Microbial associates and social behavior in ants

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Abstract
Current research in life sciences provides advances on how animal-associated microbes affect behavior and its underlying neurophysiology. However, studies in this field are often limited to individuals outside of their social context and neglect social dynamics. Contrarily, animals and humans develop and live in complex societies where they constantly adjust physiology and behavior to social interactions. To improve our understanding of how microbes and hosts interact and produce phenotypes at social and group levels, we need to broaden our experimental approaches to a group-level dimension. Here, we point out that eusocial insects, and ants in particular, are ideal models for this purpose. We first examine the most common types of microorganismal associations that ants engage in, and then briefly summarize what is known about the role of symbiotic microbes in ant social behavior. Finally, we propose future directions in the field, in the light of recent technical advances in behavior measuring techniques.

Keywords Social evolution · Commensal microbes · Primary endosymbionts · Ants · Group-level behavior

1 Introduction
In the last two decades, the microbes associated with animals, including humans, have been at the center of a multidisciplinary scientific revolution. In life sciences, correlations and causal links have been established between microbiota and a plethora of aspects relevant to animal biology and human health. A key concept emerging from this conspicuous body of scientific work is the existence of an intimate relationship between gut microbes, gut, and brain, which is usually defined as the “microbiota–gut–brain axis” [1–3]. According to this model, the communities of bacteria living in the animal gut communicate with the gut itself and the brain through multiple routes. In humans, these include the vagus nerve and the hypothalamic–pituitary axis, as well as host- and bacteria-produced neurotransmitters, cytokines, and other bacterial metabolites [4]. This network of exchanges affects individual neurophysiology and the resulting behavioral output (Fig. 1a). Although gut microbes attract most of the interest in the scientific community, relevant connections between brains and animal-associated microbes can be broadened to include bacteria dwelling in all locations of the host body [5–8].

Effects of microbiota on behavior are frequently described, but little is known about the relationships between the microbiota–gut–brain axis and the social life of animals. This is surprising, because dialogs between animal-associated bacteria and nervous systems, similar to any other organismal trait, are expected to coevolve with the social systems of their animal hosts. The microbiota is therefore likely to influence brains and behaviors across levels of biological organization, and in multiple contexts. For example, in animals with parental care, parents sometimes drive developmental trajectories of brood (e.g., eusocial insect larvae developing in workers vs reproductive individuals), playing a role in determining their adult phenotypes [9–11] (Fig. 1b). If associated microbes consistently affect behaviors of parents and developing offspring, these are also likely to affect their interactions, with consequences on individual and social phenotypes.

Microbial effects on behavior are not limited to developmental dynamics. Contrarily, when the development of a group-living animal is accomplished, its life still largely
depends on iterative adjustments of behavior and underlying physiology to interactions with its social partners, where all involved parties are affected by their associated microbiota (Fig. 1b). Finally, group-level properties emerge from microbiota-affected individual traits and social interactions, and the resulting group performance contributes to the pool of genes passing to the next generations (Fig. 1c). Is it possible to determine to what extent symbiotic microbes affect lives of group-living animals across levels of biological organization?

2 Model systems

Conducting experimental work on animals seems a reasonable way to explore the roles of microbiota in social dynamics. However, the models to be used for this purpose need to have three essential features: first, they must live in relatively stable groups; second, they must consistently establish symbioses with microorganisms; third, they must be suitable for experimental manipulations in the laboratory or in natural conditions. The classic laboratory-adapted animals of contemporary behavioral neuroscience [12] do not always fulfill these conditions. For example, Drosophila fruit flies are easy to rear and very well characterized, and gut microbes affect their physiological and behavioral traits. These include specific appetites for certain foods [13], reproductive choices and mating [14–17], “social recognition” of related individuals [18], and even locomotion [19]. However, fruit flies have limited interactions with conspecifics, only forming relatively ephemeral gatherings in the proximity of food sources, where individuals interact to compete for territories or mate [20]. Contrarily, rodents such as mice and rats, commonly used in life sciences to model human systems, live in familial groups relatively similar to those of humans, and are employed in a variety of behavior-relevant microbiome studies [21, 22]. However, laboratory-adapted rodents often host microbial communities that differ from those of natural populations [23] and have relatively complex brains, with relatively high cognitive abilities and important effects of experience on behavior. Such traits complicate experimental approaches.

The ideal models to investigate group-level behavior effects of associated microbes are group-living animals with relatively simple brains and behaviors. From this perspective, eusocial insect societies can be considered as systems of elementary cooperative modules acting altruistically to maximize the reproductive output of their groups. In their colonies, cohesion and stable division of reproductive labor are maintained because of genetic homogeneity and group-level benefits [24], while simple and stereotypical behavioral patterns of individuals generate complex group-level phenotypes. Such emerging dynamics characterize key features of insect eusocial life, including, for example, the ability of finding and exploiting food sources or to build nests with sophisticated architectures.

From a more practical point of view, eusocial insect individuals perform working tasks that are not always directly correlated with their appetitive needs (i.e., a non-starving worker would altruistically engage in foraging if its colony needs food [25]), and communicate extensively with each other across a variety of contexts. These traits make them suitable for laboratory-based behavioral experiments in which individuals and groups are challenged with operational tasks. In addition, eusocial insects host relatively simple communities of symbiotic bacteria compared to...
other animals (and including humans) [26, 27], which is ideal when roles of specific microbial strains need to be investigated.

Honeybees are by far the best characterized species among eusocial insects, due to their economic importance as pollinators and producers of honey. Therefore, their microbiome has already attracted a significant interest from the scientific community. However, ants offer different experimental advantages compared to honeybees. For example they are relatively easy to work with in laboratory conditions, as they do not fly and can be hosted in limited space. Contrarily, honeybees form very large colonies and usually need to forage in natural settings, which in temperate climates limits experimental work to around half of the year. Finally, ants are a large and diverse group of insects comprising > 12,000 described species. Research questions about the role of microbes in ant behavior can thus be tailored based on a variety of species-specific features.

3 Ants: microbial associations and social behavior

3.1 Types of symbiosis

Insect associations with microbes are distributed along a broad spectrum, with bacterial parasitism and obligate mutualism at the two extremes. Along the non-parasitic side, bacteria are classified based on the degree of intimacy of their association with insects. For example, “commensals” live on the insect cuticle, in exocrine glands or in the gut lumen [28], and their activity sometimes provides advantages to their hosts [29, 30]. Insect symbionts are different from commensals in that their presence is usually necessary to the survival of the host, on which they may even depend completely. Some of these microbes, called primary endosymbionts, live in specialized host cells (bacteriocytes), from where they provide nutritional benefits [31]. Primary endosymbionts are strictly vertically transmitted, i.e., from mother to offspring, and have simplified small genomes resulting from a long process of erosion throughout evolutionary time [32]. This extreme genomic simplification prevents them from living anywhere else than the bacteriocytes of their hosts, and for this reason, they are considered as intermediate stages between free-living bacteria and endosymbiotic cellular organelles [33]. To some extent similar to primary endosymbionts, secondary endosymbionts can be found in the insect hemolymph or organs, and are sometimes vertically transmitted from mother to offspring. However, these are less intimately linked to their insect hosts and their transition from commensals to symbionts is generally more recent than that of primary endosymbionts [34].

3.2 Ant commensals and behavior

Across eusocial insects, individuals belonging to different castes within the same society generally harbor distinct microbiomes (reviewed in [35]). In ants, differences in the communities of associated microbes are found between workers and queens [36, 37] and among groups of workers [38] (but see [39]). Based on such differences, it has been hypothesized that the composition of gut microbes communities within insect societies varies according to the functional roles of colony members, ultimately resulting in increased fitness benefits [35]. Effects of insect-associated microbes are usually investigated via comparing control and microbiota-remodeled individuals, with the latter most often treated with antibiotics. Commensal microbes of eusocial insects have been shown to serve nutritional upgrading roles for their hosts [30, 40] and are thought to be also involved in socially relevant dynamics.

In humans, commensals largely contribute to the biosynthesis of neurotransmitter neuroactive molecules [41–46]. Although, in principle, this may also occur in ants, at present the only evidence of microorganismal effects on neurotransmitter biosynthesis comes from parasitic fungi such as Ophiocordyceps unilateralis, which manipulates ant behavior to reach the fruiting stage and propagate [47]. Similarly, bacteria-produced semiochemicals (chemicals conveying signals to other organisms, affecting their behavior) are widely distributed across insects (reviewed in [48]), but mechanisms underlying the putative roles of ant-associated microbes in the production of such chemicals are largely unknown. There is some evidence for a direct role of cuticle-dwelling microbes on cuticular hydrocarbons, with a single study on Pogonomyrmex harvester ants (Fig. 2a) in which topical administration of antibiotics was found to alter cuticular hydrocarbon (CHC) profiles of workers [49]. Such findings are corroborated by work on fruit flies, suggesting that CHC may be used by cuticular microbes as sources of carbon [16]. Another related study on ant semiochemicals that is unique in its scope shows that pyrazines, serving as trail pheromones in leaf-cutting Atta sexdens rubropilosa (Fig. 2b), are produced by the ant-associated microbe Serratia marcescens [50].

Recent work on Acromyrmex leaf-cutting ants (Fig. 2c) proposes that indirect effects of microbe metabolism on the production of semiochemicals may also be possible [51]. For example, insect cuticular hydrocarbons are produced in the oenocytes, which are also the center of the insect intermediate metabolism [52]. The availability of compounds involved in the biosynthesis of cuticular hydrocarbons may therefore be affected by gut microbes, which would in principle result in effects on the composition of ant CHC blends [51].
3.3 Ant primary endosymbionts and their putative roles in social behavior

Only few ant taxa are known to harbor primary endosymbionts. Among those, *Cardiocondyla obscurior* (Fig. 2d) (subfamily Myrmicinae), which hosts *Westeberhardia cardiocondylae* [53, 54], and genera within the tribe Camponotini (subfamily Formicinae, Fig. 2e), which live in symbiosis with *Blochmannia* [55]. Closely related to one another, *Westeberhardia* and *Blochmannia* are both γ-proteobacteria belonging to the family Enterobacteriaceae, upgrading the nutrition of their ant hosts by contributing to the biosynthesis of amino acids. In particular, the 530 kb genome of *Westeberhardia* encodes for enzymes of the shikimate pathway. These ultimately lead to 4-hydroxyphenylpyruvate, which is likely converted into tyrosine by the ant host metabolic machinery [54]. The 700 kb genome of *Blochmannia* [28] includes genes involved in nitrogen recycling (urease and glutamine synthetase) and the biosynthesis of aromatic and branched amino acids (tyrosine and tryptophan and leucine, isoleucine, and valine) [56, 57]. For *Westeberhardia* and *Blochmannia*, the contribution to the biosynthesis of tyrosine, involved in the establishment of cuticle in ants [54, 58] and in insects in general, is thought to be a key feature of the symbiosis. The role of endosymbionts in cuticle formation is also supported by the fact that both *Westeberhardia* and *Blochmannia* peak across the end of metamorphosis and the beginning of adult life, when the ant cuticle is established. Accordingly, long-term antibiotic treatment on developing *Camponotus* workers results in individuals with a lighter and probably less sclerotized cuticle [58].

The metabolic activity of primary endosymbionts provides chemicals required for the formation of a functional cuticle. This protects the ant from dehydration, but is also coated with...
a blend of long-chain hydrocarbons mediating social interactions such as nestmate recognition [59], the process by which individuals discriminate colony mates from strangers. If cuticle thickness and structure affect cuticular hydrocarbon blends qualitatively and/or quantitatively, the activity of *Westeberhardia* and *Blochmannia* might be indirectly involved in social interactions within ant colonies. In addition, the metabolic activity of these endosymbionts might further affect social interactions, because compounds deriving from aromatic amino acids (i.e., tyrosine and tryptophan) are precursors of neurotransmitters such as dopamine and serotonin, which affect animal neurophysiology and behavior. In *Blochmannia* and *Westeberhardia*, this would be especially relevant, because they achieve their peaks during the late pupation and early adulthood of their ant hosts, when ants establish their colony-specific blend of cuticular hydrocarbons, learn to discriminate nestmates from non-nestmates based on such blends [34] and begin developing their behavioral profile through early life experiences [60]. Nevertheless, it must be stressed that, at present, there is hardly any evidence for primary endosymbionts to affect ant social behavior, and all the stated hypotheses have not yet been experimentally investigated.

The scarcity of studies explicitly linking ant primary endosymbionts and behavior is partly due to technical limitations. Experimental manipulations of microbial communities (microbiota remodeling) can be conducted via rearing individuals in sterile conditions, or administering specific microbial strains via the food. However, experiments involving ants often rely on the use of antibiotics. While these provide invaluable help in selecting and controlling culturable symbiotic microbes of insects in *in vitro* assays [61], they tend to result in undesired effects when used to suppress microbes within live insects. For example, antibiotics usually do not target a single bacterial strain, potentially resulting in the suppression of the targeted endosymbionts but also of commensal species, making impossible to disentangle the role of specific bacteria. In addition, the experimental suppression of primary endosymbionts should target the phases in which these are most active, such as metamorphosis and early adult life. However, ants do not feed in these phases, which precludes antibiotics administration via the food. Finally, some antibiotics are known to interfere with animal host physiology, for example by affecting mitochondrial function [62, 63]. To limit such side effects that potentially impair physiology and behavior, a reasonable alternative approach would be silencing genes of primary endosymbionts via RNAi.

### 3.4 Incidental effects of microbial partners on social dynamics

Microbial partners do not necessarily need to affect the function of nervous systems to influence the behavioral dynamics of animals, including ants. For example, microbe-suppressed *Acromyrmex* leaf-cutting ants were recently found to have low levels of some microbe-produced antibiotic chemicals [64], and were aggressed in social recognition assays with their nestmates [51]. In that case, microbe suppression disrupted colony-level cohesion and triggered an effect on social behavior. Such an increase of aggression probably occurs because these immunocompromised individuals would increase the risk of disease within the colony, and are kept at bay via the aggressive behavior of their nestmates.

In principle, incidental effects of microbes on social life can be extended to any ant trait that is potentially affected by microbial partners. For example, if ant-associated gut commensals affect host appetites for specific foods as it is found in fruit flies [13, 29, 65], the food consumption of an ant will depend on its microbiota. As a result, given that the colony-level nutrient homeostasis depends on individual feeding [66], the colony-level foraging investment is also likely to depend on the microbiota. Similarly, maternally inherited primary endosymbionts support larval growth and upgrade nutrition during metamorphosis [54, 67]. As the metabolic activity of primary endosymbionts contributes to the ant adult phenotypes, this might in principle contribute to individuals’ behavior in the long term, ultimately affecting social interactions.

### 4 Experimental approaches to microbial effects on social behavior

Since the 1980s, researchers in biology have been implementing techniques to automatically measure the activity of animals in experimental settings [68]. Such methods rely on computer algorithms extracting quantitative measures of behavior via pattern analysis applied to recorded images. The rise of these systems has improved experimental reliability, because computer algorithms are not subject to variations typical of human performance [68]. Initially, automatic behavior observation was found to be particularly suited for quantifying animal locomotion [69, 70], behaviors occurring unpredictably [71] or over long periods [72, 73]. With progress in camera resolution and integration of code identification, tracking technology became suitable to study the behavior of animal groups. Automatic tracking is usually conducted on groups of insects moving on 2D arenas [74]. In recent systems, individuals are tagged with unique markers (matrix codes such as QR or ArUco [75–78], Fig. 3a; or combinations of color dots painted on ants [79–82], Fig. 3b), and images are taken by video cameras placed on top of the arenas. Systems based on automatic identification of individual codes allow determining the positions of all insects within a given colony at pre-set time intervals, which are used to determine basic attributes of individual behavior.
such as speed, distance traveled, or activity levels. Interaction networks within insect colonies can be reconstructed by measuring inter-individual distances over time.

In the last few years, the integration of behavioral pattern recognition based on machine learning and computer vision has allowed to automatically classify not only individual identities, but also different behaviors and interactions among individuals [83, 84]. These next-generation systems do not rely anymore on the positions of individual tags, but perform analyses of videos based on the recognition of insect shapes and movements. These innovations are ideal to investigate the behavior of ants at individual and group levels, which is in turn the best approach to evaluate effects of symbiotic microbes on such dynamics. From an individual perspective, it is in principle possible to monitor microbiota-remodeled individuals over long periods of time, which allows detecting even slight modifications on their movement patterns. Concerning social behaviors, it is possible to quantify different types of interactions (e.g., antennal contacts or trophallaxis [85]) and for example correlate those with the spread of horizontally transmitted commensal microbes or pathogens. Finally, the effects of symbiotic microbes on group-level phenotypes can be explored by tracking groups of ants engaging in cooperative behaviors. This can be done, for example, by measuring the performance of colonies including known proportions of microbiota-remodeled individuals, or including/excluding specific bacterial strains from the microbial communities of all individuals within colonies. Testable group-level behaviors include structure construction, cooperative transport of large prey items, nest choice, etc.

To conclude, it is important to emphasize that most studies still rely on the offline processing of the data. This enables experimenters to compensate for errors and employ more advanced and expensive computational techniques, or even apply future knowledge to previous experiments by reprocessing previously collected data. However, it does not allow for rapid insights into the experiment itself. Recent progresses in technology, such as the use of Graphics Processing Units to accelerate computation, will allow to integrate the traditional way of conducting these studies with real-time data. In this way, while individuals are tracked, data are recorded for further processing and at the same time analyzed, and preliminary results are made available to scientists. By combining real-time processing with traditional experimental methods, it will be possible to identify individual behavioral attributes and group emerging properties before the end of an experiment, therefore increasing the rate of discovery.

5 Conclusion

Microorganisms associated with group-living animals potentially impact the social interactions of their hosts, but knowledge in this field is still limited. On the other hand, determining whether and how microbial partners affect social dynamics is key to contextualize the evolution of the interactions between microbiota and brains. Future research effort in the field should be conducted in multiple directions. First, by addressing the direct roles of microbes in semiochemical production, with a focus on eusocial Hymenoptera; second, by disentangling the indirect effects of bacterial partners, such as gut commensals, on the production of semiochemicals. This should be especially focused on the relationship between chemicals produced by microbes and the metabolism in the insect oenocytes; third, by exploring effects of microbes that do not directly impact socially relevant traits but incidentally affect social dynamics. Next-generation video tracking systems based on artificial intelligence and real-time data analysis will be instrumental to delineate how microbial effects on individuals are mirrored at social and group levels.
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