The gut microbiota - brain axis of insects

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Highlights

The gut microbiota is emerging as a regulator of neurophysiology and behavior.

Similar processes may govern the gut microbiota - brain axis across mammals and insects.

The honey bee allows disentangling microbial effects on behavior in a eusocial animal.

Abstract

Research on the connections between gut microbes and the neurophysiology and behavior of their animal hosts has grown exponentially in just a few years. Most studies have focused on mammalian models as their relevance to human health is widely established. However, evidence is accumulating that insect behavior may be governed by molecular mechanisms that are partly homologous to those of mammals, and therefore relevant for the understanding of their behavioral dysfunctions. Social insects in particular may provide experimentally amenable models to disentangle the contributions of individual bacterial symbionts to the gut microbiota - brain axis. In this review, we summarize findings from recent research on the neurological and behavioral effects of the gut microbiota of insects and propose an integrated approach to unravel the extended behavioral phenotypes of gut microbes in the honey bee.

Introduction

Research on symbiotic microorganisms associated with eukaryotic hosts has expanded dramatically in recent years, because advances in sequencing technologies allow rapid characterization of unculturable – and thus previously unknown – microbial diversity. An emerging avenue in this field is that of the neurophysiological consequences of microbial symbionts, which is rapidly changing the way we understand key aspects of symbiosis and animal behavior. Such interdisciplinary research operating at the interface of neuroscience, microbiology, and medicine is becoming a major subfield of biology, holding promise for the treatment of diseases affecting millions worldwide [1].

The gut microbiota has well-established roles in animal nutrition and immunity [2,3]. However, gut microorganisms also hold a previously underestimated potential to contribute to host processes beyond those occurring in the intestinal tract. For example, they can produce neuroactive compounds that influence brain function and behavior [4], with numerous implications for disorders of the central nervous system [1,5-7]. Research on the gut microbiota - brain axis in mammalian models (i.e. rodents) is unraveling contributions of bacterial taxa to the etiology of neurodegenerative diseases such as Alzheimer [8] and Parkinson's disease [9] and in the modulation of emotional states, including anxiety and depression (reviewed in [6]). Recent studies also suggest a link between the gut microbiota and social behavior, connecting microbial dysbiosis in the gut with social dysfunctions, such as autism-spectrum disorders (ASD) [10,11] and schizophrenia [12].

So far, experimental investigations of the connections between gut bacterial strains, their metabolic output, the induction of gene expression in the host brain, and the ensuing effects

on behavioral traits, have mostly focused on a few established vertebrate model organisms (mostly mice and rats) [6,13]. This implies that the evolutionary history of the gut microbiota - brain axis has remained elusive, and we lack knowledge about the conservation of the underlying mechanisms by which hosts and microbes interact. Moreover, animals vary substantially in the diversity and stability of their microbial gut communities, as well as in the extent to which they engage in social behavior. Little is known about how these traits are regulated along the gut microbiota - brain axis, i.e. how microbial community structure impacts host brain and behavior and how social interactions shape the assembly of microbial communities in return. Insects provide experimentally amenable models that vary tremendously in the characteristics of their gut microbiota as well as in degree of sociality, but research in this field is still in its early stage. The exploitation potential of the gut microbiota - brain axis to manage invertebrate species of economic interest, and the suitability of insect species as pharmacological models for microbiota-induced neurological and behavioral dysfunctions have thus remained largely unexplored. Filling such knowledge gaps is now feasible owing to technological breakthroughs in DNA sequencing, genome engineering, metabolomics, and behavioral tracking, and the amenability of a few insect model organisms to manipulation of their gut microbiota composition.

Social insects in particular hold promise for disentangling the contributions of individual bacterial strains and their synergistic effects on social behavior. Recent discoveries suggest that homologous molecular mechanisms may underlie responsiveness to social stimuli across bees and humans [14,15] (Figure 1). Honey bee workers that do not engage in brood care and defense of the hive, and solitary individuals in a halictid bee species characterized by a social polymorphism, both show brain gene expression differences compared to their social

97 counterparts for genes implicated in ASD in humans [14,15]. This implies that social insects
98 could provide excellent model organisms to understand the role of gut microbes on the
99 evolution of social behavior and its dysfunctions.
100
101 In this review, we will summarize recent investigations on microbially-induced alterations of
102 neurophysiology and behavior across insects and propose an integrated approach to
103 characterize the gut microbiota - brain axis in the honey bee, a social insect in which the
104 understanding of brain physiology and social behavior [16], as well as the composition and
105 function of the gut microbiota [17-20], are well-advanced. Further, a suite of assays to track
106 cognitive performance and social interactions in social insects, including honey bees, has
107 recently become available [21-24] (Box 1 and Figure 2A).
108
109 **The extended behavioral phenotypes of symbiotic microorganisms in insects**
110 The first appreciation that symbiotic microorganisms can alter the behavioral repertoire of
111 their insect hosts derived from studies looking into how microbes manipulate their hosts to
112 enhance their own transmission. Examples include *Wolbachia* bacterial symbionts modifying
113 the mating preferences of their hosts [25], or *Ophiocordyceps* parasitic fungi turning infected
114 ants into "zombies" that abandon their maternal nest to die where conditions are most
115 favorable for fungal sporulation [26]. More recently, researchers have started investigating the
116 specific neurological and behavioral effects of the bacterial communities associated with the
117 intestinal tract of insects, identifying their contributions in numerous processes, including
118 chemical communication, development, cognition, and social interactions.
119

120 Gut microbes can alter the odorant profiles and the olfactory behavior of their insect hosts
121 [27], consequently regulating how individuals interact through chemical communication,
122 aggregate in social groups, or make decisions about foraging and mating. For example in the
123 lower termite *Reticulitermes speratus*, conspecific intruders are more easily recognized and
124 aggressed when they are colonized by foreign gut bacteria promoting unfamiliar scents [28].
125 In *Acromyrmex echinatior* leaf-cutting ants, suppression of the gut microbiota seemingly
126 promotes aggression between non-nestmates, possibly through changes in the cuticular
127 hydrocarbon profiles (CHCs) [29]. German cockroaches that lack gut bacteria have lower
128 amounts of volatile carboxylic acids in their feces, which mediate aggregation responses.
129 These feces become less attractive to conspecifics than those from conventionally colonized
130 or re-inoculated (after antibiotic treatment) individuals [30]. Similarly, the production of the
131 pheromone guaiacol by gut microbes mediates the aggregation of locusts into swarms [31]. In
132 *Drosophila*, gut microbes influence olfactory-guided foraging decisions by making hosts
133 prefer food patches seeded with specific (beneficial) bacterial strains, although these decisions
134 are traded against the need to balance the flies' nutritional intake [32,33]. Similarly, when
135 *Bactrocera dorsalis* oriental fruit flies are depleted of their gut microbes, they prefer food
136 containing a full complement of amino acids over other less nutrient-rich options even when
137 this food is less readily accessible [34].
138
139 The gut microbiota can have profound effects on the neurophysiological development of the
140 host [35], aiding in cognition by potentiating its capacity to learn and memorize. Axenic
141 *Drosophila* flies perform worse in an aversive phototactic assay of learning and memory than
142 flies reared with a conventional gut microbiota [36]. The co-inoculation of two commensal
143 microbes, *Lactobacillus* and *Acetobacter* (but neither of those in mono-inoculations), is

144 required and sufficient to recapitulate the cognitive performance of fully-colonized flies [36].
145 Likewise, several cognitive-enhancing effects of the gut microbiota have been described in
146 rodent models (reviewed in [6]). For example antibiotic-treated rats suffer reduced spatial
147 memory abilities, which can be reversed by gut colonization of *Lactobacillus*
148 *fermentum* NS9 [37].
149
150 Recent findings also show that insect models may be appropriate for understanding the
151 development of neurodegenerative diseases and the potential for their probiotic treatment.
152 *Drosophila* null mutants of the *parkin* gene (a gene whose mutations are strongly associated
153 with early onset of Parkinson's disease in humans) have 5-fold higher bacterial loads and an
154 altered community structure in their guts compared to wild-type control flies [38]. These flies
155 are also more sensitive to paraquat (a neurotoxin whose chronic exposure increases the risk of
156 developing Parkinson's disease) as compared to germ-free *parkin* mutants [38]. Selective
157 RNAi knockdown of *parkin* in gut enterocytes increases bacterial load but does not cause
158 changes in paraquat sensitivity. However, sensitivity to paraquat is altered if the knockdown
159 occurs throughout the entire fly, suggesting that dysbiosis of the gut microbiota can influence
160 sensitivity to toxins in distal tissues [38]. These results suggest that *parkin* regulates microbial
161 homeostasis in the gut of fruit flies, and conversely, that the gut microbiota impact fruit fly
162 traits that are associated with Parkinson's disease in humans. These findings are intriguing
163 because recent studies in mice have linked the gut microbiota with the etiology of this disease
164 [9] and suggest that at least some forms of Parkinson's disease may represent autoimmune
165 diseases starting in the gut years before any motor deficit occurs [39].
166

Three recent studies also linked the gut microbiota with markers of Alzheimer's disease in a *Drosophila* model. Together these studies show that dysbiosis results in exacerbated progression of the disease as modeled in the fly [40] and that probiotic supplementation with distinct *Lactobacillus* and *Bifidobacterium* strains can ameliorate several symptoms [41], possibly mediated by the production of short-chain fatty acids (SCFAs) such as acetate [42]. Gut dysbiosis and associated changes in SCFA abundance in the gut are common markers of Alzheimer's disease in mammals, including humans (reviewed in [8]). Further, initial therapeutic attempts with probiotics composed of *Lactobacillus* and *Bifidobacterium* strains had positive effects on disease symptoms [43,44].

A recent study [45] showed that *Drosophila* are hyperactive in axenic conditions compared to conventionally-inoculated flies. These effects could be reversed by colonization with *Lactobacillus brevis*, a common gut symbiont of fruit flies, but not *Lactobacillus plantarum*. The study gained some mechanistic understanding of these interactions by showing that xylose isomerase was responsible for the locomotor effects by modulating trehalose levels, and that thermogenetic activation of octopaminergic neurons or exogenous administration of octopamine abrogated its effects, implicating octopaminergic neurons as mediators of cues from the gut microbiota. Mice lacking a microbiota are similarly hyperactive [35] and have increased anxiety-like behavior [46]. Moreover, recent studies showed that ASD symptoms in mice [10,11] and human children [47] can be improved through microbiota transplantations. ASD symptoms include hyperactivity (i.e. attention deficit hyperactivity disorder) and anxiety, in addition to gastro-intestinal and autoimmune disorders, depression and obsessive-compulsive disorder [48,49]. Therefore, it has been suggested that there could be a mechanistic link between these results in *Drosophila* and

191 mammals [50]. One potential mechanism has been recently identified. Reducing the
192 expression of histone demethylase KDM5 genes in *Drosophila* (whose loss-of-function
193 mutations are associated with ASD in humans and mice) causes intestinal barrier dysfunction
194 and induces changes in gut microbiota composition and social behavior that can be partly
195 rescued by feeding a *Lactobacillus* strain [51]. KDM5 histone demethylases regulate
196 transcription of genes in the immune deficiency signaling pathway [51]. The functions of
197 these enzymes are evolutionary conserved, indicating that they may play a key role in
198 maintaining gut microbial homeostasis across a wide range of host species [51]. Epigenetic
199 modifications such as DNA methylation and histone modifications are broadly implicated in
200 neurodegenerative diseases in humans [52], so future comparative work should detail the
201 extent to which these processes are conserved.
202
203 An interesting aspect emerging from this body of research is that, in spite of gut communities
204 being comprised of substantial bacterial diversity, in several instances mono-inoculations with
205 individual bacterial strains appear to be sufficient to recapitulate the cognitive, social, and
206 locomotor abilities of fully-colonized individuals [11,36,45]. This may point towards general
207 mechanisms of host-microbe interaction that are redundant across multiple gut symbionts.
208 Indeed empirical evidence so far suggests that several neurophysiological effects of gut
209 microbes can be induced by molecules that are broadly produced via bacterial fermentation in
210 both insects and mammals, such as SCFAs [9,42,53], or by the activity of enzymes encoded
211 by genes present across multiple bacterial genomes [45]. Taken together the recent studies on
212 insects are encouraging, as they provide support for the hypothesis that homologous processes
213 underlie the regulation of neurodevelopmental diseases by the gut microbiota across
214 mammals and insects. If this hypothesis will be substantiated by additional empirical

evidence, it would suggest that these diseases are deeply rooted in evolution and represent by-products of ancient and complex interactions between gut microbes and the host nervous system. However, a full appreciation of homology in these interactions will require a much better mechanistic understanding of the extended phenotypes of gut bacteria on their insect hosts. Most studies have so far focused on the fruit fly gut microbiota, which consists of few bacterial species that for the most part only transiently colonize the gut [reviewed in 54, but see 55]. While *Drosophila* provides a good model to dissect the proximate mechanisms that mediate host responses to bacterial colonization, it is sub-optimal to understand how more complex and persisting bacterial communities impact neural functioning and regulate the interaction dynamics of host social networks, questions that are highly relevant for human psychology and medicine.

A research primer to characterize the gut microbiota - brain axis in the honey bee

The honey bee is a promising model to investigate the neurological and behavioral effects of bacterial symbionts for a number of reasons. The gut microbiota is well characterized and known to consist of eight to ten predominant bacterial phylotypes (clusters of bacterial strains sharing ≥ 97% sequence identity in the 16S rRNA gene; Figure 1), five of which represent the core microbiota found in every honey bee worker, independently of sub-species and geography [56]. This represents a remarkably simple gut community that can be easily manipulated (see Box 1) compared to vertebrate models, yet that is both more complex and stable than that of a fruit fly [54]. The bacterial lineages present in the honey bee gut are comprised of several sequence-discrete populations (SDPs, which can be considered as bacterial species [20,57]), each of which contains high levels of strain diversity [20] (Figure 2B). Each bee harbors a unique combination of strains, indicating that the functional

repertoire of the gut community varies across bees even within the same hive [20]. Distinct behavioral groups characterized by division of labor coexist within the hive, and these show differences in gut microbiota composition and structure [58-60]. This system therefore represents a unique opportunity to understand how gut bacterial diversity affects variation in individual cognition and behavior and how the cumulative effect of these microbe-host interactions shapes the colony's social network structure. Communication between host and microbes is bi-directional and social interactions can have profound effects on how gut bacteria are distributed between hive members and how the microbiota assembles in individual bees. These dynamics could be investigated using tracking technologies as recently done to assess how ants modify social interaction to slow down transmission of a fungal pathogen [23]. These technologies are already applicable to honey bees [24].

The physiological impact of honey bee gut symbionts has recently been investigated. So far, the focus has mostly been restricted to roles for nutrition [18,61] and immunity [62-64] in gut tissues. However, these first explorations are encouraging as they also suggest that the gut microbiota alters worker behavior towards increased sugar intake, likely by modulating insulin sensitivity [61] (Figure 1), and that specifically *Bifidobacterium asteroides* induces juvenile hormone III and prostaglandins in the host gut [18], which may be instrumental for gut - brain communication. The study of the neurophysiological effects of gut microbes is still in its infancy, but as honey bees are major pollinators of invaluable importance to secure food production, it could make vital contributions to ensure hive health.

Conclusions

Studies of the extended behavioral phenotypes of microbial gut symbionts have implications across biological and medical disciplines. They are also contributing to a shift in perspective of organismal function to one in which the behavioral repertoires of animals result from interactions between symbiotic species spanning multiple domains of life. So far our proximate and ultimate understanding of these interactions has been limited by the use of only a handful of model organisms, rodents for the most part. This has precluded understanding when and how such gut microbe - brain interactions evolved, as well as the generality of the proximate mechanisms involved. To fully appreciate the role of bacterial symbionts in the evolution of the social brain, future research should contrast these interactions across multiple taxa representing different degrees of sociality. Nevertheless, encouraging first investigations have begun to suggest that homologous gut microbiota - brain interactions in mammals and insects may exist, pointing to a deep evolutionary origin of the gut microbiota - brain axis. Establishing the role of gut microbes in cognition and behavior as well as the suitability of probiotic supplementation as a mean to adjust behavioral traits of species of strategic importance has the potential to open up a different perspective on how bees and other insects will be managed in the future.

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Figure captions

Figure 1 Comparative summary of studies that previously investigated the gut microbiota - brain axis in mammals (including humans) and the physiological responses to gut microbes in honey bees, highlighting parallels between these systems, as well as knowledge gaps for honey bees (in bold) and recently discovered expression overlap with brain genes involved in autism spectrum disorders (ASD).

Figure 2 Schematic summary of experimental approaches to investigate the effect of gut microbes on the neurophysiology and behavior of the honey bee host. (A) The gut microbiota composition can be manipulated in any desired way (see Box 1), after which colonized and microbiota-depleted bees can be used in gene expression, metabolomics, brain imaging, or behavioral tracking experiments with 'fiducial' ARTags – unique matrix-like markers that are glued to the thorax of each bee. (B) Each bee harbors a unique combination of gut microbe strains [20] and the panel depicts a hypothetical example of strain distributions across bees, whose presence is shown by gray quadrants on top of orange and purple dashed lines separating bees belonging to distinct behavioral groups. Interactions between bees are shown

by gray arcs towards the top. The distinct behavioral groups (e.g. foragers and nurses, depicted in different node colors) cluster separately in a hypothetical social interaction network (C), where nodes represent individual bees and gray edges report interactions between bees, with edge width being proportional to the number of interactions between individuals through time. SDPs = sequence-discrete populations, as defined in [20,57].

Box 1 Research approaches to characterize the gut microbiota - brain axis in the honey bee.

The production of gnotobiotic honey bees is rather simple, as bees can be deprived of gut symbionts via elimination of their oral-anal transmission route by isolating mature pupae in sterile rearing boxes and allowing adults to emerge in incubators [18]. This avoids the potentially confounding effects of the antibiotic exposure often required to produce germ-free individuals in other organisms and results in bees colonized only by transient, environmental bacteria at very low abundance, which are referred to as microbiota-depleted (MD) [56]. All bacterial strains associated with the honey bee can be cultured in the laboratory and re-inoculated in MD bees by the simple addition of bacterial cultures to the food or by 'pipette-feeding' defined quantities of bacteria in sugar water, producing bees colonized by any combination of bacterial strains [18]. The bees whose microbiota composition has been experimentally manipulated can be subjected to neurotranscriptomic analyses to identify brain gene expression changes upon bacterial colonization, and metabolomics studies to track bacterial metabolites [18,61] as they travel through the host body and possibly reach the brain, also with the aid of stable-isotope labeling. Brain regions and neuronal populations involved in the interactions can be identified via fluorescence *in situ* hybridization and microscopy. Phenotypic effects on behavior can be quantified by assays of learning and memory abilities [21], flight performance and responses to sensory stimuli [65]. Moreover, advanced tracking

technologies that allow the full quantification of social interactions in observation boxes are now available [22-24] and can be used to quantify whether gut bacteria influence the position of each bee in the hive interactome and the number of times each bee interacts with other individuals and engages in more complex behaviors such as nectar/pollen handling, brood rearing, or trophallaxis.

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•• The authors demonstrated that the gut microbiota of *Drosophila melanogaster* influences the fruit fly's locomotor behavior by interacting with the central nervous system. To do this, they compared fruit flies with and without their conventional gut flora showing that germ-free flies are hyperactive, and went on to test flies that were only inoculated with individual *Lactobacillus* strains, showing that *Lactobacillus brevis* but not *Lactobacillus plantarum* can recapitulate the effects of the conventional gut flora. Using single-gene mutation experiments, they identified xylose isomerase (Xi) as the enzyme mediating these effects by modulating trehalose levels. By selective activation of specific neuronal populations the authors showed that *L. brevis* and Xi downregulated neuronal pathways for octopamine production and that the addition of octopamine to conventional flies or *L. brevis* increased fly activity to the levels of axenic flies, gaining in-depth understanding of the proximate mechanisms by which hosts and microbes interact.

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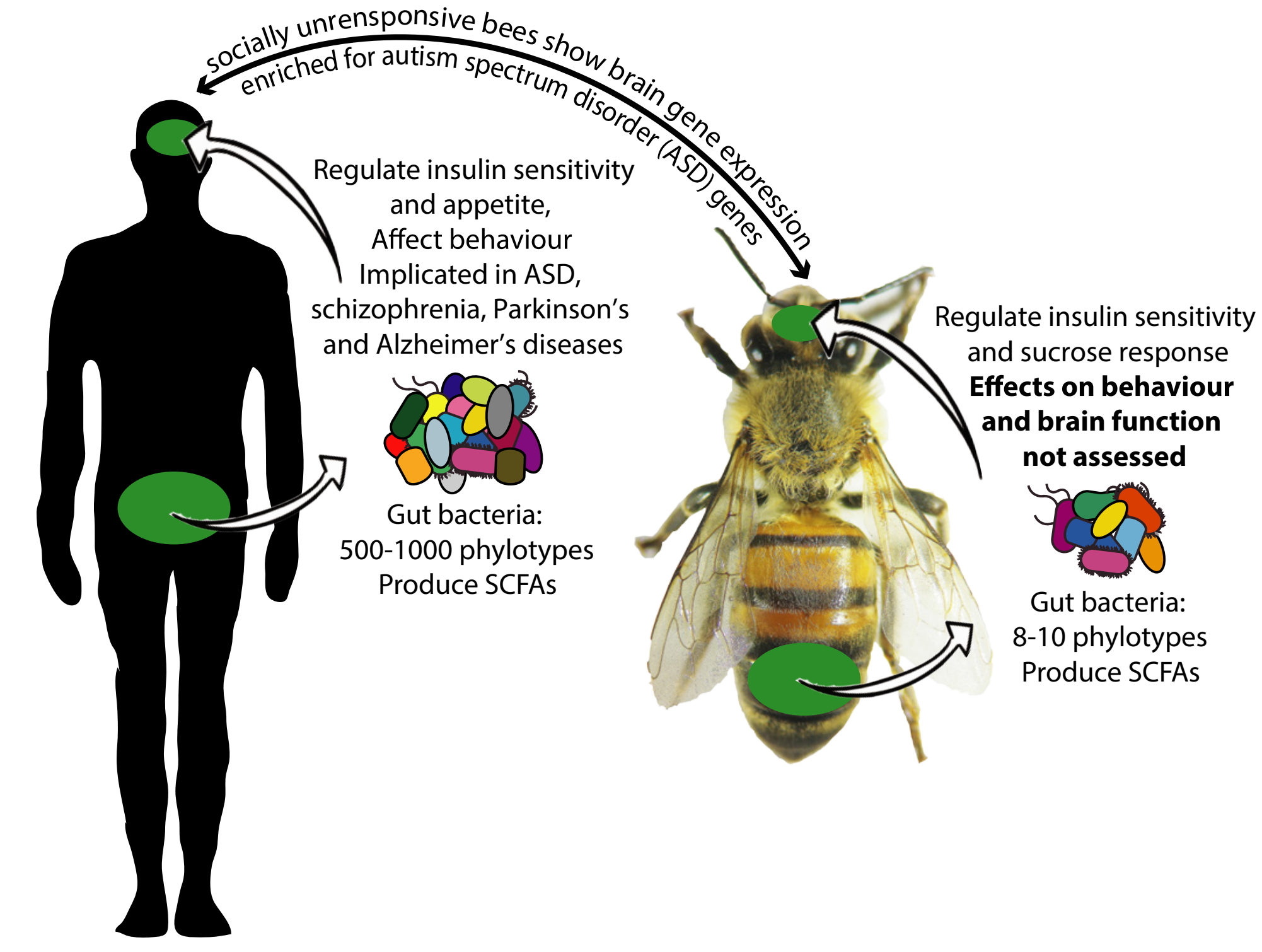
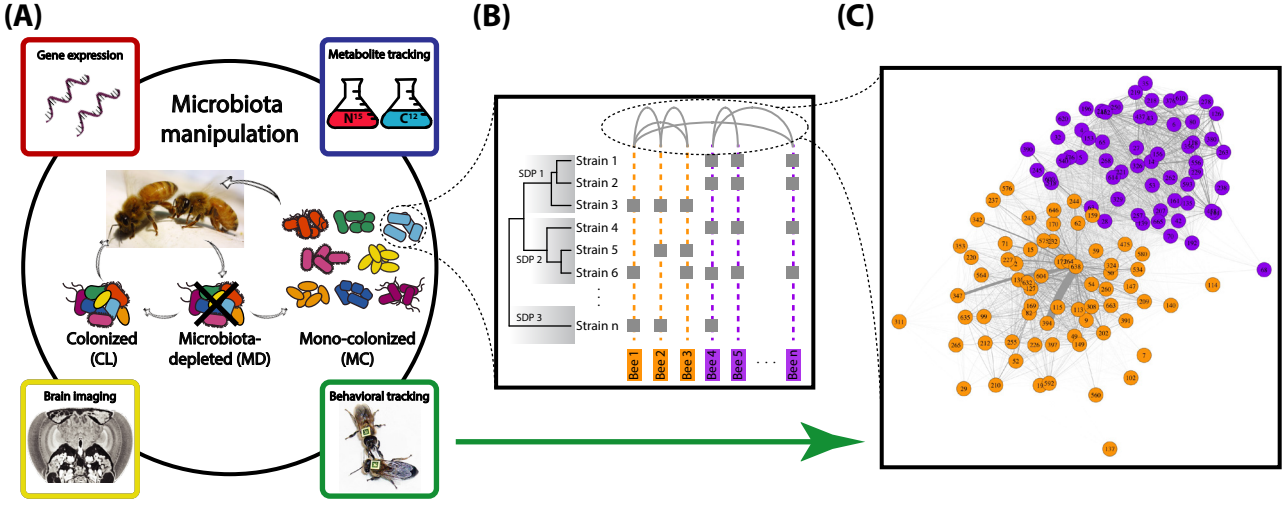

socially unrensponsive bees show brain gene expression
enriched for autism spectrum disorder (ASD) genes
Regulate insulin sensitivity
and appetite,
Affect behaviour
Implicated in ASD,
schizophrenia, Parkinson's
and Alzheimer's diseases
Gut bacteria:
500-1000 phylotypes
Produce SCFAs
Regulate insulin sensitivity
and sucrose response
Effects on behaviour
and brain function
not assessed
Gut bacteria:
8-10 phylotypes
Produce SCFAs


(A)
Gene expression
Metabolite tracking
Microbiota manipulation
Colonized (CL)
Microbiota-depleted (MD)
Mono-colonized (MC)
Brain imaging
Behavioral tracking
(B)
SDP 1
SDP 2
SDP 3
Strain 1
Strain 2
Strain 3
Strain 4
Strain 5
Strain 6
Strain n
Bee 1
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