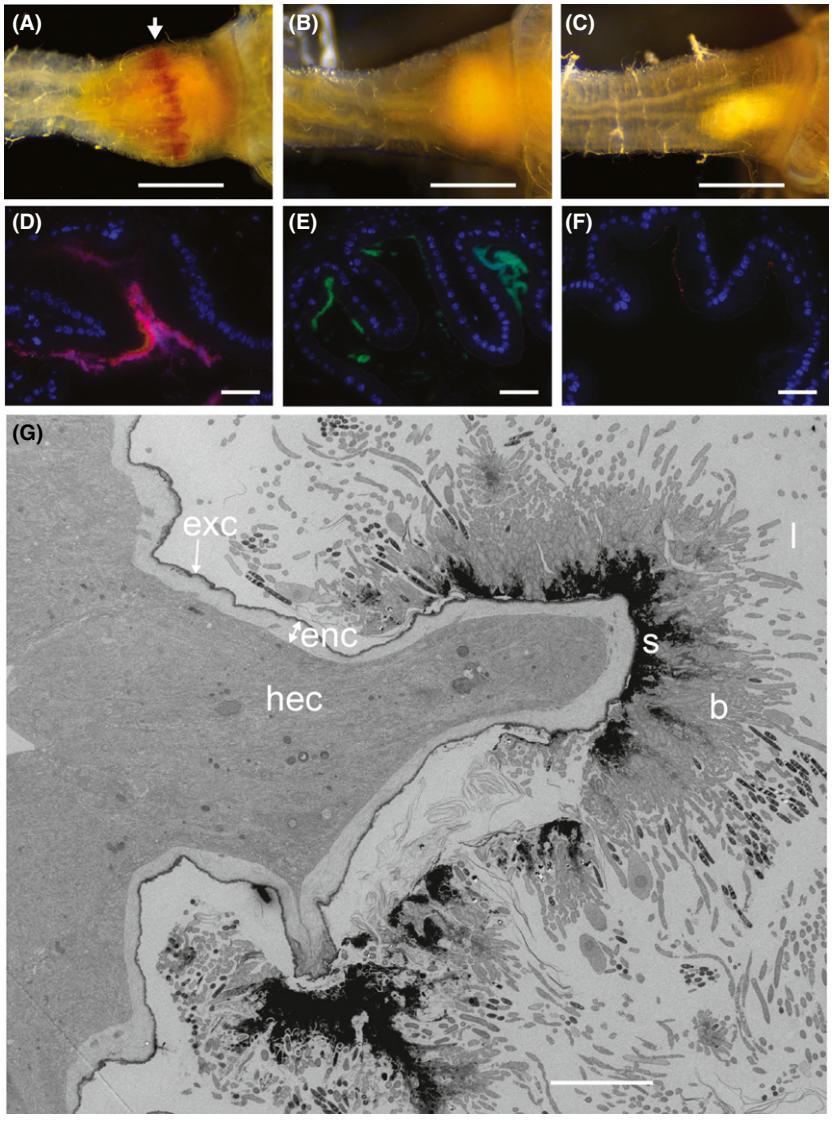


Fig. 1 The scab phenotype in the pylorus of honey bees. (A, B, C) The pylorus region of 10-day-old bees that were either colonized with (A) *Frischella perrara* or (B) *Snodgrassella alvi* or (C) that were left untreated (i.e. noncolonized bees). The white arrow indicates the scab phenotype in (A). Scale bars, 0.5 mm. (D,E,F) FISH microscopy images of cross sections through the pylorus of 10-day-old bees that were either colonized with (D) *Frischella perrara* or (E) *Snodgrassella alvi* or (F) that were left untreated (i.e. noncolonized bees). Signals for *F. perrara* and *S. alvi* are shown in red and green colour, respectively. No bacterial signals can be detected in noncolonized bees. DAPI signal for DNA is shown in blue. Scale bars, 50 μm. (G) Electron micrograph showing the scab phenotype on the gut epithelial surface of the pylorus of a female worker bee sampled from a honey bee colony. hec, host epithelial cells; enc, endocuticle; exc, exocuticle; l, lumen; b, bacteria; s, scab. Scale bar, 10 μm.

tissue and the formation of the scab. The origin of the scab, that is whether it is produced by the bacterium or the host, has so far remained elusive. However, in line with similar phenotypes observed in other insects (Hillyer *et al.* 2003; Cerenius *et al.* 2008; Seisenbacher *et al.* 2011; Binggeli *et al.* 2014), it is conceivable that the scab results from a melanization response of the host upon colonization by *F. perrara*. Together with the production of antimicrobial peptides (AMPs), melanization is part of the humoral immune response of insects (Lavine & Strand 2002). Melanin is a biopolymer that is produced from the oxidation of phenolic compounds such as dopamine (Nappi & Christensen 2005). It has antimicrobial properties as it causes oxidative stress and leads to the encapsulation of invading parasites (Cerenius & Söderhäll 2004). Further, it is involved in wound healing processes upon tissue damage and also plays an important role in the development (coloration and sclerotization) of the cuticle layer of the exoskeleton (True 2003; Galco & Krasnow 2004; Andersen 2010). While it is well known that pathogens induce melanization responses of their insect hosts (Marmaras *et al.* 1996; Söderhäll & Cerenius 1998; Cerenius *et al.* 2008), a spatially restricted host response to a resident gut microbiota member in the gut lumen, as possibly triggered by *F. perrara*, has to our knowledge not been observed yet.

The aim of this study was to reveal the host response underlying the specific interaction with *F. perrara* and the formation of the scab phenotype in the pylorus. To this end, we monitored transcriptome changes between bees experimentally colonized with *F. perrara* and *S. alvi* and between age-controlled hive bees with and without the scab phenotype. Our results show that *F. perrara* causes a characteristic immune response in the gut that seems to be responsible for the development of the scab phenotype. These findings provide important new insights into the impact of this bacterium on honey bees and highlight the need to better understand the cross-talk between gut microbes and their host.

Materials and methods

Experimental colonizations of newly emerged honey bees

To rear honey bees (*Apis mellifera carnica*) that lack the dominant gut bacteria of the pylorus, brood frames were removed from a healthy colony located at the University of Lausanne, Switzerland. In the laboratory, wax cell caps were carefully removed using sterile toothpicks, and tan-coloured pupae with black eyes were pulled out and placed on their back on a moistened cotton pad in a plastic cage. Nine cages containing 30 pupae were kept in an incubator at 32 °C under a relative humidity of 75–85% for 2 days. Bees that emerged during this period were distributed among nine new cages and were provided 1:1 (wt:wt) sucrose–water *ad libitum*, and approximately 700 mg of sterile pollen. Pollen was treated with a 10 MeV electron beam (LEONI Studer AG, Switzerland). The sterility of pollen was ensured by plating homogenized aliquots on different solid media and incubating these plates at 37 °C in air, microaerophilic or anaerobe conditions.

Newly emerged laboratory bees were colonized within the first 24 h after emergence with either *F. perrara* strain PEB0191 (Engel *et al.* 2013) (FP bees) or *S. alvi* strain wkB2 (Kwong & Moran 2012) (SA bees), or they were left noncolonized (NC bees). All three conditions were performed in triplicates. *F. perrara* and *S. alvi* were grown on brain–heart infusion agar (BHIA) at 37 °C under anaerobic conditions and on tryptic soy agar (TSA) at 37 °C in 5% CO_2, respectively. After harvesting in 1 mL 1× PBS, the optical density at a wavelength of 600 nm was adjusted to 1 and cells were resuspended in 1× PBS plus sucrose–water (1:1). 400 μL of this solution was evenly distributed on the pollen provided to the newly emerged bees. For NC bees, 400 μL of 1× PBS plus sugar–water (1:1) without bacteria was applied to the pollen. Bees were then provided sterile sugar–water (1:1) *ad libitum* and were placed in an insect chamber at 32 °C under a relative humidity of 75–85% for 10 days before sampling.

Sampling of hive bees with and without scab

To sample age-controlled female hive bees with and without scab phenotype, brood frames with capped brood were collected after brushing off bees from their surface, and kept in a closed polystyrene box in an incubator overnight at 32 °C under a relative humidity of 75–85%. Bees that emerged from the wax cells were collected and marked with a dot of paint on their thorax. 280 marked bees were then put back in the hive for 10 days before sampling and extraction of RNA and DNA for transcriptome analysis and quantification of *F. perrara* and *S. alvi* abundance, respectively. This procedure was repeated three times to have sufficient high quality samples with scab and without scab.

RNA and DNA extractions from honey bee gut tissues

The region of the scab, that is the posterior part of the pylorus, was dissected from CO_2-anesthetized bees by making a first perpendicular cut with a sterilized scalpel after the Malpighian tubules and a second perpendicular cut 1–2 mm away towards the rectum (see

Fig. 1A–C for the region that was dissected). For each replicate, a pool of seven pylori was added to a bead-beating tube containing about 200 μL of glass beads (0.75–1 mm in diameter, Roth) and 750 μL of TRI Reagent® (Sigma-Aldrich). For age-controlled hive bees, the pylori were pooled according to the presence/absence of the scab phenotype (Scab+/Scab− bees). The content of the bead-beating tubes was homogenized using a MagNA lyser instrument (Roche Molecular Diagnostics) three times 30 s at 7000 rpm with 30 s pauses on ice between homogenizations. Then, simultaneous RNA and DNA extractions were performed following the manufacturer's instructions, including an overnight precipitation at −20 °C, with an elution in 45 μL of nuclease-free water. After DNase treatment, the RNA was purified with the Nucleospin RNA clean-up XS kit (Macherey-Nagel, Germany). RNA quantity and integrity were assessed with a UV spectrophotometer (NanoDrop, Thermo Fisher Scientific) and a Bioanalyzer instrument (Agilent Technologies), respectively. For DNA extraction, DNA pellets suspended in 100 μL of 8 mM NaOH were subjected to a DNA clean-up procedure using the PCR and gel cleanup kit (Macherey-Nagel) according to the manufacturer's instructions with a final elution volume of 30 μL of nuclease-free water.

RNA sequencing

Total RNA samples for sequencing were selected based on RNA quality. Samples were sent to the Lausanne Genomic Technology Facility (LGTF, Switzerland) where TruSeq stranded mRNA libraries were generated (Illumina) including a polyA selection step in order to enrich for host mRNA. Fifteen libraries (three replicates of each of the following conditions: FP, SA and NC bees, and Scab+ and Scab− bees) were prepared and sequenced in two lanes on a Illumina HiSeq 2500 instrument to obtain single end 100-bp reads.

Differential gene expression analysis

Raw FASTQ files provided by the LGTF contained all reads and corresponding tags indicating whether they were accepted or filtered out according to the CASAVA 1.82 pipeline (Illumina). We kept only the accepted reads from CASAVA for further analysis. We controlled the quality of the data using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then used CUTADAPT v1.5 (Martin 2011) to trim adapters. Differential gene expression analysis was carried out using the Tuxedo pipeline (Trapnell *et al.* 2012) on filtered 100-bp-long reads. In short, we aligned the reads to the *A. mellifera* genome (Amel_4.5 assembly) (Honey Bee Genome Sequencing Consortium, 2006; Elsik *et al.* 2014) using TOPHAT v2.0.11 (Trapnell *et al.* 2009; Kim *et al.* 2013) while incorporating the official gene set annotation version 3.2 in GFF format (OGS_3.2). Transcriptomes were reconstructed by aligning the reads of each replicate using CUFFLINKS v2.2.1 (Trapnell *et al.* 2010; Roberts *et al.* 2011). Then, a merged assembly was computed based on OGS_3.2 and the individual transcriptomes using the CUFFMERGE package of CUFFLINKS. To determine significantly differentially expressed genes (SDEGs), we used the CUFFDIFF algorithm, which is part of the CUFFLINKS software suite. Differential gene expression analysis was assessed between all pairwise comparisons using CUFFDIFF and the merged transcriptome assembly (merged.gtf) with a FDR adjusted *P*-value threshold of 0.05. CUFFDIFF results were retrieved and visualized in R using the 'CUMMERBUND' package (Goff *et al.* 2013).

Annotation of significantly differentially expressed genes

A BLASTX analysis was performed on all coding nucleotide sequences from our data set using a local database containing protein sequences from *A. mellifera* and closely related species from NCBI (other *Apis* species, and multiple species from the genera *Bombus*, a total of 177′538 sequences). BLASTX results with an e-value threshold of 10^{-6} were loaded into BLAST2GO to carry out annotation of the coding sequences. Briefly, known protein domains were retrieved using INTERPROSCAN (IPS) with default options and corresponding GO terms from BLASTX and IPS results were merged in the final annotation. In cases where the gene product description was too vague (e.g. 'isoform A', 'partial'), we manually annotated the gene product based on BLASTX results. The same annotation steps were executed on all coding sequences with a local database consisting of protein sequences from *D. melanogaster* and closely related species (*Drosophila yakuba* and *Bactrocera dorsalis*, a total of 247′461 sequences) so as to obtain additional information. Finally, we retrieved the gene product and chromosomal location from BEEBASE based on the OGS3.2 gene ID (i.e. identifiers starting with 'GB') and chose either the gene product information from BEEBASE or from the BLAST2GO results manually. To determine whether certain functional gene categories were enriched among SDEGs, we used the BLASTX results in combination with the BLAST2GO-automated annotation pipeline (Conesa *et al.* 2005; Conesa & Gotz 2008) to perform a GO enrichment analysis. The implemented analysis consists of a Fisher exact test that tests whether the proportion of SDEGs associated with a given GO term is

significantly different than the proportion of the total number of genes associated with this GO term in the *A. mellifera* genome.

Quantitative PCR to determine colonization levels

Quantitative PCR (qPCR) was carried out on a StepOnePlus instrument (Applied Biosystems) to determine the bacterial loads of *S. alvi* and *F. perrara* in the pylori of experimentally colonized bees and in that of hive bees. The following run method was used a holding stage consisting of 2 min at 50 °C followed by 2 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 65 °C. A melting curve was generated after each run (15 s at 95 °C, 20 s at 60 °C and increments of 0.3 °C until reaching 95 °C for 15 s) so as to confirm the production of a single amplification product. qPCRs were performed in 10 μL reactions in triplicates in 96-well plates, and each reaction consisted of 1 μL of DNA, 3.2 μL of nuclease-free water, 0.4 μL of forward primer, 0.4 μL of reverse primer, and 5 μL of SYBR green 'Select' master mix (Applied Biosystems). For each target, standard curves were generated for absolute quantification using serial dilutions (from 10^7 to 10 copies) of the target amplicon cloned into the vector pGEM®-T (Promega AG).

Quantitative reverse transcription–PCR to confirm differential gene expression

To confirm the differential expression of genes identified by RNA sequencing, we conducted quantitative reverse transcription–PCR. To this end, we carried out two independent colonization experiments, which were set up in the same way as the RNAseq experiment. RNA was isolated as described above, and 300 ng of RNA was reverse-transcribed into cDNA (M-MLV Reverse Transcriptase; Promega AG) with either gene-specific primers for a subset of the SDEGs (B-gluc2, DOPA decarboxylase, defensin, IRP30, PGRP-S2) and *A. mellifera* actin, or with random primers (Promega AG). The cDNA was subsequently used as template to quantify expression of selected genes using the same qPCR protocol as above. Gene-specific primers were designed with the online tool OligoCalc (Kibbe 2007). To determine the fold change of gene expression between FP and SA bees, we used the Livak method (also known as $\Delta\Delta C_T$ method) with *A. mellifera* actin as reference gene. A list of all primers used in this study can be found in Table S1 (Supporting information).

Fluorescence and electron microscopy

For fluorescence *in situ* hybridization (FISH) microscopy, newly emerged laboratory bees were colonized as described above and the pylorus region sampled 10 days after colonization. Tissue fixation, embedding, sectioning and hybridization were carried out as described in Engel *et al.* (2015a). Species-specific probes fluorescently labelled with Cy3 and Cy5 were used to detect *F. perrara* and *S. alvi*, respectively. DNA was stained with DAPI (4′,6-diamidino-2-phenylindole).

For electron microscopy, pylorus regions were dissected in glutaraldehyde solution (EMS, Hatfield, PA, USA) 2.5% in phosphate buffer (PB 0.1M pH7.4) (Sigma-Aldrich) and fixed during 2 h at room temperature (RT). After washing three times in PB buffer, they were postfixed by a fresh mixture of osmium tetroxide 1% (EMS, Hatfield, PA, USA) with 1.5% of potassium ferrocyanide (Sigma-Aldrich) in PB buffer during 2 h at RT. The samples were then washed three times in distilled water and dehydrated in acetone solution (Sigma-Aldrich) at graded concentrations (30%–30 min; 70%–30 min; 100%–1 h; 100%–2 h). This was followed by infiltration in Epon (Sigma-Aldrich) at graded concentrations (Epon 1/3 acetone-1 h; Epon 3/1 acetone-1 h, Epon 1/1–2 h; Epon 1/1–12 h) and finally polymerized for 48 h at 60 °C in oven. Ultrathin sections of 50 nm were cut transversally on a Leica Ultracut and picked up on a copper slot grid 2 × 1 mm (EMS, Hatfield, PA, USA) coated with a polystyrene film (Sigma-Aldrich). Sections were poststained with uranyl acetate (Sigma-Aldrich) 4% in H2O followed by Reynolds lead citrate during 10 min. Micrographs were taken with a transmission electron microscope FEI CM100 at an acceleration voltage of 80 kV with a TVIPS TemCam-F416 digital camera. Large montage alignments were performed using blendmont command-line program from the IMOD software (Kremer *et al.* 1996).

Results

Genome-wide host response in the pylorus upon F. perrara colonization and scab formation

To determine the impact of the gut symbiont *F. perrara* on the honey bee, we identified transcriptome changes in the pylorus between 10-day-old bees that were monocolonized with either *F. perrara* or *S. alvi* (FP or SA bees) or left untreated (noncolonized bees, NC bees). qPCR analysis with species-specific primers confirmed that the experimental colonizations with *F. perrara* and *S. alvi* were successful. For both monocolonizations, similar numbers of the respective species were detected in the three replicates subjected to RNAseq analysis (Fig. S1, Supporting information). Importantly, qPCR signals in NC bees were below the detection threshold for both *F. perrara* and *S. alvi*. Moreover, all bees that were colonized with *F. perrara*, but none from the other

two conditions, showed the characteristic scab phenotype (Fig. 1) at day 10 of colonization. FISH microscopy of colonized bees further showed that both bacteria colonized the host epithelium in the pylorus region, while no signals for these bacteria could be detected in NC bees (Fig. 1A–C).

Illumina sequencing of the RNA yielded a total of 456′910′246 filtered reads (average of 30′460′683 reads per replicate), which were mapped to the *A. mellifera* genome with satisfactory overall mapping rates for all replicates (average over all replicates of 84.9%, see Table S2, Supporting information). A noticeable exception was the mapping rate of NC2 which was the lowest with 64.2% although still in the range of other recent RNAseq studies on honey bees (Corby-Harris *et al.* 2014b; McNeill *et al.* 2016).

Substantial host gene expression changes occurred in the pylorus region upon colonization with *F. perrara* relative to NC bees when compared to the changes arising from colonization with *S. alvi* relative to NC bees (Fig. 2A and Fig. S2 and Table S3, Supporting information). Colonization with *F. perrara* significantly changed the expression of 181 host genes (132 upregulated and 49 downregulated), while *S. alvi* colonization significantly altered the expression of only 41 host genes (32 upregulated and nine downregulated). In total, 27 genes (24 upregulated and three downregulated) were differentially expressed under both treatments (Table S3, Supporting information). All significantly differentially expressed genes (SDEGs) across laboratory conditions were plotted in a heatmap to represent their respective fold changes among replicates relative to the average RPKM (reads per kilobase per million mapped reads) value obtained for NC bees (Fig. 2B). The heatmap shows that not only FP bees have more SDEGs than SA bees, but also that these have higher fold changes. Hierarchical clustering places all three replicates of FP bees within a single cluster, whereas NC and SA bees form a mixed cluster. Analogous results were obtained using other clustering methods such as principal component analysis (PCA) and multiple dimension scaling (MDS, Fig. S3, Supporting information).

To focus the analysis on genes specifically differentially regulated between bees colonized with *F. perrara* and *S. alvi*, we normalized host gene expression between these two treatments. This direct comparison yielded 141 SDEGs in FP bees compared to SA bees (113 upregulated and 28 downregulated, Fig. 2A and Table S1, Supporting information). About 63.2% (n = 72) of the SDEGs between FP and SA bees were also differentially expressed between FP and NC bees. Overall, the genome-wide transcriptome analysis shows that *F. perrara* leads to a more pronounced host gene expression response than *S. alvi*, which is consistent with the morphological changes that develop in the pylorus (i.e. the scab phenotype) upon colonization with *F. perrara*, but not with *S. alvi* (Fig. 1A and B).

Functions involved in immune responses, transport and extracellular processes are enriched among the genes upregulated in F. perrara-colonized bees

To identify functional categories overrepresented among the SDEGs between FP and SA bees, a GO enrichment analysis was conducted. The analysis on significantly downregulated genes did not yield any enrichment, probably due to the relatively small

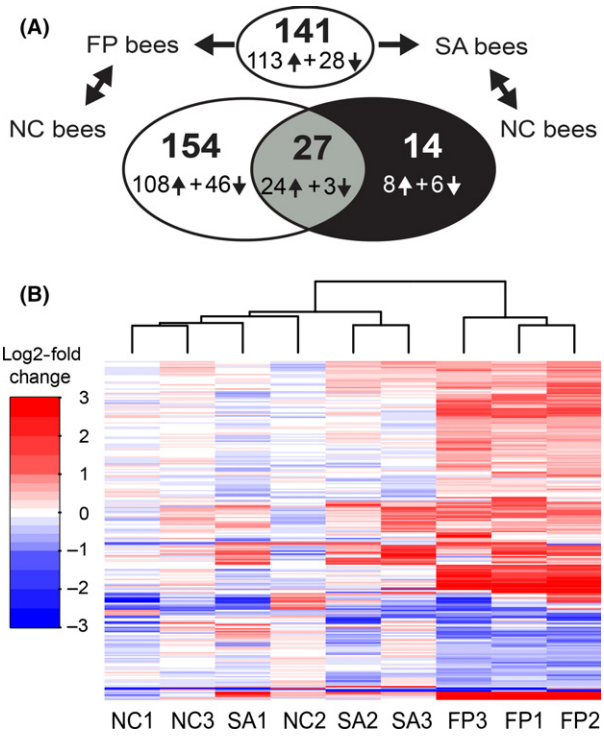


Fig. 2 Differential gene expression between honey bees monocolonized with either *Frischella perrara* (FP) or *Snodgrassella alvi* (SA) relative to noncolonized (NC) bees. (A) Venn diagram representing the number of significantly differentially expressed genes (SDEGs) between SA and FP bees, relative to NC bees. Numbers in the ellipse above the Venn diagram show the number of SDEGs for the direct comparison between FP and SA bees. Numbers in bold show the total number of SDEGs for each comparison, with the sum of upregulated and downregulated genes indicated below. The intersection corresponds to genes that were significantly differentially expressed in both comparisons. (B) Heatmap showing the log2-fold changes of gene expression between each replicate and the average RPKM value for NC bees. Rows correspond to the 240 unique SDEGs obtained from the three possible comparisons of the data sets shown in (A). The dendrogram was built by hierarchical clustering based on these 240 SDEGs using the Jensen–Shannon distance as metric.

number of genes in this set (28 genes), of which several had no significant BLAST hit or encoded proteins of unknown function (Fig. 3). In contrast to the downregulated gene set, several GO terms were enriched among the 113 genes upregulated in FP bees compared to SA bees (Fig. 3). These included 'Immune system process' (one-sided Fisher's exact test, FDR 2.4×10^{-2}, *P*-value 4.1×10^{-4}), 'Extracellular region' (FDR 1.2×10^{-2}, *P*-value 1.6×10^{-4}) and three GO terms related to localization and transport ('Establishment of localization' FDR 1.2×10^{-2}, *P*-value 1.3×10^{-4}, 'Transport' FDR 1.2×10^{-2}, *P*-value 1.3×10^{-4} and 'Localization' FDR 1.2×10^{-2} *P*-value 4.3×10^{-5}).

Upregulation of an immune-responsive protein, several antimicrobial peptides and pattern recognition receptors

The six genes with the highest fold changes (*irp30*, *cdc2c*, *apid73*, *abaecin*, *def1*, *b-gluc2*) all correspond to immune-related genes, in particular genes known to be activated in response to bacteria (Fig. 3 and Table S3, Supporting information). The gene coding for the immune-responsive protein 30 (IRP30) was the most highly upregulated gene between FP and SA bees, with an expression fold change of 137×. IRP30 [formerly HP30 (Randolt *et al.* 2008)] is a glycoprotein specific to social hymenopterans. It was shown to be induced in honey bees upon bacterial challenge or exposure to bacterial cell wall components (Albert *et al.* 2011). *apid1* (116×), *apid73* (113×), *abaecin* (111×) and *def1* (33×) are genes coding for the production of the antimicrobial peptides (AMPs) apidaecin type 14, apidaecin type 73, abaecin and defensin 1, respectively. In insects, the expression of AMPs is typically controlled by one of the two major immune signalling pathways, the immune deficiency (Imd) or the Toll pathway (Buchon *et al.* 2014). These pathways are initiated by the recognition of microbial cell wall components through a class of pattern recognition receptors (PRRs). Strikingly, we detected three upregulated genes coding for PRRs (Fig. 3 and Table S3, Supporting information): β-1,3-glucan recognition protein 2 (B-Gluc2, 38×), the peptidoglycan recognition protein S2 (PGRP-S2, 6×) and the peptidoglycan recognition protein S3 (PGRP-S3, 1.5×). In particular, the gene for B-Gluc2 was highly upregulated in FP bees compared to SA bees with a fold change of 38×. B-Gluc2 is a homolog of Gram-negative bacteria-binding proteins (GNBPs) from *D. melanogaster* with highest similarity to *Dm*_GNBP1 (from now on we will use the prefix '*Dm*_' to indicate gene or protein names from *D. melanogaster*). *Dm*_GNBP1 has been shown to bind lipopolysaccharides and β-glucan structures and to function in the Toll pathway of *Drosophila* in the recognition of Gram-positive bacteria and fungi (Gobert *et al.* 2003; Wang *et al.* 2006). Similarly, PGRP-S2 and PGRP-S3 are homologs of several peptidoglycan recognition proteins from *D. melanogaster*. PGRP-S2 shares the highest similarity to *Dm*_PGRP-SC2 which was shown to promote gut immune homeostasis to limit dysbiosis and extend lifespan (Guo *et al.* 2014), while PGRP-S3 is most similar to *Dm*_PGRP-SA which participates in the detection of Gram-positive bacteria and the activation of the Toll pathway in conjunction with GNBP1 (Michel *et al.* 2001; Gobert *et al.* 2003; Wang *et al.* 2006). Two additional genes encoding putative PRRs were identified to be subtly upregulated upon colonization with *F. perrara*, including *dscam* (Down syndrome cell-adhesion molecule, 1.6×) and *tep3* (complement-like thioester-containing protein 3, 1.3×). The upregulation of AMP and PRR genes upon colonization with *F. perrara* compared to colonization with *S. alvi* was confirmed by RT-qPCR in two independent colonization experiments and resulted in similar fold changes as in the RNAseq analysis (Fig. S4, Supporting information). The reproducibility of these

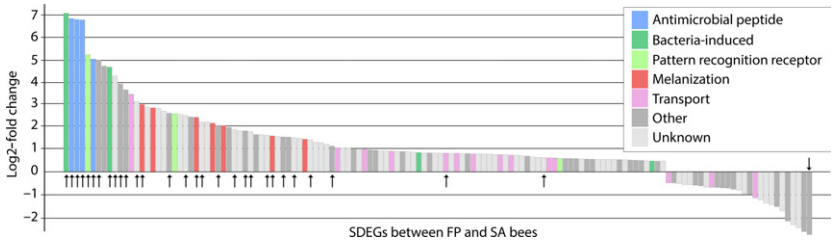


Fig. 3 Log2-fold changes of significantly differentially expressed genes (SDEGs) between honey bees monocolonized with *F. perrara* (FP) and *S. alvi* (SA). Genes from enriched GO categories (i.e. immune-related and transport-related) are highlighted in colour according to the legend. Arrows indicate genes that are also differentially expressed between hive bees with and without scab phenotype (see Fig. 5). Of note, pattern recognition receptors (light green) may also be part of the melanization cascade (red). Two genes were expressed exclusively in FP bees; and one gene was expressed exclusively in SA bees, resulting in positive and, respectively, negative infinite fold changes. These were excluded from the figure for practical reasons. A complete list of SDEGs with gene names, log2-fold changes and annotation information is available in Table S3 (Supporting information).

results shows that *F. perrara* triggers a highly specific and robust immune response in the pylorus.

Upregulation of the melanization cascade

In line with the hypothesis that the scab phenotype stems from a melanization response of the host, several genes involved in the production of melanin were found to be upregulated between FP and SA bees (Figs 3 and 4, Table S3, Supporting information). This included the two enzymes tyrosine hydroxylase [Krishnakumar *et al.* 2000) (*Dm_pale*, 3.1×)] and DOPA decarboxylase (*ddc*, 8.1×), which convert tyrosine to DOPA and further into dopamine. Dopamine is used as a substrate by the enzyme phenoloxidase (PO) to produce quinone, which in turn is polymerized into melanin (González-Santoyo & Córdoba-Aguilar 2012). Tyrosine hydroxylases need the cofactor tetrahydrobiopterin which is synthesized by the enzyme GTP cyclohydrolase (*Dm_Punch*) and which was also upregulated (4.2×) in FP vs. SA bees. We did not find any phenoloxidase homolog to be significantly upregulated in FP bees. However, these enzymes are typically stored as zymogens (pro-POs) and are activated by proteolytic cleavage rather than by *de novo* gene expression (Cerenius & Söderhäll 2004; Cerenius *et al.* 2008). The activation occurs through a stepwise process involving PRRs, a serine protease activation cascade and serine protease inhibitors (i.e. serpins). As mentioned above, several PRRs were upregulated upon *F. perrara* colonization (Fig. 3 and Table S3, Supporting information). In particular, *b-gluc-2* (38×) and *pgrp-s3* (1.5×) are homologous to *Dm_gnbp1* and *Dm_pgrp-sa*, respectively, which are known to induce the Toll pathway and subsequent PO activation in *D. melanogaster* (Binggeli *et al.* 2014). Moreover, we found three homologs of serine proteases and one gene encoding a serpin-like protein to be upregulated in FP bees (2.8–7.3×). As for other immune response genes, we confirmed the upregulation of the melanization response gene DOPA decarboxylase in FP bees in independent colonization experiments (Fig. S4, Supporting information). Overall, these findings provide first evidence at the transcriptional level that the scab phenotype indeed originates from a melanization response of the host (Fig. 4).

Upregulation of transporters, extracellular matrix proteins and detoxification functions

In addition to immune-related genes, we found a relatively large number of genes ($n = 20$) implicated in transport mechanisms to be upregulated in FP bees compared to SA bees (Fig. 3 and Table S3, Supporting information). These included genes coding for sugar transporters (three genes encoding facilitated trehalose transporters, 2.1–3.1×, and one encoding a glucose transporter, 1.8×), amino acid transporters (3 genes, 1.7–11.1×), cation transporters (3 genes, 1.8× and 2.9×) and solute transporters (7 genes, 1.7–2.5×).

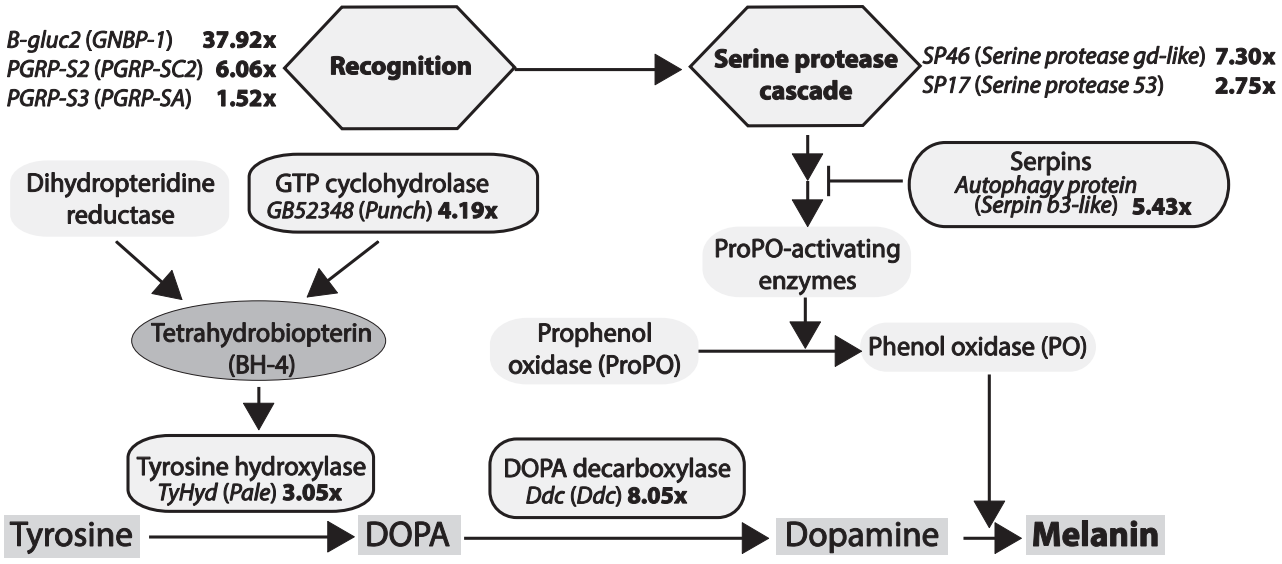


Fig. 4 The *Drosophila melanogaster* melanization cascade, adapted from De Gregorio *et al.* (PNAS, 2001). Processes that are upregulated in bees colonized with *F. perrara* compared to bees colonized with *S. alvi* are indicated with a black frame, and the gene expression fold change is given. The names of upregulated genes are indicated with their respective homolog in *D. melanogaster* in parentheses.

Three genes upregulated in FP bees (1.5× and 5.5×) contain chitin-binding domains with a putative role in extracellular processes. Chitin is a linear biopolymer of *N*-acetyl-glucosamines (GlcNAc), which is a crucial component of the insect exoskeleton, the peritrophic matrix in the midgut, and the cuticle lining in the fore- and hindgut and the trachea. One of these upregulated genes encoded a peritrophin-like protein (5.5×). Peritrophins are major components of the peritrophic matrix (PM) with putative roles in formation and remodelling of the extracellular structure (Zhu *et al.* 2016). The other two genes belong to protein families which are less well characterized, but which have been implicated in similar processes: a member of the obstructor gene family (Behr & Hoch 2005) (2×) and a chitin deacetylase (Zhao *et al.* 2010) (1.5×) (Table S3, Supporting information).

We further found two genes involved in detoxification processes to be upregulated: the esterase FE4-like (13.0×) which confers insecticide resistance in the peach–potato aphid (Field *et al.* 1989) and the cytochrome Cyp6bd1 which is related to *Dm*_Cyp6 g2 (2.0×). The latter leads to resistance to the insecticides diazinon and nitenpyram (Daborn *et al.* 2007). Another markedly induced gene (6.1×) was the multicopper oxidase 1 (MCO1) that was shown to be upregulated upon bacterial challenge in *Anopheles gambiae* (Gorman *et al.* 2008) (Table S3, Supporting information). Further, this enzyme seems to also participate in iron metabolism in *D. melanogaster* by transforming ferrous iron to ferric iron which can then be bound by transferrin thereby leading to iron storage, iron withholding from pathogens, regulation of oxidative stress and/or epithelial maturation (Lang *et al.* 2012). Interestingly, the gene *tsf1* encoding transferrin 1 was also markedly upregulated in Fp bees compared to SA bees (25.8×). However, it needs to be noted that certain multicopper oxidases are known to also have laccase activity oxidizing o- and p-diphenols in which case MCO1 could also participate in the melanization response (Dittmer & Kanost 2010). Overall, the large number of genes involved in transport processes, modulation of the extracellular space or stress responses provides evidence that the tissue homeostasis in this part of the gut may be disturbed in the presence of *F. perrara*.

Differentially expressed genes between hives bees with and without scab

To determine the relevance of the characteristic host response triggered by *F. perrara* under laboratory conditions, we investigated whether similar responses could be found in 10-day-old hive bees that acquired their gut microbiota through contacts with nestmates and hive components, but differed in the prevalence of *F. perrara* and the presence of the scab phenotype. While *F. perrara* was detected in all samples obtained from hive bees, qPCR analysis confirmed that the three replicates of bees without scab (Scab− bees) all had lower levels of *F. perrara* than those with scab (Scab+ bees) (Fig. S1, Supporting information) confirming previous results (Engel *et al.* 2015a). By contrast, the levels of *S. alvi* did not much differ between the two groups (Fig. S1, Supporting information).

For differential gene expression, we conducted the same analysis as for the experimentally colonized bees: we first compared gene expression in Scab+ and Scab− bees relative to NC bees. This resulted in a substantially higher number of SDEGs genes than the transcriptome analysis of monocolonized, laboratory-raised bees (Fig. S5, Supporting information): we detected 794 SDEGs (549 upregulated and 245 downregulated genes) and 752 SDEGs (515 upregulated and 237 downregulated) in Scab+ and Scab− bees, respectively, of which 509 genes were differentially regulated in both conditions compared to NC bees (362 upregulated and 147 downregulated). We then directly compared Scab+ to Scab− bees (i.e. without using NC bees as a reference) to highlight the differences between hive bees with and without the scab phenotype. This comparison resulted in only 135 SDEGs (75 upregulated and 60 downregulated, Fig. 5) of which 30 genes were also differentially regulated between FP bees and SA bees (see arrows in Fig. 3). Strikingly, many of the immune genes specifically upregulated by *F. perrara* under laboratory conditions were also among the 135 SDEGs in hive bees, including the immune-responsive protein IRP30, all previously detected AMPs and genes of the melanization cascade. Most of them were upregulated in hive bees with scab compared to hive bees without scab, which is consistent with the higher prevalence of *F. perrara* in these bees. For example, genes coding for DOPA decarboxylase, tyrosine hydroxylase and a serpin b3-like were induced both, under laboratory conditions in FP bees compared to SA bees and under hive conditions in Scab+ compared to Scab− bees, suggesting key roles in the host response towards *F. perrara* and the formation of the scab phenotype. However, a number of the serine proteases were actually downregulated in hive bees with scab, which may indicate the presence of possible negative feedback loops in the melanization cascade.

Discussion

Frischella perrara colonizes a restricted region in the pylorus and induces the so-called scab phenotype (Fig. 1) (Engel *et al.* 2015a). Our transcriptome analysis

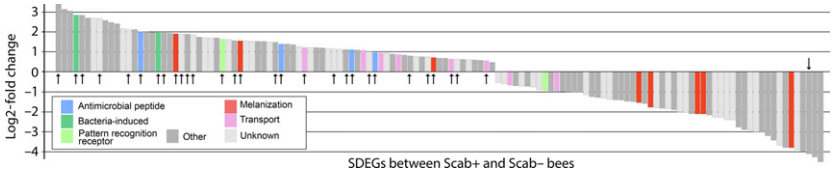


Fig. 5 Log2-fold changes of significantly differentially expressed genes (SDEGs) between 10-day-old hive bees with (Scab+) and without scab phenotype (Scab−). Immune-related and transport-related genes are highlighted in colour according to the legend. Arrows indicate genes that are also differentially expressed in bees colonized with *F. perrara* compared to bees colonized with *S. alvi*. Four genes were expressed exclusively in Scab− bees resulting in negative infinite fold changes. These were excluded from the figure for practical reasons. A complete list of SDEGs with gene names, log2-fold changes and annotation information is available in Table S3 (Supporting information).

provides first insights into the host response underlying this specific interaction within the bee gut. Compared to the gut symbiont *S. alvi*, we find that *F. perrara* causes a strong transcriptional response in the pylorus. More host genes were differentially regulated in FP bees than in SA bees, and the fold changes were generally higher (Figs 2 and 3 and Table S3, Supporting information). We find functions linked to immune system, transport and extracellular processes to be overrepresented among the differentially regulated genes, which is in line with the morphological changes elicited by *F. perrara* on the epithelial surface. Specifically, key steps of the melanization cascade were induced providing first evidence at the transcriptional level that the scab phenotype corresponds to a melanization response of the host (Fig. 4). Moreover, AMPs, pattern recognition receptors, and bacteria-induced proteins of unknown function (e.g. *irp30*) belonged to the genes with the highest fold changes (Fig. 3 and Table S3, Supporting information). This shows that *F. perrara* not only induces a melanization response that leads to the scab phenotype, but also activates other parts of the host immune system including signal perception and effector functions.

Moreover, our data provide evidence that the physiological conditions in the pylorus are altered, as metabolite transporters, matrix proteins and stress-related genes were induced. Melanin is often produced upon tissue damage and is accompanied by the generation of reactive oxygen species (Nappi & Christensen 2005). This can rupture gut homeostasis resulting in the induction of the identified genes.

Overall, the transcriptional changes induced by *F. perrara* seem to be reminiscent of a host response towards a pathogen infection rather than a beneficial or commensal gut symbiont (Casteels *et al.* 1993; Evans 2004; Evans *et al.* 2006; Buchon *et al.* 2009; Vieira *et al.* 2014). Intriguingly, a recent study on the effect of diet quality showed that the consumption of aged pollen resulted in a marked increase of the abundance of *F. perrara* compared to other gut bacteria in the hindgut, which was correlated with impaired host development and increased mortality (Maes *et al.* 2016). However, whether *F. perrara* is the direct cause of these detrimental effects has not been investigated.

Despite the vast difference between hive and laboratory conditions, we found that a relatively large fraction of the genes induced in laboratory bees upon colonization with *F. perrara* were also differentially regulated between hives bees with and without scab phenotype. In particular, many of the immune functions induced by *F. perrara* in laboratory bees were also found to be upregulated in hive bees with scab phenotype (Figs 3 and 5). However, the fold changes were generally lower. This may be explained by the presence of *F. perrara* not only in hive bees with scab, but also in hive bees without scab, though at lower abundance (Fig. S1, Supporting information). In addition, the presence of other gut bacteria, the natural diet and social interactions with nest mates may dampen the host response to *F. perrara* in hive bees compared to laboratory conditions. Yet, our comparison shows that the specific host response to *F. perrara*, measured under laboratory conditions, also occurs under hive conditions. Hive bees with and without scab seem to have distinct immune activation states in the pylorus. This may have important implications for the host: on one side, immune responses are energetically expensive and hence harbouring high numbers of *F. perrara* may be disadvantageous for the host. On the other side, pre-activation of the immune system can enhance protection against subsequent pathogen challenges. This phenomenon is generally referred to as immune priming and has been shown to exist in a wide range of insects, including bees (Sadd & Schmid-Hempel 2006; Rodrigues *et al.* 2010; Milutinović *et al.* 2016). Several characteristics make it conceivable that the immune response induced by *F. perrara* may serve as a priming response for the host. First of all, *F. perrara* colonizes the gut immediately after adult emergence ensuring immune system priming early in life. Second, the pylorus marks the transition between the midgut and the hindgut.

Immune activation in this narrow, funnel-like region of the gut may hinder foreign microbes from invading the hindgut environment. Third, several of the immune genes induced by *F. perrara* encode AMPs, which have a broad antimicrobial activity (Bulet *et al.* 1999) and are thus likely to act not only against *F. perrara,* but also against a wide range of other microbes. Moreover, the upregulated PRRs typically function as epithelial receptors that activate or modulate immune responses upon binding of microbe-associated molecular patterns (such as peptidoglycan) (Royet & Dziarski 2007). Increased expression of these receptors may raise the host's sensitivity towards microbial encounters facilitating adequate immune responses.

Notably, some of the induced PRRs may also act as negative regulators to avoid overactivation of the immune system. In *D. melanogaster*, for example, homologs of PRGP-S2 (which was 6× fold upregulated in FP bees) impair immune pathway activation by binding or cleavage of peptidoglycan, thereby inhibiting further AMP induction and preventing host damage from overstimulation of the immune system (Royet *et al.* 2011; Guo *et al.* 2014). Future studies will show whether the immune response in the pylorus is specifically targeted towards *F. perrara* or whether other microbes are also affected.

In a previous study, we found that the number of *F. perrara* bacteria in the pylorus rapidly increases until day 5 postemergence and then stays more or less constant (Engel *et al.* 2015a). Even old forager bees still harbour high numbers of *F. perrara* in the gut. We thus hypothesize that the immune response elicited by *F. perrara* does not clear the bacteria from the gut, but rather plays a role in limiting colonization levels. In support for this hypothesis, a previous study using the silkworm *Bombyx mori* showed that the inhibition of the melanization response using phenylthiourea (PTU) through feeding increased bacterial levels in the insect's faeces where the melanization was diminished (Shao *et al.* 2012). Similarly, impeding the honey bee host immune response in response to *F. perrara* using melanization inhibitors, or by targeting specific host genes via RNAi will provide further insights concerning the impact of these immune subsystems in controlling *F. perrara* colonization or persistence. In fact, host-mediated regulation of the endogenous microbiota via the activation of AMPs and PRR is a well-established concept and has been shown to play key roles for maintaining gut homeostasis in a wide range of animals (Royet *et al.* 2011). For example, weevils control the level of their intracellular symbionts via the production of a specific AMP (Login *et al.* 2011). In *D. melanogaster*, the microbiota composition is altered when the IMD pathway is inactive, suggesting that immune genes under the control of IMD regulate microbiota composition (Broderick *et al.* 2014). In consistence with our findings, several AMPs were found to be upregulated in flies with microbiota compared to axenic flies providing further evidence that these immune effectors play an important role in regulating gut bacteria levels (Broderick *et al.* 2014). This seems also to be the case in mammals as exemplified by several studies conducted in mice (Cash *et al.* 2006; Salzman *et al.* 2010). The fact that some of the AMP genes are also moderately upregulated in bees monocolonized with *S. alvi* (Table S3, Supporting information) suggests that this may be a general mechanism of honey bees to regulate their microbiota. Another colonization control more specifically targeted towards *F. perrara* may be conferred by two other genes, encoding transferrin 1 and multicopper oxidase 1, both of which were only induced in the pylorus upon colonization with *F. perrara*. Homologs of these proteins have been shown to facilitate sequestration of free iron thereby withholding this essential nutrient from pathogens and limiting infection (Skaar 2010). Interestingly, iron acquisition genes were recently shown to be critical fitness factors for bacteria in the honey bee gut (Powell *et al.* 2016) indicating that iron indeed constitutes a limiting nutrient in this environment and that lowering its availability may be an efficient mechanism to control bacterial growth.

Despite the fact that *F. perrara* induces a strong host response in the pylorus, the bacterium is a persistent member of the honey bee gut microbiota. It can be found in every colony of *A. mellifera* worldwide and has also been detected in other species of the genus *Apis* (Ahn *et al.* 2012; Engel *et al.* 2015a; Saraithong *et al.* 2016) . Other gut bacteria do not seem to outcompete *F. perrara,* although they are much more abundant, colonize the same gut regions and harbour similar metabolic capabilities (Engel *et al.* 2015a,b). These observations may suggest that either the host or other microbiota members profit from the presence of *F. perrara*. Such beneficial effects may be linked to the induction of the immune response (e.g. immune priming, see above), but could also involve other functions of *F. perrara* such as breakdown of dietary compounds, nutrient complementation or the engagement in syntrophic networks with other gut bacteria.

Another important point that remains to be addressed is why *F. perrara* but not the tested strain of *S. alvi*, induces the scab phenotype. Possibly, *F. perrara* encodes a specific 'virulence' factor that causes tissue damage or stress in the pylorus and in turn activates the melanization response of the host. Alternatively, high loads of *F. perrara* may result in an overstimulation of the immune system, for example by shedding large amounts of bacterial peptidoglycan. This could cause

immunopathology in the gut upon which the host activates the melanization response. Monitoring the different host responses over time upon bacterial colonization will provide further insights into this possibility. Finally, specialized host cell populations may be responsible for inducing the melanization response in the pylorus, but not in other parts of the gut. The timing of the colonization or the bacterial density may also be factors that can explain the specific induction of the melanization response by this bacterium.

In conclusion, our study shows that different gut bacteria trigger distinct host responses in the honey bee gut. Specifically, *F. perrara* causes a strong immune activation that leads to the development of the scab phenotype. The wide distribution of this gut symbiont in honey bee populations worldwide suggests that this specific immunomodulation may be of relevance for bee health and disease. Future studies should focus on the impact of *F. perrara* on gut homeostasis and dissect the molecular mechanisms underlying the specificity of this symbiont–host interaction. Assessing the diversity of different *F. perrara* strains and their respective contribution to scab formation and to host immune response in *A. mellifera* and other bee species also remains to be investigated. Because *F. perrara* can be cultured, and controlled infections can be established, this host–microbe interaction presents a very promising system to study the role of the immune system in regulating symbiosis.

Acknowledgements

We would like to thank the staff of the Lausanne Genomic Technologies Facility (University of Lausanne), in particular Johann Weber and Keith Harshman for their help in the design of the RNA-seq experiment and for performing the sequencing. We are grateful to Bruno Humbel and Jean Daraspe (Electronic Microscopy Facility) for providing technical assistance to generate the electronic microscopy pictures and to Berra Erkosar Combe for her constructive feedback that allowed to improve this manuscript. The computations were performed at the Vital-IT (http://www.vital-it.ch) Center for high-performance computing of the SIB Swiss Institute of Bioinformatics. This research was funded by the Swiss National Science foundation grants PZ00P3_148264 and 31003A_160345 (to P.E.).

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P.E. and O.E. designed research and wrote the manuscript. P.E., O.E. and K.S. conducted experiments. P.E and O.E analyzed the data. All authors contributed to the final version of the manuscript.

Data accessibility

Raw multiplexed FASTQ files and associated metadata are available on the NCBI Sequence Read Archive (SRA) under the Bioproject Accession no. PRJNA343067 (SRA Accession nos SRR4292727, SRR4292728, SRR4292734, SRR4292735, SRR4292736, SRR4292737, SRR4292738, SRR4292739, SRR4292740, SRR4292741, SRR4292729, SRR4292730, SRR4292731, SRR4292732 and SRR4292733).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Number of bacteria in the pooled pylorus samples subjected to RNAseq as determined by qPCR using species-specific primer pairs. NC, non-colonized bees; SA, *S. alvi* colonized bees; FP, *F. perrara* colonized bees; Scab−, hive bees without scab; Scab+, hive bees with scab.

Fig. S2 Scatterplots of gene expression.

Fig. S3 Multidimensional reduction of the RNAseq gene expression data.

Fig S4 Confirmation of significantly differentially expressed genes (SDEGs) by RT-qPCR.

Fig. S5 Venn diagram indicating the number of significantly differentially expressed genes (SDEGs) in the pylorus of 10-day old bees between Scab+ bees and non-colonized bees (white and grey areas) and between Scab− bees and non-colonized bees (black and grey areas).

Table S1 Primers used in this study.

Table S2 Overview of the RNAseq experiments.

Table S3 Lists of significantly differentially expressed genes (SDEGs) between different treatments.

Supporting information

The supporting information for the above publication can be found in the online version of the article or can be downloaded from the following link :
https://drive.switch.ch/index.php/s/YrfuNQnedQjMvTc
Which can also be accessed by scanning the following QR code :

Filename	Description
mec14058-sup-0001-SupInfo.pdf *PDF document, 572.8 KB*	**Fig. S1** *Number of bacteria in the pooled pylorus samples subjected to RNAseq as determined by qPCR using species-specific primer pairs. NC, non-colonized bees; SA, S. alvi colonized bees; FP, F. perrara colonized bees; Scab−, hive bees without scab; Scab+, hive bees with scab.* **Fig. S2** *Scatterplots of gene expression.* **Fig. S3** *Multidimensional reduction of the RNAseq gene expression data.* **Fig S4** *Confirmation of significantly differentially expressed genes (SDEGs) by RT-qPCR.* **Fig. S5** *Venn diagram indicating the number of significantly differentially expressed genes (SDEGs) in the pylorus of 10-day old bees between Scab+ bees and non-colonized bees (white and grey areas) and between Scab− bees and non-colonized bees (black and grey areas).* **Table S1** *Primers used in this study.* **Table S2** *Overview of the RNAseq experiments*.
mec14058-sup-0002-TableS3.xlsx *application/msexcel, 246.4 KB*	**Table S3** *Lists of significantly differentially expressed genes (SDEGs) between different treatments.*

1.2) Host survival experiments

Introduction

Our transcriptome analyses showed that *F. perrara* induces a significant immune response in the pylorus compared to *S. alvi.* Thus, we wondered whether this bacterium has a negative impact on the host. To this end, we tested whether colonization of bees with *F. perrara* would lead to a decreased lifespan of honey bees compared to bees colonized with *S. alvi* or non-colonized bees. We conducted survival experiments so as to compare the host lifespan under a protein-rich and protein-free diet.

Materials and methods

Rearing of microbiota-depleted bees

Honey bees in the pupa stage were collected from a brood frame and incubated for 2 days as described in chapter 1.1. In total, 18 cages containing ~20 adult microbiota-depleted bees were set up.

Bacterial colonization of microbiota-depleted bees

F. perrara and *S. alvi* were grown in 6 replicates on brain–heart infusion agar (BHIA) at 37°C under anaerobic conditions and on tryptic soy agar (TSA) at 37 °C in 5% CO2, respectively. After two days of growth, bacteria were

restreaked onto new plates for an additional day. Bees from six cages were colonized with *F. perrara* (FP treatment), bees from six cages were colonized with *S. alvi* (SA treatment) and six cages contained non-colonized bees (NC treatment). For bacterial colonization, microbiota-depleted honey bees from one cage were first anesthesized by cooling at 4°C for 15 min and placed directly on top of the plate containing bacteria covered with a holed plastic cup for 1h, and placed back into their cage.

Survival experiments and dietary conditions

In order to assess if different diets may affect the lifespan of bees in response to bacteria, we set up two different diets. Half of the cages from each treatment (i.e. 3x FP, 3x SA, 3x NC) included pollen in troughs and sugar water *ad libitum* in a 2 mL tube with small holes. The other half of the cages contained only a source of sugar water (also provided *ad libitum*) but no pollen. Survival data was analyzed using Cox Proportional Hazards Model in R using *coxph* function in *coxme* package (Therneau, 2015). Pairwise comparisons were done using Tukey's Post-Hoc Test with *glht* function in *multcomp* package (Hothorn et al., 2008). P-values were adjusted for multiple comparisons using the "single-step" method.

Results

Colonization with F. perrara does not reduce the lifespan of laboratory-raised honey bees whether bees were fed pollen or not

Survival experiments with mono-colonized (i.e. FP and SA treatments)and NC bees were performed under two different diets: sugar water + pollen and sugar water only. Overall, these experiments indicate that *F. perrara* colonization does not have a deleterious impact on honey bee survival under laboratory conditions (**Fig. 6**). When pollen was included in the diet (**Fig. 6A**), bees colonized with *F. perrara* had a slightly higher overall survival rate compared to non-colonized bees and, to a lesser extent, also to bees colonized with *S. alvi*. The ANOVA indicated that there was a significant effect of treatments (FP, SA or NC) on the survival (p-value = 0.002046, df=2). The tests for general linear hypotheses showed a significant difference between the FP and NC treatments (adjusted p-value=0.00266) and between FP and SA treatments (adjusted p-value = 0.03194) but not between the SA and NC treatments (adjusted p-value = 0.61381).

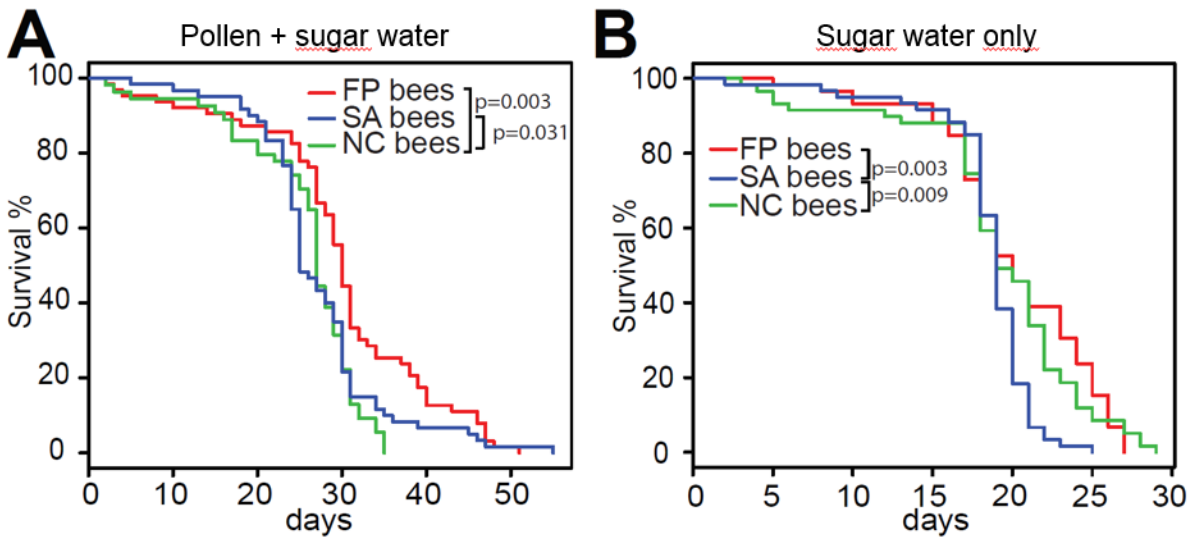

Fig. 6 *Survival curves of bees colonized with F. perrara (FP) or S. alvi (SA) or non-colonized (NC) bees fed sugar water in the presence (B) or absence (C) of pollen (~20 bees in triplicates for each curve). Pairwise comparisons with significant differences (Tukey's post-hoc test) are indicated with their respective p-values.*

In the presence of only sugar water (**Fig. 6B**), bees had an overall lower survival than bees which were also fed pollen, with all bees from a given treatment being dead by day 25 to 30 of age (compared to day 34 to 53 for the pollen diet). As before, the ANOVA indicated a significant effect of treatment (p-value = 0.002089, df=2). However, statistically significant differences were only found between SA and FP treatments (adjusted p-value = 0.00257), as well as between SA and NC treatments (adjusted p-value = 0.00887), but not between FP and NC treatments (adjusted p-value =

0.91638). Interestingly, in the case of the diet consisting of only sugar water, bees colonized with *S. alvi* had a slightly shorter survival than NC bees.

Conclusions

We found that colonization with *F. perrara* does not reduce host lifespan relative to colonization with *S. alvi* or to non-colonized bees under laboratory conditions. Furthermore, this was observed for both diets, suggesting that even under a protein-free diet, *F. perrara* does not reduce host lifespan. While our survival experiments show that *F. perrara* does not have a significant deleterious effect on honey bee survival under laboratory conditions, we cannot rule out that *F. perrara* affects honey bee fitness under natural conditions (i.e. in the hive). In particular, it remains to be determined if *F. perrara* colonization affects host fitness in the context of invasion by certain pathogens.

1.3) Melanization inhibition experiments

Introduction

Melanization plays an important role in the insect immune system in response to pathogens and leads to the formation of highly reactive species (Ayres and Schneider, 2008; Nappi and Christensen, 2005; Cerenius and Söderhäll, 2004; Marmaras et al., 1996; Söderhäll and Cerenius, 1998). In the honey bee, the gut symbiont *F. perrara* causes the scab phenotype which corresponds to melanization and co-localizes with *F. perrara* in the pylorus region of the gut (Engel et al., 2015a). We have previously shown that colonization of bees with *F. perrara* leads to the upregulation of host genes involved in the melanization cascade as well as other immune-related genes such as antimicrobial peptides and pattern recognition receptors (Emery et al., 2017). Our RNA-seq analysis of the pylorus region upon colonization witrh Fp provides clear evidence that the scab phenotype presents a melanization response to the colonization of *F. perrara*. However, the importance of this melanization for establishing, maintaining or controlling the symbiosis with this particular gut symbiont is still unclear. Melanization may allow bees to tolerate *F. perrara* by limiting bacterial outgrowth in the pylorus. Another possibility is that *F. perrara* needs the melanization reaction to colonize and persist in the host niche by yet unknown mechanisms. Since melanization produces cytotoxic compounds, *F. perrara*

may have a competitive advantage in the pylorus relative to other gut microbiota members which may be more susceptible to this environment.

Inhibiting the melanization response experimentally while allowing F. perrara to establish colonization may provide insights into the role of melanization in the symbiosis between *F. perrara* and the honey bee. Another PhD student in the laboratory, Konstantin Schmidt, first attempted to use RNA interference technology (RNAi) to knock down two host genes involved in the melanization cascade (i.e. tyrosine hydroxylase and DOPA decarboxylase) but did neither obtained consistent reductions in scab production nor significant reduction of the transcription of the targeted genes (data not shown). RNAi seems to be notoriously difficult in bees as also other groups have experienced similar problems (personal communications at conferences). Therefore, I considered other possibilities. One option is to use chemical inhibitors of enzymes involved in the melanization cascade such as phenylthiourea (PTU), a competitive inhibitor of DOPA (Ryazanova et al., 2012). However, despite successful melanization inhibition with PTU in several insects (Shao et al., 2012; Zlotkin et al., 1973; Beresky and Hall, 1977), PTU has also been shown to have a wide array of side effects, including increased mortality (MacDonald et al., 2015; Dixit and Perti, 1965; Ogita, 1958).

Here, I attempted to knock down the melanization response of honey bees colonized with *F. perrara* by feeding bees with a range of different PTU concentrations. I used the percentage of bees with a scab at day 10 post colonization as a readout for melanization inhibition. I also tested for the toxicity of PTU at different concentrations in non-colonized honey bees and found toxicity effects on bees for the highest PTU concentrations.

Materials and methods

Rearing of microbiota-depleted bees

Honey bees in the pupa stage were collected from a brood frame and incubated for 3 days as described in chapter 1. In total, 12 cages containing ~25 adult microbiota-depleted bees were set up.

Colonization of microbiota-depleted bees with *F. perrara*

F. perrara ESL0034 (wild type) was plated from stock on tryptone yeast extract agar (TYG) plates and was incubated at 35°C under anaerobic conditions for two days. *F. perrara* was then restreaked onto new TYG plates for an additional day. In order to make the inoculation solution, cells were harvested in 1 mL 1x PBS and the optical density at a wavelength of 600 nm (OD600) was measured. The bacterial solution was adjusted to an OD600 of 0.1 in 1x PBS plus sugar–water (1:1). Microbiota-depleted honey bees were

starved for 2 hours by removing the sugar water solution from their cages. Bees were then anesthetized by cooling at 4°C for 15 min and transferred onto ice. Each bee was then placed at room temperature in a 2 ml Eppendorf tube modified with a hole at the bottom to let only the head of the bee out of the tube. Actively moving bees were individually fed 5 µl of the inoculation solution containing *F. perrara*. Successfully colonized bees were placed back into their respective cages while bees that did not ingest the entire inoculum were excluded from the experiment. Bees were then were placed in an insect chamber at 32 °C under a relative humidity of 75–85% and daily checked for dead bees until day 10 post colonization when they were dissected and the percentage of scabs for each condition recorded.

PTU dilutions and feeding to bees

In order to test the effect of PTU over a broad range of concentrations, we performed sequential 3x dilutions from water-saturated PTU in water (i.e. 16.4mM, 5.5mM, 1.8mM, 608µM and 202 µM). Each PTU concentration mixture (or pure water for the negative control without PTU) was mixed 1:1 with sugar water and put in 2mL feeding tubes in the cages containing the bees. Feeding tubes were replaced every two days until the end of the experiment.

Survival experiment

We conducted a survival experiment to test if the range of PTU concentrations chosen had an effect on bee survival for two groups of bees: non-colonized bees and bees colonized with *F. perrara*. We assessed the survival of these two groups under the five PTU concentrations described above and the negative control for a duration of 10 days. Survival data was analyzed using Cox Proportional Hazards Model in R using *coxph* function in *coxme* package (Therneau, 2015). Pairwise comparisons were done using Tukey's Post-Hoc Test with *glht* function in *multcomp* package (Hothorn et al., 2008). P-values were adjusted for multiple comparisons using the "single-step" method.

Results

None of the non-colonized bees sampled at day 10 developed a scab, independently of the PTU concentration received. By contrast, 40-75% of bees colonized with *F. perrara* sampled at the same time point had a scab (**Fig. 7**). Only 75% of bees colonized with *F. perrara* but which did not receive PTU developed a scab which is lower than the percentages usually obtained for this condition at this time point (i.e. 90-100%). There seems to be a trend for lower scab percentages in response to higher PTU concentrations except for the PTU concentrations at 1.82 mM and 16.42 mM. The smallest

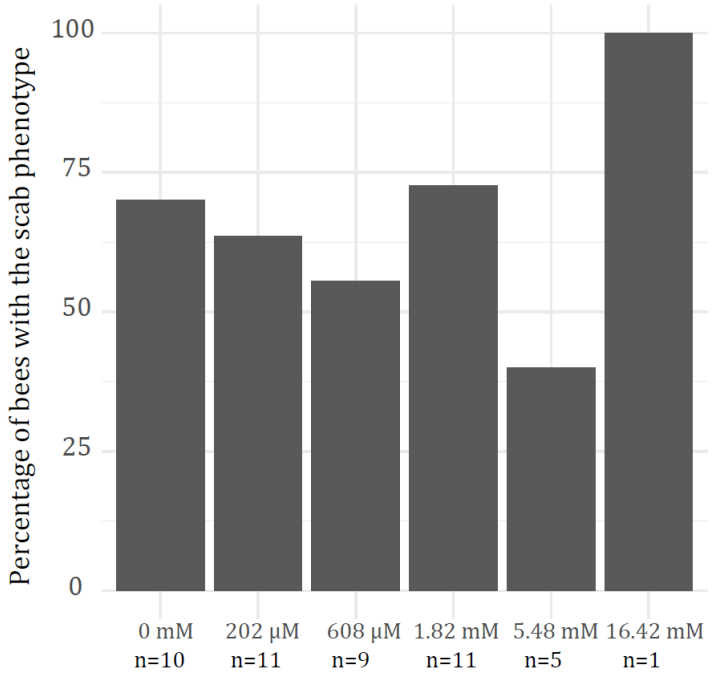

Fig. 7 *Percentage of bees with the scab phenotype in F. perrara colonized bees fed different PTU concentrations. Each bar corresponds to the percentage of bees with the scab phenotype in the group fed with the corresponding PTU initial concentration. The number of bees sampled at day 10 for each treatment group is indicated below its corresponding percentage plot.*

percentage of scabs was 40% and was obtained at a PTU concentration of 5.48 mM but was derived from only 5 bees since all other bees in this group died before sampling. Hence, although PTU seems to inhibit the melanization response, the achieved reduction in melanization is modest and based on only a few replicates.

The survival of *F. perrara* colonized and non-colonized bees in function of PTU concentration was also monitored during 10 days (**Fig. 8**). The most

concentrated PTU solution (16.4 mM) had a toxic effect on bees whether they were colonized with *F. perrara* or not (i.e. >80% of bees dead by day 6 for both groups), indicating an intrinsic toxicity of PTU for honey bees.

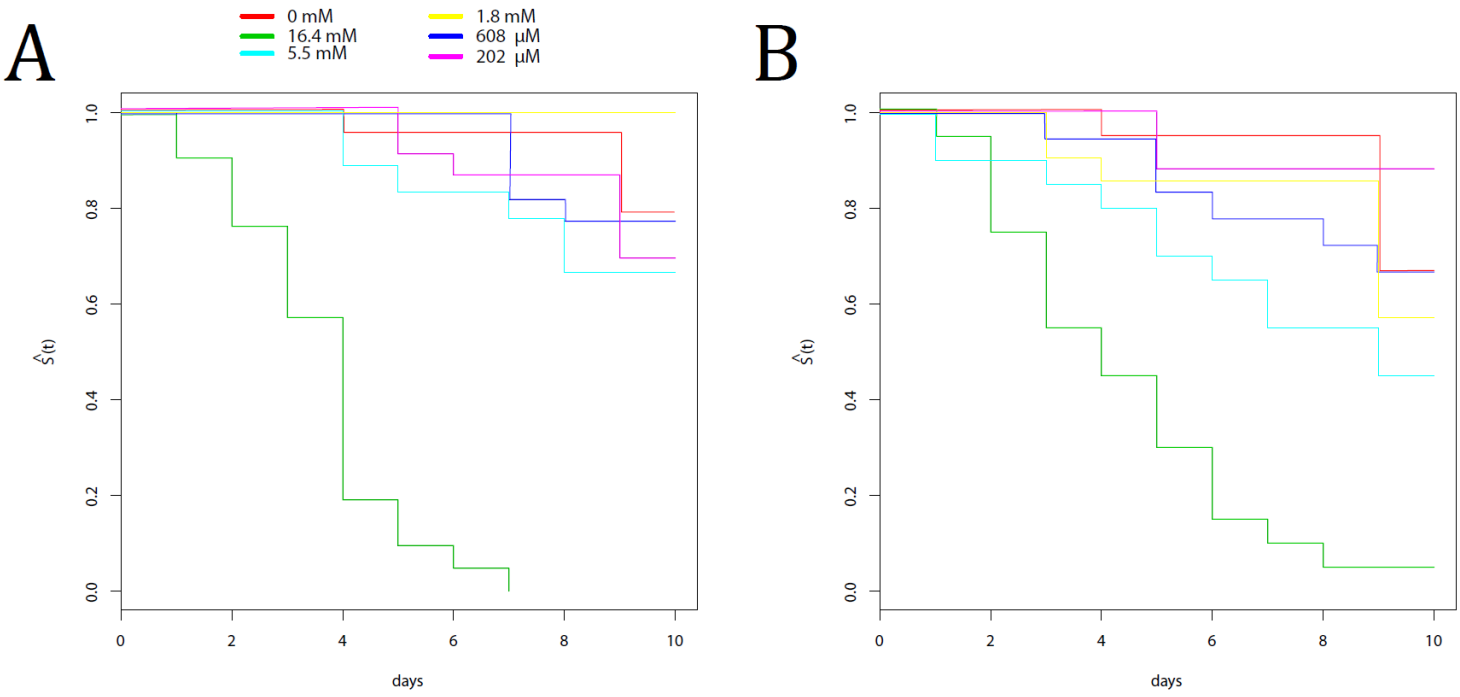

Fig. 8 *Survival curves of non-colonized bees (A) or colonized with F. perrara (B) in function of PTU concentrations (~25 bees for each curve). S(t) is the estimator of the survival function which corresponds here to the proportion of bees alive at time t. Each curve represents the survival of bees through time from a cage which received a certain concentration of PTU that corresponds to the color code in (A).*

Nevertheless, we observed lower survivals in *F. perrara* colonized bees relative to colonized bees for the PTU concentrations of 608 µM and 5.5 mM,

suggesting that the combination of PTU and *F. perrara* colonization is more deadly to bees than PTU alone at these concentrations.

Discussion

Feeding bees with increasing concentrations of PTU tended to lower the percentage of scabs but this was at the price of higher toxicity towards honey bees. The achieved reduction in scab development was relatively small and more replicates would be needed to confirm the inhibition of the melanization response based on scab occurences. Interestingly, the 608 µM and 5.48 mM PTU concentrations resulted in reduced survival for *F. perrara* colonized bees relative to non-colonized bees. However, it is not clear if the additional bee deaths caused by *F. perrara* colonization in these PTU concentrations were due to a reduced melanization response, or if they were attributable to the combined effect of PTU toxicity and bacterial colonization.

Overall, these results indicate that melanization inhibition in vivo using PTU feeding in honey bees needs further development and may not be the ideal method to study the role of the melanization response on *F. perrara* colonization. Although treated bees had only access to PTU solutions and no other sugar water source, individual bees may have ingested different total amounts of PTU over the 10 days. In particular, the most highly concentrated

PTU solution had a strong smell that may have repelled bees from drinking it. It is also possible that ingested PTU does not reach the location where the chemical reactions leading to melanization take place. The use of other inhibitors of melanization enzymes (i.e. the DOPA decarboxylase inhibitor L-α-methyl-DOPA or the tyrosine hydroxylase inhibitor 3-iodo-tyrosine) could help disentangle the role of melanization from other host immune responses in this symbiosis, provided they are effective and less toxic/repulsive for the bees. Alternatively, editing the honey bee genome using CRISPR/Cas-9 to knock out specific melanization genes in honey bees could also be used towards this goal, however this technique is still in its infancy for honey bees and has only been applied once so far (Kohno et al., 2016).

2) The transcriptomes of *F. perrara* during gut colonization

Summary

Compared to other honey bee gut symbionts, *F. perrara* engages in a relatively unique interaction with the host. It colonizes a restricted region in the gut, the pylorus, and causes the scab phenotype. Together with another PhD student, Konstantin Schmidt, we thus aimed to understand which bacterial factors are involved in the colonization, the formation of the scab phenotype, and the response to the strong immune response in the pylorus. To this end, we conducted an RNA-seq experiment that focused on the bacterial transcriptome rather than that of the host. We compared *F. perrara* during gut colonization at two different time-points (at the onset of scab formation and after scab formation) to *F. perrara* grown in vitro. The analysis of this RNA-seq data showed that many bacterial genes are differentially expressed between *in vivo* and *in vitro* conditions. By contrast, the in vivo samples had little to no genes differentially expressed between the two time points, indicating little adaptive changes to the presence of the scab phenotype.

Genes upregulated in *F. perrara* in the *in vivo* conditions were enriched in genes coding for carbohydrate and ion transporters, and for tryptophan biosynthesis. Carbohydrate and ion transport may be involved in nutritional

intake from the host diet, while we speculate that tryptophan biosynthesis may provide phenolic substrates for melanization contributing to the formation of the scab phenotype. Downregulated genes were enriched in genes related to cell motility and sulfur metabolism. Our study provides the first insights into the interaction between *F. perrara* and its host from the perspective of the symbiont. It also provides a list of candidate genes that could be targeted by genetic approaches to establish causal links between specific genes and host colonization or scab formation.

Introduction

We have shown that *F. perrara* colonization results in the upregulation of host genes encoding recognition receptors, antimicrobial peptides, and different components of the melanization cascade, indicating that the host mounts a specific immune response against *F. perrara* (Emery et al. 2017). Despite these defensive mechanisms, *F. perrara* is capable of colonizing and persisting in pylorus where the host immune response occurs. Specific genes of *F. perrara* may be upregulated in vivo to colonize and persist under such conditions but have not yet been experimentally tracked. In addition, while a number of potential 'virulence' factors have been detected in the genome

of *F. perrara*, it has remained elusive if specific gene fucntions are responsible for inducing the scab phenotype.

The only member of the honey bee gut microbiota for which genes contributing to host colonization have been investigated is *Snodgrassella alvi*. This honey bee gut symbiont colonizes a similar region as *F. perrara*, the pylorus and ileum, but does not cause the scab phenotype. High-thoughput sequencing of a saturated transposon mutant library (Tn-seq) revealed a genome-wide array of genes important for colonization in vivo (Powell et al. 2016). Genes promoting gut colonization were classified into three main categories: extracellular interaction (including genes coding for O antigens, adhesion factors and type IV pilus), metabolism (including genes involved in oxygen sensing, acetate and lactate metabolism, the TCA cycle, nucleic acids and amino acid biosynthesis and iron uptake) and stress response. The study of Powell et al. also used RNA-seq to investigate changes in gene expression upon colonization of the gut. Some of the genes promoting colonization in the Tn-seq screen were upregulated such as genes responsible for branched-chain amino acid synthesis, iron acquisition and short-chain fatty acid utilization. Other upregulated genes which did not significantly affect colonization in mutants included genes coding for nutrient acquisition transporters and the type VI secretion system, implicated in interbacterial competition.

Although bacterial factors important for *F. perrara* colonization have not yet been determined, the initial biochemical characterization of *F. perrara* PEB0191^T (Engel et al. 2013) may provide some cues to explain how this bacterium is able to colonize and persist in the gut. Catalase activity was found to be present and may contribute to withstand the oxidative environment generated by melanization. *F. perrara* was shown to have β-glucosidase activity which could contribute to the degradation of carbohydrates from the host diet (Singhania et al., 2013). This would be consistent with the fact that *F. perrara* is anaerobe, lacks a complete TCA cycle and relies on sugar fermentation for its energy intake. The colibactin genomic island is present in the *F. perrara* genome and is also found in symbiotic bacteria associated with coral, but also in human gut *Escherischia coli* strains that are linked to colorectal cancer (Cougnoux et al., 2014; Dalmasso et al., 2014). Colibactin is a genotoxic compound and, due to its presence in diverse symbioses, may play a role in the symbiosis of *F. perrara* with the honey bee (Engel et al., 2015b). The genome of *F. perrara* harbours other genes potentially important for colonization and persistence in the gut, such as genes coding for type VI secretion systems which mediate cell-cell interactions and bacterial competition, and genes involved in aryl-polyene production. Bacterial pigments of the aryl-polyene type are related to

carotenoids and help protect bacteria against oxidative stress (Schöner et al., 2016).

The genetic tools to perform Tn-seq in *F. perrara* are currently lacking. To get a first idea of the genes of *F. perrara* specifically involved in the symbiosis with its host, the honey bee, we performed RNA-seq. We compared whole-genome transcriptomes of *F. perrara* from in vitro culture conditions to in vivo growth in the honey bee gut at 5 and 10 days post colonization using RNA-seq. Two time points were used in order to assess gene expression changes at different scab development stages. Our results showed many significantly differentially expressed genes (SDEGs) between in vivo and in vitro conditions but no significant differences between the two time points of vivo samples. Overall, genes upregulated in vivo were enriched in genes involved in carbohydrate transport and tryptophan biosynthesis; while downregulated genes were enriched in genes related to cell motility, and to a lesser extent sulfur metabolism. Our findings provide new insights from the perspective of the symbiont on the symbiosis between *F. perrara* and its honey bee host.

Materials and methods

Rearing of microbiota-depleted bees

Honey bees (*Apis mellifera carnica*) brood frames were removed from a healthy colony located at the University of Lausanne, Switzerland. In order to obtain microbiota-depleted bees, wax cell caps were carefully removed in the laboratory using sterile toothpicks after wiping the surface of cells with ethanol, and tan-colored pupae with black eyes were pulled out and placed on their back on a moistened cotton pad in a plastic cage. Ten cages containing 50 pupae were kept in an insect chamber at 35 °C under a relative humidity of 75–85% for 2 days with Eppendorf tube caps filled with 1:1 (weight:weight) sugar:water. Bees that emerged during this period were transferred into ten new plastic cup cages (0.3l PET cups) in which they stayed for an extra day before experimental colonization with *F. perrara*.

Experimental colonization of honey bees with *F. perrara*

F. perrara ESL0034 (wild type) was plated from stock in three replicates on tryptone yeast extract agar (TYG) plates and was incubated at 35°C under anaerobic conditions for two days. *F. perrara* was then restreaked onto five new TYG plates for an additional day. Half of each plate was then harvested with a loop and directly transferred into a 2 ml tube containing TRI reagent

(Sigma-Aldrich, Merck) and ~200 µl zirconia/silica beads (0.1mm diameter, Roth). These samples were immediately snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. These samples form the in vitro samples. The rest of the *F. perrara* plates was used for the colonization of honey bees. In order to make the inoculation solution, cells were harvested in 1 mL 1x PBS and the optical density at a wavelength of 600 nm (OD600) was measured. The bacterial solution was adjusted to an OD600 of 0.1 in 1x PBS plus sugar–water (1:1). Microbiota-depleted honey bees were starved for 2 hours by removing the sugar water solution from their cages. Bees were then anesthetized by cooling at 4°C for 15 min and transferred onto ice. Each bee was then placed at room temperature in a 2 ml Eppendorf tube modified with a hole at the bottom to let only the head of the bee out of the tube. Actively moving bees were individually fed 5 µl of the inoculation solution containing *F. perrara*. Successfully colonized bees were placed back into their respective cages while bees that did not ingest the entire inoculum were excluded from the experiment. Bees were then provided sterile filtered sugar–water (1:1) *ad libitum* and were placed in an insect chamber at 32 °C under a relative humidity of 75–85% before sampling at day 5 and day 10 post colonization.

Preparation of in vivo samples

Whole guts of honey bees previously colonized with *F. perrara* were removed by gently pulling the stinger with a forceps and placed on a 1x PBS drop. Using a stereomicroscope, a gut region including the pylorus and ~half of the ileum was dissected and the presence or absence of the scab phenotype was determined. For each in vivo sample 10 pylori-ileum regions from one cage were pooled and the corresponding percentage of scabs was recorded.

The other half of the ileum was kept for each of the 10 bees and these gut parts were pooled for each sample as well (hereafter "quality control samples"). The quality control samples were used to confirm *Varroa destructor* virus 1 (VDV-1) absence in the ileum and as a proxy to determine *F. perrara* colonization success using qPCR.

All samples (i.e. in vivo and quality control samples) were put into 2 ml bead beating tubes kept on ice containing 750 µl of TRIzol reagent (Invitrogen) and ~200 µl of glass beads (0.75-1mm diameter, Roth) and zirconia/silica beads (0.1mm diameter, Roth). Once sample collection was finished, samples were immediately flash frozen in liquid nitrogen and kept at -80°C until RNA extraction.

RNA extractions

Bead beating tubes containing samples were thawed briefly on ice, placed in a CoolPrep adapter (MP Biomedicals) filled with dry ice and homogenized on a Fast-Prep24™5G homogenizer (MP Biomedicals) at 7 m/s for two cycles of 40 s with a 30 s pause in between. Tubes were then held 5 min at room temperature before adding 150 µL of 100% chloroform, manually shaken for 15 s and held at room temperature for 2.5 min before being centrifuged at 12'000 x g for 15 min at 4°C. The top aqueous phase (~400 µL) was placed in a new tube followed by the addition of 400 µL of 100% isopropanol, manual inversion of the tube six times and storage for 2 h at -20°C for RNA precipitation. Next, tubes were centrifuged at 12'000 x g and 4°C for 10 min, the supernatant was removed and the pellet was washed with 1mL 70% ethanol (RNAse free). Tubes were vortexed briefly, centrifuged 5 min at 12'000 x g and 4°C for 5 minutes and the supernatant was discarded. Tubes were then placed on an Eppendorf heat block at 37°C for 5-10 min to dry the leftover ethanol. 45 µL of RNAse free water were rinsed over the pellet several times, tubes were placed on an Eppendorf heat block at 37°C for 5-10 min with shaking to facilitate RNA pellet resuspension and kept on ice afterwards before DNAse treatment. To remove DNA contamination, 5 µL of 10x DNase I buffer and 1 µL of rDNAse I were added to each tube. The content of the tube was spun down and tubes were incubated at 37°C for 30 min. 1

µL of rDNAse I was added again followed by an incubation at 37°C for 30 min. RNA clean-up was performed using a NucleoSpin RNA clean-up XS kit (Macherey-Nagel) following the manufacturer's instruction with an elution with 30 µL of RNAse-free water. RNA concentrations were assessed using a Qubit fluorometer (Invitrogen) with the Qubit RNA BR (broad range) assay kit and RNA quality was determined using a Bioanalyzer instrument (Agilent).

Reverse transcription and quantitative PCR

A common RNA virus of honey bees is VDV-1 which can reach high numbers and thereby lower the ratio of bacterial RNA sequencing reads from a contaminated sample. In order to exclude the presence of the VDV-1 virus in the in vivo samples and to have a proxy for *F. perrara* colonization, the RNA of each quality control sample was first reverse transcribed using the M-MLV reverse transcriptase RNase H minus point mutant enzyme (Promega) before performing qPCR on the resulting cDNAs with VDV-1 and *F. perrara* specific primers. For each reverse transcription reaction, 1 µl of random primers (250 ng/µL), 1 µL of dNTP mix and a volume corresponding to 300ng of RNA from the sample were added to a PCR tube and completed with nuclease-free water to reach a volume of 15 µl. This mixture was then heated to 65°C for 5 min, cooled down to 4°C and briefly centrifuged. 4 µl of M-MLV

reverse transcriptase 5X reaction buffer and 1 µl of M-MLV reverse transcriptase were added to the tube and gently mixed by pipetting. Tubes were then subjected to the following in a thermocycler: 10 min at 25°C, 50 min at 42°C, 15 min at 70°C and a hold stage at 4°C until retrieval. The reverse transcribed samples were then diluted 5 times by adding 80 µl of nuclease-free water.

The relative levels of VDV-1 and *F. perrara* 16S rRNA gene using the *Apis mellifera* actin gene for normalization were determined using qPCR on a StepOnePlus real time PCR machine. qPCR reactions were performed in 10 µl volumes in triplicates consisting of: 1 µl of cDNA from the sample, 5 µl of SYBR 'Select' Master mix (x2 concentrated, Applied Biosystems), 0.4 µl of forward primer, 0.4 µl of reverse primer and 3.2 µl of nuclease-free water. The following forward/reverse primer pairs were used: AGCTTATCGGTCTTTGGGTTC / ATCATAGCTCTCTGCCTCCAC for *F. perrara*, GTATATATGGCTAATCGACGTAAAG / AGTACTAATCTCTGAGCCAACAC for VDV-1 virus and TGCCAACACTGTCCTTTCTG / AGAATTGACCCACCAATCCA for the *A. mellifera* actin gene. The following qPCR program was used: 2 s at 50°C and 2 s at 95°C followed by 40 cycles of 15 s at 95°C and 1 s at 60°C followed by the default melting curve stage. Relative levels of *F. perrara* and of VDV-1 relative to actin were computed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

RNA sequencing

In vivo RNA samples were selected for sequencing based on RNA quality assessed with a Bioanalyzer instrument (Agilent). Each sample at day 10 post colonization was taken from the corresponding cage at day 5. Four biological replicate samples for each time point were sent to the Lausanne Genomic Technology Facility (LGTF, Switzerland) where TruSeq stranded mRNA libraries were generated (Illumina) following a poly-A depletion in order to enrich for bacterial mRNA and a Ribo-zero rRNA depletion step to deplete both prokaryotic and eukaryotic rRNAs. The eight resulting libraries were then sequenced on an Illumina HiSeq 2500 instrument to obtain single end 125 bp reads.

For the in vitro RNA samples, four libraries corresponding to the *F. perrara* cultures used for colonizations were generated and sequenced on an Illumina MiniSeq instrument at the Department of fundamental Microbiology configured to obtain 125 bp single end reads. In order to check that in vivo samples would give consistent results on both instruments despite their different sequencing depths, RNA from one in vivo replicate at day 10 (sample identifier D10_3M) and from one replicate at day 5 (sample identifier D5_1M) post colonization previously sequenced on the HiSeq were also re-sequenced on the MiniSeq.

RNA-seq differential gene expression analysis

Raw reads in FASTQ format from all libraries were filtered to only include reads passing the standard Illumina quality control pipeline (Casava), which were then trimmed and adapters removed using Trimmomatic (version 0.36) with the following command:

```
trimmomatic SE -phred33  RawFile.fastq.gz TrimmedFile.fastq.gz
ILLUMINACLIP:TruSeq3-SE.fa:2:30:10 LEADING:3 TRAILING:3
SLIDINGWINDOW:4:15 MINLEN:42
```

Genome indices were built for *F. perrara* PEB0191 and for the *Apis mellifera* genome (version 4.5) was built using Bowtie2 (version 2.3.4.1). We also used Bowtie2 to map trimmed reads to the *F. perrara* genome, using the genome file in FASTA format and its corresponding annotation file in GTF format with the following command:

```
bowtie2 -p 16 -q TrimmedFile.fastq.gz -x FpGenomeIndexPrefix >
alignment.sam
```

Alignments were then converted from SAM to BAM format and sorted using Samtools (version 1.8). Read counts mapping to genes were obtained for each replicate with the HTseq-count command of HTseq (version 0.7.2) as follows :

```
htseq-count -t CDS -i gene_id -s reverse -f bam alignment.bam
FpGenomeAnnotation.gtf > output.count
```

For in vivo samples, we also mapped the reads against the host genome using Tophat2 (version 2.2.1) with the *A. mellifera* GTF annotation file from the original gene set version 3.2 with the following command:

```
tophat -p 8 -G amel_OGSv3.2.gff3 -o OutFolder AmGenomeIndex
trimmed_reads.fastq.gz
```

Mapping computations with Bowtie or Tophat2 were performed at the Vital-IT (http://www.vital-it.ch) Center for high-performance computing of the SIB Swiss Institute of Bioinformatics.

Differential gene expression analysis was conducted with the Bioconductor package EdgeR (version 3.20.9) in R (version 3.4.3), mostly as specified in the EdgeR user guide for generalized linear models. Read counts and a design matrix specifying the different conditions and replicates were imported into R. Lowly expressed genes (55 out of 2267 genes) were filtered out by removing read counts with a threshold of 2 counts per million (cpm) in at least 4 replicates across conditions (4 corresponding to the number of replicates per condition). Normalization by the method of trimmed mean of M-values (TMM, Robinson and Oshlack, 2010) was performed using the calnormFactors() function which produce scaling factors used by edgeR to determine effective library sizes. After obtaining dispersion estimates, negative binomial generalized linear models for each condition were fitted using the glmQLFit() function. We used quasi-likelihood F-tests for each defined contrast (i.e. pairwise comparison between conditions) to assess the

significance of differentially expressed genes at a false discovery rate (FDR) cutoff of 0.05 and a p-value significance level of 0.05. We also applied a conservative significance cutoff value of 2 for absolute log2 fold changes in gene expression so as to focus on most differentially expressed genes.

Gene ontology (GO) enrichment analysis

In order to identify significantly enriched or depleted gene classes or functions among significantly differentially expressed genes, we performed a gene set enrichment analysis (GSEA) with two-sided Fisher exact tests in Blast2GO (version 5.2.4, Conesa and Götz, 2008; Götz et al., 2008; Conesa et al., 2005) at a significance level of 0.05.

Pathway analysis of SDEGs

In order to display SDEGs within specific molecular pathways, we extracted Kyoto Encyclopedia of Genes and Genomes (KEGG) identifiers from the different lists of SDEGs and pasted them into the online pathway mapping tool KEGGmapper, and uploaded *F. perrara* specific pathway information (https://www.genome.jp/kegg/tool/map_pathway1.html last updated: June 10, 2014).

Results

Colonization of honey bees with *F. perrara* and sample selection

Honey bees were colonized with *F. perrara* and in vivo samples and corresponding quality control samples were collected at 5 and 10 days post colonization in ten replicates. The percentage of bees with the scab phenotype from each in vivo sample at day 5 ranged from 20 to 60% while it was 100% for all in vivo samples at day 10, as expected from previous research on scab phenotype development (Emery et al., 2017; Engel et al., 2015a). A previous pilot experiment to isolate and sequence *F. perrara* RNA in vivo had resulted in ~40% of the all sequenced reads mapping to the *Varroa destructor* virus 1(VDV-1) virus thereby minimizing the quantity of reads mapping to *F. perrara* to less than 1% (data not shown). We hence decided to control for the presence of the VDV-1 virus with qPCR in ileums (i.e. the other half of the 10 ileums from the guts used for in vivo samples, hereafter "quality control samples") following reverse transcription. We used the neighboring gut region of the gut so as to spare the actual in vivo samples (i.e. pooled pylorus+ileum regions) in order to obtain sufficient amounts of RNA for sequencing. If the virus is present in the in vivo samples we also expect it to appear in the corresponding quality control sample. We also screened for *F. perrara* by qPCR in the quality control samples. Although *F. perrara* levels in the ileum are much lower in the ileum than in the pylorus

(Engel et al., 2015a), we expected to also detect *F. perrara* in these samples when the colonization was successful. The qPCR results confirmed that, in all sampled guts, VDV-1 was absent (**Fig. 1A**) with much lower levels relative to the positive control and similar levels relative to the negative controls (i.e. RNA from whole gut samples from bees in which the virus was known to be absent, or for a mock reverse transcription without reverse transcriptase). Regarding *F. perrara* relative qPCR levels, the quality control samples contained higher levels than in the water negative control (**Fig. 1B**). The lower *F. perrara* levels observed in the quality control samples at day 5 and day 10 compared to the positive control can be explained by the fact that the positive control corresponded to a bee gut including the pylorus region in which *F. perrara* is more abundant than in the ileum. The mock reverse transcription of a quality control sample resulted in *F. perrara* levels almost identical to the lowest levels found after reverse transcription, indicating that *F. perrara* DNA was amplified in this control. RNA was extracted from all in vivo samples and four replicates per time point were chosen for RNA sequencing based on their RNA quality.

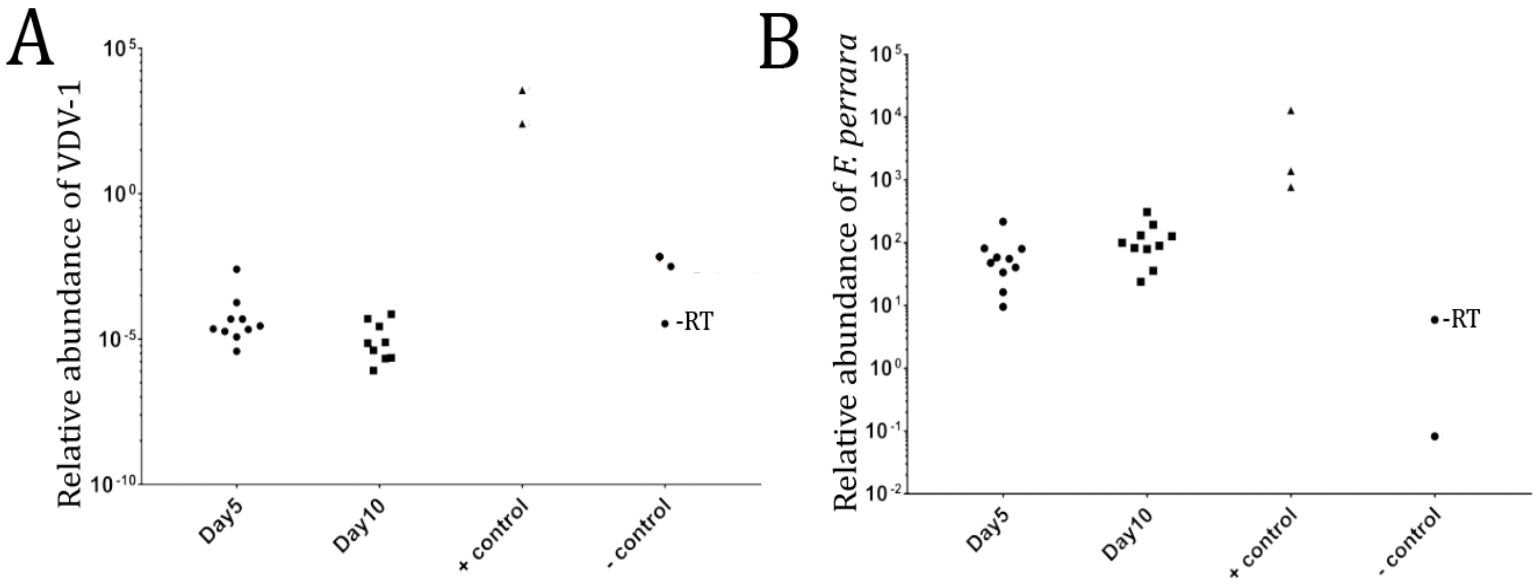

Figure 1 *Quantification of VDV-1 virus and F. perrara in quality control samples (10 pooled ileums/sample) from Day5 and Day10 by qPCR using the A. mellifera actin gene for normalization (A) Relative abundance of VDV-1 virus with reverse transcribed RNA from honey bee guts previously determined not to contain VDV-1 or mock reverse transcription as negative controls. (B) Relative abundances of F. perrara with nuclease-free water or mock reverse transcription (indicated by* –RT *next to the corresponding point) as negative controls. For both qPCR targets, positive controls corresponded to DNA isolated from honey bee guts (including the pylorus) previously determined to be positive for the qPCR target.*

Overview of sequencing results

RNA sequencing of in vitro samples on the MiniSeq instrument yielded a total of 10'766'585 filtered reads with an average of ~2.7 million reads per replicate with >99% of reads mapping to *F. perrara* genome (**Table 1**). For in vivo samples sequenced on the HiSeq instrument, we obtained 254'690'234 filtered reads in total with an average of ~32 million reads per replicate. For each in vivo replicate, 42-67% of all reads mapped to *A. mellifera* genome, despite poly-A depletion. We nevertheless obtained 3-7 million reads per replicate mapping to the *F. perrara* genome at day 5, and 4-13 million reads at day 10. The higher average number of reads mapping to *F. perrara* at day 10 relative to day 5 is consistent with the fact that there are higher numbers of *F. perrara* cells at this time point, while the quantity of RNA from the host tissue should not be significantly different (Engel et al., 2015a). Reads from in vivo replicates that were sequenced on both the MiniSeq and on the HiSeq instruments (i.e. replicates D5_1M and D10_3M and replicates D5_1 and D10_3, respectively) had similar mapping rates to *A. mellifera* and to *F. perrara*. This indicates that despite the fact that the MiniSeq samples had ten times less reads mapping to *F. perrara* relative to their corresponding HiSeq samples, each matching pair of replicates from the two sequencing instruments produced similar mapping results.

Sequencing instrument	Condition	% of bees with scab in sample	Sample identifier	Time point	Number of raw reads	Number of trimmed reads	Number of reads mapped to *F. perrara* genome	% of reads mapped to *F. perrara* genome	Number of reads mapped to *A. mellifera* genome	% of reads mapped to *A. mellifera* genome
MiniSeq	in vitro	NA	Fp1	NA	2 634 464	2 584 650	2 569 142	99.40	NA	NA
MiniSeq	in vitro	NA	Fp2	NA	2 641 352	2 582 749	2 563 894	99.27	NA	NA
MiniSeq	in vitro	NA	Fp3	NA	2 955 274	2 897 703	2 880 316	99.40	NA	NA
MiniSeq	in vitro	NA	Fp4	NA	2 754 109	2 701 483	2 685 814	99.42	NA	NA
MiniSeq	in vivo	50	D5_1M	Day5	3 271 308	3 190 590	289 067	9.06	2 294 034	71.90
MiniSeq	in vivo	100	D10_3M	Day10	3 778 349	3 690 073	1 299 275	35.21	1 867 177	50.60
HiSeq	in vivo	50	D5_1	Day5	42 062 252	32 646 017	3 052 670	9.35	21 740 268	66.60
HiSeq	in vivo	30	D5_2	Day5	37 702 890	29 530 119	4 520 836	15.31	18 196 064	61.60
HiSeq	in vivo	50	D5_3	Day5	43 761 575	34 210 475	6 988 012	20.43	19 794 862	57.90
HiSeq	in vivo	60	D5_4	Day5	44 631 540	35 141 025	4 033 254	11.48	23 234 887	66.10
HiSeq	in vivo	100	D10_1	Day10	29 886 535	23 354 183	4 285 981	18.35	13 106 975	56.10
HiSeq	in vivo	100	D10_2	Day10	49 083 096	38 391 445	9 882 859	25.74	17 267 644	45.00
HiSeq	in vivo	100	D10_3	Day10	48 670 328	38 296 384	13 341 871	34.84	18 313 594	47.80
HiSeq	in vivo	100	D10_4	Day10	29 636 169	23 120 586	5 635 519	24.37	9 707 539	42.00

Table 1 *Sequencing and read mapping summary.* NA = *not applicable.*

Differential gene expression between *F. perrara* grown in vitro or in vivo

In order to determine global patterns of gene expression of in vitro and in vivo replicates relative to each other, we used a multidimensional scaling (MDS) representation of the dataset with the MDSplot() function in R (**Fig. 2**). MDS displays a 2-D representation of the distances between samples and provides gives an overview of similarities and differences between samples. In vitro and in vivo samples were clearly separated from each other on the MDS plot, indicating large gene expression differences between the two conditions. In vivo samples at day 5 and day 10 mostly clustered by time point, with the exception of two replicates (D5_3 and D10_1). Most of the difference between time points was attributable to the leading fold change of

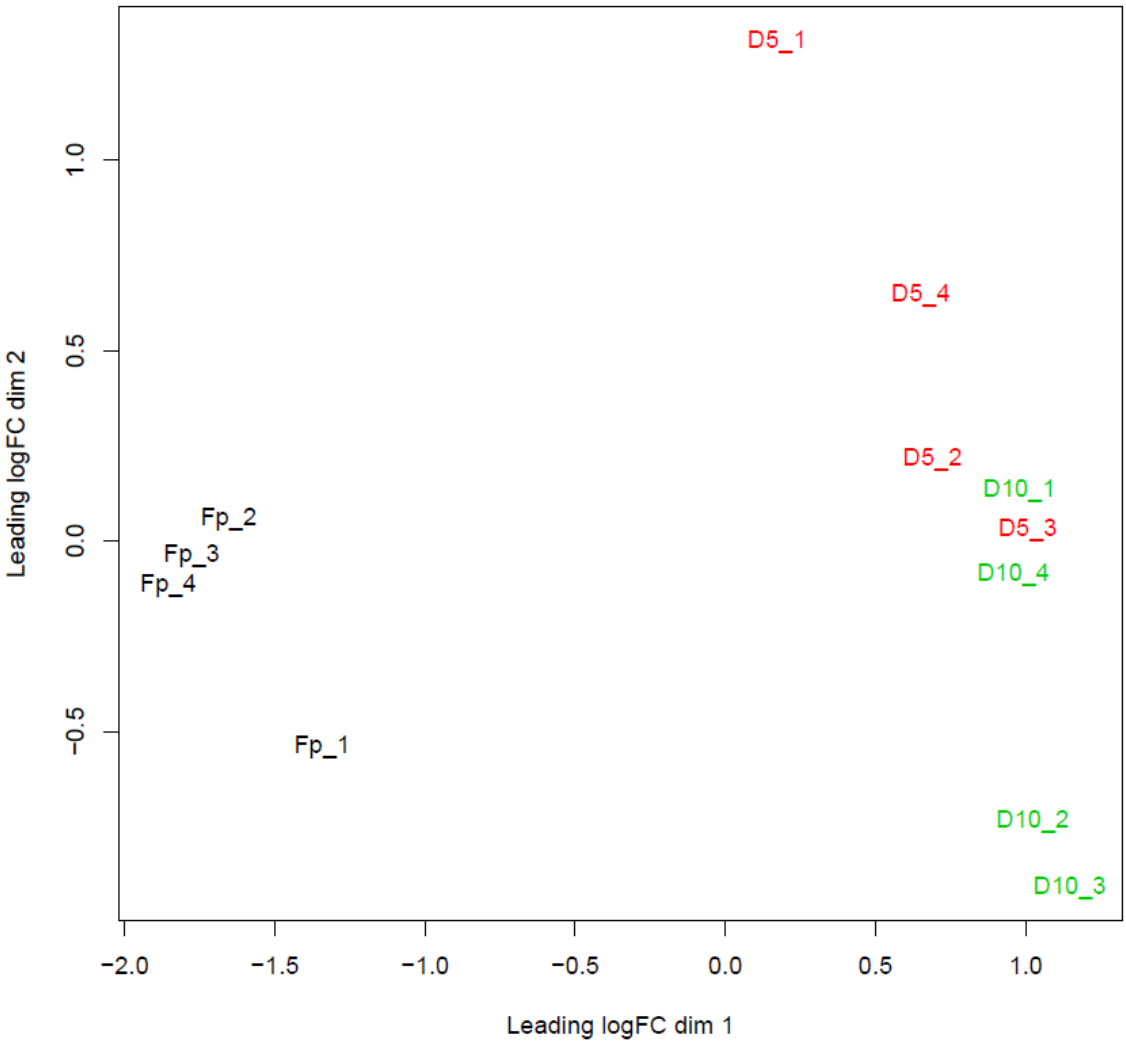

Figure 2 *Multidimensional-scaling (MDS) plot showing global gene expression patterns of the in vivo and in vitro samples used for differential gene expression analysis. In vivo samples at day 5 (D5_1, D5_2, D5_3, D5_4) and day 10 (D10_1, D10_2, D10_3, D10_4) post colonization, in vitro samples (Fp_1, Fp_2, Fp_3, Fp_4).*

the second dimension (i.e. vertical differences on the plot). Overall the MDS plot indicated that in vivo samples had a much different gene expression profile than in vitro samples, with day5 and day10 samples being more

similar (i.e. less distant in the plot) to each other than to in vitro samples. We also produced a MDS plot which included in addition data from the in vitro replicates sequenced on the MiniSeq (**Fig. S1**). This confirmed that the same in vivo replicates sequenced on instruments with different sequencing depths had similar global gene expression profiles, indicating an appropriate normalization of library sizes across samples of different sequencing depths. In line with the MDS results, there were many significantly differentially expressed genes (SDEGs) when comparing *F. perrara* in vivo at either time point to in vitro conditions (262 and 301 SDEGs at day 5 and day 10, respectively) while the comparisons of in vivo samples between day 5 and day 10 yielded no SDEGs satisfying the log2 fold change cutoff of 2. There were 198 genes that were differentially expressed both at both time points, 64 genes that were differentially expressed only at day5 and 103 differentially expressed only at day 10 (**Fig. 3A**).

In order to investigate the gene expression changes and the putative functions of the identified SDEGs, we determined their log2 fold changes in expression and retrieved gene functional annotations for *F. perrara* from the Integrative Microbial Genomes database (https://img.jgi.doe.gov). These were mapped to SDEGs identified at day 5 and day 10 (**Fig. 3B** and **C**, respectively). Genes upregulated in vivo at both time points included genes coding for transporters of sugar and various ions, oxidative stress related genes, genes

involved in tryptophan biosynthesis and some phage-related sequences. Genes downregulated in both time points included genes involved in the type VI secretion system and motility-related genes. The top 10 genes with the highest fold changes had vague or no annotations and included some phage-related sequences.

We then conducted a gene set enrichment analysis in Blast2GO in order to determine if there were differentially enriched GO categories among SDEGs. We separately tested for enriched categories in significantly upregulated genes from day 5 (**Fig. 4A**) and day 10 (**Fig. 4B**) relative to in vitro samples, as well as for downregulated genes at day 5 (**Fig. 4C**) and day10 (**Fig. 4D**). Genes coding for sugar or ion transporters as well as gene coding for the metabolism of tryptophan were enriched among upregulated genes for both time points, with more transport subcategories enriched at day 5 (**Fig. 4A** and **Fig. 4B**). Genes downregulated at day 5 (**Fig. 4C**) and at day 10 (**Fig. 4D**) relative to in vitro conditions (i.e. upregulated in vitro relative to in vivo) were significantly enriched in genes involved in cell motility and flagellar formation. Downregulated genes in vivo at day 10, but not at day 5, were enriched in genes coding for sulfate transport.

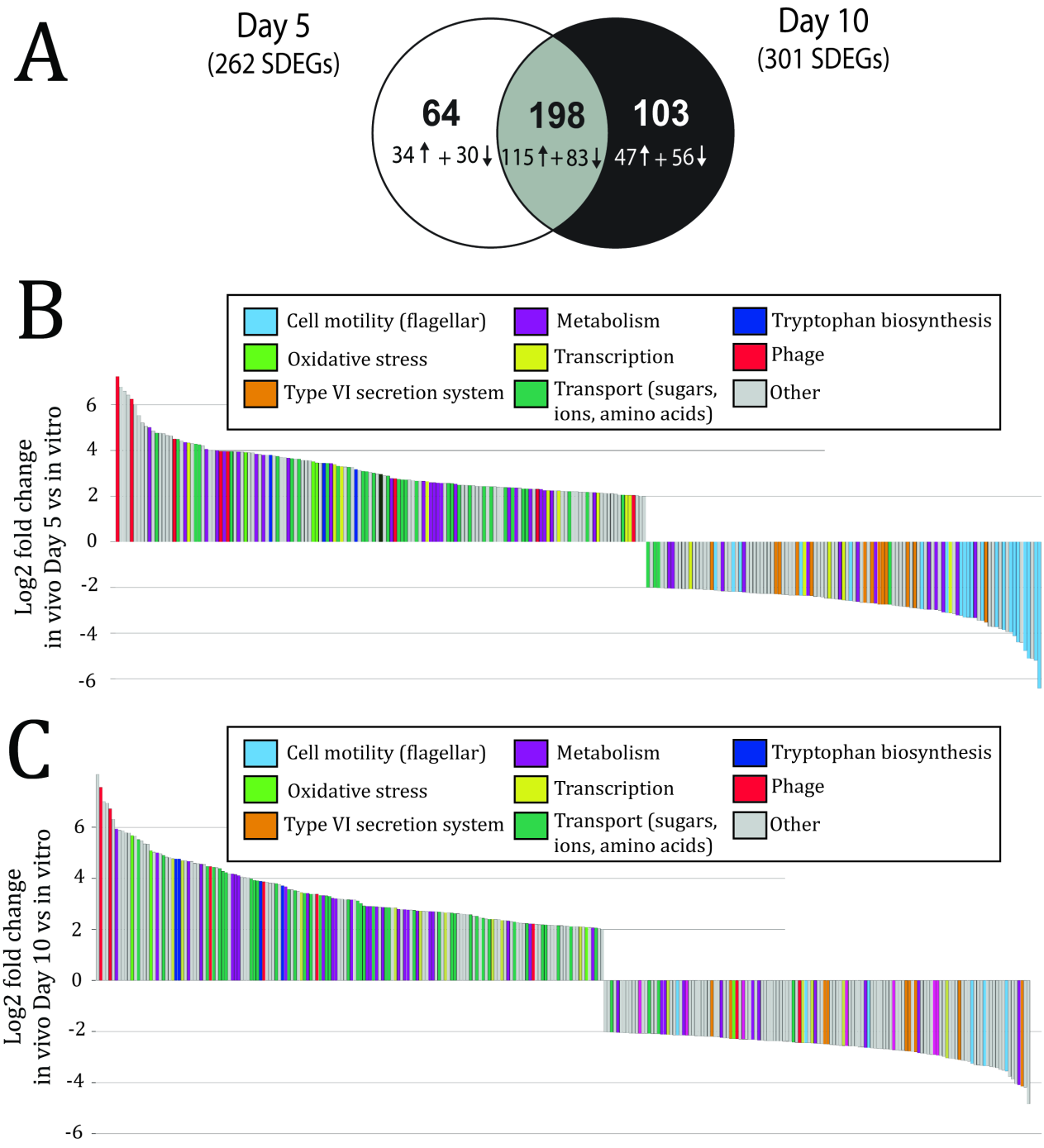

Figure 3 *Differential gene expression between in vivo samples and in vitro samples at day 5 and day 10 post colonization with F. perrara. A) Venn diagram showing shared and specific SDEGs in different comparisons. The white-filled circle (including the grey part) represents SDEGs between in vivo Day 5*

samples and in vitro samples (i.e. in vivo at day 5, in vivo at day10 and in vitro. The black-filled circle (including the grey part) represents SDEGs between in vivo Day 5 samples and in vitro samples. The intersection of circles in grey represents SDEGs that were differentially expressed in both comparisons. Numbers of genes from each set are indicated in their corresponding area of the plot and the corresponding number of upregulated and downregulated genes indicated below (upwards and downwards pointing arrows, respectively). Log2 fold changes of SDEGs from in vitro samples at day 5 (B) and day 10 (C) relative to in vitro samples. Each bar represents a gene with a height corresponding to its log2 fold change and colored according to the functional gene categories shown in the legend.

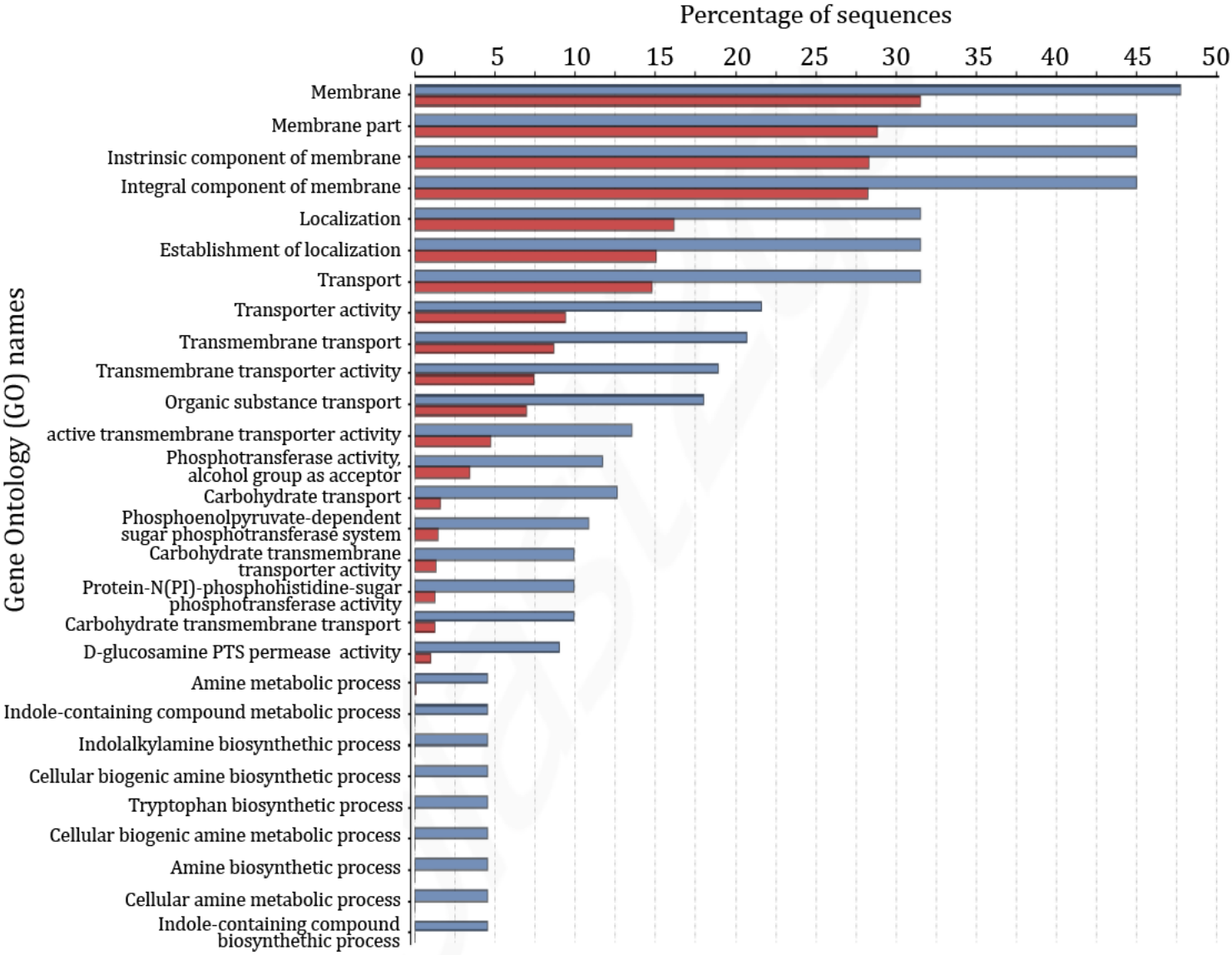

Figure 4 A) *GO enrichment analysis of upregulated genes in vivo at day 5 post colonization with F. perrara obtained with Blast2GO. The proportion of each GO term from the set of upregulated genes (i.e. the test set, in blue) is compared to the proportion of all genes with this the GO term in the F. perrara genome (i.e the reference set, in red). Only significantly enriched GO terms in the test set following Fisher's exact test are shown.*

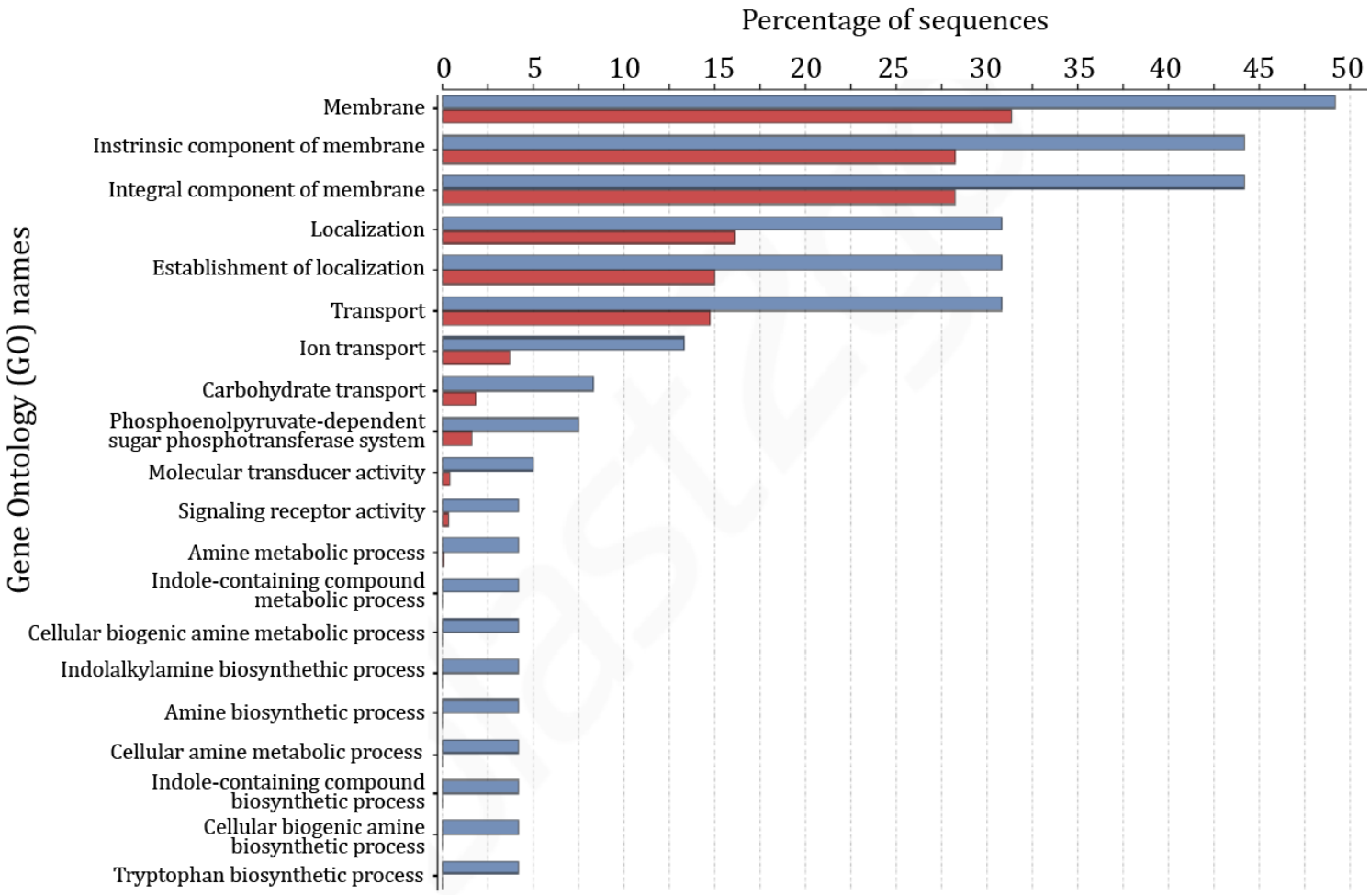

Figure 4 B) *Enrichment analysis of upregulated genes in vivo at day 10 post colonization with F. perrara. See legend in Fig. 4A.*

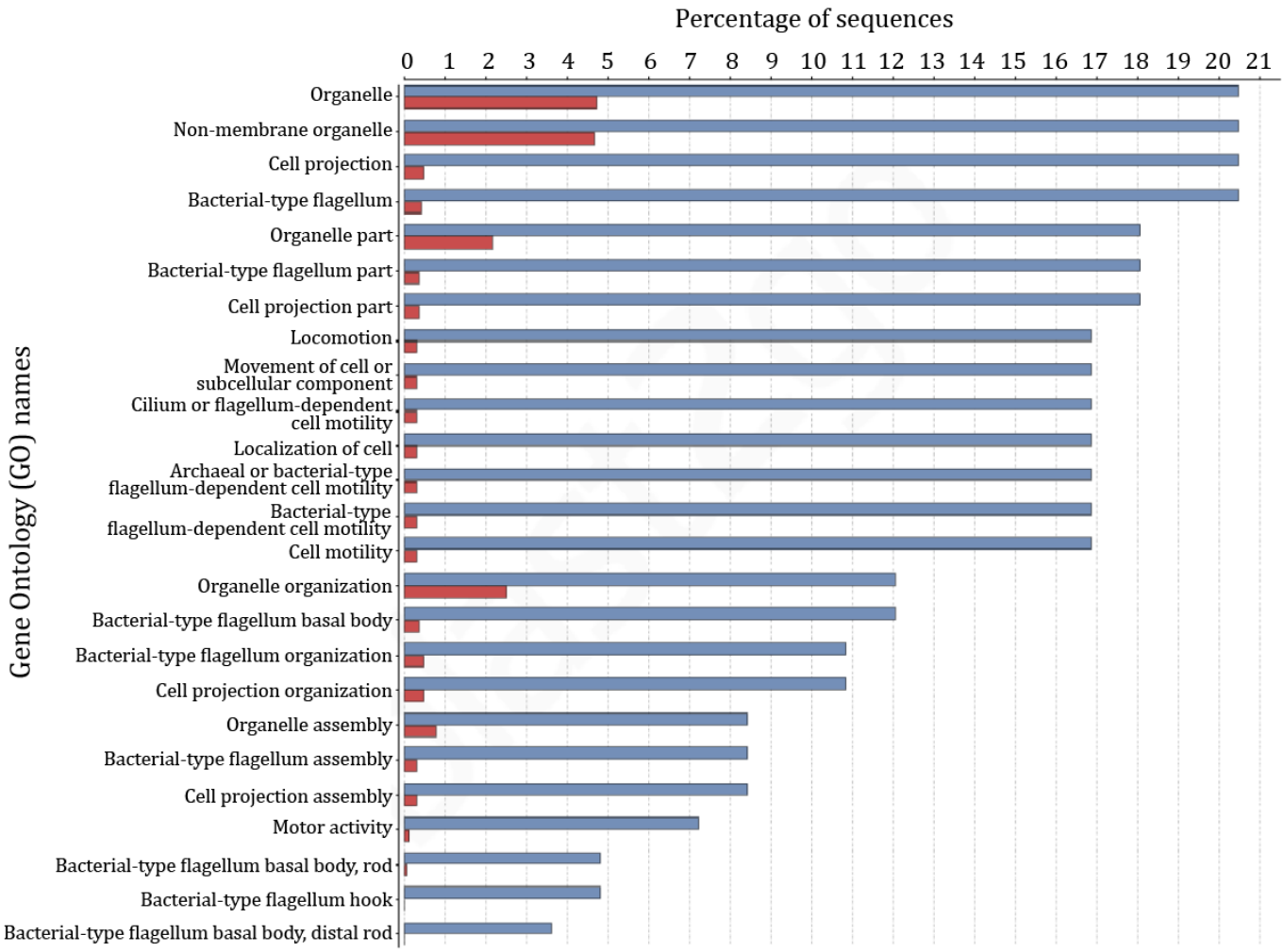

Figure 4 C) *Enrichment analysis of downregulated genes in vivo at day 5 post colonization with F. perrara. See legend in Fig. 4A.*

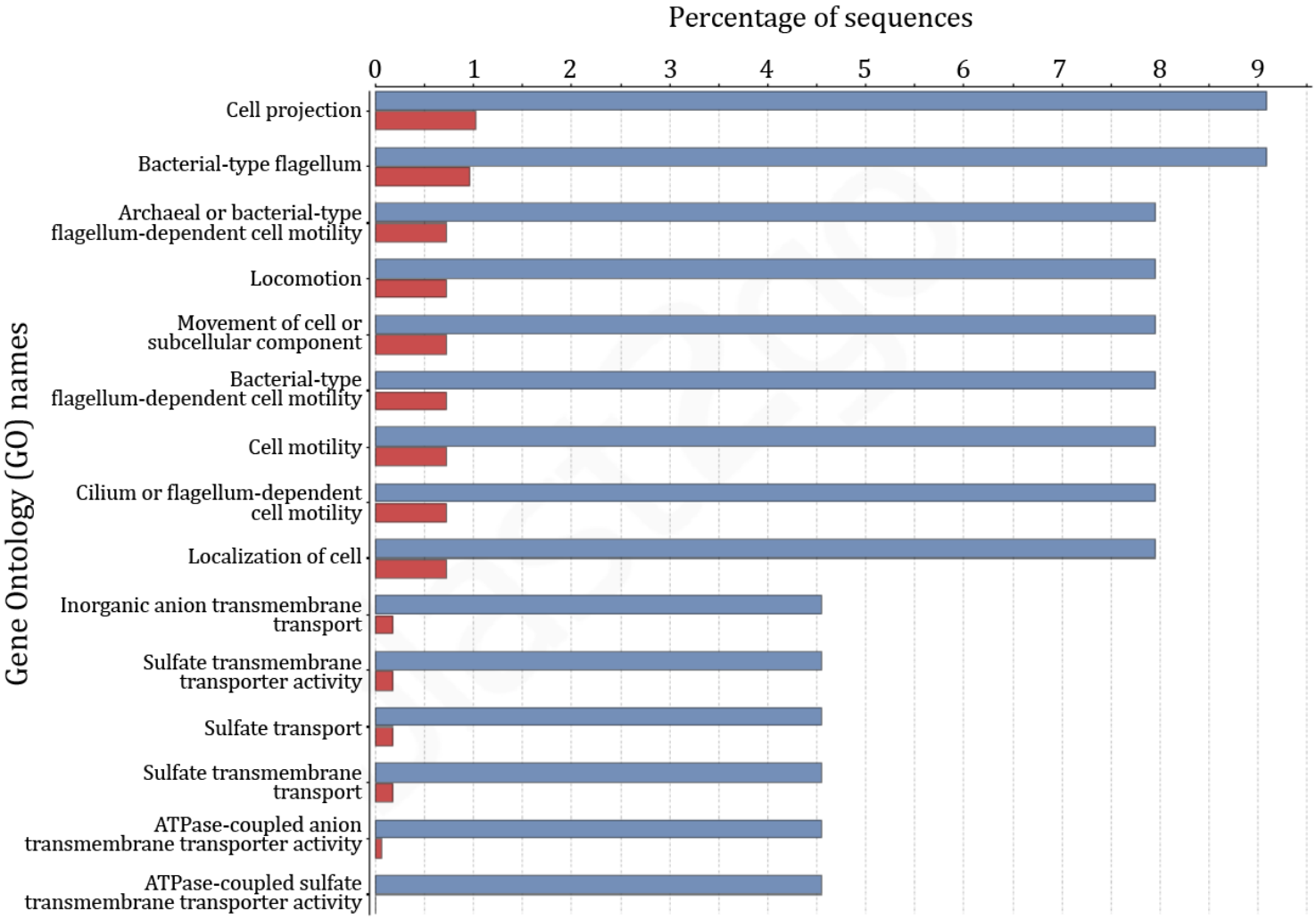

Figure 4 D) *Enrichment analysis of downregulated genes in vivo at day 10 post colonization with F. perrara. See legend in Fig. 4A.*

Next a pathway analysis was performed with KEGGmapper in order to determine how SDEGs were distributed within different molecular pathways. We found that multiple SDEGs that were upregulated at both day 5 and day 10 belonged to the tryptophan biosynthesis pathway (**Fig. 5A**). Several genes downregulated at both time points were part of the sulfur metabolism pathway (**Fig. 5B**). Several SDEGs downregulated at day 5 were found to be part of the flagellar assembly pathway (**Fig. 5C**).

Expression patterns of other genes of interest for this symbiosis

We next investigated the expression patterns of specific genes which potentially play a role in this symbiosis. The melanization of the host leads to the formation of highly reactive species including hydrogen peroxide (H_2O_2) which cause oxidative stress. We hence looked at the gene expression of catalase which can split the H_2O_2 molecule into H_2O and O_2 and hence lower oxidative stress (Zamocky et al., 2008). Catalase was found to be significantly upregulated at day 5 (`~11 x relative to in vitro) and day 10 (~34 x). Most genes involved in aryl-polyene were not significantly differentially expressed in vivo but were constitutively expressed in all conditions. The colibactin gene cluster has been found in different symbiotic bacteria and has been shown to cause DNA damage (Engel et al., 2015b). We found that genes of this cluster were significantly downregulated at day 10 but not at day 5.

We investigated the gene expression of type VI secretion systems (T6SS) which are have been shown to mediate exchanges with the host (Jani and Cotter, 2010). Several genes encoding for effectors of the T6SS were downregulated in vivo, although most of the genes involved in the T6SS were constitutively expressed in both conditions. Although *F. perrara* was determined to have β-glucosidase activity, we did not find significant changes in the genes related to β-glucosidase activity between conditions. Finally, we analyzed the expression of genes involved in iron acquisition and found them to be upregulated in vivo, in line with the fact that iron is important for bacterial metabolism (Faraldo-Gómez and Sansom, 2003) and that iron acquisition related genes in *S. alvi* promote gut colonization and are upregulated in vivo (Powell et al, 2016).

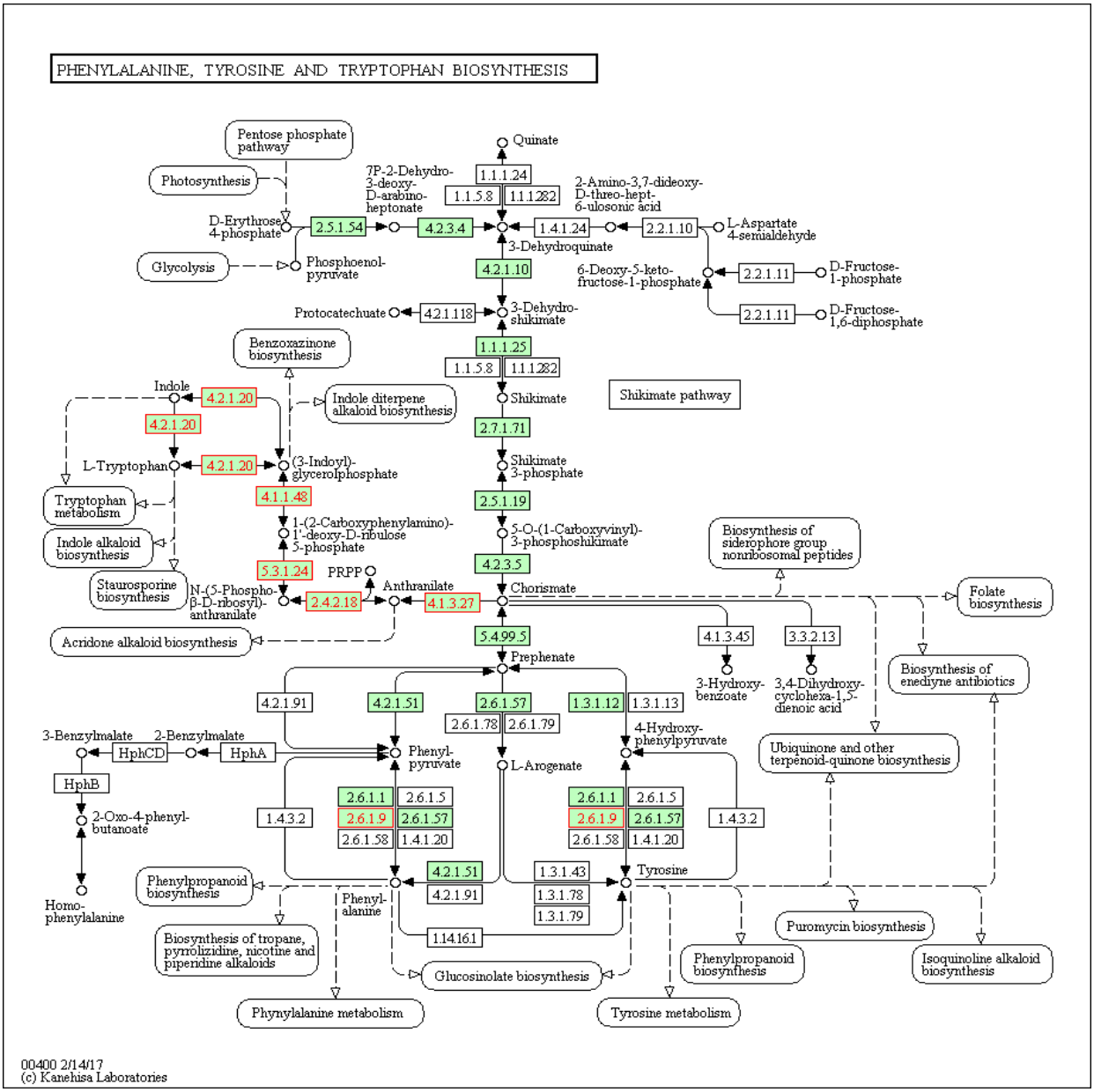

Figure 5 A) *Localization of SDEGs upregulated on both day 5 and day10 relative to in vitro samples on the phenylalanine, tyrosine and tryptophan biosynthesis KEGG pathway. Green boxes indicate gene presence in the genome of F. perrara with the Enzyme Commission (EC) code of the corresponding enzyme inside of the box. Boxes highlighted in red correspond to genes that were present among the SDEG set tested.*

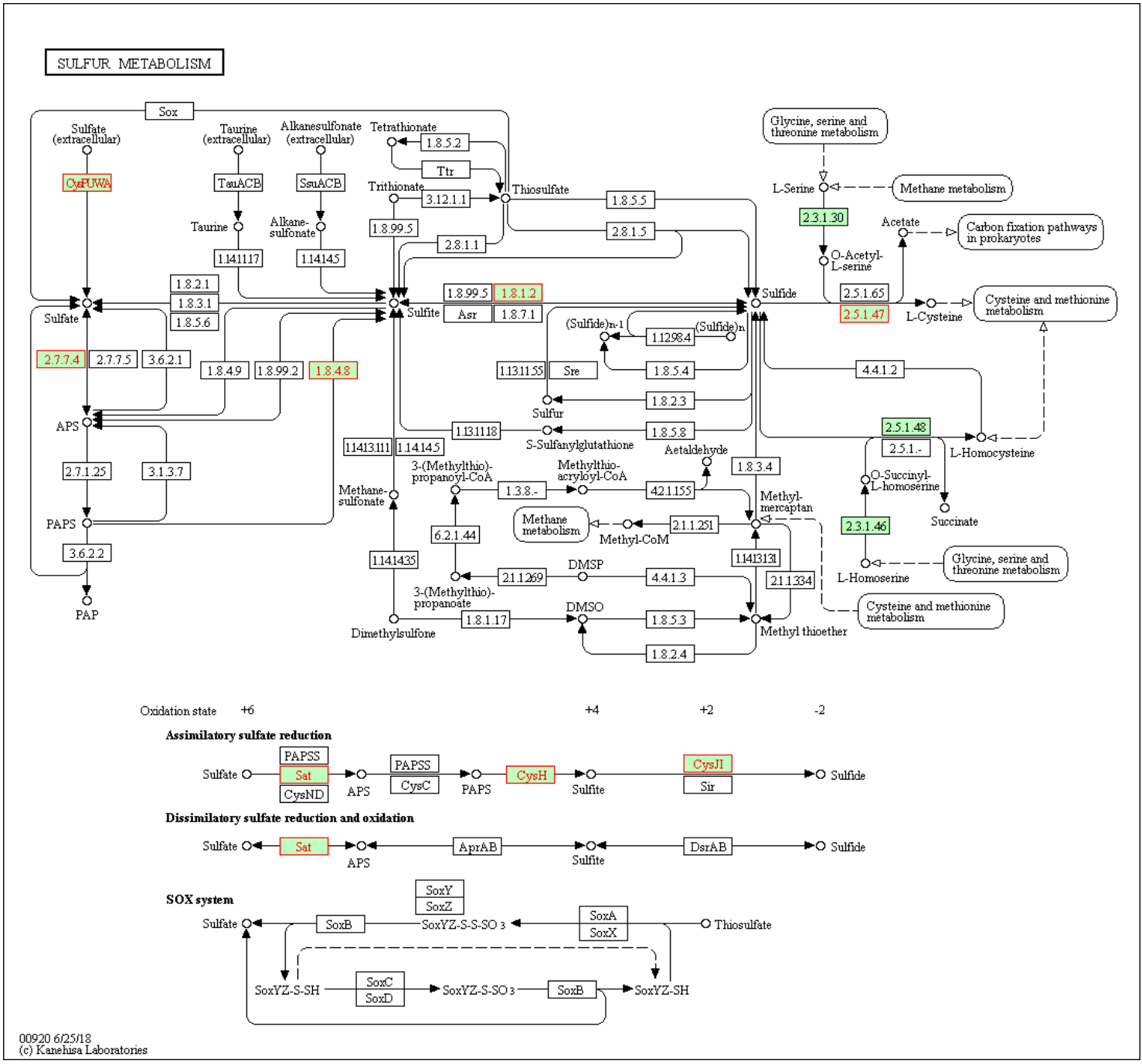

Figure 5 B) *Localization of SDEGs downregulated on both day 5 and day10 relative to in vitro samples on the KEGG pathway for sulfur metabolism. Coloring scheme as in Fig. 5A.*

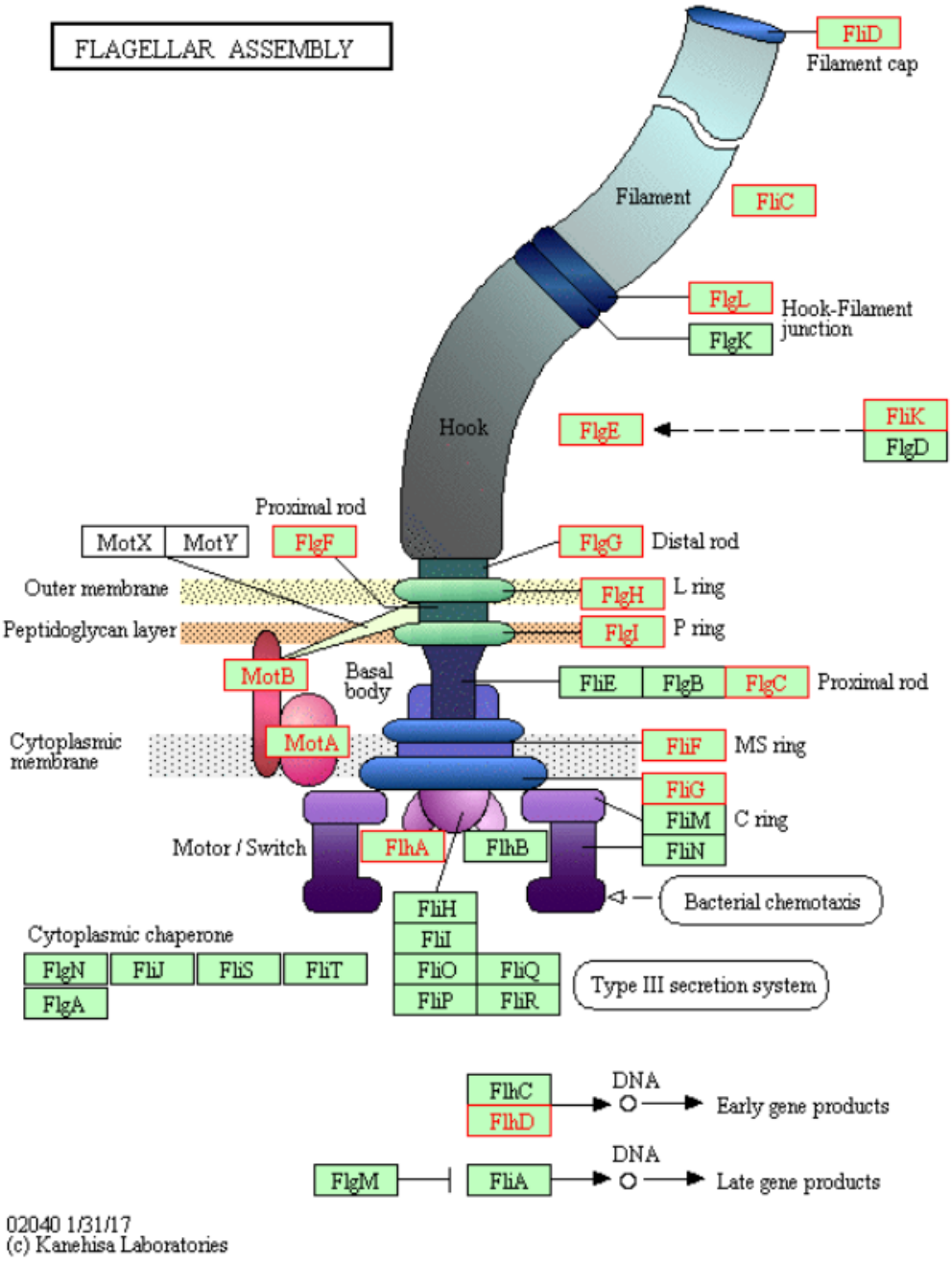

Figure 5 C) *Localization of SDEGs downregulated at day 5 relative to in vitro samples on the KEGG pathway for flagellar assembly. Coloring scheme as in Fig. 5A.*

Discussion

Our transcriptome analysis on *F. perrara* sheds light on how this bacterium responds to in vivo conditions in the bee gut relative to in vitro conditions. Despite the high abundance of other RNA molecules, we were able to sufficiently enrich for *F. perrara* mRNA so that we could obtain an appropriate amount of sequencing depth for the in vivo samples to perform differential gene expression analysis (Haas et al., 2012). We found similar numbers of SDEGs of *F. perrara* in vivo at day 5 (n=262) and at day 10 (n=361) relative to *F. perrara* grown on plate, with most of the SDEGs in each comparison differentially expressed at both time points (n=198, **Fig. 2A**). Contrary to our expectations, we found no SDEGs when comparing day 5 directly to day 10 in vivo with the log2 fold change greater than 2 (and only 33 SDEGs when not applying this cutoff). This indicates that despite different *F. perrara* levels and different scab percentages at day 5 and day 10, the transcriptomes at both time points were similar and there were no density-dependent differential gene regulation between these two time points. It would be interesting to determine if the transcriptome of *F. perrara* in vivo is different at even earlier time points during colonization, when absolutely no scab is observed yet. However, this will be difficult to assess, because the levels of *F. perrara* at such early timepoints may be simply too low to obtain sufficient sequencing reads from bacteria.

Compared to in vitro growth, carbohydrate and ion transporters were found to be overrepresented among the upregulated genes in vivo, which is consistent with nutrient exchange occurring between the bacteria and the host. Furthermore, genes upregulated in vivo were significantly enriched in genes involved in tryptophan biosynthesis. Tryptophan is an essential amino-acid and amino acid production has been shown to be important for *S. alvi* colonization, in particular for essential amino acids (Powell et al. 2016). Tryptophan biosynthesis has been shown to have protective effects for Mycobacteria against CD4 T-cell-mediated killing (Zhang et al., 2013) but since insects do not have an acquired immune system, these effects may not be applicable to *F. perrara* in the bee gut. Although we do not know if tryptophan is exported from *F. perrara* cells to the host or if the upregulation observed is to complement low tryptophan levels found in the gut, tryptophan supply to the host from symbionts has been shown for the intracellular symbionts of aphids that provide essential amino acids to their host that cannot synthesize them (Hansen and Moran, 2011; Douglas and Prosser, 1992). Determining the fate of tryptophan (i.e. whether it is used by *F. perrara* or exported to the host, and its localization) will be important to assess its role in this symbiosis. Tryptophan is a precursor to a large number of complex microbial products, almost every atom can be enzymatically modified, and tryptophan can undergo spontaneous, non-enzyme catalyzed

reactions (Alkhalaf and Ryan, 2015). Tryptophan biosynthesis is part of a pathway connected to tyrosine biosynthesis (**Fig. 5A**) and tyrosine is a substrate used by the melanization cascade (Ellango et al., 2018; González‐Santoyo and Córdoba‐Aguilar, 2012). Hence, the upregulation of tryptophan biosynthesis genes we observed in vivo may ultimately lead to the production of tyrosine used as a substrate in the host melanization response. Under this hypothesis, *F. perrara* may contribute to the formation of the scab phenotype directly by increasing the tyrosine levels available for the host melanization cascade. Further work would be needed in order to confirm this hypothesis. In particular, mutants of *F. perrara* for the SDEGs involved in tryptophan biosynthesis could be used to test if the scab is still formed after colonization with these mutants or if there are general fitness effects on gut colonization. Alternatively, tryptophan may affect honey bee behavior if it reaches the brain, as evidenced from lower neuronal activation in honey bee mutants of the kynunerine pathway for tryptophan metabolism and behavioral changes including reduced locomotion in mutant *Drosophila* of this pathway (Smirnov et al., 2006, 2007; Zakharov et al., 2012).

The catalase gene of *F. perrara* was upregulated in vivo and may provide a protection against peroxide formation during melanization of the host. Interestingly this gene was more upregulated at day 10 than at day 5 when the scab is more developed and reactive oxygen species may be more

abundant. Contrary to what was found in *S. alvi* by Powell et al., we did not observe upregulation of T6SS genes in vivo, and even determined that several *F. perrara* genes coding for T6SS effectors were downregulated. Nevertheless, most genes coding for T6SS elements were constitutively expressed both in vivo and in vitro (data not shown), suggesting that under the in vitro growth condition of *F. perrara*, these functions may be already active. This highlights an important limitation of RNA-seq analysis: genes important for environmental adaptation may not be differentially expressed but rather be constitutively expressed (Evans, 2015). Accordingly, there was little overlap in the study of Powell et al. in *S. alvi* between genes differentially expressed and genes determined to be promoting gut colonization based on Tn-seq (i.e. 22 out of 369 genes). Besides constitutive expression, undetected transitory expression of genes at earlier time points may explain the incongruence between transcriptional responses and gene essentiality.

Genes involved in cell motility and, to a lesser extent (i.e. only for day 10 samples), sulfur metabolism were enriched among SDEGs downregulated in vivo or, correspondingly, enriched in upregulated genes in vitro. Cell motility may not be needed anymore once *F. perrara* has colonized the bee gut, while on agar plates, this may allow cells to move towards higher nutrient patches if the local nutrient concentration surrounding cells diminishes. Consistent

with our results, cell motility has been shown to be important for host colonization and to cease once the symbiosis is established in the association between luminescent *Vibrio fisheri* bacteria and the sepiolid squid *Euprymna scolopes* (Ottemann and Miller, 1997). Concerning sulfur metabolism, we may speculate that sulfur is taken up by *F. perrara* from the medium on plates to form cysteine. Cysteine has been shown to be important for *Staphylococcus aureus* under stress conditions (Lithgow et al., 2004).

Other experimental settings and techniques may provide additional information on this symbiosis. For example, determining the transcriptomes of in vivo *F. perrara* from bees co-colonized with another gut microbiota species, may reveal other gene expression differences such as genes involved in inter-bacterial competition relative to the mono-colonization experiments conducted here. Furthermore, the development of efficient genetic tools to produce *F. perrara* mutants is needed in order to assess the contribution of different genes to colonization success and scab formation. The random insertion mutagenesis screening technique Tn-Seq used for *S. alvi* in (Powell et al., 2016) or for *Streptococcus pneumoniae* (van Opijnen et al., 2009) could then be used to determine which *F. perrara* genes are important in order to colonize the host. While we have separately assessed the transcriptomes of the host (Emery et al., 2017) and its symbiont (this work) in response symbiosis, recently available methods for dual RNA-seq in which both host

and bacterial gene expression are evaluated at the same time (Westermann et al., 2017) could be performed to assess differential gene expression simultaneously in the honey bee and in *F. perrara*. Furthermore, determining the dynamics of transcriptomes from the host and from the symbiont using time resolved dual RNA-seq could provide important cues to further understand the interplay between these two organisms.

Supporting information

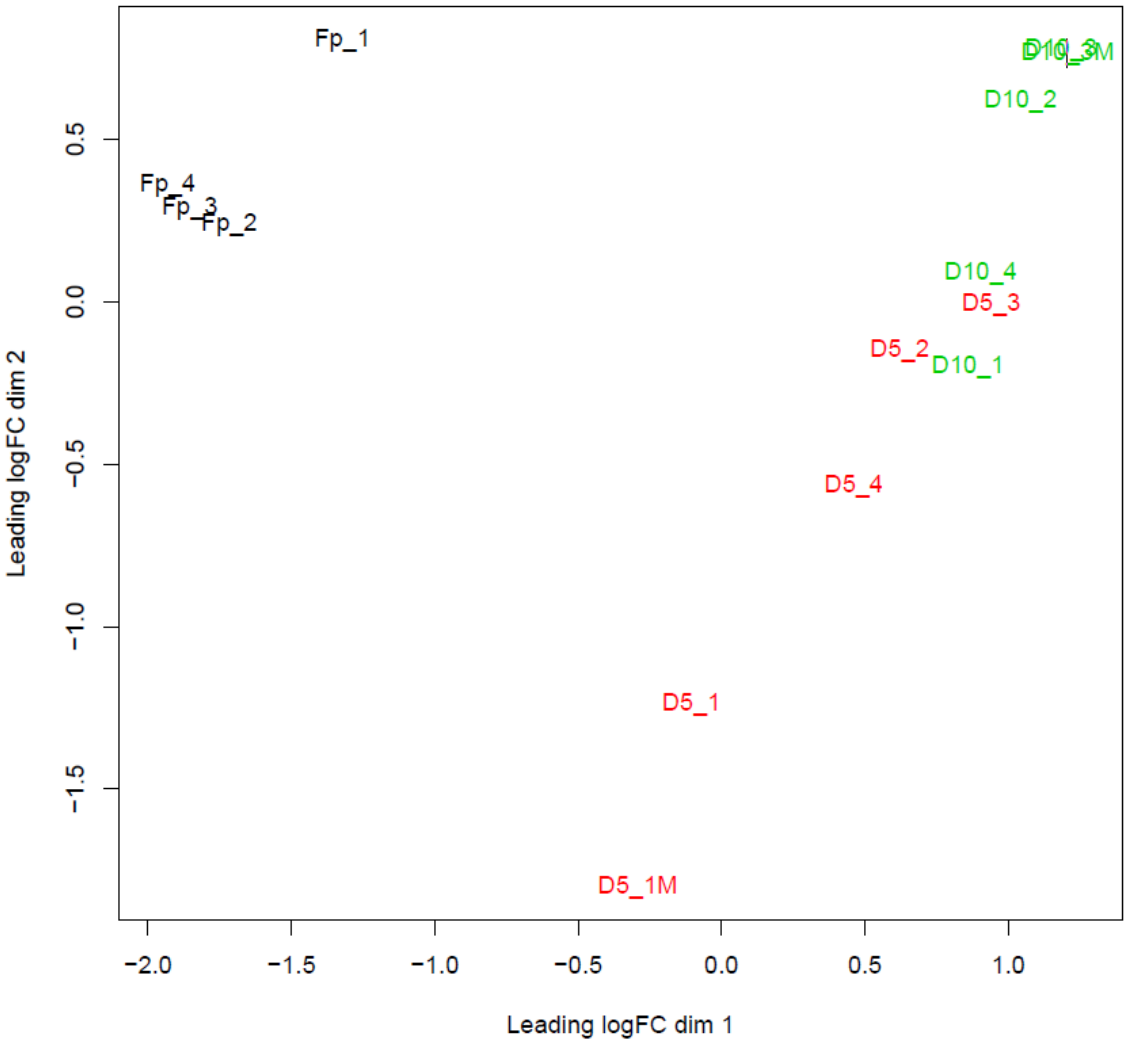

Figure S1 *MDS plot showing global distribution patterns of all sequenced samples. In vivo samples sequenced on the HiSeq instrument from day 5 (D5_1, D5_2, D5_3, D5_4) and day 10 (D10_1, D10_2, D10_3, D10_4) post colonization, in vitro samples (Fp_1, Fp_2, Fp_3, Fp_4) and in vivo replicates sequenced on the MiniSeq instrument (D5_1M, D10_3M). Please note that D10_3 and D10_3M legends are overlapping on the right top corner of the plot.*

3) *F. perrara* in the context of the hive

Summary

Why *F. perrara* can be found in every hive but not in every bee remains unclear. One possibility is that its presence correlates (positively or negatively) with the presence of other community members. To test this hypothesis, I monitored the honey bee gut microbiota composition and the levels of two common bee pathogens from individual bees sampled monthly from a single hive during two years. As this project involved a lot of sampling, DNA extractions, and qPCR assays, and covered a broad range of scientific questions, the project was carried out in collaboration with Lucie Kešnerová, another PhD student in the lab. We sampled foragers returning with pollen to the hive during the foraging season, and winter bees taken from inside the hive in winter. In a series of follow-up experiments, we (i) included younger nurse bees during the foraging season to our analysis, which have a more similar diet to winter bees than forager bees, (ii) expanded our screening to a larger number of hives to test for the generality of the observed patterns, and (iii) included a laboratory experiment to test whether diet can explain the differences observed in microbiota composition. Data analysis was conducted in collaboration with Dr. Berra Erkosar (post-doctoral researcher at the Department of Ecology and Evolution, University of Lausanne). Although we could not detect clear patterns of correlations between *F. perrara* and other microbiota members or changes over time, we found that winter bees showed a markedly distinct gut microbiota

community relative to foragers and nurses. While both winter bees and nurse bees had higher total bacterial loads than nurse bees, the community of winter bees were heavily dominated by Firm-5 and *B. apis*. Strikingly, these differences between winter bees and forager and nurse bees were found across hives sampled in different localities of the canton de Vaud suggesting that this is general charactersitic of the gut microbiota of winter bees. The laboratory colonization experiments allowed us to link the overall higher bacterial loads to the dietary differences between winter and nurse bees (pollen/bee bread) compared to forager bees (mostly honey and nectar). However, the extremely high levels of *B. apis* in winter bees could not be recapitulated in this experiment suggesting that other factors could be at play. The fact that winter bees have a different microbiota than bees during the summer months may have important implications in colony mortality over winter and to devise bee probiotic formulations.

The main results of this analysis, focusing on the differences in the gut microbiota between foragers, winter bees and nurses, are summarized in chapter 3.1 as a manuscript in preparation for submission to the journal Molecular Ecology. Chapter 3.2 provides additional data about *F. perrara* levels and scab intensity across the entire dataset and about the occurrence of the scab phenotype along seasons.

3.1) Gut microbiota characterization of summer and winter honey bees reveals distinct community structures

Olivier Emery[1], Lucie Kešnerová[1], Michaël Troilo, Berra Erkosar , Philipp Engel*

Department of Fundamental Microbiology, University of Lausanne, CH-1015 Lausanne, Switzerland

[1]equally contributed

*Correspondence:
Prof. Philipp Engel
Department of Fundamental Microbiology
University of Lausanne, CH-1015 Lausanne, Switzerland
Tel.: +41 (0)21 692 56 12
e-mail: philipp.engel@unil.ch

Keywords: (6) Microbiota, honeybee, *Apis mellifera*, qPCR, pathogens, pollen, winter

Abstract

Independent studies have shown that honey bees harbor a relatively simple gut microbiota that is globally distributed. Yet, the dynamics of the relative composition of this community over time remain largely unexplored. Here we investigated the honey bee gut microbiota composition through seasons by performing longitudinal sampling of individual nurses, foragers and winter bees of a single colony over consecutive years. Using qPCR, we quantified absolute abundance of the major gut microbiota community members and revealed that winter bees had a distinct gut microbiota community than foragers and nurses. This was characterized by higher total bacterial loads and by high relative abundances of the phylotypes Firm-5 and *B. apis* which dominated the community in winter bees. We also monitored two common pathogens (i.e. *Nosema ceranae* and trypanosomatids) and found lower prevalence and abundance of *N. ceranae* in winter bees while trypanosomatids were similarly present and abundant across bee types. By analyzing gut samples of pooled bees from 14 different hives from two different geographic sites, we confirmed that the main differences observed in the seasonal structure of the gut microbiota is a general trend that can be observed across colonies. Finally, we performed laboratory experiments with gnotobiotic bees to show that diet may be one factor that affects the seasonal gut microbiota structure. In summary, our study highlights that

winter bees can have a markedly different gut microbiota composition relative to foragers and nurse bees. Further research is needed in order to determine the reproducibility of the observed patterns under larger geographical ranges and to determine their putative functional role in bee health.

Introduction

In contrast to many other animals, community analyses have shown that the gut microbiota of the Western honey bee is of low taxonomic complexity. In female worker bees the community is dominated by only 7-9 phylotypes, (i.e. clusters of strains sharing ≥97% sequence identity over the analyzed 16S rRNA gene fragment) making up >95% of all bacterial cells in the gut (Raymann and Moran, 2018; Kwong and Moran, 2016; Corby-Harris et al., 2014; Moran et al., 2012; Sabree et al., 2012; Martinson et al., 2011; Cox-Foster et al., 2007). These phylotypes have been consistently detected in honey bees, regardless of geographic location, life stage or sampling season (D'Alvise et al., 2018; Corby-Harris et al., 2014; Moran et al., 2012); and are acquired horizontally through contact with nest mates and hive components (Powell et al., 2014). They include five core members (*Snodgrassella alvi, Gilliamella apicola,* Lactobacillus Firm-4 and Firm-5, and *Bifidobacterium asteroides*) that are present in all bees, and other non-core members such as *Frischella perrara* or *Bartonella apis,* which are not found in all bees (Kwong and Moran, 2016).

A balanced gut microbiota is an important factor for honey bee health (Raymann and Moran 2018; Anderson et al. 2011; Hamdi et al. 2011). Recently, gnotobiotic bee studies have elucidated some key effects of the gut

microbiota on the host, such as increased weight gain, enhanced responsiveness to sucrose (Zheng et al. 2017) and immune system stimulation (Kwong et al. 2017; Emery et al. 2017). Moreover, disruption of the healthy gut microbiota composition by antibiotic treatment or dietary manipulations was associated with increased rates of mortality (Raymann, Shaffer, and Moran 2017; Maes et al. 2016). Therefore, understanding the dynamics of gut microbiota composition in honey bees is crucial for determining the factors affecting bee health.

The honey bee is considered as a superorganism (Emerson 1939) with a social organization that involves division of labor between and within castes, and dense communication networks ensuring group level coordination (Johnson and Linksvayer 2010). Newly emerged worker bees (nurses) stay inside the hive, take care of larvae and feed on nutrient-rich pollen, while 1-2 weeks old workers become foragers that feed mainly on nectar and honey to fuel their flights (Brodschneider and Crailsheim 2010). From late autumn to early winter, winter bees that have an extended lifespan are produced to ensure the colony survival in cold temperatures in the absence of brood during winter (Winston 1991). These bees form a tight cluster for thermoregulation inside the hive during winter, feed strictly on food stores (pollen beebread and honey) and retain their feces all winter (Pavlovsky and Zarin 1922). Overall, honey bee nutrition is dependent on seasonal changes

that determine the type and quantity of available nutrient sources (i.e. pollen and nectar). Because diet is a major factor contributing to changes in the gut microbiota in many animal species (Smits et al., 2017; Ben Guerrero et al., 2016; David et al., 2014b; Muegge et al., 2011; Zoetendal and de Vos, 2014), the community structure and function of the honey bee gut microbiota are susceptible to changes in time. However, these changes remain largely unexplored.

To date, most of the knowledge on the adult honey bee gut microbiota composition has been derived from single time points from different hives (Moran et al., 2012; Sabree et al., 2012) or between only two time points in different seasons (Corby-Harris et al., 2014) and point towards a stable gut microbiota composition. One study provided bacterial composition data from six consecutive months during foraging season but was restricted to the midgut/pylorus (Ludvigsen et al. 2015). Another study investigated bacterial community composition from whole guts for a period of four months before and during the almond pollination in California, in the presence or absence of supplemental forage. This study showed that supplemental forage did not strongly affect gut microbiota composition; and that the microbial communities of individual bees from the same colony could exhibit as much variation as those from different colonies (Rothman et al. 2018). Despite these studies, the extent to which the honey bee gut microbiota composition

stays stable throughout seasons remains to be determined over longer periods.

In contrast to composition dynamics in honey bee gut microbiota, the prevalence through seasons of several bee pathogens including the *Varroa destructor* mite has been reported to vary throughout the year (Antúnez et al., 2015; Copley et al., 2012; Runckel et al., 2011; Tentcheva et al., 2004). Temporal infection patterns of the microsporidian pathogen *Nosema ceranae* have been previously characterized but with conflicting results: some studies reported a peak of infection in summer (Mulholland et al., 2012; Runckel et al., 2011; Traver et al., 2012) while other studies found infections to peak during winter (Retschnig et al. 2017; Traynor et al. 2016; Fries et al. 2013). *N. ceranae* causes lesions of the host intestinal epithelial layer and negatively affects the normal process of digestion (Dussaubat et al. 2012; García-Palencia et al. 2010) and correlates with bee hive depopulation (Higes et al. 2008; Martín-Hernández et al. 2007). However, the direct or indirect impact of pathogen abundance on gut microbiota composition (or vice versa) by community invasion or by modulation of host immunity is not yet known.

Here, we quantified the abundances of each gut microbiota member in individual honey bees that were collected monthly in different seasons over a period of two years. We also assessed the levels of two common bee pathogens (i.e. *Nosema ceranae* and trypanosomatids). We determined the

bacterial community structures of foragers, nurse bees and winter bees from the same hive the following year, experimentally confirmed that diet can affect gut community composition and expanded our analysis to multiple hives. Absolute quantification of gut microbiota phylotypes in the monthly sampling revealed differences in microbial community structure and pathogen prevalence between winter bees and foragers. Nurse bees have a pollen-based diet as winter bees, and we found similarities between the gut microbiota structure of nurses and winter bees. We further determined experimentally with bees colonized with an artificial community that diet can impact the honey bee gut microbiota structure. Altogether, our results indicate that each bee type possesses its own specific gut microbiota composition structure and that diet plays a role in shaping gut microbiota structure although other yet undetermined factors are likely involved in this process.

Materials and Methods

Sampling of honey bees

Apis mellifera carnica bees were collected from a healthy colony located at the apiary of the University of Lausanne, Switzerland. For the monthly monitoring of the honey bee gut microbiota, ~24 bees were collected

monthly from a single hive (named “Dent de Morcles”) during two years. We sampled either bees returning to the hive entrance with pollen on their legs during the foraging season (hereafter “foragers”) or, during cold months when bees did not forage, bees on top of frames from inside the hive (hereafter “winter bees”) Each sampling time point took place at the middle of each month (+/- 3 days) between April 2015 and April 2017 with the exception of July 2017 due to a manipulation error during DNA extraction. Additional sampling took place in the same hive in July 2017 and January 2018 to compare the gut microbiota of foragers, nurses and winter bees. For this, we sampled both forager bees returning to the hive with pollen and bees inside of the hive considered to be mostly nurse bees in July 2017, and winter bees were sampled from inside the hive on top of frames in January 2018. For samples from multiple hives, we pooled 20 bee guts per sample and collected samples from foragers and nurses in August 2018 and winter bees in January 2019 from 11 hives located on the UniL campus and from 3 hives located in Yens, about 17 km from the University of Lausanne. For all dissections, bees were anesthetized via exposure to CO2 for 10 seconds and each individual bee gut including crop, mid- and hindgut and Malpighian tubules was dissected using sterile forceps. Individual gut samples were then placed in a drop of PBS, scored for the scab phenotype, weighted in case of additional sampling, and placed in a bead beating tube containing

approximately 150mg of glass beads (0.75-1 mm in diameter, Carl Roth), 750 µL of CTAB lysis buffer (0.2 M Tris-HCl, pH 8; 1.4 M NaCl; 0.02 M EDTA, pH 8; 2% CTAB, w/v, dissolved at 56°C overnight; 0.25% β-mercaptoethanol, v/v). Tubes were flash frozen in liquid nitrogen immediately after adding guts and stored at -80°C before DNA extraction. For each pooled sample, 20 bee guts were dissected and placed inside a 50 mL Falcon tube placed on ice which was then flash frozen in liquid nitrogen and kept at -80°C until DNA extraction.

Experimental colonization of honey bees to investigate the effect of diet

Generation and colonization of newly emerged bees was performed as described in Kešnerová et al. (2017). In fact, the colonized bees fed on pollen diet were the same as in this study. The experiment comprised two groups of bees, the first group (SW) was fed *ad libitum* on sterilized sugar water (50% sucrose) while the second group (SW+P) had access to both sugar water and pollen. Bees from both groups were colonized with the selection of 11 strains which were provided as a 300 µl inoculum in a feeder (a 6-well piece cut out of a 96-well plate) in the presence or absence of 1 mg of sterilized pollen (10 MeV electron beam). Bees from the SW+P group (n=26) were generated in five treatment boxes (~20 bees) during spring and

autumn 2016. Bees from SW group (n=20) were generated in three treatment boxes during autumn 2016.

DNA extraction from honey bee gut tissues

The DNA extraction protocol and qPCR analysis correspond to the method described in Kešnerová et al. (2017) with modest modifications: samples with individual guts were thawed on ice and homogenized in a Fast-Prep24™5G homogenizer (MP Biomedicals) at 6 m/s for 45 s, briefly centrifuged, and 1 ml of Roti®-Phenol (Carl Roth, pH 7.5-8) was added. After mixing thoroughly, samples were incubated in a water bath at 64°C for 6 min with occasional shaking. Samples were then transferred to a new tube containing 400 µl of chloroform, mixed and the phases were separated by centrifugation at 16000x g for 10 min at room temperature. The upper aqueous phase (500 µl) was transferred and mixed with 500 µl phenol:chloroform:isoamyl alcohol (Fischer Bioreagents, pH 6.5). After centrifugation at 16000x g for 3 min at room temperature, the upper aqueous phase was transferred and mixed with the same volume of chloroform. After another centrifugation, 300-350 µl of the upper aqueous phase was transferred to a new tube, mixed with 900 µl of pre-cooled 100% RNase-free ethanol, and incubated overnight at -80°C for precipitation of nucleic acids. Precipitated nucleic acids were pelleted at 16000x g at 4°C for

30 min. Pellets were washed with 900 µl of 70% ethanol, dried for 5-15 min and resuspended in 200 µl of nuclease-free water (Invitrogen) by shaking in a thermo-mixer (64°C, 400 rpm, 10 min). The resulting 200 µl of RNA/DNA mix was split into two tubes à 100 µl. One tube was stored at -80°C while the second was processed with the Nucleospin PCR Clean-up kit (Macherey-Nagel, Germany) according to the manufacturer's instructions in order to obtain clean DNA for qPCR analysis. DNA concentrations were assessed with Qubit™ (Thermo Fisher) and ranged usually between 7 – 15 ng/µl.

For pooled samples containing 20 guts, 2 ml of glass beads and 15 ml CTAB lysis buffer were added to each sample. Samples in Falcon tubes were then homogenized in a Fast-Prep24™5G homogenizer at 6 m/s for 40 s, briefly centrifuged, and an aliquot of 750 µl was transferred to a new 2 ml bead beating tube with beads and homogenized again. These aliquots should represent an average bee since they correspond to the same volume as when an individual gut was processed. Further steps in DNA extractions of such pooled samples were performed as described above.

Quantitative PCR (qPCR) to determine bacterial and pathogen loads

All qPCR reactions were carried out in a 96-well plate on a StepOnePlus instrument (Applied Biosystems) with the thermal cycling conditions as follows: denaturation stage at 50°C for 2 min followed by 95°C for 2 min, 40

amplification cycles at 95°C for 15 s, and 60°C for 1 min. Melting curves were generated after each run (95°C for 15 s, 60°C for 20 s and increments of 0.3°C until reaching 95°C for 15 s) to compare dissociation characteristics of the PCR products obtained from gut samples and positive control. Each reaction was performed in triplicates in a total volume of 10 µl (0.2 µM of each forward and reverse primer; and 1x SYBR® Select Master Mix, Applied Biosystems) with 1 µl of DNA. Each plate contained a positive control and a water control.

To determine the absolute quantity of each target in the samples we performed standard curves on serial dilutions of plasmids (pGEM®-T Easy vector; Promega) containing the target sequence. qPCR conducted on genomic DNA from the bee gut or on pure plasmid DNA results in different primer efficiencies (*E*) (due to the complexity of DNA samples, different methods of extraction, presence of inhibitors, etc.) (Brankatschk et al. 2012). Therefore, to assess more realistic primer efficiencies plasmid dilutions were mixed 1:1 with DNA isolated with the above CTAB-based protocol from the gut of a newly emerged microbiota-free bee that was negative for all investigated targets. The final concentrations of the plasmid in these template samples ranged from 10^7 – 10^1 copies per µl. The plasmid copy number was calculated from the molecular weight of the plasmid and the DNA concentration of the purified plasmid measured with Qubit™ (Thermo

Fisher). The slope and intercept of standard curves were calculated based on the Cq values (quantification cycle; (Bustin et al. 2009)) obtained from the dilutions 10^7 – 10^2 copies. Frequently, no amplification was obtained at the highest dilution or the amplification came up at a similar Cq as the negative control. As the *limit of detection* (LOD) of a given primer pair, we consider the Cq value of the highest plasmid dilution, at which the target was detected. The *E* values were estimated from the slopes according to the equation: $E = 10^{(-1/slope)}$ (Pfaffl 2001). Primer characteristics and their performance are summarized in **Table 1**.

The MIQE guidelines (minimum information for publication of qPCR experiments) were followed throughout the data analysis of the qPCR experiments (Bustin et al. 2009). A uniform detection value of fluorescence intensity was set for each target and kept the same across all qPCR plates of the study. Only samples with dissociation curves matching the curves of the positive control were kept. Technical outliers from each triplicate were eliminated and mean *quantification cycle* (Cq) and SD values were calculated. Then, the data was exported from the StepOnePlus qPCR instrument for further processing in R. We only considered data from plates for which no signal in the negative control was detected or for which the Cq value of the negative control was higher than the highest dilution (10 copies) included in the standard curve. All samples for which the Cq value of actin was >24 were

excluded from the analysis, as the extracted DNA was considered to be not of sufficient quality. For each DNA sample that passed the initial quality check, we determined the number of bacterial cells per gut as follows. We first calculated the 'raw' copy number of each target in 1 µl of DNA from the Cq value and the standard curve using the formula $n = E^{(\text{intercept} - Cq)}$ (Gallup 2011). Then, we normalized the bacterial 16S rRNA gene copies to the median number of actin gene copies by dividing by the 'raw' copy number of actin for the given sample and multiplying by the median number of actin gene copies across all samples. Normalization with the actin gene was carried out to reduce the effect of gut size variation and extraction efficiency. To infer the number of microbial cells from the normalized 16S rRNA gene copy number we divided by the number of rRNA loci present in the genome of the given phylotype (as listed in **Table 1**) and multiplied by 200 as we only analyzed 1 µl of the 200 µl of DNA obtained from each sample. In the qPCR screen of the present study, all honey bee gut trypanosomatids (i.e. both *C. mellificae* and *L. passim*) were quantified indiscriminately. For each bee gut sample, the total number of 16S rRNA gene copies were determined with universal bacterial primers. To determine the total number of bacterial cells per sample while accounting for the varying numbers of 16S rRNA gene copies and the relative abundances between bacterial phylotypes, we estimated the weighted average number of 16S rRNA genomic loci per

Target (gene name and accession no.)	Sequence (5' to 3')	Tm (°C)[a]	Amplicon size	# Gene loci per genome[b]	Standard curve			Reference and notes
					Efficiency, R^2	Slope, intercept	LOD Cq[c] (# copies)	
Apis mellifera (*actin*, AB023025)	**F:** TGCCAACACTGTCCTTTCTG **R:** AGAATTGACCCACCAATCCA	58.4 56.4	156 bp	-	1.896 = 89.6 %, 1.0	-3.6, 37.579	33.726 (10)	(Zufelato et al. 2004)
Gilliamella apicola (*16S rRNA*, JQ936674)	**F:** CTTTGTTGCCATCGGTTAGGCC **R:** CCGCTTGCTCTCGCGAGG	64.2 62.9	160 bp	4	1.86 = 86 %, 0.997	-3.709, 40.807	37.026 (10)	(Engel et al., 2015a)
Frischella perrara (*16S rRNA*, JX8783306)	**F:** GGAAGTTATGTGTGGGATAAGC **R:** CTATTCTCAGGTTGAGCCCG	60.1 60.5	185 bp	4	1.946 = 94.6 %, 0.995	-3.457, 38.042	31.704 (100)	(Kešnerová et al., 2017)
Snodgrassella alvi (*16S rRNA*, JQ746651)	**F:** CTTAGAGATAGGAGAGTGCCTT **R:** AACTTAATGATGGCAACTAATGACAA	60.1 60.1	132 bp	4	1.966 = 96.6 %, 0.999	-3.406, 38.236	31.256 (100)	(Martinson et al., 2011), modified
Bartonella apis (*16S rRNA*, KP987885)	**F:** GTGGGAATCTACCTATTTCTACG **R:** AACGCGGGCTCATCTATCTC	60.9 60.5	103 bp	2	2.051 = 105.1 %, 0.997	-3.205, 36.771	30.752 (100)	(Kešnerová et al., 2017)
Bifidobacterium asteroides (*16S rRNA*, AB437355)	**F:** ATGCAAGTCGAACGGGATCC **R:** CATCCCATRCCGGTAAACCC	60.5 60.5 – 62.5	174 bp	2	1.948 = 94.8 %, 0.997	-3.453, 38.983	34.906 (10)	(Kešnerová et al., 2017)
Lactobacillus Firm-4 (*16S rRNA*, DQ837632)	**F:** AGTCGAGCGCGGGAAGTCA **R:** AGCCGTCTTTCAACCAGCACT	61.6 61.2	169 bp	4	1.912 = 91.2 %, 0.999	-3.554, 37.196	29.966 (100)	(Kešnerová et al., 2017)
Lactobacillus Firm-5 (*16S rRNA*, JX099547)	**F:** GCAACCTGCCCTWTAGCTTG **R:** GCCCATCCTKTAGTGACAGC	60.5 60.5 – 62.5	118 bp	4	2.144 = 114.4 %, 0.998	-3.02, 36.306	29.94 (100)	(Kešnerová et al., 2017)
Universal bacteria (*16S rRNA*)	**F:** AGGATTAGATACCCTGGTAGTCC **R:** YCGTACTCCCCAGGCGG	62.9 60 – 62	variable	-	1.946 = 94.6 %, 0.993	-3.457, 37.986	33.546 (10)	(Kešnerová et al., 2017)
Universal Trypanosomatid (*18S rRNA*)	**F:** GTGCAGTTCCGGAGTCTTGT **R:** CTGAGCTCGCCTTAGGACAC	60.5 62.5	103 bp	2	2.075 = 107.5 %, 0.998	-3.155, 36.116	29.694 (100)	(vanEngelsdorp et al., 2009)
Nosema ceranae (Microsporidia) (*18S rRNA,* KC680656)	**F:** AAGAGTGAGACCTATCAGCTAGTTG **R:** CCGTCTCTCAGGCTCCTTCTC	64.1 65.3	104 bp	10	1.955 = 95.5 %, 0.994	-3.436, 40.947	33.287 (100)	(Bourgeois et al. 2010)

Table 1. Primers used in this study and standard curve characteristics.

[a]Melting temperatures were calculated with the online tool described in (Kibbe 2007). [b]The number of loci of the target gene per genome of the corresponding organism was assessed based on JGI database annotations. [c]*LOD* refers to the limit of detection of primers sets, expressed here as the lowest number of plasmid copies reliably detected by qPCR when standard curves were performed. [c]NTC refers to negative water control.

sample based on previous qPCR results with phylotype specific primers. For each sample, the weighted average number of 16S rRNA gene copies was computed as follows: the number of 16S rRNA genomic gene copies for each phylotype was multiplied by the corresponding phylotype relative abundance, summed over all phylotypes. The total number of bacterial cells was then determined by dividing the total number of 16S rRNA copies obtained with universal primers by the weighted average number of 16S rRNA gene copies in each sample.

Gut microbiota diversity analyses

We measured alpha diversity of the seven gut microbiota phylotypes screened in this study using the effective number of species as metric. The effective number of species refers to the number of equally abundant species (or in this case phylotypes) needed to obtain the same mean proportional species abundance as that observed in the dataset of interest, where all species may not be equally abundant (Jost 2006). The Shannon diversity index (H') was first computed for each sample (i.e. individual bee gut microbial community) in the R statistical platform using the function "diversity" from the R package "vegan". The effective number of species of each sample was obtained by computing the exponential of H' and values were then grouped by either bee type (i.e. foragers or winter bees).

We performed principal components analysis (PCA) to determine how similar the bacterial communities of foragers and of winter bees from the

monthly monitoring were using absolute cell numbers from the seven gut microbiota phylotypes. To perform PCA, we used the *prcomp* function of the R package "stats". PCA is an ordination method which reduces the complexity of high dimensionality data by geometrically projecting the data onto lower dimensions called principal components (PC) while minimizing the distance between the data and their projections. The PCs are defined as linear combinations of the data's original variables with the first PCs explaining the highest variance in the data (Lever, Krzywinski, and Altman 2017).

Statistics

All the statistical analyses were performed using R version 3.4.3. We tested the effect of bee type (i.e. categorical variable consisting in foragers/winter bees or foragers/nurses/winter bees depending on the tested dataset) on the following variables using linear models: individual gut microbiota phylotype levels detected by qPCR, effective number of species, total bacterial loads (i.e. universal 16S rRNA copy number), and gut wet weight. Since residuals of certain models exhibited overdispersion, we used a permutation approach to test the significance of the effects. We randomized the values of the response variable 10'000 times and computed the F-values for the tested effect for each randomized dataset. The p-values corresponding to the effects were calculated by the proportion of 10'000 F-values that were equal or higher than the observed one. The R script for the custom function was

written by modifying a previously published method (Lavanchy et al. 2016). Pairwise comparisons between different factors were performed by Tukey's HSD using "multcomp" package using *glht* function on the model. P-values were adjusted using the Bonferroni method. We used the same statistical approach to test the effect of pollen on the universal 16S rRNA copy number, wet gut weight, *A. mellifera* actin copy number and individual gut microbiota phylotype levels detected by qPCR of lab-raised honey bees.

Spearman rank correlations between all different pairs of gut microbiota phylotypes and pathogen species in which both pair members were detected by qPCR were computed by the *rcorr* function of the "Hmisc" package and p-values were corrected for multiple inference using the Holm method as implemented in the *rcorr.adjust* function of the "RcmdrMisc" package.

Results

Gut community size and composition is stable during the foraging season but differs in winter bees

To monitor absolute abundance of major honey bee gut microbiota members, we performed qPCR on DNA extracted from ~24 individual bee guts sampled from the same healthy colony every month over a period of two years starting at the beginning of the foraging season. We used phylotype-specific primers to quantify the five core members (*G. apicola*, *S. alvi, B. asteroides*, Firm-4 and Firm-5) and two non-core members (*F. perrara* and *B. apis*). Together these seven phylotypes typically constitute >95% of all bacteria in the hindgut (Cox-Foster et al., 2007; Martinson et al., 2011; Sabree et al., 2012) meaning that we obtained absolute abundance data for the vast majority of the bee gut bacterial community. To normalize across DNA samples, we also quantified the *Apis mellifera* actin gene. A few samples were removed due to low DNA quality (see methods), resulting in a total of 566 individual bees that were analyzed in the current study.

In accordance with previous reports, the core members *G. apicola*, *S. alvi*, Firm-5 and *B. asteroides* were present in all analyzed bees, and the vast majority of bees (557/566, 98.4%) also had detectable levels of the fifth core member Firm-4 (**Fig. S1**). Unexpectedly, we found that the non-core member

B. apis was also present at relatively high prevalence in the studied hive, ranging from 57-100% per month with an overall prevalence of 94.7% (536/566 bees). The other non-core member *F. perrara* was less prevalent ranging from 42-100% per month with an overall prevalence of only 73.1% (414/566 bees). Consistent with previous results, *F. perrara* also showed a strong correlation with the presence of the scab phenotype (**Fig. S2**), a local melanization response in the pylorus region of the gut, previously shown to be induced upon experimental colonization with *F. perrara* (Engel et al., 2015a).

We calculated the cell number for each bacterial phylotype per bee and visualized the corresponding relative abundances. We observed major fluctuations in relative abundances of bacteria during winter and early spring, when winter bees were sampled inside the hive rather than foragers during the rest of the year (**Fig. 1A**). We therefore determined bee type (winter bees versus foragers) as a major factor that could affect the gut community structure in honeybees and analyzed how bacterial abundances change between foragers and winter bees. We observed a 10 to 100 fold increase in the levels of the core species Firm-4, Firm-5, and *B. asteroides*, as well as the non-core species *B. apis* in winter bees (**Fig. 1B**, df=1, P=0.001 for the latter four species). We observed a smaller but significant increase for *S. alvi* in winter bees (**Fig. 1B**, df=1, $P=4x10^{-4}$), but no significant difference for

G. apicola (**Fig. 1B**, df=1, P=0.6). In contrast, we observed a significant decrease in the abundance of *F. perrara* (**Fig. 1B**, df=1, $P=10^{-4}$). The increased levels of the four former gut microbiota members in winter bees were also reflected in the increase in total bacterial load per gut as based on the summed abundances of the seven members ("Total" in Fig. 1B, df=1, $P=10^{-4}$) and on the absolute abundance determined with universal 16S rRNA qPCR primers (**Fig. S3**, df=1, $P=10^{-4}$). In both years, Firm-5 and *B. apis* dominated the community in winter bees (**Fig. 1A**) resulting in a significantly lower alpha-diversity compared to foragers (**Fig. 1C**, df=1, $P=10^{-4}$). In line with this, principal component analysis showed that bees cluster separately according to bee type (**Fig. 1D**) and that this separation is driven by the abundances of Firm-4, Firm-5, *B. apis* and *B. asteroides*, i.e. the phylotypes that show increased abundance in winter bees.

In order to identify potential negative or positive associations between gut microbiota members, we computed Spearman correlation coefficients between phylotype pairs separately for foragers and winter bees (top and bottom panels respectively in **Fig. 1E**). *B. asteroides* was positively correlated with Firm-4, Firm-5, *B. apis* and *G. apicola* in both bee types with higher r values in winter bees. Similarly, Firm-5 was positively correlated with Firm-4 and *G. apicola* in both foragers and winter bees, with higher r values in winter bees. By contrast, *S. alvi* was positively correlated with *G. apicola*,

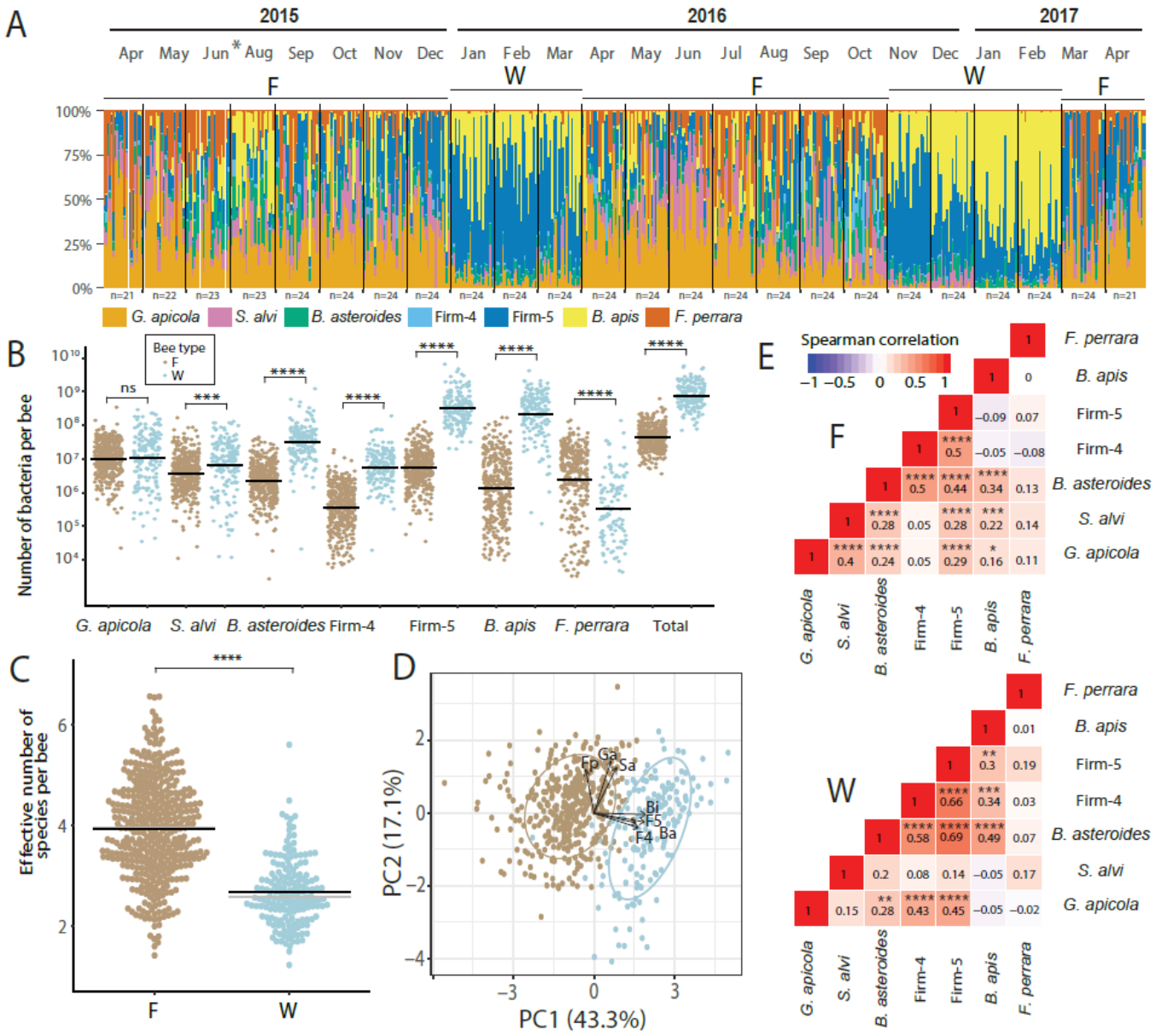

Figure 1 Gut bacterial communities change between foraging season and winter.

(A) *Relative community composition in individual bees based on the absolute abundance of the seven monitored phylotypes. F indicates samples from forager bees and W indicates winter bee samples. Asterisk indicates missing month (July 2015) due to sampling error.* ***(B)*** *Number of cells per individual honey bee gut in F and W bees for each of the seven bacterial phylotypes and their sum ("Total") based on qPCR results. For each gut microbiota member, only bees with detectable levels were considered and their mean cell number is shown as a black horizontal line. Asterisks indicate significance levels of each linear model*

to compare cell numbers between F and W bees, ns non significant. ***(C)*** *Effective number of species in F and W bees (linear model, df=1, P=10^{-4}). Black and grey lines show mean and median values, respectively.* ***(D)*** *Principal component analysis of F and W bees based on the cell numbers of the 7 monitored bacterial phylotypes. (**E**) Heatmap of Spearman correlations between gut microbiota members in F (top panel) and W (bottom panel) bees. For each pair of phylotypes, the correlation value is indicated in the corresponding square. Asterisks indicate correlation significance levels while absence of asterisk indicates no significance.*

B. asteroides, Firm-5 and *B. apis* in forager bees but was not correlated to any species in winter bees. *B. apis* was mildly positively correlated with *G. apicola* in foragers (r=0.16) but not in winter bees. We also found positive correlations present only in winter bees for Firm-4 and *G. apicola*, as well as for *B. apis* and Firm-4/Firm-5. Of note, the non-core bacteria *F. perrara* had no significant correlation with any other species in either foragers or winter bees, and no significant negative correlations were found for any pair of species. Taken together, these results suggest that the gut microbiota composition, the bacterial loads and association patterns between gut microbiota members are different in winter bees compared to foragers, with a few phylotypes dominating the community in winter bees.

Community size and composition are linked to bee type and diet

The different dietary habits of foragers and winter bees may account for the observed differences in microbiota loads and community structure between the two bee types in our dataset. While winter bees feed on stored pollen in the form of bee bread, forager bees typically feed on nectar and honey and have less pollen in their guts. Winter bees thus resemble young nurses rather than older foragers in terms of their dietary habits. Despite this similarity, winter bees differ from nurses in several aspects including feces retention over winter (Pavlovsky and Zarin 1922), tight clustering for thermoregulation (Fahrenholz, Lamprecht, and Schricker 1989), longer lifespan (Münch and Amdam 2010), a modified immune system (Gätschenberger et al. 2013; Hystad et al. 2017; Cristian et al. 2016; Steinmann et al. 2015) and an altered protein metabolism (Karl Crailsheim 1986).

If pollen diet is the main driver of the observed differences in gut microbiota composition and bacterial load between winter bees and foragers, we would expect to see similar differences between nurses and foragers. We thus sampled foragers and nurse bees at the same time point during summer (July 2017), and an additional batch of winter bees from the same hive in January 2018 to compare their gut microbiota composition and gut properties such

as wet weight, actin gene copy number and bacterial loads. Dissected guts of winter bees and nurses possessed yellow rectums filled with pollen, while the rectums of foragers appeared translucent indicative of the absence of pollen (**Fig. 2A-C**). Gut wet weights were significantly different between bee types (df=1, P=0.0075) with winter bees having the heaviest guts while foragers and nurse bees had lower gut weights which were not significantly different from each other (**Fig. 2D**). Since the gut wet weight includes both gut content and the gut tissue itself, we determined the actin gene copy number as a proxy for gut tissue size for each bee type in order to assess the contribution of gut size to gut wet weight. The actin gene copy numbers were significantly different between bee types (df=1, $P=4x10^{-4}$) with guts from nurses and winter bees having similar values while guts from foragers had less actin copies (**Fig. 2E**). The difference in the average number of actin copies in foragers relative to other bee types was relatively small compared to the average difference in gut weight. This indicates that the guts of winter bees were heavier than those of nurses or foragers and that this does not result from different gut tissue sizes.

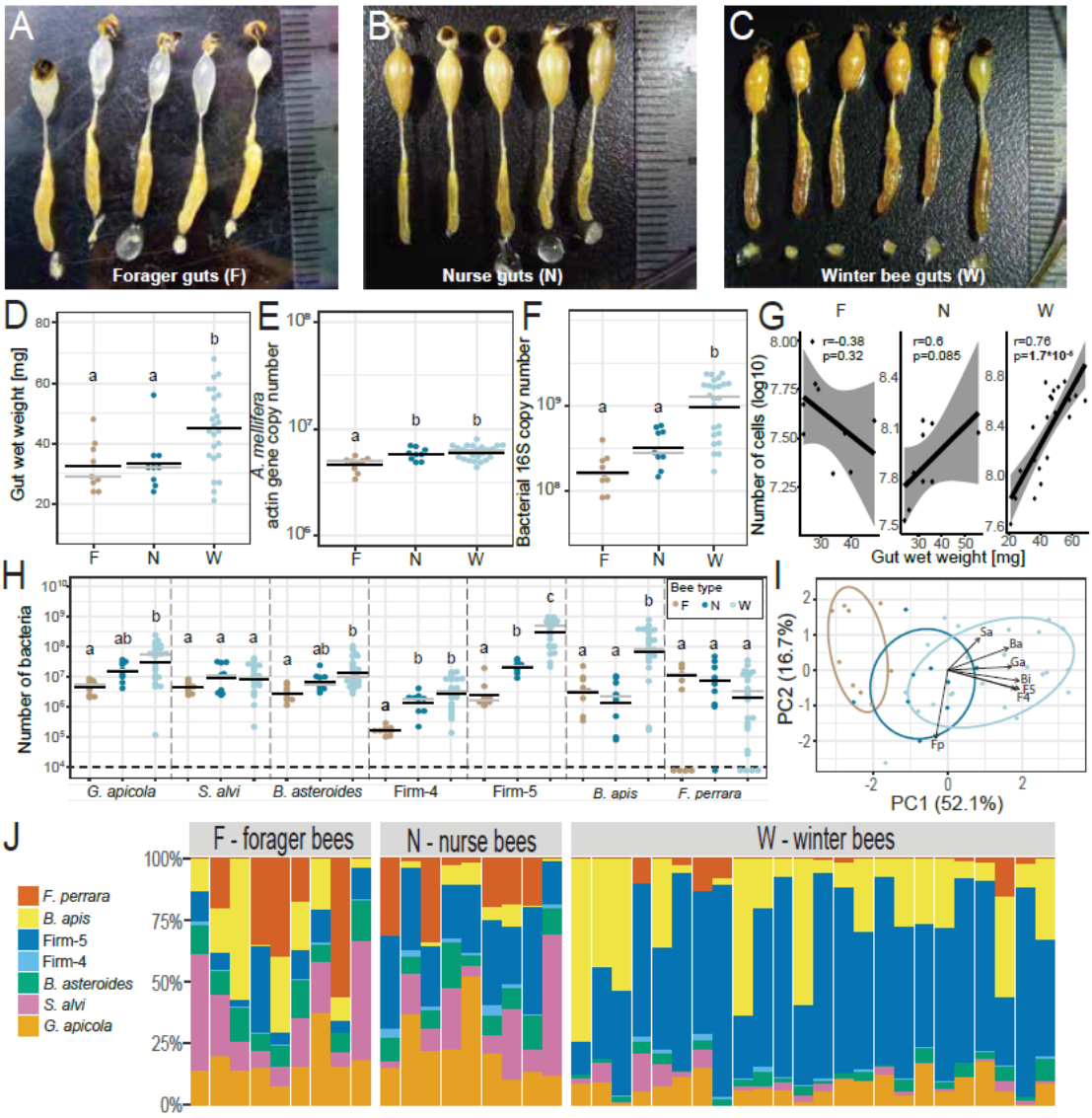

Figure 2 Gut content and community differs between bee types. (A) *Dissected guts of forager bees (F, n = 9) show transparent rectums (upper part of guts with attached sting and last tergite) suggest the switch to the nectar-based diet while rectums of nurse bees (N, n = 9)* ***(B)*** *and winter bees (W, n = 24)* ***(C)*** *are filled with a pollen-based diet.* ***(D)*** *Gut wet weight (mg) in F, N and W bees. Different letters indicate different significance levels between bee types.* ***(E)*** *A. mellifera actin gene copy numbers used as normalizer between samples and as a DNA extraction quality control.* ***(F)*** *Total bacterial loads determined with universal 16S rRNA gene primers, normalized to median actin value. (**G**) Gut weight vs total bacterial load (sum of cell number of all phylotypes) in F, N and W bees. The Spearman*

correlation (r) is indicated for each bee type with its associated p-value. The grey area indicates 95% confidence interval. ***(H)*** *The bacterial loads of the seven predominant community members. Black and grey lines show mean and median detected values, respectively. Undetected species (samples with <10^4 bacterial cells per gut) are shown below the black dashed line, representing the threshold of detection for most targets.* ***(H)*** *Relative community composition in individual sampled guts of F, N and W bees.* ***(I)*** *Principal component analysis based on cell numbers of microbiota species from individual bees (points) colored according to bee type (F,N and W bees).*

When assessing bacterial abundance by qPCR, we found that the bee type had an influence on bacterial abundance (df=1, P=10^{-4}), and that winter bees had significantly higher total bacterial loads than foragers, which is consistent with our previous observation. Contrary to our expectations, bacterial loads from nurse bees were not significantly different from those in foragers, despite a trend towards higher levels in nurses (**Fig. 2F**). Interestingly, we found that gut weight was significantly positively correlated with total bacterial loads for winter bees. The gut weight of nurses also increased with total bacterial loads but the correlation between the two was under the significance threshold. By contrast the gut weight of foragers was mostly independent of total bacterial loads (**Fig. 2G**). The quantification of individual gut microbiota members revealed similarities but also differences between bee types (**Fig. 2H**). *S. alvi* and *F. perrara* levels were not

significantly different between bee types while *G. apicola*, *B. asteroides*, Firm-4, Firm-5 and *B. apis* levels were significantly higher in winter bees relative to foragers bees. In particular, Firm-5 levels were significantly different between the three bee types with nurses having intermediate levels. Likewise, *G. apicola* and *B. apis* displayed levels between those of foragers and winter bees in nurses, but were not significantly different from either of these. The levels of Firm-4 were similar between nurses and winter bees, with higher levels relative to foragers. *B. apis* was the only phylotype showing a different trend in winter bees and nurses relative to foragers bees. While it was 100x more abundant in winter bees than in foragers, it showed no difference in abundance between nurses and foragers.

Despite similar trends in nurses and winter bees, the much higher loads of Firm-5 and *B. apis* in winter bees resulted in rather distinct community profiles (**Fig. 2J**). This was evident from the PCA analysis, where forager and winter bee communities cluster separately and communities of nurse bees are located somewhere in between (**Fig. 2I**).

Taken together, these results show that the bacterial communities from nurses and winter bees display similar trends towards higher bacterial loads relative to foragers.

Compositional differences in gut microbiota structure between nurses, foragers and winter bees are conserved across hives

Since our study focused on a single hive, we next assessed whether the observed patterns in gut community composition and total bacterial loads between foragers, nurses and winter bees would be consistent across multiple hives. For this, we quantified the main gut microbiota phylotypes from pooled honey bee gut samples consisting of 20 guts per hive and per bee type (n=14 hives x 3 bee types = 42 pooled samples). The actin copy number per pooled sample was significantly different between bee types (df=1, $p=10^{-4}$) with nurses having the highest value while foragers and winter bees had lower values which were similar between each other (**Fig. 3A**). The total bacterial load was also significantly different between bee types (df=1, $p=10^{-4}$), with foragers having the lowest bacterial 16S rRNA copy number after normalization to the actin gene, while nurses and winter bees had similar values of $\sim 10^9$ copies (**Fig. 3B**). The non-core phylotype *B. apis* and *F. perrara* were detected by qPCR in all pooled samples, indicating that all samples had at least some bees in which these species were present. The levels of individual bacteria were significantly different between bee types (df=1, $10^{-4} < p < 3x10^{-4}$) for all species except for *S. alvi* (df=1, p=0.547). The bacterial levels of *G. apicola*, *B. asteroides* and Firm-4 were similar between nurses and winter bees but higher than in foragers (**Fig. 3D**). Firm-5 and

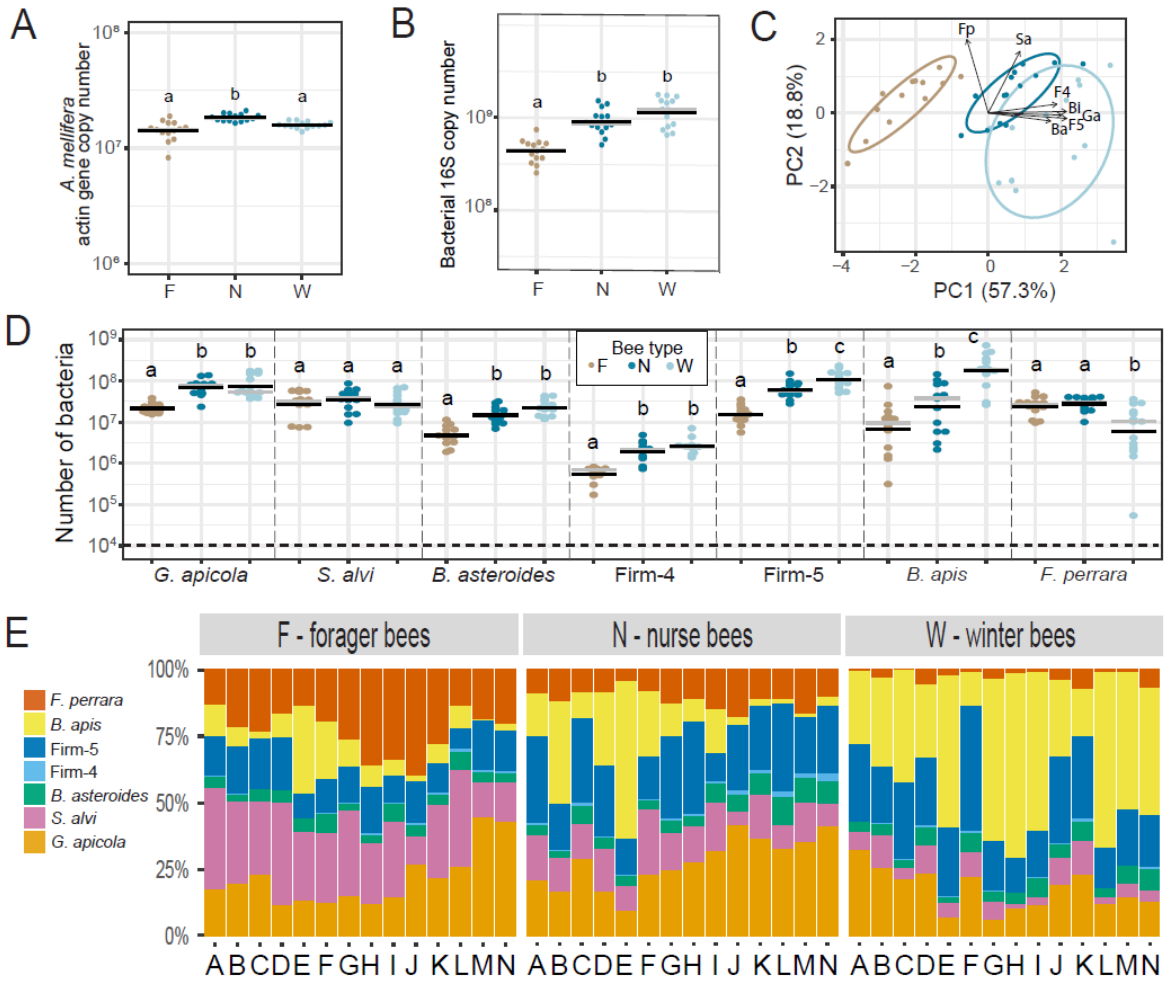

Figure 3 Gut community structure from pooled honey bee guts of multiple hives also differ between bee types. ***(A)*** *A. mellifera actin gene copy numbers of pooled samples consisting of 20 bee guts from foragers (F), in-hive nurses (H) or winter bees (W) in function of bee type (n=14 different hives)* ***(B)*** *Total bacterial loads of pooled samples determined using universal 16S rRNA gene primers, normalized to median actin value.* ***(C)*** *Principal component analysis based on cell numbers of microbiota species obtained from pooled samples (points) colored according to bee type (F,N and W bees).* ***(D)*** *The bacterial loads of the seven predominant community members (species-specific primers) in pooled samples. Black and grey lines show mean and median values, respectively.* ***(E)*** *Relative community composition in pooled gut samples based on qPCR data in function of bee type. Capital letters below the percent stacked plots indicate the hive of origin of the corresponding pooled sample, with L,M,N for hives from Yens and the rest from hives located at the University of Lausanne campus.*

B. apis levels were different for all three bee types, with winter bees having the highest values, foragers the lowest and nurses showing an intermediate level between foragers and winter bees. The only species found at a lower level in winter bees relative to foragers and nurses was *F. perrara*.

Overall these results together with the PCA clustering of the different bee types (**Fig. 3C**) and the relative abundances of phylotypes in different bee types (**Fig. 3E**) indicate that each bee type has a characteristic gut microbiota profile across colonies. These findings largely recapitulate the results obtained from individual gut samples from a single colony over three consecutive years, and in addition, suggest more similarities in gut community composition and total bacterial loads between nurses and winter bees than between either of these and foragers.

Pollen diet increases bacterial loads in experimentally colonized honey bees

We have previously determined that the gut microbiota structure of nurses and winter bees share similarities with higher bacterial loads for most phylotypes relative to foragers. This could be explained by the fact that both winter bees and nurses follow a pollen-based diet while the diet of foragers consists mostly of nectar. In order to provide more direct evidence that diet accounts for the increased bacterial loads in nurses and winter bees, we

analyzed bacterial levels in bees that were experimentally colonized with a community of 11 cultured gut microbiota strains (comprising all studied phylotypes) and that were fed *ad libitum* either sterile pollen and sugar water (SW+P treatment), or sugar water only (SW treatment). Ten days after colonization, total bacterial loads in SW+P bees were significantly higher than in SW bees (**Fig. 4A**, df=1, $p=10^{-4}$). The gut weight and the actin gene copies were both significantly higher in SW+P bees (**Fig. 4B**, df=1, $p=10^{-4}$ and **Fig. 4C**, df=1, p=0.0296, respectively). However, the fold change in actin numbers between treatments was minor compared to the changes in gut weight, indicating that the content of the gut most likely explained most of the difference in weight observed. Only *B. apis* and *S. alvi* were at similar levels in SW and SW+P bees, while all other gut microbiota members exhibited increased levels in the presence of pollen. The most dramatic increase was observed for Firmicutes and *B. asteroides*. Only *F. perrara* and to a lesser extent *B. apis* were unable to colonize all SW bees (**Fig. 4D**) while still being able to colonize all SW+P bees, suggesting dependence of these two non-core members on pollen for successful colonization. These results provide experimental evidence that pollen diet leads to an overall increase in the bacterial loads in the honey bee gut. The observed increases of Firm-4, Firm-5 and *B. asteroides* in SW+P bees relative to SW bees mirror the

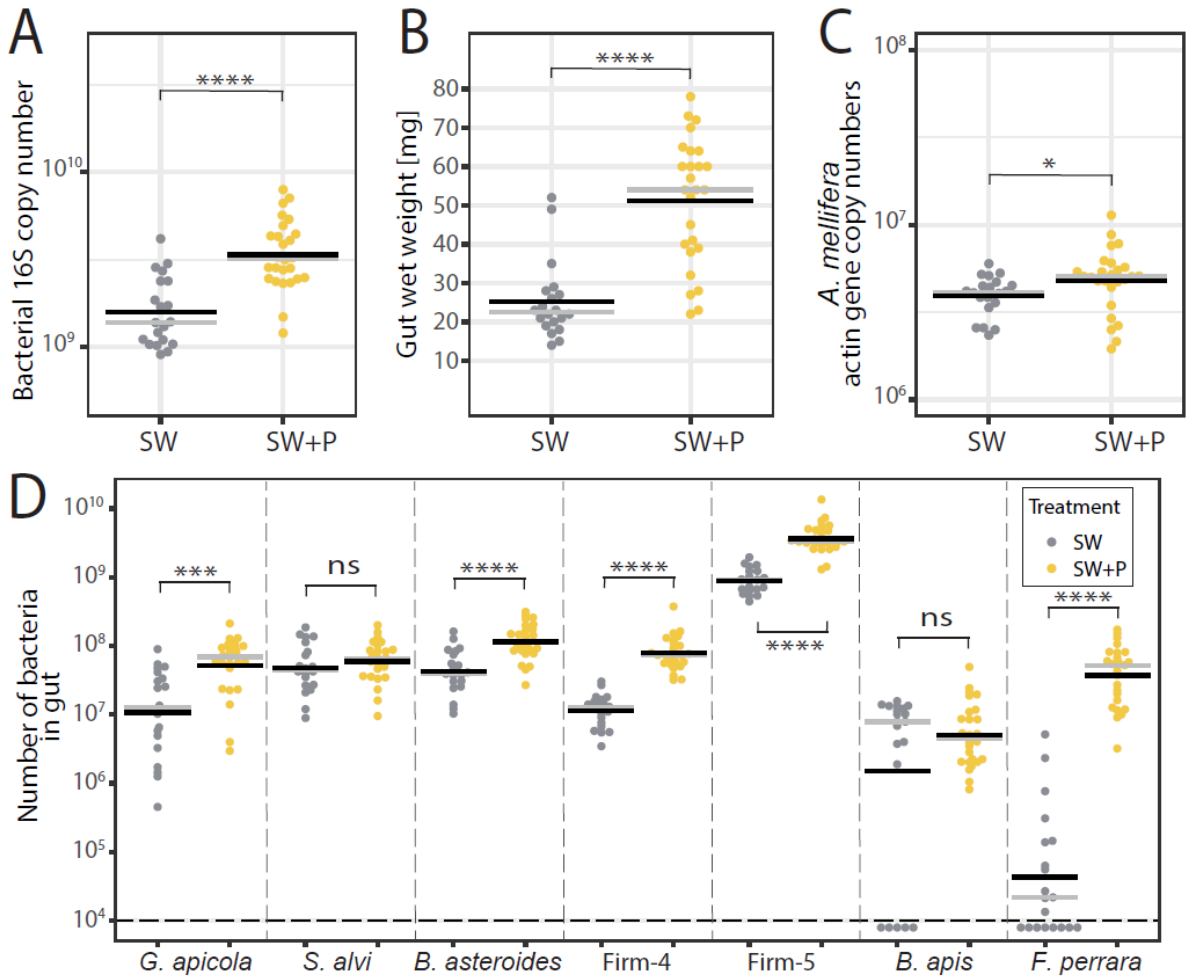

Figure 4 Diet effect on experimentally colonized bees. ***(A)*** *Total bacterial loads in the guts of bees fed sterile sugar water (SW, n = 20) or sterile sugar water and sterile pollen (SW+P, n = 26) based on qPCR with universal 16S rRNA gene primers normalized to median actin copies.* ***(B)*** *Gut wet weight (mg) of SW bees (n = 20) and of SW+P bees (n = 26).* ***(C)*** *A. mellifera actin gene copy numbers in the guts of SW bees (n = 20) and of SW+P bees (n = 26).* ***(D)*** *The bacterial loads of the seven predominant community members (phylotype-specific primers). Black and grey lines show mean and median values detected by qPCR, respectively. Samples with <10^4 bacterial cells per gut are shown below the black dashed line, representing the threshold of detection for most targets.*

increases observed in the monthly sampling between winter bees which eat pollen and foragers which don't, providing support that diet is responsible for the differences observed between winter bees and foragers. However, while *B. apis* levels were significantly higher in winter bees relative to foragers, we did not observe a significant difference in the levels of *B. apis* between SW+P and SW bees. Furthermore, *F. perrara* levels tended to be lower in winter bees relative to foragers while SW+P bees had much higher levels than SW bees. These discrepancies between the comparison of SW+P and SW bees and the comparison of winter bees and foragers indicate that other factors than diet play a role in shaping the gut microbiota of winter bees.

Common Trypanosomatids and *Nosema ceranae* have non-overlapping associations with gut microbiota species from different bee types

In addition to diet, another factor that may contribute to community differences between bee types is the prevalence of pathogens. We carried out an initial pathogen screen on a subset of the analyzed bees and found that two gut-associated pathogens, the microsporidia *Nosema ceranae* and trypanosomatids (the primers used here do not distinguish between *Lotmaria passim* and *Crithidia mellificae*) were commonly present in our

monthly sampling dataset. We thus decided to systematically assess the levels of these two types of pathogens and test for correlations with single microbiota member levels.

N. ceranae was found at high prevalence (77.7%) in the analyzed hive across the two years. However, in winter bees, as well as foragers that were collected in colder months (Nov, Dec 2015 and Nov 2016) levels of this pathogen were overall lower and less variable. In particular, all bees from December 2016 and January 2017 were negative for *N. ceranae*. The prevalence of *N. ceranae* was significantly different between foragers (89.2%) and winter bees (50.6%, X^2=101.71, df=1, P < $2.2*10^{-16}$, **Fig. 5A**). In addition, the levels of *N. ceranae* above the qPCR limit of detection in winter bees were significantly lower than for foragers (**Fig. 5B,** df=1, P=10^{-4}).

With an overall prevalence of only 50.3%, trypanosomatids were less prevalent than *N. ceranae* in the studied hive with a high degree of variation between bees and sampling times. Contrary to *N. ceranae*, trypanosomatids were similarly prevalent in foragers (48.2%) and winter bees (55.3%, X^2=2.3929, df=1, P = 0.12, **Fig. 5A**). In addition, trypanosomatid levels detected with qPCR were not significantly different between bee types (df=1, P=0.63, **Fig. 5B**).

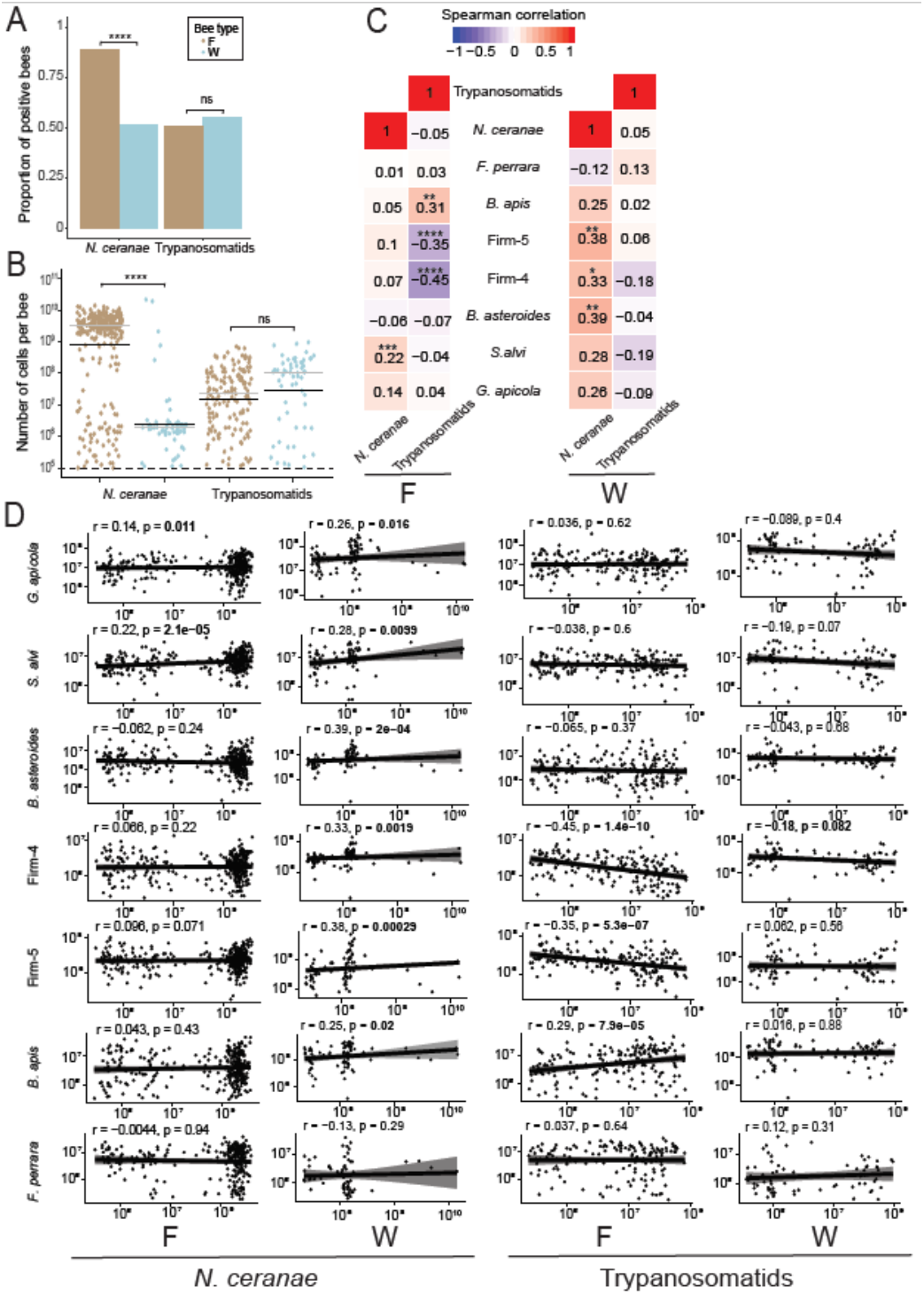

Figure 5 Pathogen levels and correlations between gut microbiota members and pathogens. ***(A)*** *Proportion of bees in which N. ceranae or trypanosomatids were detected by qPCR in F and W bees.* ***(B)*** *Number of N. ceranae or trypanosomatid cells in F and W bees.* ***(C)*** *Spearman correlations between gut microbiota species and N. ceranae or trypanosomatids in F or in W bees.* ***(D)*** *N. ceranae or trypanosomatid cells plotted against each gut microbiota species in foragers (F) or in (W) bees.*

To determine correlative patterns between pathogens and microbiota members, we computed Spearman pairwise correlations between each pathogen species and each gut microbiota species (considering only pairs in which both species were detected by qPCR), separately for foragers and winter bees (**Fig. 5C-D**). In foragers, *N. ceranae* levels correlated positively with *S. alvi* only, no negative correlations were found. In winter bees, *N. ceranae* levels were positively correlated with *B. asteroides*, Firm-5 and Firm-4. Trypanosomatid levels correlated positively with *B. apis* and negatively with Firm-4 and Firm-5 in foragers. No significant correlations were found in winter bees between trypanosomatids and any gut microbiota species. Overall, these data indicate that the two bee pathogens have different associations with the gut community structure from different bee types.

Discussion

Previous studies have shown that the honey bee gut microbiota consists of a simple yet specific bacterial community. However, the relative species composition dynamics of this community have not been investigated over extended time periods. By monitoring the honey bee gut microbiota composition of individual bees monthly over two years from a single hive, we found that winter bees had a distinct gut community composition relative

to foragers, with higher bacterial loads for most bacterial species (**Fig. 1**). In particular, we observed a sharp increase in *B. apis* and Firm-5 cell numbers in winter bees relative to foragers (**Fig. 1B**) which resulted in the relative abundances of these two species representing most of the gut microbiota community of winter bees (**Fig. 1A**). *B. apis* has been previously described as a non-core species (Raymann and Moran, 2018; Kwong and Moran, 2016; Martinson et al., 2011) and, while we did detect certain months in which some bees had undetected *B. apis* levels, the prevalence of *B. apis* was very high when considering all bees sampled (94.7%) in contrast to that of *F. perrara* (73.1%), the other non-core species that we monitored (**Fig. S1**). Furthermore, *B. apis* consistently dominated the gut community of winter bees together with Firm-5. Hence, depending on the definition used, *B. apis* could be considered as a core species.

Since nurses and winter bees eat pollen while foragers feed only on nectar, we tested whether the gut communities of nurses would resemble that of winter bees. We found that nurse bees had a distinct gut microbiota community relative to foragers and to winter bees, although this gut community was more similar to that of winter bees. While the common pollen diet of nurses and winter bees is consistent with the similarities in their gut microbiota composition, other differences between bee types are likely to explain their specific gut microbiota structure. In particular, winter

bees have a longer lifespan, feed only on stored reserves and retain their feces the entire winter until the outside temperature is high enough to perform a cleansing flight early in spring (Winston 1991; K. Crailsheim et al. 1993; Pavlovsky and Zarin 1922). Host age has been shown to correlate with higher bacterial loads in *Drosophila* (Ren et al. 2007) and may also play a role in shaping the gut microbiota structure of winter bees differently than in the short-lived nurses and foragers. The consumption of an aged diet has been previously shown to affect the gut microbiota composition of nurse bees, causing dysbiosis and higher mortality (Maes et al. 2016). Whether the consumption of the aging food reserves by winter bees affects their gut microbiota composition is unknown but we did not observe major microbiota composition differences between winter bees collected early or late in winter. The fact that the intestinal transit is stopped in winter bees may explain why species residing in the rectum (ie. Firm-5, *B. apis*, Firm-4 and *B. asteroides*), where the digested pollen is located, showed consistently higher levels in winter bees. Winter pollen stores accumulating in the gut may provide more substrate for the bacteria, which would be consistent with the higher gut weights and bacterial loads observed in winter bees (**Fig. 2D,F,G**).

We quantified the gut microbiota of pooled samples of nurses, foragers and winter bees from different hives and confirmed that winter bees have higher

total bacterial loads relative to foragers (**Fig. 3B**). While certain differences for specific phylotypes could not be fully recapitulated across hives, the overall trend was the same as in data from individual samples. The results from pooled samples confirm that the gut microbiota of foragers, nurses and winter bees are distinct and that community structures from nurses and winter bees have a greater overlap (**Fig. 3C**). D'alvise et al. (2018) found similar differences between bees sampled before and after winter in Germany, although it remains to be determined if this pattern is conserved among bees from other geographical locations.

Consistent with the role of pollen in explaining the higher bacterial loads of winter bees relative to foragers, we found that laboratory raised bees fed with pollen established higher bacterial loads and higher gut weights than bees with a pollen-free diet (**Fig. 4A,B**). However, contrary to what we observed between winter bees and foragers, *B. apis* levels were similar between bees fed with pollen or not. This indicates that the extremely high *B. apis* levels found in winter bees cannot be explained by pollen ingestion alone. Another discrepancy between bees collected from the hive and experimentally colonized bees was found for the levels of *F. perrara*: these dramatically increased in experimentally colonized bees fed pollen relative to bees fed only sugar water while we found that winter bees had lower levels than foragers. Winter bees have a specific physiology and lifestyle that

cannot be fully recapitulated in a controlled setting which could explain these differences. Furthermore, winter bees possess multiple strains of each phylotype while our experimental colonization used only one strain which may not correspond to the ones sampled in winter bees.

Quantification of pathogens showed that *N. ceranae* had a higher prevalence and number of cells in foragers relative to winter bees while no differences were found for trypanosomatids between bee types (**Fig. 5A,B**). Correlations between individual gut microbiota species and pathogens did not overlap for foragers and winter bees (**Fig. 5C**), indicating that no consistent association could be found that persisted across bee types. This suggests that the gut microbiota of each bee type had different associations with pathogens but, based on the relatively low correlation values obtained, these pairwise associations appear to be modest. Despite previously reported similar annual dynamics between *L. passim* and *N. ceranae* in naturally coinfected hives (Vejnovic et al. 2017), the trends we observed for *N. ceranae* and trypanosomatids seem to be independent. However, this could be due to the different methodologies used: Vejnovic et al. used spore counts for *N. ceranae* and ten pooled samples of 60 bees for each of ten hives for each sampling point.

Although the qPCR approach we carried is a targeted approach which may miss atypical bacteria which are not part of the set of chosen targets, numerous reports indicate that the bacterial species that we screened form the vast majority of the honey bee gut microbiota (Raymann and Moran, 2018; Kwong and Moran, 2016; Corby-Harris et al., 2014; Sabree et al., 2012 Martinson et al., 2012, 2011;). In addition, invasion of the microbiome by atypical bacterial species seems unlikely after we measured bacterial loads with universal primers and compared them to the sum of cells obtained from the different species-specific qPCR targets (**Fig. S3**). As in the case of 16S rRNA profiling, it should be emphasized that our method does not discriminate between bacterial strains within species, which have been shown to be numerous and to possess highly diverse metabolic capabilities (Ellegaard and Engel, 2019; Engel et al., 2014).

So far studies investigating the honey bee gut microbiota of winter bees are scarce. Anecdotal evidence from microscopy pictures of the rectum of winter bees indicate, as we observed in our study, that the bacterial loads of overwintering bees from the USA are higher than in foragers (Landim 1972). To our knowledge, only one recent study investigated the gut microbiota composition of winter bees and of foragers using 16S rRNA sequencing at two time points, while investigating the effect of winter feed type on the honey bee gut microbiota composition and on pathogen resilience (D'Alvise

et al., 2018). Although there was no significant effect of winter feed type on the gut microbiota community in that study, authors found that the most striking difference in gut community composition was observed between winter bees and foragers. Consistent with our study D'alvise et al. found higher levels of *Lactobacillus* and lower *F. perrara* levels in winter bees, and higher *N. ceranae* abundance in summer bees. By contrast to our study, they found that the levels of *S. alvi* and trypanosomatids were lower in winter bees and that *Rhizobiales* (*Bartonella*) levels were not generally different between summer and winter bees. We found significant positive correlations between Firm-4, Firm-5, and *B. asteroides* whether they were computed from winter bees or foragers suggesting a general pattern independent of bee type, this was also observed by D'Alvise et al. between summer and winter bees fed with honey who suggested that the positive correlation between lactobacilli and bifidobacteria may result from their similar substrate requirements or a mutualistic relation. While our results are consistent with what was found by D'alvise et al. in large terms, the results that differ between our studies could come from environmental differences between the apiaries or bee genetic background of the two studies, or from the different quantification and analysis methods that were used.

It would be interesting to determine if the gut microbiota of winter bees is acquired differently early in life relative to summer bees, or if young winter

bees have a similar gut microbiota relative to nurses which shifts in time as winter bees age. Since foragers and winter bees will coexist in the hive in late fall or early winter, it is possible that the gut microbiota composition of bees at this time depends on each bee type. We only observed a strong shift in our sampling but since we were limited to one time point per month we may have missed a more gradual transition from the gut microbiota of foragers to that of winter bees.

In conclusion, the microbiota composition datasets obtained in this study have allowed to uncover a marked difference in the gut microbiota community of foragers and winter bees. The winter bee gut community was characterized by higher total bacteria loads relative to foragers and dominated by Firm-5 and *B. apis*. Whether this community is consistent across winter bees from other geographical regions or of different genetic backgrounds will require further investigations of the so far under-studied gut microbiota of winter bees. Since winter is a crucial period for winter survival of honey bee colonies, the shift of microbiota structure between foragers and winter bees may be relevant to help bees withstand the harsh winter conditions and be used as an indicator of bee health.

Supporting information

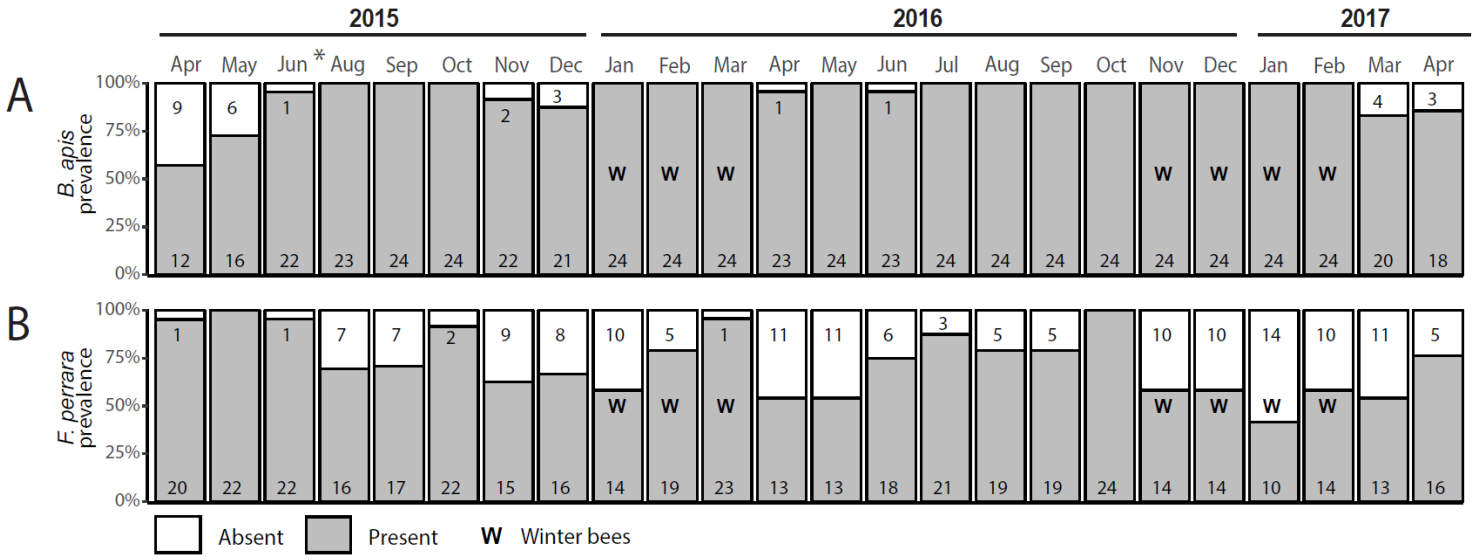

Figure S1 Non-core bacteria show no strong temporal pattern in prevalence. *Monthly prevalence (i.e. the proportion of bees in which the species was detected in a given month) B. apis* ***(A)*** *and F. perrara* ***(B)****. The targetted phylotype was considered as present or absent whether the qPCR signal was above or below the detection limit of the primer set, respectively (see Table 1). Absolute levels of qPCR-detected bacteria in bees are available in Fig. 1. The numbers in the upper and lower part of the bar plots indicate the number of bees in which the target is absent or present, respectively. Asterisk indicates missing month (July 2015) due to sampling error. W indicates winter bees.*

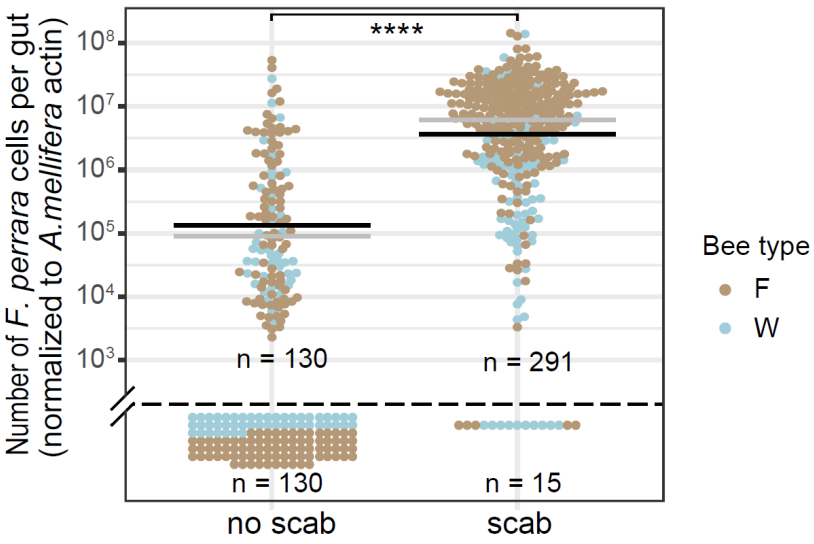

Figure S2 F. perrara loads correlate with scab presence. *F. perrara loads in guts with or without scab phenotype in the pylorus region. Data points under the black dashed line correspond to samples with the qPCR signal below the detection limit of primers. Black and grey lines show mean and median detected values, respectively. F, forager bees; W, winter bees.*

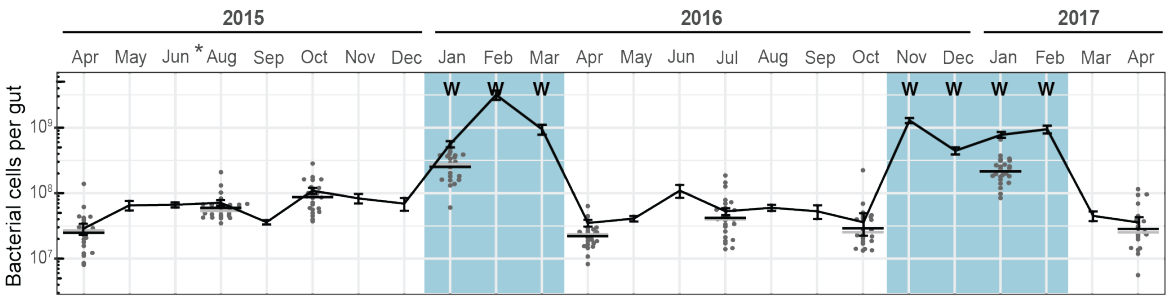

Figure S3 The majority of the gut community is composed of honey bee-specific species. *Total bacterial loads obtained using universal bacterial primers (grey data points with mean value and median shown with horizontal black and grey bars, given for selected months) recapitulate results obtained using the sum of the cell numbers from the seven monitored species (black line ±SE) assessed by qPCR with species-specific 16S rRNA primers. Winter months when sampling occurred inside of the hive are highlighted in blue. Asterisk indicates missing month (July 2015) due to sampling error.*

3.2) Additional characteristics of *F. perrara* and the scab in the hive

Introduction

F. perrara has been previously shown to cause the scab phenotype in honey bees (Engel et al. 2015). Based on our monthly sampling microbiota monitoring using 566 bees sampled over two years, we confirmed that *F. perrara* levels are indeed correlated to the presence of the scab phenotype. However, it is not known if the intensity of the scab depends on the number of *F. perrara* cells present or not. In order to investigate the relationship between scab intensity and *F. perrara* cell numbers, we visually assigned a scab intensity score and assessed the corresponding levels of *F. perrara* for each scab intensity score.

We also determined wether there were seasonal patterns in scab occurrence or similar patterns between hives by sampling additional honey bee gut samples from five hives and recorded the percentage of scabs from ~20 bees monthly for 21 consecutive months.

Materials and methods

Honey bee samples

Honey bee guts sampled from the monthly sampling over two years (n=566) were used to measure scab intensity and *F. perrara* levels by qPCR (see chapter 1 for details on honey bees and qPCR methods). A subset of these bees (i.e. those from March 2015 to November 2016, including those from July 15, n=185) were used to determine monthly scab percentages for this hive (i.e. samples "Dent de Morcles MM"). Monthly scab percentages from ~24 bees were also determined for an additional sampling of this hive (i.e. samples "Dent de Morcles"), as well as from four other hives located on the campus of the University of Lausanne (i.e. "Grand combin", "Les droites", "Grammont" and "Christophs") from March 2015 to November 2016.

Scab scoring

The scab phenotype can vary in color and size between individual bees: a weak scab is less dark and covers a smaller area than a strong scab. We assigned a scab intensity score to each honey bee gut visually with the following scheme : 0 in the absence of the scab phenotype, 0.5 in case there was a doubt whether the scab was present, 1 for a weak scab, 2 for a strong scab and 3 for a very strong scab. For the analysis between scab intensity and

F. perrara levels, samples with scab intensity score of 0.5 (n=13) were not included.

Statistics

Kruskal-Wallis non-parametric test was used to determine if there was a significant difference in the number of *F. perrara* cells in function of scab intensity score (set as categorical variable). Post-hoc tests on pairwise comparisons were carried using Dunn's test with Bonferroni correction for multiple testing.

Results

Higher scab intensity scores are linked to higher *F. perrara* loads

F. perrara levels were significantly different between bees with different scab scoring scores (Kruskal-Wallis $X^2 = 271.78$, df = 3, $p < 2.2*10^{-16}$).

Bees without scab had a significantly lower number of *F. perrara* cells relative to bees which had scabs independent of their scab intensity ($p<2.10^{-16}$ for all corresponding pairwise comparisons following Dunn's post hoc tests). Bees with a weak scab had significantly less *F. perrara* cells than bees with a strong (p=0.0057) or very strong (p=0.0019) scab. However, bees with a strong scab had similar *F. perrara* levels relative to bees with a very strong scab (p = 1).

Together these results indicate that the scab intensity is different in function of the number of *F. perrara* cells, with higher cell numbers linked to stronger scab intensity categories and that our scoring of strong and very strong scabs had similar *F. perrara* levels.

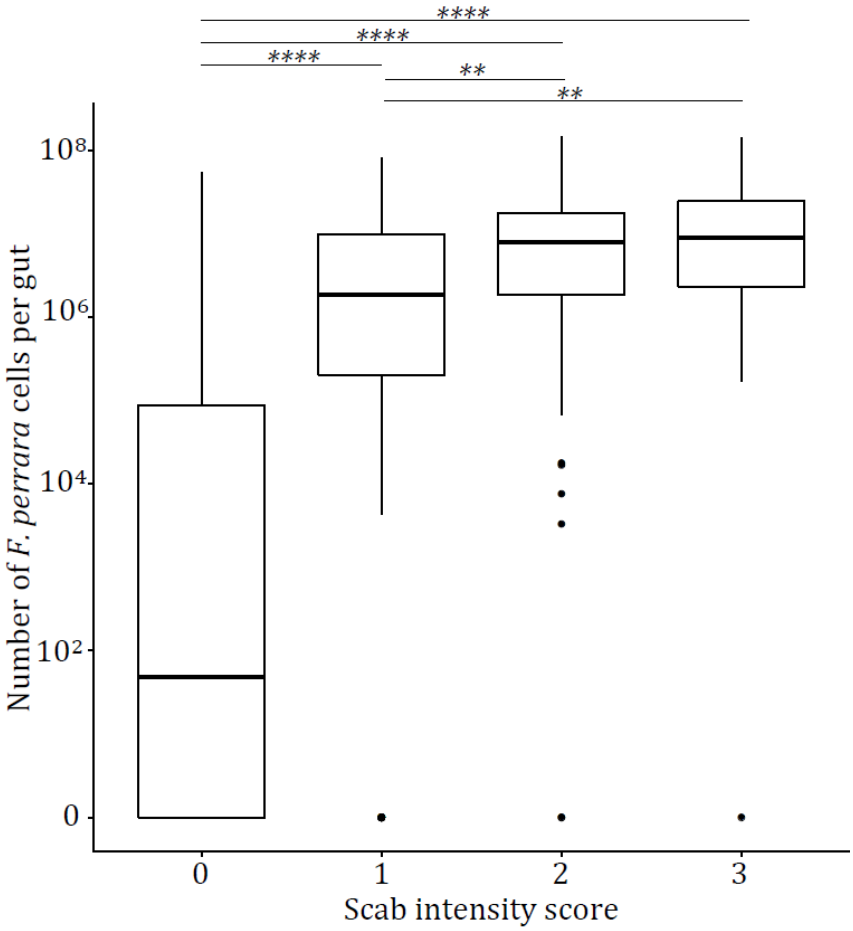

Fig. 1 *F. perrara levels in function of scab intensity scores. 0 = no scab, 1 = weak scab, 2 = strong scab, 3 = very strong scab. Bars show significantly different pairwise comparisons following Dunn's post hoc test and asterisks above indicate the respective significance levels with **** for $p<10^{-4}$ and ** for $10^{-3}<p<10^{-2}$.*

Monthly percentage of bees with scab varies within and between hives

In order to determine if and how the prevalence of the scab phenotype varies among hives, I dissected monthly the guts of ~20 bees from each of five hives and recorded the percentage of bees which had the scab phenotype for each hive from March 2015 to November 2016 (21 months). In particular, bees from the hive "Dent de Morcles" were sampled twice (24 bees from the monthly sampling and an additional 24 bees from each month, collected the same day as for the monthly sampling). The percentage of bee guts with a scab ranged from 20.83% to 87.5% across all hives and time points (**Fig. 2**). There were important variations between hives at the same time point, but also in the same hive across time points (See fluctuations from the hive "Grand Combin" in red in **Fig. 2** for example). While percentages for a given time point were usually similar for the two sets of bees collected from the hive "Dent de Morcles" from the same month (see august 2015 – January 2016), we also observed high variation (e.g. in April 2016). Based on these results, it appears that scab prevalence does not follow a clear seasonal pattern although there was a trend for lower values in winter (January-March 2016).

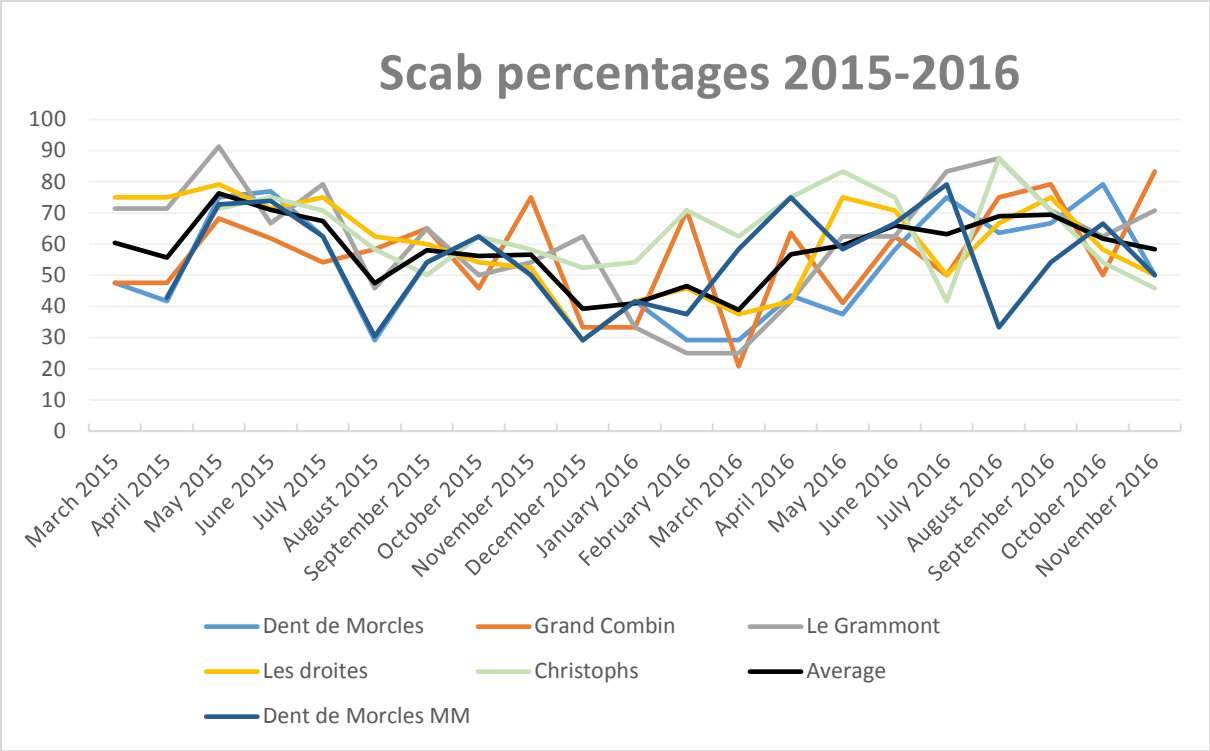

Fig 2*. Monthly percentages of bees with the scab phenotype from different hives. The percentages of bees with scab from five hives located on the campus of Lausanne University were determined monthly. Colored lines indicate scab percentages for individual hives and the average scab percentage from all hives is shown in black. The scab percentages from the monthly sampling (Dent de Morcles MM, dark blue) are shown as well as scab percentages of the same hive taken from additional bees (Dent de Morcles, light blue).*

Discussion

F. perrara was previously shown to cause the scab phenotype (Engel et al.) but whether the size and color intensity of the scab are linked remained unexplored. Here, despite the fact that our scab intensity scoring system was visual and approximate, we were able to find differences in *F. perrara* cell numbers between most intensity categories. This indicates that determining the scab intensity allows to roughly estimate the number of *F. perrara* cells

in the honey bee gut. Performing a more elaborate automated image analysis-based scoring to determine scab intensities in relation to *F. perrara* loads may provide more resolution (i.e. more scab intensity categories).

Since *F. perrara* is not present in each and every bee but is found in all hives, the prevalence of the scab phenotype may be hive specific or follow seasonal patterns. Scab percentages varied greatly within and between hives. By sampling twice the same hive (i.e. “Dent de Morcles” and “Dent de Morcles MM” samples in **Fig. 2**), we found that the overlap in monthly scab percentage was generally high between the two samplings but that variation can occur at some time points (such as in March-April 2016). It is possible that sampling more bees would reduce these differences. We did not find obvious seasonal patterns for scab prevalence, with the exception for a trend for lower percentages in percentages in winter (December-March 2016). Interestingly, this is consistent with the fact that these are winter bees and we have previously determined that winter bees have lower *F. perrara* values relative to winter bees. It remains to be determined whether winter bees are able to eliminate *F. perrara* and the scab, and if this is due to their longer lifespan, to their different diet or to other differences specific to winter bees.

General discussion

For the concluding discussion, I would like to come back to the questions we posed in the beginning of this thesis (see Summary). The first question we asked was whether the scab is a melanization response of the host? Based on our host RNA-seq analysis (chapter 1), we have now supporting evidence that the scab phenotype caused by *F. perrara* indeed corresponds to a melanization response from the host, because we found upregulation of host genes of the melanization cascade upon *F. perrara* colonization. Electron microscopy revealed no obvious epithelial cell lesions and it appeared as the melanization occurs extracellularly between the epithelial cell layer and the bacteria. We thus hypothesize that melanization may be an important host response to keep *F. perrara* in check, i.e. to control its colonization levels. It would be interesting to determine (i) if *F. perrara* would replicate out of control in the absence of melanization and reduce host fitness, (ii) if melanization is a prerequisite for successful colonization of *F. perrara* or (iii) if melanization provides an advantage to *F. perrara* to colonize the gut in the presence of other gut microbiota members. Although I tried to inhibit the melanization response in bees colonized with *F. perrara* using PTU to assess the role of melanization in this symbiosis, I found toxicity effects of PTU on the bees and little inhibition of the scab phenotype, which suggests that this method is not effective in honey bees. Deleting or knocking down host

melanization strategies using other chemical inhibitors (Chen et al., 2018; Liu et al., 2015) or genetic tools such as CRISPR-cas9 (Kohno et al., 2016; Hsu et al., 2014) or RNAi (Costa et al., 2016; Burand and Hunter, 2013; Scott et al., 2013; Jarosch and Moritz, 2012) would be ideal to better understand the role of melanization in this symbiosis. However such methods and approaches need further methodological development in honey bees.

Melanin is a biopolymer composed of aromatic compounds (Riley, 1997). Where the substrates for melanin production come from in the bee gut remains an open question. As the scab forms in the luminal space, it is possible that such substrates may not only come from the host, but also from the bacteria or from diet. Pollen, the major diet of honey bees, contains substantial amounts of different aromatic compounds (Rzepecka-Stojko et al., 2015) that could serve as substrates for melanin. In addition, our RNA-seq analysis of chapter 2 showed that genes of *F. perrara* involved in the tryptophan biosynthesis pathway were upregulated in vivo, which led us to speculate if *F. perrara* by itself provides certain substrates for melanin production. Dietary manipulations or gene knockouts of the amino acid biosynthesis pathways may be two possibilities to investigate this further in the future.

A second question that was in the focus of this thesis was which genes of *F. perrara* are important for colonization or may trigger the scab formation.

Genetic tools, such as targeted knockouts or genome-wide transposon screens would be the ideal choice for addressing this question. However, this has turned out to be difficult in the case of *F. perrara* (personal communication of Philipp Engel), and while another PhD student is working on these aspects, the methods were not yet available for this thesis. Therefore, we approached this question with an RNA-seq experiment focusing on the bacterial transcriptome of *F. perrara* during gut colonization. While this analysis revealed a large number of genes that were upregulated during gut colonization, we did not find any of the putative virulence factors (e.g. colibactin) to be specifically induced in the gut. One possibility is that these genes are also expressed in vitro and therefore did not show up in our differential gene expression analysis (Evans, 2015). Alternatively, the scab phenotype may not be triggered by tissue damage induced by a specific virulence factor but by an overstimulation of the immune system, or by higher shedding of peptidoglycan relative to other bacterial members, or a specific modification of the LPS that binds to immune receptors and activates pathways that lead to melanization. The fact that several host immune receptors were among the most upregulated genes during *F. perrara* colonization would be in favor of such hypotheses, making them interesting targets for knock-down/knock-out experiments.

A third question that we wanted to address in these thesis was why only a subset of all bees are colonized by *F. perrara* and form the scab phenotype. Our hypothesis was that specific environmental conditions or the presence of other gut symbionts may influence *F. perrara* colonization. While I did not find strong correlations between *F. perrara* levels and the levels of other gut microbiota species or pathogens, the monthly monitoring of honey bee gut microbiota of bees from the hive (in chapter 3) revealed that overwintering bees had a distinctive gut microbiota composition. In particular, *F. perrara* was the only species to be at significantly lower levels in winter bees relative to foragers. This is in contrast with the fact that in bees colonized with an artificial gut community and in absence of pollen (i.e. mimicking a forager diet), *F. perrara* was less abundant relative to bees fed pollen (i.e. mimicking a winter bee diet), and even failed to colonize the honey bee gut. While the experiment testing for the effect of pollen indicates that *F. perrara* colonization success and growth are dependent on nutrients contained in the honey bee diet, winter bees (which feed on pollen) had lower levels than foragers (which do not eat pollen) in our monthly sampling. Hence the low levels of *F. perrara* in winter bees cannot be explained by diet. An alternative explanation could be that the particular physiology of winter bees or their longer lifespan leads to a gradual elimination of *F. perrara* due to the prolonged exposure to immune effectors and melanization. However, it is not

known if the host immune response to *F. perrara* is kept over time or whether it also occurs in winter bees, qPCR on specific immune genes in differently aged summer and winter bees could be used to test this.

The final and probably most important question was whether *F. perrara* impacts host health. In addition to host genes of the melanization cascade, several immune-related host genes including genes coding for antimicrobial peptides and pattern recognition proteins were highly upregulated upon *F. perrara* colonization, indicating a specific and strong immune response of the host. Despite this response, which is considered to be costly for the host (Moret and Schmid-Hempel, 2000), the survival experiments of bees colonized with *F. perrara* did not show a reduced host lifespan under laboratory conditions. However, we cannot rule out that *F. perrara* has a positive or negative fitness effect on individual bees in the hive or under particular conditions. Other insect gut symbionts have been shown to provide beneficial traits to their respective hosts which we have not assessed for *F. perrara*, including detoxification of pesticides (Cheng, 2018; Kikuchi et al., 2012), enhanced tolerance to cold (Henry and Colinet, 2018) or adaptations to new diets (Hosokawa Takahiro et al., 2007). In particular, we can speculate that the host immune activation induced by *F. perrara* may inhibit the subsequent invasion of specific pathogens in the honey bee gut and ultimately benefit the host. Studies in bumble bees have shown an analogous

mechanism across generations: immune challenged queens produce offspring with higher antibacterial activity than the offspring of non-challenged queens, a process called trans-generational immune priming (Barribeau et al., 2016; Sadd Ben M et al., 2005). In addition, the fitness at the level of individual bees does not necessarily reflect the fitness at the level of the colony. This can be exemplified by the following simple case: when an individual bee stings a potential intruder, it will die shortly afterwards (i.e. drastic individual fitness reduction) whereas the fitness of the colony will be increased thanks to the protection provided by this bee among the tens of thousands that form the hive.

Despite environmental differences and the presence of other gut microbiota species in hive bees, host genes that were upregulated by *F. perrara* in laboratory-raised bees showed an overlap with the genes that were upregulated in hive bees with scab (i.e. higher levels of *F. perrara*) versus hive bees without (i.e. lower levels of *F. perrara*). This indicates that the specific gene expression changes induced by *F. perrara* in the pylorus were robust enough to also be detected in the presence of other gut microbiota species and highlights the relevance of our findings from the experimental manipulations for understanding the impact of *F. perrara* under natural conditions, i.e. in worker bees in the hive.

Overall, while *F. perrara* induced an immune response in its host which included melanization, we found no obvious negative effects on host health in response to *F. perrara* under controlled conditions. This warrants for further studies assessing the fitness of the host in function of *F. perrara* colonization in order to determine if *F. perrara* has either beneficial or detrimental effects on the host under certain conditions, in particular in the context of infection with different bee pathogens. *F. perrara* could be a successful parasite based on its prevalence and be tolerated by the host. Alternatively, *F. perrara* may be a mutualist and the benefit(s) provided to the host may explain why this bacterial species is found within bee hives globally.

It remains to be determined how *F. perrara* and other gut microbiota species are able to persist in the gut in the presence of the antimicrobial peptides produced by the host. Another venue for further research is to determine diversity and distribution of *F. perrara* at the strain level and their effect on the host as strain diversity has been show to occur in other honey bee gut bacteria (Ellegaard and Engel, 2019; Engel et al., 2012b). In conclusion, this thesis brings novel insights onto the symbiosis between *F. perrara* and its honey bee host from the perspective of both species under natural and experimental settings, and contributes to a better understanding of this extraordinary host-symbiont association.

Abbreviations

DNA: deoxyribonucleic acid
FP: *F. perrara* mono-colonization treatment
NC: non-colonized treatment
PO: phenoloxidase
proPO: prophenoloxidase
PRP: pattern recognition protein
PTU: phenylthiourea
qPCR: quantitative polymerase chain reaction
RNA: ribonucleic acid
RNA-seq: RNA sequencing
SA: *S. alvi* mono-colonization treatment
SDEG: significantly differentially expressed gene
T6SS: type VI secretion system

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Curriculum vitae

Olivier Emery
Born 1st July 1984
Lausanne
Switzerland

Education

PhD student in Life Sciences Department of Fundamental Microbiology University of Lausanne	2014 – present
Master of Science in Biology Bioinformatics and data analysis in Biology University of Geneva	2012 - 2014
Master of Science in Biology Genomics and Experimental Biology University of Lausanne	2008 - 2010
Bachelor of Science in Biology University of Lausanne	2005 - 2008

Conferences and presentations

Talk title: Overwintering honey bees exhibit a distinct gut microbiota composition
Authors: Olivier Emery, Lucie Kešnerová, Berra Erkosar and Philipp Engel
Event name: 7th Swiss Microbial Ecology meeting (SME)
Date: January 30th – February 1rst 2019
Location: Aquatis conference center, Lausanne

Talk title: Immune stimulation by the gut symbiont *Frischella perrara* in the honey bee (*Apis mellifera*)
Authors: Olivier Emery, Konstantin Schmidt and Philipp Engel
Event name: EurBee8 8th congress of apidology
Date: September 18-20th 2018
Location: Ghent University, Belgium

Poster title: Immune system stimulation by the gut symbiont *Frischella perrara* in the honey bee
Authors: Olivier Emery, Konstantin Schmidt and Philipp Engel
Event name: Swiss Society for Microbiology meeting (SSM)
Date: August 30th – September 1st 2017
Location: Basel, Switzerland

Poster title + flash presentation: Immune system stimulation by the gut symbiont *Frischella perrara* in the honey bee
Authors: Olivier Emery, Konstantin Schmidt and Philipp Engel
Event name: Joint EPFL/ETHZ PhD winter school,
Date: March 12-17th 2017
Location: Fiesch, VS, Switzerland

Swiss French radio show "CQFD"
Details (in french)
https://pages.rts.ch/la-1ere/programmes/cqfd/22-05-2017#8603631
Audio (in French): https://rtsww-a-d.rts.ch/la-1ere/programmes/cqfd/2017/cqfd_20170522_standard_dossier_139f63c9-00a8-4580-ab71-ee9291a19e8f-128k.mp3?mediaId=8603631
Date: May 22nd 2017
Location: RSR radio station studio, Lausanne, VD, Switzerland

Poster title: The gut symbiont *Frischella perrara* induces a specific immune response in its honey bee host
Authors: Olivier Emery and Philipp Engel
Event name: 6th Swiss Microbial Ecology meeting (SME)
Date: September 10-12th 2015
Location: Ascona, TI, Switzerland

Event name: Bee microbiome meeting at the National Evolutionary Synthesis Center (NESCent), USA
Participants: Philipp Engel, Waldan K. Kwong, Quinn McFrederick, Kirk E. Anderson, Seth Michael Barribeau, James Angus Chandler, R. Scott Cornman, Jacques Dainat, Joachim R. de Miranda, Vincent Doublet, Olivier Emery, Jay D. Evans, Laurent Farinelli, Michelle L. Flenniken, Fredrik Granberg, Juris A. Grasis, Laurent Gauthier, Juliette Hayer, Hauke Koch, Sarah Kocher, Vincent G. Martinson, Nancy Moran, Monica Munoz-Torres, Irene Newton, Robert J. Paxton, Eli Powell, Ben M. Sadd, Paul Schmid-Hempel, Regula Schmid-Hempel, Se Jin Song, Ryan S. Schwarz, Dennis vanEngelsdorp, and Benjamin Dainat
Event name: BeeBiome meeting: Omic approaches for understanding bee-microbe relationships
Date: October 21-23rd 2014
Location: Durham, North Carolina, United States of America

List of publications

Emery, O., Schmidt, K., and Engel, P. (2017). Immune system stimulation by the gut symbiont *Frischella perrara* in the honey bee (*Apis mellifera*). *Mol. Ecol.* 26, 2576–2590.

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