Microbial Modulation of Host Locomotion

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ABSTRACT

Coordinated locomotor behavior is critical for the survival and propagation of an individual and is modulated by internal and external sensory inputs. The microbiota regulates host metabolism, which is closely intertwined with motor behavior. However, little is known regarding influences by the gut microbiome on host locomotion, or the pathways involved. The work presented in this thesis examines microbial regulation of locomotor behavior from both bacterial and host perspectives. Removal of the microbiota results in hyperactivity in female *D. melanogaster*, which is reversible through colonization with specific bacteria or administration of bacterial-derived products, including xylose isomerase (Xi) from *Lactobacillus brevis*. We found that Xi modulates host speed via sugar metabolism and octopamine signaling in flies. Additionally, aspects of microbial regulation of host locomotion appear to be conserved in mice. This work suggests that microbial modulation of host physiology extends beyond local intestinal effects to locomotor behavior through alterations in energy-related pathways.

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OVERVIEW OF *DROSOPHILA MELANOGASTER*

1.1 INTRODUCTION

In order to understand host-microbe interactions, it is important to consider the microbial species and metabolites that influence the host in addition to the corresponding pathways that mediate these effects. A limited microbial community and the ability to perform high-throughput genetic screens facilitate this type of examination into both sides of host-microbe interactions. The microbiome of vinegar fruit fly *Drosophila melanogaster* contains 5-20 bacterial species in laboratory strains. Therefore, along with its genetic tractability and ease of behavioral methodology[1,2], it is uniquely positioned for examining host-microbe interactions.

1.2 A BRIEF DESCRIPTION OF *D. MELANOGASTER*

Drosophila melanogaster originated in sub-Saharan Africa and is commonly associated with rotting fruit and fermented beverages[3,4]. This ability to survive and reproduce on sources commonly produced by humans is thought to contribute to the dispersal of *D. melanogaster*[4]. The developmental period of *D. melanogaster* is relatively quick compared to mice and humans, with only 9-10 days needed to go from an egg to a sexually mature adult[4]. During its life cycle, *D. melanogaster* metamorphoses from a larval stage into its adult form, undergoing eclosion, or the emergence of a pupa to an adult, in this time period. Once in its adult stage, its average life span is between 34 to 53 days[5].

Although *D. melanogaster* was first described by Johann Wilhelm Meigen in 1830, the first reported cultivation in the laboratory was in the 1900s by Harvard entomologist Charles Woodworth[3,4]. In the laboratory, flies are grown on standardized media that usually contain mold inhibitors along with antibiotics[3]. However, there is a large variability in the specific ingredients used in fly media recipes leading recent researchers to develop chemically defined media that have produced more consistent experimental results[6,7]. In addition to this difference in food from the natural environment, *D. melanogaster* is also reared at a constant temperature and humidity in the laboratory.

In part due to its adaptability, short life cycle, and widely available genetic toolkits, *D. melanogaster* has become a model organism used throughout biology. The utilization of *D.*

melanogaster has led to major breakthroughs, from the work of Thomas Hunt Morgan in genetics to studies by Jeffrey C. Hall, Michael Rosbash, and Michael W. Young in circadian rhythms.

1.3 LINKS BETWEEN THE INTESTINE AND CENTRAL BRAIN IN ARTHROPODS

Arthropods have an open circulatory system, which contains hemolymph[8]. Separating the circulatory system and the lumen of the gastrointestinal tract are epithelial cells, the peritrophic matrix in the midgut, and the cuticle in the foregut and hindgut[9]. Molecules that pass through or are transported across these barriers can enter the body cavity, or hemocoel[8]. As invertebrates lack a true endothelium with intercellular junctions, the hemocoel only restricts the hemolymph through the extracellular matrix lining the vessels[8].

Changes in the gastrointestinal tract can also impact neurological function through their effects on the enteroendocrine cells (EECs) and neurons that innervate the gut. Similar to vertebrates, stem cells in the adult fly gastrointestinal tract give rise to EECs in addition to enterocytes[10,11]. Gustatory receptors are expressed by EECs in the midgut, which may relay information about changes in nutrient levels to the central nervous system[12]. In addition to signaling through EECs, information can be relayed from the gastrointestinal tract through immune pathways in the epithelial layer, such as the IMD pathway[13,14]. Neuronal innervation of the adult fly gastrointestinal tract comes from the stomatogastric nervous system (SGS), the corpus cardiacum, and the central nervous system[9]. Sensory neurons innervate predominantly the anterior and posterior regions of the intestine with their cell bodies predicted to reside primarily in the stomatogastric ganglia[9]. The functions of these intestinal innervating neurons span from facilitating peristalsis to sensing nutrient and fluid levels[15–18]. Recently, two neuroendocrine peptides and their receptors have been described as gut-brain peptides with expression in both the brain and gastrointestinal tract: CCHamide-1, CCHamide-2, and Diuretic hormone 44 (Dh44) along with its receptors[17,19]. Although the exact function of CCHamide-1 has not been fully enumerated, CCHamide-2 has been found to affect food intake and locomotor behavior[19]. Neurons that produce Dh44 act as nutritive sugar sensors, responding to sugar levels in the hemolymph independent of taste[17]. The hemolymph, EECs, and enteric neurons link the complex milieu within the gastrointestinal tract to other body sites throughout the organism, including the central nervous system.

1.4 THE MICROBIOME OF *DROSOPHILA*

A mixture of bacteria, viruses, and fungi reside inside of the gastrointestinal tract, and collectively constitute the gut microbiota. The microbiota of laboratory-reared *D. melanogaster* contains between 5 – 20 different bacterial species, with the most common commensals from the *Lactobacillus* and *Acetobacter* genera[1]. When compared to that of mice and humans, the bacterial community of *D. melanogaster* is relatively limited; however, many of these core commensals are found in mammals, including *Lactobacillus plantarum* and *Lactobacillus brevis*[1,20]. The four dominant bacterial species identified in laboratory strains of *D. melanogaster* are: *L. plantarum*, *L. brevis*, *Enterococcus faecalis*, and *Acetobacter pomorum*[20].

Multiple factors influence the microbial composition within *Drosophila*. In the wild, *Drosophila* populations associate with a wider diversity of bacterial species, which is proposed to be due in part to their diet[21]. Additionally, shifts in the carbohydrate:protein ratio of laboratory fly media dramatically influence the proportions of specific bacterial genera[21–23]. In addition to dietary influences, the microbiota also changes throughout the life cycle of *D. melanogaster*. During the larval stage, *L. fructivorans* and *L. plantarum* are the two dominant species, with the former retaining this status in young adults[1,24]. Microbial dysbiosis can occur during old age (30 days post-eclosion) with an expansion in Gammaproteobacteria, similar to that found in elderly humans[25–27]. Within adult flies, seasonal changes in the microbiota do occur, and *L. brevis* is a species that particularly displays this temporal variation[23]. Furthermore, the composition of the microbiota differs between males and female flies, which is another feature also found in mammals[1,24,28].

In addition to bacteria, *Drosophila* harbor fungal commensals and endosymbionts, some of which are removed in common fly media preparations. The connection between the diet and the microbiota in flies extends to fungi, as diet outweighs host species in its contribution to fungal diversity[29]. In addition to fungi, the endosymbiont Wolbachia is an Alphaproteobacteria that has a range of effects on host physiology, including cytoplasmic incompatibility[30]. Furthermore, roughly 30% of all stocks at the Bloomington *Drosophila* Stock Center harbor Wolbachia[31]. Multiple factors influence the microbial composition within *Drosophila*. The use of flies lacking a

microbiota, also known as axenic flies, facilitates the examination of the aspects of host physiology that microbes modulate.

1.5 HOST-MICROBE INTERACTIONS IN *D. MELANOGASTER*

Due to the genetic tools available and its limited microbial community *D. melanogaster* represents an ideal model organism to unravel the complexities of host-microbial interactions. The primary focus of such studies previously centered on bacterial activation of immune pathways, such as immune deficiency (IMD) and Toll. However, more recent research has uncovered commensal regulation of various aspects of host physiology, including growth and development[32–35]. Axenic larvae exhibit a significant delay in development under nutrient-poor conditions compared to conventional controls bearing a complete microbiota[36]. Metabolic products from the commensal *A. pomorum* activate systemic insulin/insulin-like growth factor signaling in *D. melanogaster*, resulting in changes in larval growth and energy metabolism[37]. Additionally, another commensal, *L. plantarum*, increases growth and development in *Drosophila* under conditions of chronic undernutrition. Association with *L. plantarum* causes these effects through enhancing the expression of larval peptidases that alter protein assimilation, and subsequently stimulate the TOR and insulin-like peptide pathways[36,38,39]. Interestingly, *L. plantarum*-mediated effects on growth are conserved in mammals, further demonstrating the importance of this bacterium and the beneficial impact of its symbiosis on various hosts[40].

The microbiota also regulates additional aspects of host physiology and function, including metabolism and behavior. Depending on the protein level within the diet, axenic female flies display significantly increased levels of triglycerides (TAG) and carbohydrates, including glucose and trehalose, compared to conventional controls[41,42]. While mono-colonization with five different commensal bacteria (*A. pomorum*, *A. tropicalis*, *L. brevis*, *L. fructivorans*, or *L. plantarum*) was able to restore glucose to levels similar to conventional controls, only *A. pomorum*, *A. tropicalis*, or to a lesser extent *L. plantarum* mono-colonization were able to lower TAG levels[42]. Related to metabolism, food consumption is reduced in axenic flies compared to conventional counterparts. However, mono-colonization with only either *A. pomorum* or *A. tropicalis* increases feeding to conventional levels in female flies[43]. No differences in food intake are found between male axenic and conventional flies[41].

Recently, commensal bacteria have been implicated in food preference, reproduction, and oviposition behaviors[44,45]. After the removal of essential amino acids from the diet, flies had an increased preference for amino-acid rich media and decreased egg laying that was diminished by *A. pomorum* and *Lactobacilli* species (*L. planatrum* and *L. brevis*), demonstrating the importance of the microbiota under nutrient-poor conditions[44]. Microbial metabolism of specific nutrients is also implicated in oviposition preference with *Enterococci* catabolism of carbohydrates and the receptors *Gr5a* and *Gr64* mediating this behavior[45]. Different compounds are produced in microbial communities depending on their composition, as bacterial and fungal members of the microbiome can catabolize products from each other. Products of one such co-culture, acetate and its metabolites, drive host preference for a mixture of *Saccharomyces* and *Acetobacter* species, partially through the olfactory receptor, *Or42b*[46]. While the microbiota and microbial-derived products are implicated in regulating host behavior, very little is known about the underlying host mechanisms facilitating these changes.

Feedback from the host is another important and only recently examined aspect of host-microbe interactions. One study using *Drosophila* Genetic Reference Panel (DGRP) lines found links between specific genes, such as those in insulin-like signaling and TOR, and microbial effects on metabolism in flies[47,48]. Furthermore, a genome-wide association study (GWAS) revealed that neuronal-related genes alter the levels of particular bacterial species, including *Acetobacter tropicalis*[48,49]. However, more research is needed in order to understand this aspect of host-microbial interactions.

The microbial community within *D. melanogaster* is relatively limited compared to that of mammals and has known effects on host physiology, making it an excellent candidate for exploring microbial contributions to conserved behaviors. As there is a close relationship between specific species and host metabolism, we chose a metabolically-related behavior, locomotion, for examination in the context of host-microbe interactions. Previous literature in mice suggested that host walking speed is regulated by the microbiota[50,51]; however, this had not been shown in *Drosophila* nor had the underlying pathways been explored.

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Chapter 2

DELVING INTO LOCOMOTION IN *D. MELANOGASTER*

2.1 INTRODUCTION TO LOCOMOTION AND ITS NEURONAL CORRELATES

At first glance, coordinated movement through an environment appears simple; however, it requires the complex integration of multiple stimuli and coordination of various organ systems throughout the body[1]. Locomotion has been studied across organisms spanning from humans and cats to ants and flies. While the number of appendages varies, there is a multitude of consistent features, including energy conservation and feedback from peripheral sensors[1]. Locomotor behavior in insects has been relatively well studied and provides a good model for examining contributions from internal and external stimuli.

As larvae, *Drosophila* crawl along a surface through coordinated peristaltic waves of muscle contractions[2]. Patterned movements are locally generated through central pattern generators (CPGs) with additional control from descending neurons in the subesophageal zone (SEZ) and central brain[2]. Within the ventral nerve cord (VNC), excitatory and inhibitory interneurons coordinate the activity of motor neurons. Nevertheless, the exact mechanisms by which sensory information is processed by VNC and integrated into motor behavior in larvae are not currently known.

Adult *Drosophila* have six legs along a body plan consisting of a head, thorax, and abdomen. Multi-joined legs are widely used among terrestrial animals for locomotion. Furthermore, as in many other invertebrates and vertebrates, CPGs constitute a critical component for the generation of rhythmic movements in adult flies[3]. For example, if the central brain is removed, CPGs can be stimulated through exogenous application of biogenic amines[4]. Regions within the central brain, including the central complex, provide inputs to CPGs that significantly alter walking behavior. Analysis of walking behavior in mutant *Drosophila* lines revealed that the central complex regulates walking initiation, step length, swing speed, and leg placement[5]. Furthermore, the ellipsoid body in the central complex is necessary for maintenance of the temporal walking pattern of adult flies[6]. In addition to the central complex, removal or inhibition of the mushroom body results in increased walking speed[7]. Such experiments in *Drosophila* demonstrate the use of both central and peripheral neuronal circuits in the execution of motor behaviors.

2.2 OVERVIEW OF THE NEUROTRANSMITTERS INVOLVED IN REGULATING LOCOMOTION

A variety of biogenic amines and small molecules have been implicated in modulating locomotor behavior in the central brain and the periphery, including dopamine, serotonin, octopamine, and tyramine. Dopamine (DA) is a catecholamine synthesized from tyrosine through the use of the enzymes tyrosine hydroxylase (TH) and DOPA decarboxylase (DDC). In both vertebrates and invertebrates, dopamine signaling is necessary for coordinated movement[4,8–11]. For example, dopamine deficient flies display decreased walking speeds and climbing behavior, both of which exhibit age-dependent declines[8,12]. Interestingly, these age-related changes in walking are associated with exploratory behavior, rather than centrophobism[12]. Similar to DA, exogenous application of serotonin to the spinal cord of cats or the VNC in insects stimulates walking behavior, implicating its involvement in locomotor regulation[4,13,14]. Serotonin (5HT) is derived from tryptophan through the use of tryptophan hydrolase (TPH) and DDC. Inactivation of serotonergic pathways in the central brain and RNAi knockdown of 5HT receptors further demonstrate the involvement of serotonin in motor behavior, specifically that of the 5HT receptors 1B, 2 and 7 in the mushroom body[15,16]. Two other monoamines involved in locomotion, octopamine (OA) and tyramine (TA), are synthesized from tyrosine and lie along the same pathway, with tyramine produced as an intermediate[17]. Tyramine was previously assumed to only act as a precursor of octopamine; however, it is now known as an independent neurotransmitter that modulates behavior and physiology through G-coupled protein receptors[18–26]. Octopamine and tyramine share homology with the adrenergic system both in terms of structure and due to their involvement in regulating metabolism and similar behaviors[17,20]. Octopamine is widely expressed within the nervous system, particularly in the ventromedial (VM) neuropil cluster along the SEZ, the AL2 cluster of the antennal lobes, and the VNC[27,28]. Additionally, it can reach other body sites through circulation in the hemolymph[17]. Tyraminergic neurons have also been identified in the central nervous system and are thought to be a distinct population as they lack octopamine expression[29]. In terms of their involvement in locomotor regulation, octopamine and tyramine have opposite effects with higher levels of octopamine driving elevated muscle contractions and locomotion, and corresponding decreases in both features associated with increased tyramine[20].

However, others have proposed a dependence on a proper balance of both neurotransmitters for normal locomotion[18].

In addition to aminergic signaling, neuropeptides and small molecules, such as GABA, also regulate locomotion in *Drosophila*. Experiments knocking down inhibitory GABAergic inputs to leg motorneurons impaired adult locomotion in flies, demonstrating their importance in motor behavior[30]. Additionally, cholinergic interneurons are involved in the regulation of locomotor behavior[31]. Other neuropeptides, including tachykinin (DTK) and short neuropeptide F (sNPF) expressed in the central complex, modulate locomotor activity[32]. Contributions from such a diverse array of neuronal subsets and neurotransmitters demonstrates the complex circuitry underling this multifaceted behavior.

2.3 REGULATION BY INTERNAL STIMULI

In order to successfully navigate a complex environment, feedback is needed from multiple sensory systems detecting internal and external stimuli[33–35]. Sensory receptors detect changes in internal metabolic state that alter various behaviors, including locomotion[33], which are mediated by certain neuromodulators[36].

Under periods of starvation, flies will execute foraging behaviors, altering their trajectory and speed[37–42]. Local search behaviors vary as the starvation period increases, with the highest activity taking place in the first three hours after starvation[41]. Foraging is mediated in part by different sets of dopaminergic neurons depending on the satiation state of the fly, with dopaminergic neurons in the PAM cluster mediating behavior in starved flies[43]. However, both of the neuronal populations employed in the two satiation states converge on the dopamine D1 receptor expressed in Kenyon cells in the mushroom body[43]. Deficits in specific nutrients, such as amino acids, also shift exploration away from a global strategy to a focused search for proteinaceous patches within defined arena, illustrating the importance of nutritional need in foraging strategies[38]. Furthermore, the bacterial populations within *Drosophila* can alter foraging patterns and preferences through olfactory cues, with an innate preference towards exploring their associated *Lactobacilli* species[44].

In addition to foraging trajectories, starvation increases the activity and speed of flies[39,40,42]. *Drosophila* originating from various locations with climates ranging from temperate to tropical all exhibit increased activity in response to starvation[42]. Recently, the involvement of octopaminergic neurons within the SEZ in starvation-induced hyperactivity was identified[39,40]. Yu et al. (2016) also found that adipokinetic hormone (Akh), similar to mammalian glucagon, mediates these effects independent of food intake. However, whether these same circuits are influenced by changes in the microbial community is currently unknown.

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Chapter 3

A GUT MICROBIAL FACTOR MODULATES LOCOMOTION IN *DROSOPHILA*

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A modified version of this chapter along with Chapter 4 was submitted in “A gut microbial factor modulates locomotor behavior in *Drosophila*.”

3.1 ABSTRACT

While research into the biology of animal behavior has primarily focused on the central nervous system, cues from peripheral tissues and the environment have been implicated in brain development and function[1–6]. Emerging data suggest bidirectional communication between the gut and the brain affects behaviors including anxiety, cognition, nociception, and social interaction, among others[4,7–17]. Coordinated locomotor behavior is critical for the survival and propagation of animals, and is regulated by internal and external sensory inputs[18–20]. However, little is known regarding influences by the gut microbiome on host locomotion, or the pathways involved. Here we report that germ-free status or antibiotic treatment result in hyperactive locomotor behavior in female *Drosophila melanogaster*. Increased walking speed and daily activity found in the absence of a gut microbiome are rescued by mono-colonization with specific bacteria, including the fly commensal *Lactobacillus brevis*. The bacterial enzyme xylose isomerase (Xi) from *L. brevis* is sufficient to recapitulate the locomotor effects of microbial colonization, likely via modulation of sugar metabolism in flies. Additionally, thermogenetic activation of octopaminergic neurons or exogenous administration of octopamine, the invertebrate counterpart of noradrenaline, abrogates Xi-induced effects on *Drosophila* locomotion. These findings reveal a previously unappreciated role for the gut microbiome in modulating locomotion, and identify octopaminergic neurons as mediators of peripheral microbial cues that regulate motor behavior in animals.

3.2 INTRODUCTION

Coordinated locomotion is required for fundamental activities of life such as foraging, social interaction, and mating, and involves the integration of multiple contextual factors, including the internal state of the animal and external sensory stimuli[18,19]. The intestine represents a major conduit for exposure to environmental signals that influence host physiology and is connected to the brain through both neuronal and humoral pathways. Recently, seminal studies have uncovered that the intestinal microbiome regulates developmental and functional features of the nervous system[4–7], though gut bacterial effects on the neuromodulators and neuronal circuits involved in locomotion remain poorly understood. Since central mechanisms of locomotion, including sensory feedback and neuronal circuits integrating these modalities, are shared in lineages spanning arthropods and vertebrates[19,21,22], we employed the fruit fly *Drosophila melanogaster* to explore host-microbiome interactions that contribute to locomotor behavior.

3.3 THE MICROBIOTA INFLUENCES HOST LOCOMOTION

Locomotion was examined in the presence (conventional; Conv) and absence (axenic; Ax) of commensal bacteria[23]. In comparison to conventionally-reared animals, axenic female adult flies exhibit increased walking speed and daily activity (Fig. 1a – b, and 1g). Axenic adult flies also display corresponding changes in locomotor coordination (Fig. 1h) that were previously correlated with elevated average walking speeds[24]. *Drosophila* locomotion is characterized by a pattern of intermittent periods of pauses and activity bouts[19,25,26], in which the average speed of the fly is above a set threshold of 0.25 mm/second. An increased average speed may be related to changes in temporal patterns, including the number and/or duration of walking bouts[25]. We discovered that axenic flies display an increased average walking bout length in addition to a decreased average pause length, while remaining indistinguishable in the number of bouts compared to animals harboring a microbial community (Fig. 1c – f). These data reveal that the microbiota modulates walking speed and temporal patterns of locomotion in *Drosophila*.

3.4 SELECT BACTERIA MODULATE LOCOMOTOR BEHAVIOR

The microbial community of *Drosophila melanogaster* contains 5 – 20 bacterial species[27–31]. In laboratory-raised flies, two of the dominant species are *Lactobacillus brevis* and *Lactobacillus plantarum*[28]. Specific bacteria in this community affect distinct features of *Drosophila* physiology, and even closely related microbial taxa can exhibit unique biological influences on the host[28,32–35]. Accordingly, we examined whether locomotor performance was impacted differentially by individual bacterial species. Despite similar levels of colonization (Fig. 2a), mono-association with *L. brevis*, but not *L. plantarum*, starting at eclosion is sufficient to correct speed and daily activity deficits in axenic flies (Fig. 1a – b, 1g, and 2b – e). However, these locomotor effects appear to be sex-specific as male flies did not exhibit significant changes in speed depending on microbial status (Fig. 2b), consistent with recent reports[36]. Varying the strain of *L. brevis* or host diet did not alter bacterial influences on host speed (Fig. 2c – e), and *L. brevis* is able to reverse changes in average pause length (Fig. 1c – f and 2f). Detailed gait analysis reveals that *L. brevis*-associated flies display comparable locomotor coordination to that of conventionally-reared flies (Fig. 1h and 2g).

As flies are usually associated with more than one bacterial species, we evaluated *L. brevis*-dependent changes in locomotion under different microbial conditions. Axenic flies co-colonized with a 1:1 mixture of *L. brevis* and *L. plantarum* display similar changes in speed to flies mono-associated with *L. brevis* (Fig. 2h). Conventional and mono-associated flies with lower speeds also appear to exhibit increased levels of *L. brevis* (Fig. 3a – b). However, a similar trend was not found in flies mono-associated with *L. plantarum* (Fig. 3c). In order to examine the locomotor effects of *L. brevis* in a complex microbial background, we supplemented conventional flies with either *L. brevis* or *L. plantarum*. Administration of *L. brevis*, and not *L. plantarum*, significantly reduced walking speeds compared to naïve conventional animals (Fig. 3d).

Due to the diversity of bacterial species within the microbiota of *Drosophila*, we tested if other commensal or non-commensal bacteria also lower host locomotion upon colonization. Of the four remaining bacteria tested (*Acetobacter pomorum, Acetobacter tropicalis, Escherichia coli, Enterococcus faecalis*), only *A. tropicalis* and *E. coli* significantly reduced host walking speed (Fig. 4). The strain of *A. tropicalis* tested was isolated from the flies used in this study; however, it is not fully sequenced. Therefore, the sequenced *Drosophila* commensal *L. brevis* and the non-

commensal *E. coli* were subsequently used to examine the involvement of specific bacterial factors.

3.5 BACTERIAL-DERIVED PRODUCTS AFFECT HOST SPEED AND ACTIVITY

Gut bacteria secrete molecular products that regulate aspects of host physiology, including immunity and feeding behavior[37,38]. To explore how microbes influence locomotion, we administered either cell-free supernatant (CFS) harvested from bacterial cultures or heat-killed bacteria to axenic flies. CFS alone from *L. brevis* (*L.b* CFS) reduces hyperactivity in axenic flies, while heat-killing bacteria ablates modulation of locomotion (Fig. 5a and 6a – e), demonstrating a requirement for metabolically active *L. brevis*. Treatment with MRS media alone or *L.p* CFS did not significantly lower host walking speed compared to axenic flies (Fig. 6a – f). The time course of administration is also important, as *L.b* CFS does not alter host locomotion if supplied less than 24 hours prior to testing (Fig. 7a). However, varying the concentration of sucrose present in the testing chamber did not alter *L.b* CFS ability to reduce host walking speed (Fig. 7b). While previous studies have revealed that *L. brevis* produces uracil[35], a molecule that affects the host immune response and may impact locomotion[37,39], administration of physiologic levels of uracil to axenic flies did not alter walking speed (Fig. 6g).

As CFS contains complex mixture of molecules, we employed a series of biochemical approaches to narrow down the bacterial factors involved. While amylase treatment did not alter the locomotor effects of *L.b* CFS, protease- or heat-treated *L.b* CFS no longer lowered host walking speed (Fig. 8a – d). CFS from *L. brevis* grown in minimal media decreased locomotion in axenic flies (Fig. 9a), limiting the complexity of the molecules potentially involved. Subsequent size-dependent fractionation of *L.b* CFS using either dialysis or fast protein liquid chromatography (FPLC) revealed eluted fractions containing molecules greater than 30 kDa reduced locomotion (Fig. 9b – c). Multiple attempts to further identify the bacterial factors in this fraction using mass spectrometry proved unsuccessful.

3.6 XYLOSE ISOMERASE FROM *L. BREVIS* ALTERS HOST LOCOMOTION

Bacterial metabolism of amino acids and carbohydrates is associated with changes in host behavior[8,16]; however, it is not known whether metabolic enzymes from bacteria influence host locomotion. Biochemical analysis of *L.b* CFS and comparative functional analysis of bacterial strains[40–42] determined that bacterial locomotor effects are mediated via proteinaceous molecule(s) present in select bacteria, including *L. brevis* and *E. coli* (Fig. 8a – e). Subsequently, a screen of *E. coli* strains containing single gene mutations related to amino acid and carbohydrate metabolism identified xylose isomerase (Xi) as a candidate factor modulating locomotor behavior (Fig. 8f). Xi is an enzyme with four 43 kDa subunits that catalyzes the reversible isomerization of certain sugars, including the conversion of D-glucose to D-fructose[43–45]. Xi is also present only in *L. brevis* and *E. coli* of the sequenced bacterial strains tested (Fig. 8e). Administration of His-tagged Xi from *L. brevis* (Xi*) reduces locomotor behavior in axenic flies to levels similar to *L.b* CFS and conventional flies (Fig. 5b – c and 8g – h). The addition of His-tagged L-arabinose isomerase (Ai*), an enzyme that is not differentially expressed among the bacteria tested, is not sufficient to influence host speed in axenic flies (Fig. 5c). Furthermore, we generated a chromosomal deletion of the xylose isomerase gene *xylA* in *L. brevis*, and demonstrate the mutant strain lacks the ability to modulate host speed and daily activity (Fig. 5d and 8g). Neither the addition of the predicted products of Xi (D-fructose, D-glucose, D-xylose, and D-xylulose) alone, nor Xi inactivated by EDTA[43] or paraformaldehyde treatment, reduces walking speed in axenic flies (Fig. 10a – d). We next sought to explore Xi activity through carbohydrate analysis of whole flies, which revealed those given Xi* exhibit increased ribose and reduced trehalose levels compared to axenic controls (Fig. 5e), with no differences in these sugars in the fly media (Fig. 10e). While EDTA-treated Xi* did not significantly alter trehalose levels, these flies still display heightened levels of ribose compared to axenic controls (Fig. 10f). Additionally, similar to previous findings[46], conventional and *L. brevis*-colonized flies show reduced levels of trehalose compared to axenic groups (Fig. 10g – h). Administration of trehalose alone abrogates microbial effects on host speed, while supplementation with arabinose or ribose did not (Fig. 5f and 10i – l). Collectively, these results demonstrate that xylose isomerase from *L. brevis* alters locomotion in *Drosophila*, likely via modulation of key carbohydrates, such as trehalose.

3.7 SUMMARY

Herein, we found that the gut microbiota modulates locomotion in female *D. melanogaster*. Removal of the microbiota results in increased locomotion, and colonization with select bacterial species, including *L. brevis*, *A. tropicalis*, and *E. coli*, decreases host speed. Furthermore, microbial-derived factors, such as xylose isomerase (Xi), mediate changes in host motor behavior, potentially through altering the carbohydrate composition in flies. While other bacterial factors may exhibit similar effects on fly locomotion, we will further use *L. brevis* and Xi to examine the host pathways involved.

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3.8 FIGURES

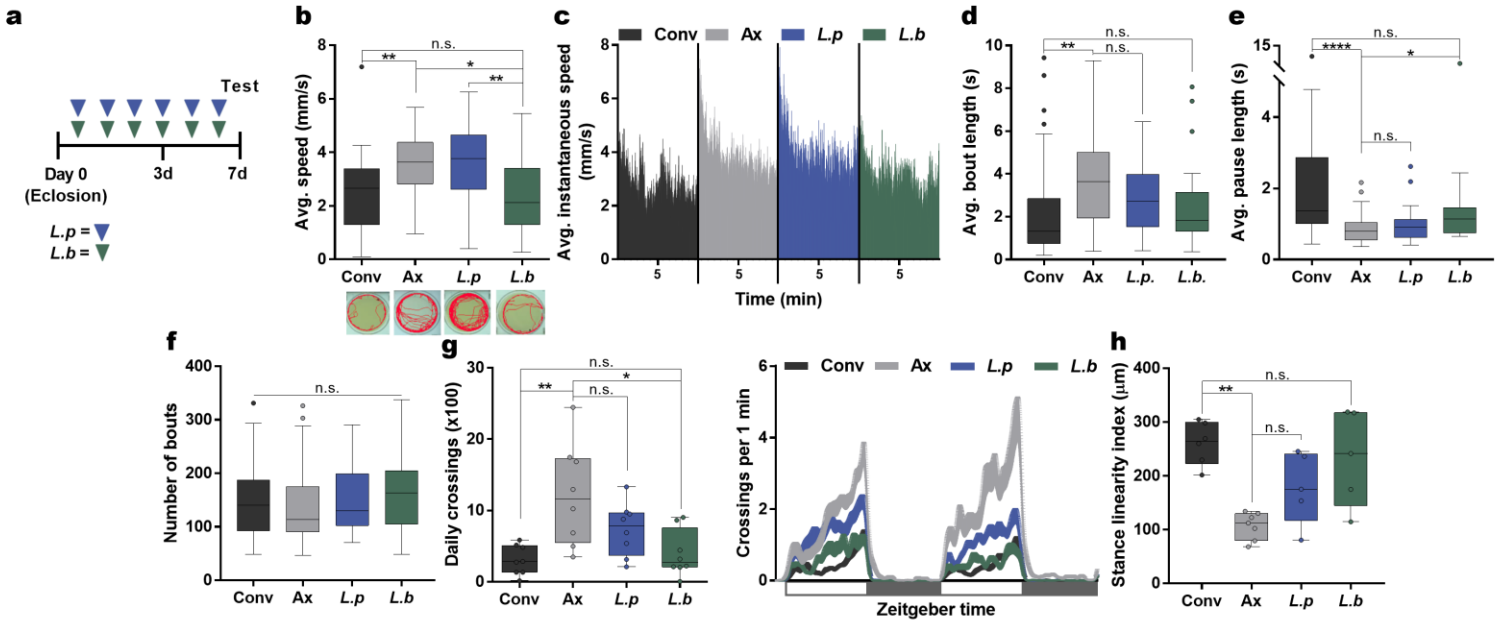

Fig. 1. Select gut bacteria modulate locomotor behavior in flies.

a, Experimental design unless otherwise stated. Flies were either left untreated or administered a single bacterial species or bacterial-derived factors. All treatments were supplied daily through application to the fly media (40 µL) for 6 days following eclosion and all assays were performed on day 7. b, Average speed of conventional (Conv), axenic (Ax), and *L. plantarum* (*L.p*) or *L. brevis* (*L.b*) mono-associated flies. Traces below are representative of individuals from each group and all groups were tested over a 10-min. period. Conv, n = 36; Ax, n = 36; *L.p*, n = 35; *L.b*, n = 36. c, Average instantaneous speed of Conv, Ax, and *L.p* or *L.b* mono-associated flies. All groups were tested over a 10-min. period and dashes below represent 5-min. mark for each group. Conv, n = 23; Ax, n = 35; *L.p*, n = 23; *L.b*, n = 21. d – f, Analysis of the average bout length (d), average pause length (e), and number of bouts (f) over a 10- min. testing period for Conv, Ax, *L.p*, and *L.b* groups. Conv, n = 32; Ax, n = 36; *L.p*, n = 22; *L.b*, n = 20. g, Daily activity of Conv, Ax, *L.p*, and *L.b* groups (virgin female Oregon^{R} flies) over a 2-day light-dark cycle period each lasting 12 hrs., starting at time 0. White boxes represent lights on and gray boxes represent lights off. n = 8/condition. h, Stance linearity index calculated for Conv, Ax, *L.p*, and *L.b* groups. Conv, n = 6; Ax, n = 7; *L.p*, n = 5; *L.b*, n = 5. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and

upper quartile, from bottom to top. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Kruskal-Wallis and Dunn's post-hoc test was applied for statistical analysis. Data are representative of at least 3 independent trials for each experiment.

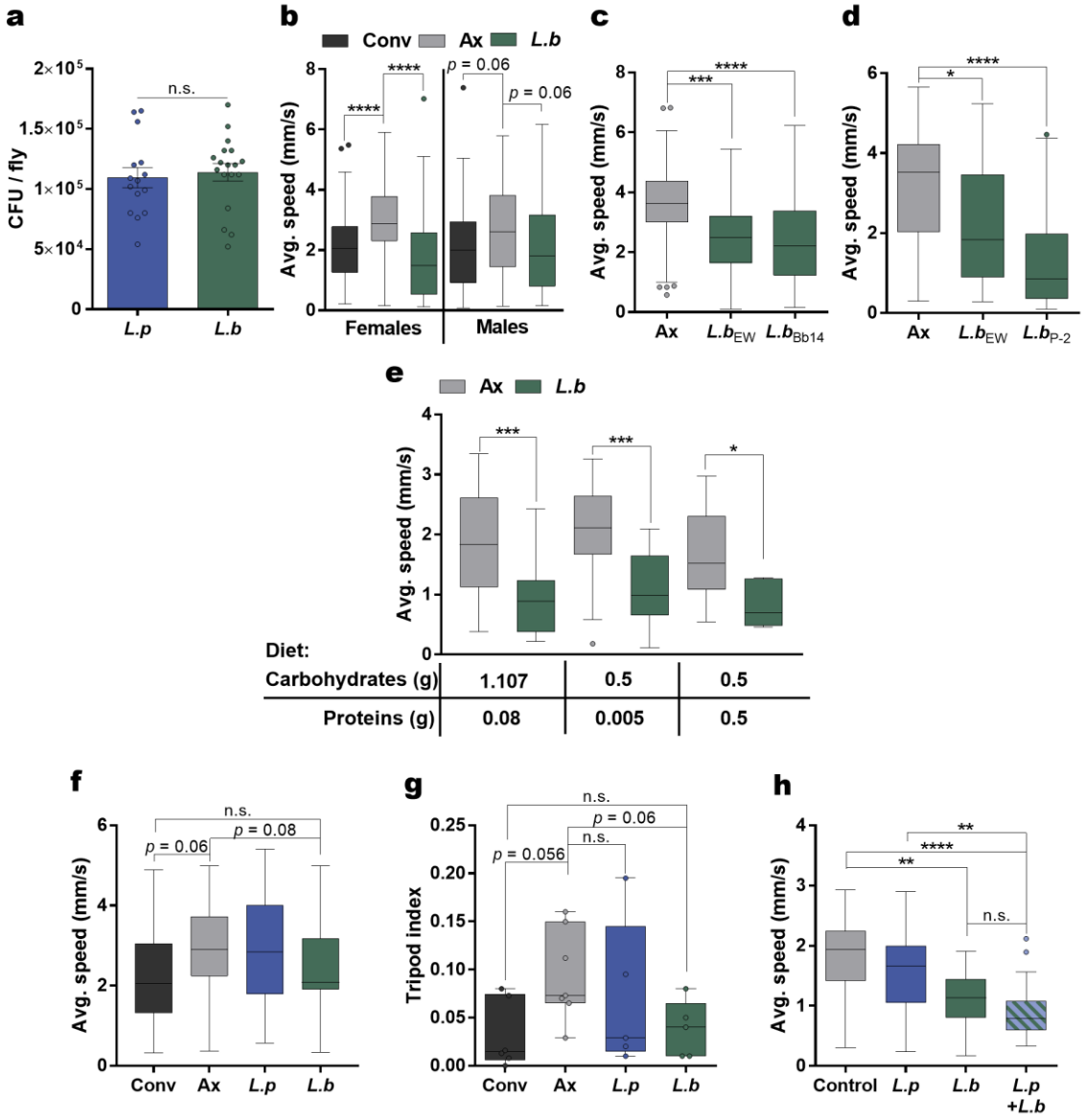

Figure 2. Effects of colonization level, bacterial strain, and host diet on *L. brevis*-modulation of locomotion.

a, Colony forming units (CFU) per individual fly for *L.p* or *L.b* mono-associated flies. Error bars represent mean +/- S.E.M. n = 16/condition. b, Average speed of Conv, Ax, and *L.b* mono-associated female or male flies. Females: Conv, n = 90; Ax, n = 92; *L.b*, n = 89; Males: Conv, n = 100; Ax, n = 100; *L.b*, n = 95. c – d, Average speed of Ax or flies mono-associated with *L.b* strains EW, Bb14, or P-2. n = 24/condition. e, Average speed of Ax or *L.b* mono-associated flies raised on different diet compositions from eclosion until day 7. n = 18/condition. f, Average speed during

walking bouts for Conv, Ax, *L.p*, and *L.b* groups. Conv, n = 32; Ax, n = 36; *L.p*, n = 22; *L.b*, n = 20. g, Tripod index for Conv, Ax, *L.p*, and *L.b* groups. Conv, n = 6; Ax, n = 7; *L.p*, n = 5; *L.b*, n = 5. h, Average speed of Ax flies or flies mono-associated with *L.p* or *L.b* alone or in combination (1:1). n = 24/condition. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Unpaired Student's t-test (a), Kruskal-Wallis and Dunn's (b – d and f – h), or Mann-Whitney *U* (e) post-hoc test was applied for statistical analysis. Data are representative of at least 3 independent trials for each experiment.

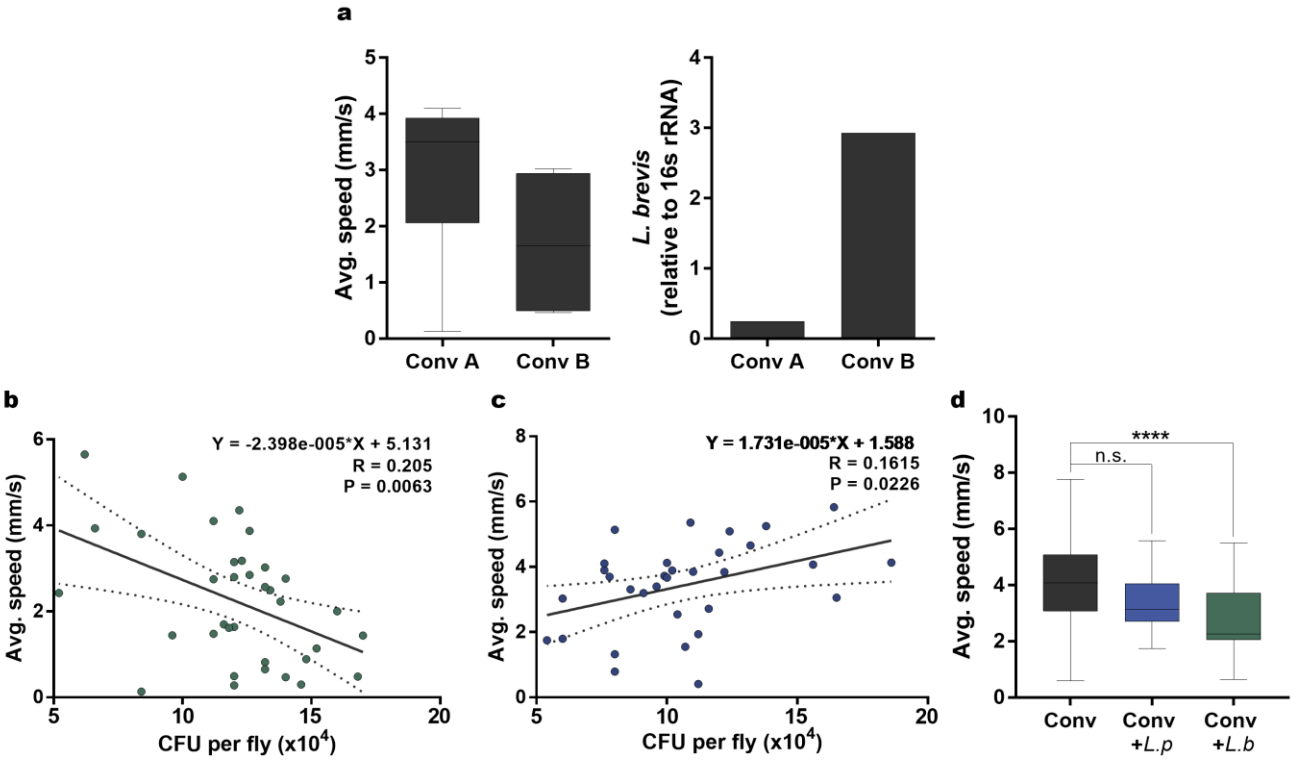

Fig. 3. *L. brevis* modulates host locomotion under conventional and mono-associated conditions.

a, Average speed of two groups of Conv flies with corresponding levels of *L.b* in each group. n = 6/condition. b – c, Correlation analysis for average speed of individual mono-associated flies with corresponding colony forming units of either *L.b* (b) or *L.p* (c). *L.b*, n = 35 (two-tailed Spearman correlation, * P = 0.02, line of best fit with 95% CI); *L.p*, n = 32 (two-tailed Spearman correlation, * P = 0.02, line of best fit with 95% CI). d, Average speed of naïve Conv flies or Conv flies supplemented with either *L.p* or *L.b*. n = 36/condition. Kruskal-Wallis and Dunn's post-hoc test was applied for statistical analysis. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. **** P < 0.0001.

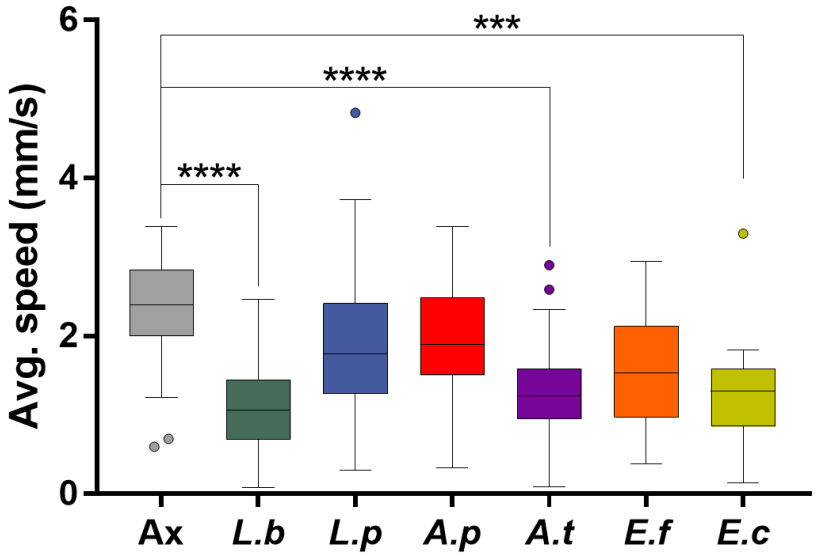

Fig. 4. Select bacteria modulate host speed.

Average speed of Ax flies and flies treated with *L.b*, *L.p*, *A. pomorum* (*A.p*), *A. tropicalis* (*A.t*), *E. faecalis* (*E.f*), or *E. coli* (*E.c*). n = 30/condition. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. *** $P < 0.001$, **** $P < 0.0001$. Kruskal-Wallis and Dunn's post-hoc test was applied for statistical analysis. Data are representative of at least 2 independent trials for the experiment.

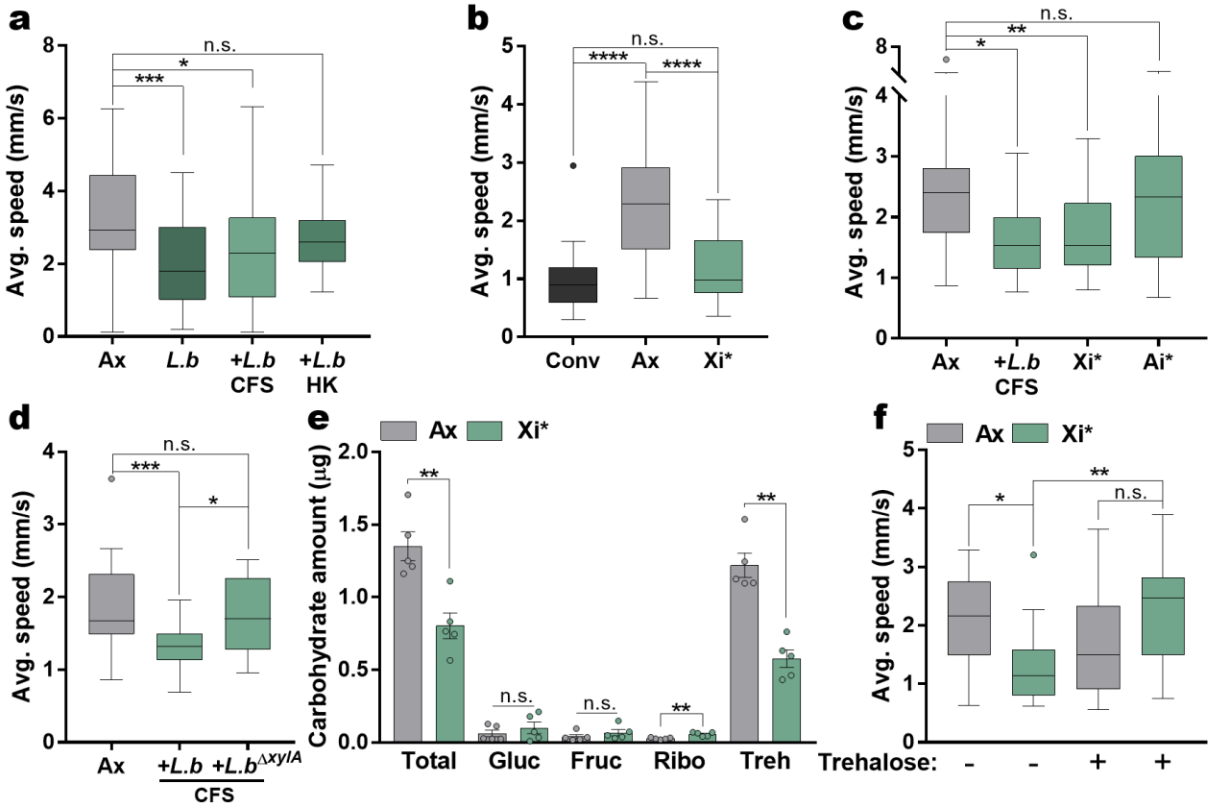

Fig. 5. Xylose isomerase (Xi) from *L. brevis* alters host locomotion.

a, Average speed of Ax, *L.b* mono-associated, and Ax flies treated with cell-free supernatant (CFS) from *L.b* or heat-killed (HK) *L.b* alone. Ax, n = 57; *L.b*, n = 42; *L.b* CFS, n = 36; *L.b* HK, n = 24. b, Average speed of Conv, Ax, and Ax flies treated with His-tagged xylose isomerase from *L.b* (Xi*, 100 µg/mL). Conv, n = 17; Ax, n = 45; Xi*, n = 29. c, Average speed of Ax and Ax flies treated with *L.b* CFS, Xi*, or His-tagged L-arabinose isomerase from *L.b* (Ai*,100 µg/mL). Ax, n = 31; *L.b* CFS, n = 12; Xi*, n = 28; Ai*, n = 13. d, Average speed of Ax and Ax flies treated with CFS from either WT *L.b* or xylA mutant *L.b* ($L.b^{\Delta xylA}$) bacterial strains. Ax, n = 28; *L.b* CFS, n = 29; $L.b^{\Delta xylA}$ CFS, n = 18. e, Carbohydrate levels in Ax and Xi*-treated flies. Each sample contains 5 flies. Error bars represent mean +/- S.E.M. n = 5 samples/condition. f, Average speed of Ax flies and Xi*-treated Ax flies either left untreated or supplemented with trehalose (10 mg/mL) for 3 days before testing. Ax, n = 16; Xi*, n = 18; Ax+Treh, n = 16; Xi*+Treh, n = 17. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$, ** $P <$

0.01, *** P < 0.001, **** P < 0.0001. Kruskal-Wallis and Dunn's (a – d and f) or Mann-Whitney *U* (e) post-hoc test was applied for statistical analysis. Data are representative of at least 2 independent trials. Gluc, glucose; Fruc, fructose; Ribo, ribose; Treh, trehalose.

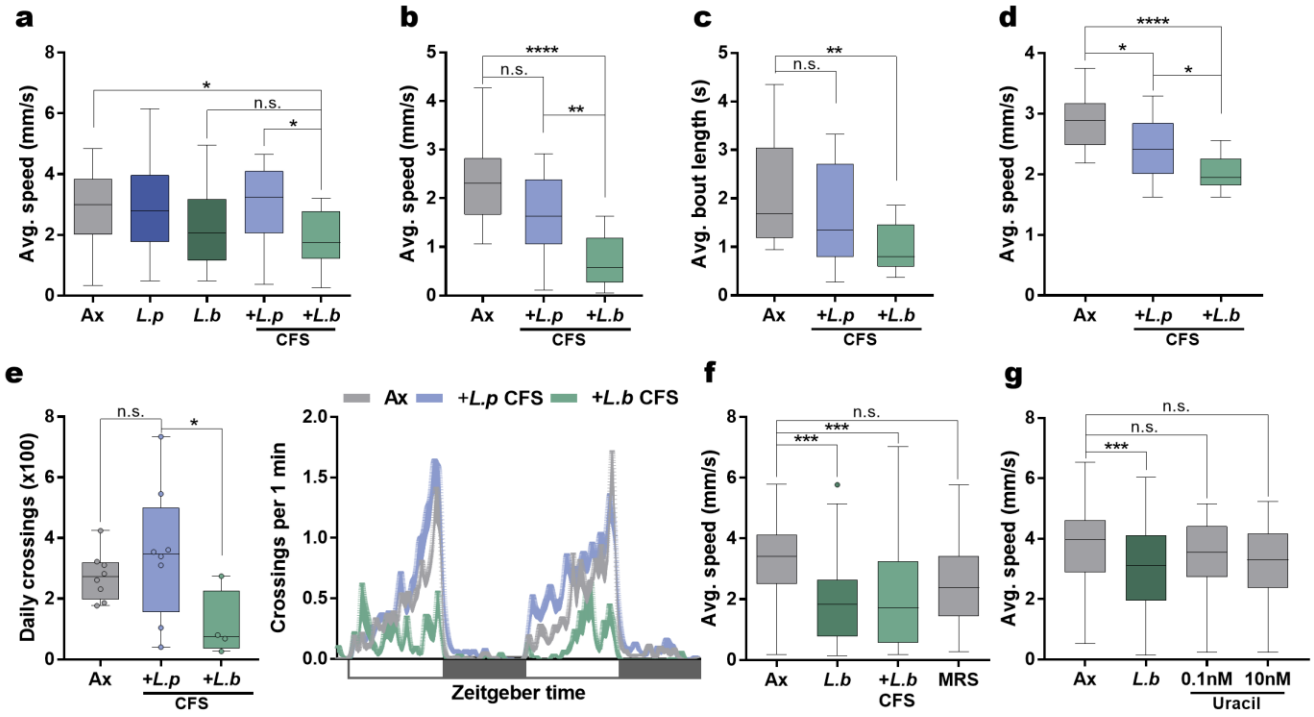

Figure 6. Bacterial-derived products from *L. brevis* alter locomotion.

a, Average speed of Ax, *L.p* or *L.b* mono-associated, and Ax flies treated with cell-free supernatant (CFS) from *L.p* or *L.b*. Ax, n = 45; *L.p*, n = 17; *L.b*, n = 42; *L.p* CFS, n = 17; *L.b* CFS, n = 16. b – e, Average speed (b), average bout length (c), average speed during walking bouts (d), and daily activity (e) of Ax and Ax virgin female Oregon^{R} flies treated with CFS from either *L.p* or *L.b*. White boxes represent lights on and gray boxes represent lights off. Speed: Ax, n = 23; *L.p* CFS, n = 20; *L.b* CFS, n = 19. Activity: Ax, n = 8; *L.p* CFS, n = 8; *L.b* CFS, n = 4. f, Average speed of Ax, *L.b*, *L.b* CFS-, and MRS-treated flies. n = 48/condition. g, Average speed of Ax, *L.b* mono-associated, and Ax uracil-treated flies. n = 24/condition. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Kruskal-Wallis and Dunn's post-hoc tests was applied for statistical analysis. Data are representative of at least 2 independent trials for each experiment.

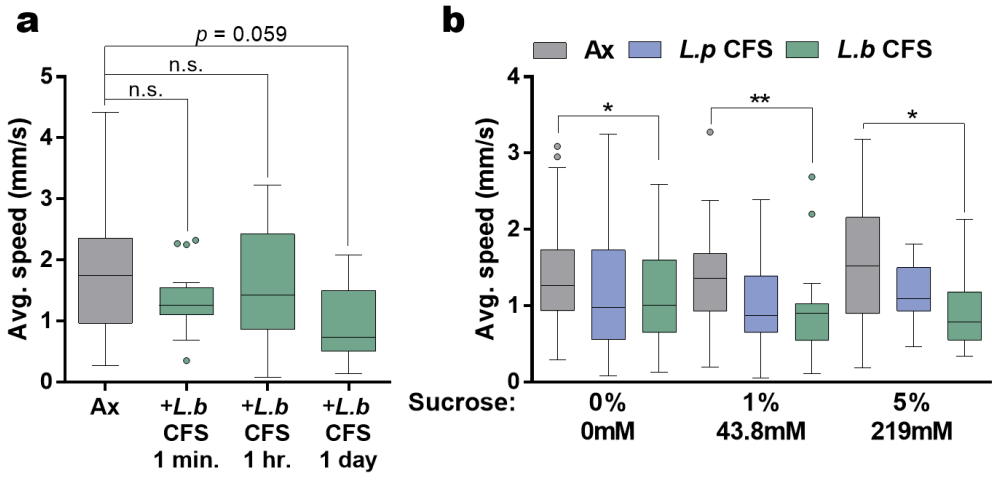

Fig. 7. The role of timing and testing environment in microbial effects on locomotion.

a, Average speed of Ax flies and flies treated with *L.b* CFS 1 min., 1 hr., or 1 day prior to testing. n = 18/condition. b, Average speed of Ax and *L.p* or *L.b* CFS-treated flies tested in arenas with 0, 1, or 5% sucrose and 1% agar. n = 24/conditions. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$, ** $P < 0.01$. Kruskal-Wallis and Dunn's post-hoc tests was applied for statistical analysis. Data are representative of at least 2 independent trials for each experiment.

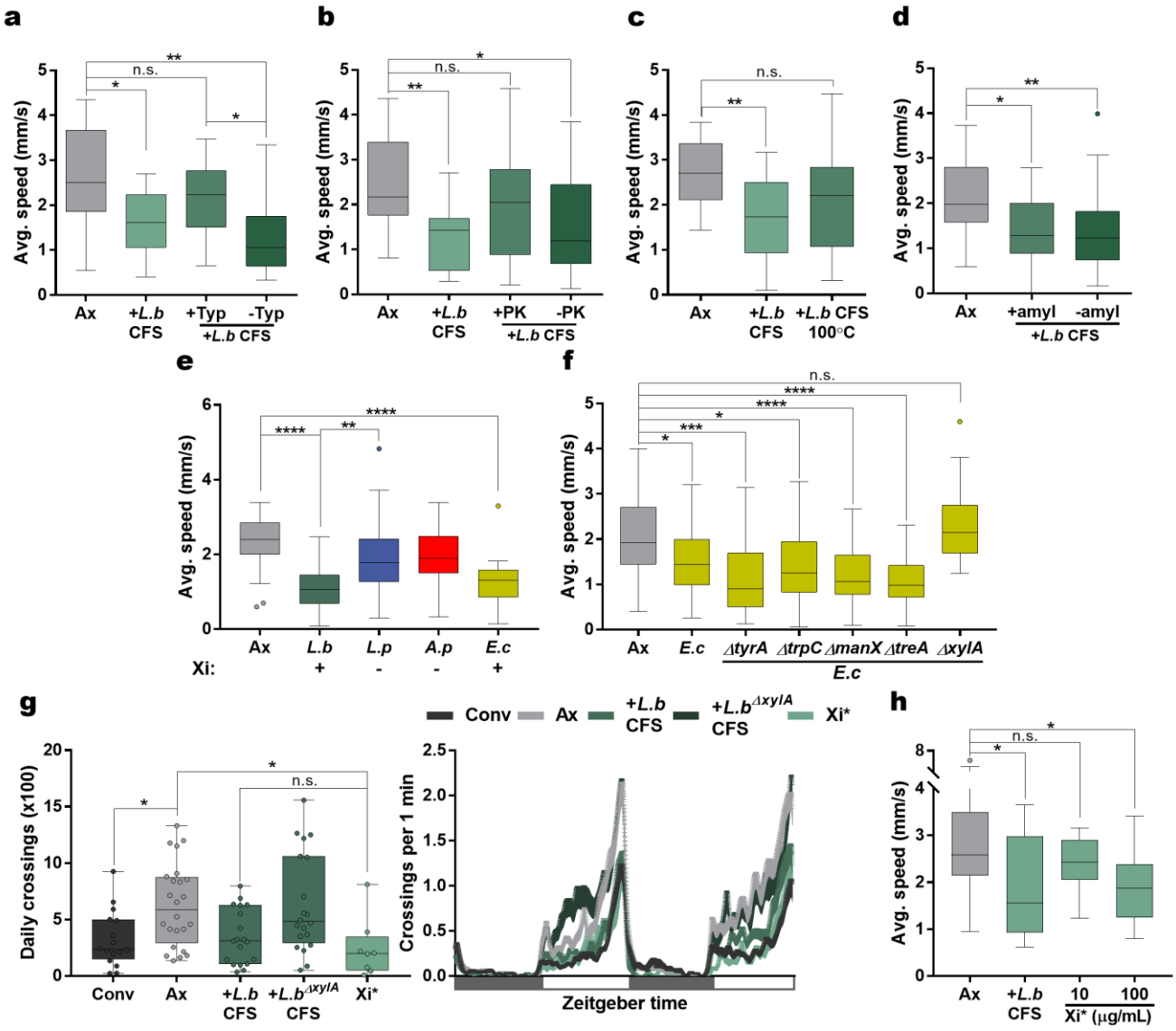

Fig. 8. Modulation of locomotion by the bacterial enzyme, xylose isomerase.

a – c, Average speed of Ax or Ax flies treated with unaltered, protease- (Typ, Trypsin; PK, Proteinase-K), or heat-treated (100°C) *L.b* CFS. n = 18/condition. d, Average speed of Ax flies administered with amylase-treated PBS (Ax), amylase-treated *L.b* CFS (+amyl *L.b* CFS), or unaltered *L.b* CFS (-amyl *L.b* CFS). n = 18/condition. e, Average speed of Ax flies or flies mono-associated with *L.b*, *L.p*, *A. pomorum* (*A.p*), or *E. coli* (*E.c*). n = 30/condition. f, Average speed of Ax and flies mono-associated with either WT *E.c* or a strain of *E.c* carrying a knockout of a single gene *(*Δ*tyrA*, Δ*trpC*, Δ*manX*, Δ*treA*, Δ*xylA*). Ax, n = 60; *E.c*; n = 60; *E.c*$^{\Delta tyrA}$, n = 24;

E.c$^{\Delta trpC}$, n = 24; *E.c*$^{\Delta manX}$, n = 48; *E.c*$^{\Delta treA}$, n = 48; *E.c*$^{\Delta xylA}$, n = 24. g, Daily activity of Conv, Ax, and Ax virgin female Oregon^{R} flies treated with *L.b* CFS, *L.b*$^{\Delta xylA}$ CFS, or Xi* over a 2-day light-dark cycle period each lasting 12 hrs., starting at time 0. White boxes represent lights on and gray boxes represent lights off. Conv, n = 16; Ax, n = 23; *L.b* CFS, n = 18; *L.b*$^{\Delta xylA}$ CFS, n = 19; Xi*, n = 8. h, Average speed of Ax and Ax flies treated with *L.b* CFS or Xi*. Ax, n = 16; *L.b* CFS, n = 11; 10 µg/mL Xi*, n = 12; 100 µg/mL Xi*, n = 14. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Kruskal-Wallis and Dunn's post-hoc test was applied for statistical analysis. Data are representative of at least 2 independent trials for each experiment.

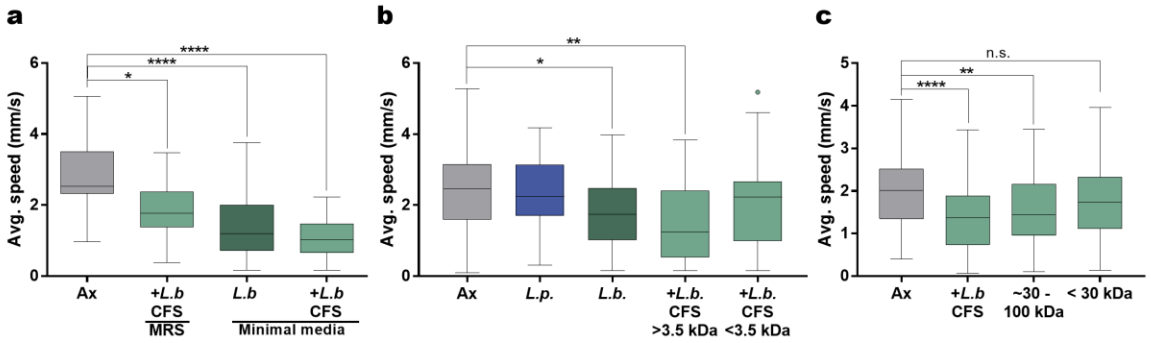

Fig. 9. Fractionation of *L. brevis*-derived products.

a, Average speed of Ax flies and flies treated with *L.b* CFS grown in MRS or minimal media. n = 24/condition. b, Average speed of Ax, *L.p* or *L.b* mono-associated flies, and flies treated with either the fraction of *L.b* CFS above or below 3.5 kDa. n = 48/condition. c, Average speed of Ax and flies treated with *L.b* CFS or the fraction of *L.b* CFS between approximately 30 to 100 kDa or less than 30 kDa. n = 96/condition. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. Kruskal-Wallis and Dunn's post-hoc test was applied for statistical analysis. Data are representative of at least 2 independent trials for each experiment.

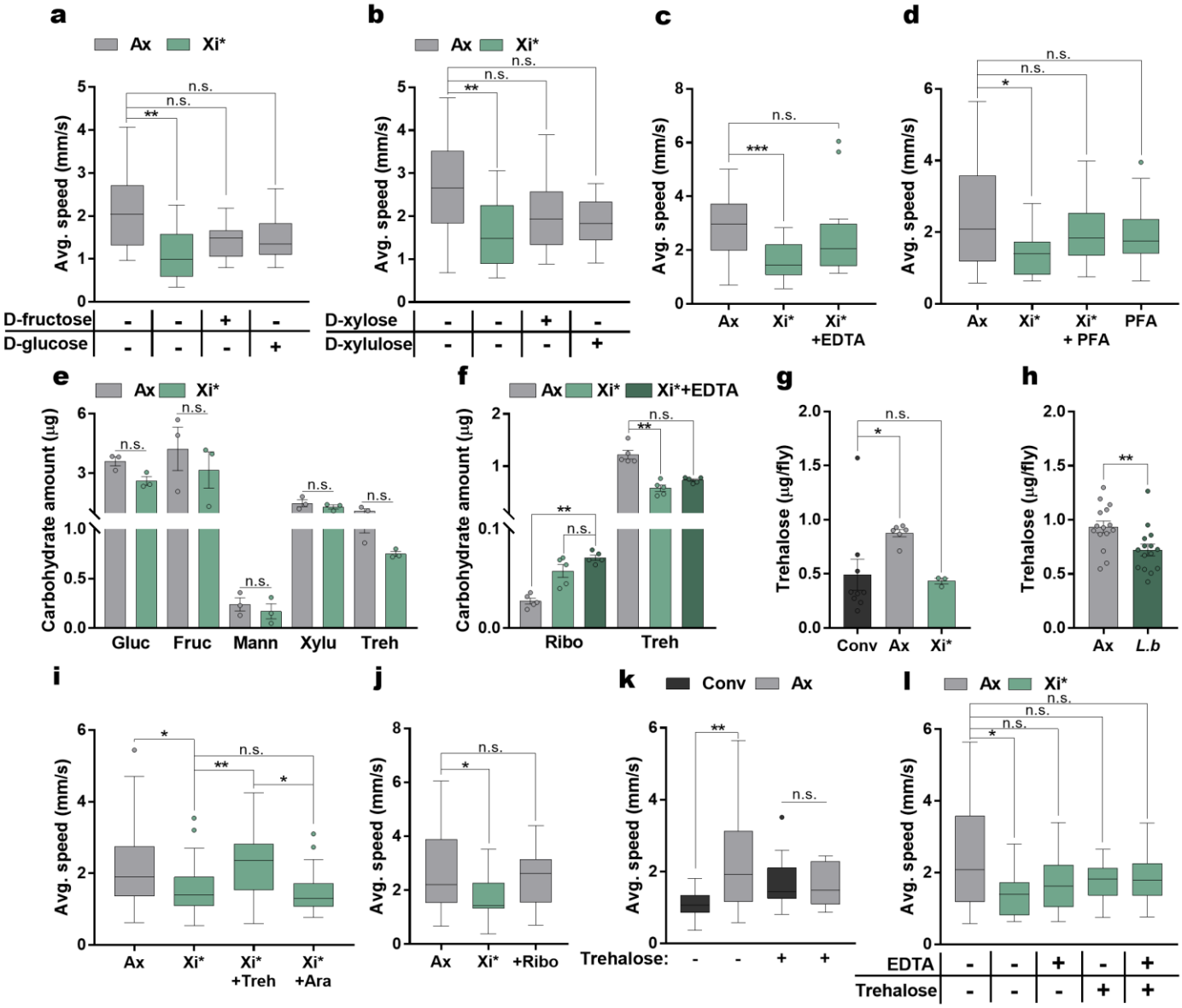

Figure 10. Xylose isomerase activity and key carbohydrates are involved in Xi-mediated changes in locomotion.

a – b, Average speed of Ax and Ax flies treated with Xi* or 100 µg of D-fructose, D-glucose, D-xylose, or D-xylulose. (a) Ax, n = 16; Xi*, n = 13; D-fructose, n = 13; D-glucose, n = 15. (b) Ax, n = 26; Xi*, n = 21; D-xylose, n = 22; D-xylulose, n = 18. c, Average speed of Ax and Ax flies treated with either Xi* or Xi* inactivated through treatment with 5 mM EDTA. Ax, n = 21; Xi*, n = 16; Xi*+EDTA, n = 18. d, Average speed of Ax and Ax flies treated with Xi*, 1.6% PFA, or Xi* treated with 1.6% PFA. Ax, n = 27; Xi*, n = 19; Xi*+PFA, n = 21; PFA, n = 21. e,

Carbohydrate levels in Ax and Xi*-treated fly media. Each sample is from 0.1 g of fly media and represents a separate vial. Error bars represent mean +/- S.E.M. n = 3 samples/condition. f, Carbohydrate levels in Ax, Xi*, and EDTA-treated Xi* flies. Each sample contains 5 flies. Error bars represent mean +/- S.E.M. n = 5 samples/condition. g, Trehalose levels in Conv, Ax, and Xi*-treated flies. Error bars represent mean +/- S.E.M. Conv, n = 9 samples; Ax, n = 6 samples; Xi*, n = 3 samples. h, Trehalose levels in Ax and *L.b* colonized flies. Error bars represent mean +/- S.E.M. n = 15 samples/condition. i, Average speed of Ax and Xi*-treated flies supplemented with either trehalose (Treh, 10 mg/mL) or arabinose (Ara, 10 mg/mL) for 3 days before testing. Ax, n = 40; Xi*, n = 40; Xi*+Treh, n = 39; Xi*+Ara, n = 18. j, Average speed of Ax, Xi*-, or ribose-(Ribo, 10 mg/mL) treated flies. Ax, n = 29; Xi*, n = 25; Ribo, n = 12. k, Average speed of Conv and Ax flies supplemented with trehalose (Treh, 10 mg/mL) for 3 days before testing. n = 30/condition. l, Average speed of Ax and Xi* or EDTA-treated Xi* Ax flies subsequently left untreated or supplemented with trehalose (Treh, 10 mg/mL) for 3 days before testing. n = 30/condition. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Kruskal-Wallis and Dunn's (a – d, f – g, i – l) or a Mann-Whitney *U* (e and h) post-hoc test was applied for statistical analysis. Data are representative of at least 2 independent trials for each experiment. Gluc, glucose; Fruc, fructose; Mann, mannose; Xylu, xylulose; Treh, trehalose; Ribo, ribose.

3.9 METHODS

Fly Stocks and Rearing

We obtained the *Canton-S* (#64349) line from Bloomington *Drosophila* Stock Center at Indiana University. Other fly stocks used were *Oregon*^{R} (kindly provided by A. A. Aravin and K. Fejes Tóth).

Flies were cultured at 25˚C and 60% humidity on a 12-hr. light:12-hr. dark cycle and kept in vials containing fresh fly media made at California Institute of Technology consisting of cornmeal, yeast, molasses, agar, and p-hydroxy-benzoic acid methyl ester. Other dietary compositions used were created through altering this standard diet or the Nutri-Fly "German Food" Formula (Genesee Scientific) and were calculated using previously published nutritional data[47]. Axenic flies were generated using standard methods[23,30,32,35]. Briefly, embryos from conventional flies were washed in bleach, ethanol, and sterile PBS before being cultivated on fresh irradiated media[23]. Axenic stocks were maintained through the application of an irradiated diet supplemented with antibiotics (500 μg/ml ampicillin, Putney; 50 μg/ml tetracycline, Sigma; 200 μg/ml rifamycin, Sigma) for at least one generation. For experiments, virgin female flies were collected shortly after eclosion and placed at random into vials (10 – 15 flies per vial) containing irradiated media without antibiotics. Vials were changed every 3 – 4 days using sterile methods. The antibiotic-supplemented diet was applied to conventional flies shortly after eclosion to generate antibiotic-treated (ABX) flies. Both antibiotic-treated and axenic flies were tested for contaminants through plating animal lysates on Man, Rogosa, and Sharpe (MRS, BD Biosciences); Mannitol (25 g/L Mannitol, Sigma; 5 g/L Yeast extract, BD Biosciences; 3 g/L Peptone, BD Biosciences); and Luria-Bertani (LB, BD Biosciences) nutrient agar plates.

Bacterial Strains

Lactobacillus brevis EW, *Lactobacillus plantarum* WJL, and *Acetobacter pomorum* were obtained from laboratory-reared flies in the laboratory of Won-Jae Lee (Seoul National University)[35,40,41]. *Lactobacillus brevis*^{Bb14} (ATCC, #14869) and *Lactobacillus brevis*$^{P-2}$ (ATCC, #27305) were isolated from human feces and fermented beverages, respectively. *Acetobacter tropicalis* was isolated from conventional flies in the Mazmanian laboratory and *Enterococcus*

faecalis was isolated from mice. *Escherichia coli*^{K12} (CGSC, #7636) was grown in LB broth and *Escherichia coli*$^{\Delta tyrA}$ (CGSC, #9131), *Escherichia coli*$^{\Delta trpC}$ (CGSC, #10049), *Escherichia coli*$^{\Delta manX}$ (CGSC, #9511), *Escherichia coli*$^{\Delta treA}$ (CGSC, #9090), and *Escherichia coli*$^{\Delta xylA}$ (CGSC, #10610)[42] were grown in LB broth supplemented with kanamycin (50 μg/mL). *Lactobacillus brevis* and *Lactobacillus plantarum* cultures were grown overnight in a standing 37˚C incubator in MRS broth (BD Biosciences). For mono-associations, fresh stationary phase bacterial cultures (OD600 = 1.0, 40 μl) were added directly to fly vials. Associations with 2 bacterial species were performed in a 1:1 mixture. For heat-killed experiments, fresh cultures of *Lactobacillus brevis* (OD600 = 1.0) were washed 3 times in sterile PBS, incubated at 100˚C for 30 min., and cooled to room temperature before administering to flies. All treatments were supplied daily through application to the fly media (40 μL) for 6 days following eclosion.

Bacterial Supernatant Preparations

Cell-free supernatants (CFS) of specified bacterial strains were harvested from bacterial cultures (OD600 = 1.0) by centrifuging at 13,000 x g for 10 min. and subsequent filtration through a 0.22-μm sterile filter (Millipore). CFS was dialyzed in MilliQ water with a 3.5 kDa membrane (Thermo Scientific) overnight at 4˚C to generate *L.b* CFS and *L.p* CFS samples. Each of these treatments was supplied daily through application to the fly media (40 μL) for 6 days following eclosion.

Heat and Enzymatic Treatment of *L.b* CFS

For heat-inactivation experiments, freshly prepared *L.b* CFS samples were incubated at 100˚C for 30 min. and cooled to room temperature before administering to flies. For proteinase K (PK) and trypsin (Typ) treatment, overnight dialysis of CFS was performed in Tris-HCl (pH 8 for PK and pH 8.5 for Typ) after which samples were treated with either PK (100 μg/mL, Invitrogen) or Typ (0.05 μg/mL, Sigma) at 37˚C for 24 or 7 hrs., respectively. A proteinase inhibitor cocktail (Sigma) was added to stop the reaction and subsequently removed through overnight dialysis (Thermo Scientific) at 4˚C in MilliQ water. Aliquots of the samples were run on a 4-20% Tris-glycine gel (Invitrogen) to confirm protein cleavage. Controls followed the same protocol except for the addition of proteinase K or trypsin. For amylase digests, 20 μl of 100 mU/mL amylase

(Sigma) was added to either freshly prepared *L.b* CFS or a PBS control for 30 min. and inhibited through lowering the pH to 4.5. Each of these treatments was supplied daily through application to the fly media (40 µL) for 6 days following eclosion.

Production of His-tagged proteins (Xi* and Ai*)

An expression plasmid for the production of His-tagged xylose isomerase from *L.b*, here termed as Xi*, was constructed by amplification of its gene and cloning the resulting PCR product in the pQE30 cloning vector (Qiagen) using SLIC ligation. The following primer sequences were used for the construct: 5'-CGCATCACCATCACCATCACGGATCTTACTTGCTCAACGTATCGATGATGTAA-3' and 5'-GGGGTACCGAGCTCGCATGCGGATCATGACTGAAGAATACTGGAAAGGC-3'. Conformation of the resulting plasmid was verified and transformed into *E. coli* (Turbo, NEB). This strain was then grown in LB containing ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL) with shaking at 220 rpm at 37˚C for 1 hr. before the addition of 0.1 mM IPTG. After 4 hrs. of shaking at 220 rpm at 37˚C, cells were pelleted and lysed using lysozyme (Sigma) and bead beating with matrix B beads (MP Biomedicals) for 45 sec. Supernatant was collected after centrifugation and the Xi* protein purified through metal affinity purification under native conditions using HisPur™ Ni-NTA Spin Columns (Thermo Scientific). Protein purification was verified through western blot using an Anti-6X His tag® antibody (Abcam) and quantified using a Pierce BCA Protein Assay kit (Thermo Scientific) after which protein was stored at -20˚C. Expression and purification of His-tagged L-arabinose isomerase from *L.b*, here termed as Ai*, was performed under the exact same conditions and the following primer sequences were used for the construct: 5'-GGGGTACCGAGCTCGCATGCGGATCATGTTATCAGTTCCAGATTATGAATTTTGG-3' and 5'-CGCATCACCATCACCATCACGGATCCTTACTTGATGAACGCCTTTGTCAT-3'. For EDTA treatment, purified Xi* was combined with 5 mM EDTA for 44 hrs. at 4°C and subsequently dialyzed prior to administering to flies through application to the fly media (40 µL) for 6 days following eclosion. For paraformaldehyde (PFA) treatment, 1.6% PFA was added to Xi* at room temperature for 2 hrs. and subsequently dialyzed prior to administering to flies through application to the fly media (40 µL) for 6 days following eclosion.

Generation of *xylA* deletion mutant (Δ*xylA*)

~1-kb DNA segments flanking the region to be deleted were PCR amplified using the following primers: 5'-ATTCCAATACTACCACTAGCAACGACATCCGTAAAGT-3'; 5'-AATTCGAGCTCGGTACCCGGGGATCCACAATCAGAATTGATCGCGGCAAC-3'; 5'-TCGTTGCTAGTGGTAGTATTGGAATCCTAAACCAGATTTCTTATCTTGATG-3'; 5'-GCCTGCAGGTCGACTCTAGAGGATCCCGCAAGTCTAGTGCGGCT-3'. The forward primers were designed using to be partially complementary at their 5' ends by 25 bp. The fused PCR product was cloned into the BamHI site of the *Lactobacilli* vector pGID023 and mobilized into *L.b*. Colonies selected for the erythromycin (Erm) resistance, indicating integration of the vector into the host chromosome were re-plated onto MRS+Erm and subsequently passaged over 5 days and plated onto MRS+Erm. Colonies selected for Erm resistance were passaged again in MRS alone over 3 days and plated on MRS. Resulting colonies were plated in replica on MRS and MRS+Erm. Erm sensitive colonies were screened by PCR to distinguish wild-type revertants from strains with the desired mutation.

Bacterial Load Quantification

Intestines dissected from surface sterilized 7-day-old adult female flies were homogenized in sterile PBS with ~100 µl matrix D beads using a bead beater. Lysate dilutions in PBS were plated on MRS agar plates and enumerated after 24 hrs. at 37˚C.

Locomotion Assays

Locomotor behavior was assayed through three previously established methods: the Drosophila Activity Monitoring System (DAMS, Trikinetics)[48,49], video-assisted tracking[26,50,51], and gait analysis[24].

Activity measurements

7-day-old individual female flies were cooled on ice for 1 min. and transferred into individual vials (25 x 95 mm) containing standard irradiated media. Tubes were then inserted and secured into Drosophila activity monitors (DAMS, Trikinetics) and kept in a fly incubator held at

25˚C. Flies were allowed to acclimate to the new environment for 1 day before testing and midline crossing was sampled every min. Average daily activity was calculated from the 2 days tested and actograms were generated using ActogramJ[49].

Video-assisted tracking

Individual female flies were cooled on ice for 1-2 min. before being introduced under sterile conditions into autoclaved arenas (3.5 cm diameter wells), which allowed free movement but restricted flight. After a 1 hr. acclimation period, arenas were placed onto a light box and recorded from above for a period of 10 min. at 30 frames per sec. All testing took place between ZT 0 and ZT 3 (ZT, Zeitgeber time; lights are turned on at ZT 0 and turned off at ZT 12) and both acclimation and testing occurred at 25˚C unless otherwise stated. Videos were processed using Ethovision software or the Caltech FlyTracker (http://www.vision.caltech.edu/Tools/FlyTracker/).

Bout analysis was performed using custom python scripts (available upon request). The velocity curve was smoothed from the acquired video at 30 frames per sec. using a 15 sec. moving average window. A minimum walking speed of 0.25 mm/s was given below which flies were moving but not walking (‘pause bouts’) and above which they were designated as walking (‘walking bouts’). Lengths were measured as time between bout onset and offset.

Gait analysis

Experiments used an internally illuminated glass surface with frustrated total internal refraction (fTIR) to mark the flies' contact with the glass[24]. The movement of the flies and their contact was recorded with a high-framerate camera, and videos were quantified using the FlyWalker software package. For further details of the parameters see [24]. All groups consisted of 7-day-old female flies and were tested at room temperature.

Measurement of carbohydrate levels

Fly (5 flies per sample) and fly media (0.1 g per sample) were homogenized in TE Buffer (10 mM Tris, pH = 8, 1 mM EDTA) using a bead beater for 45 sec. followed by centrifugation at

7,000 x g for 3 min. The supernatant was heat treated for 30 min. at 72°C before being stored at -80°C before subsequent clean-up steps prior to running on HPAEC-PAD.

100 µL of fly or fly media homogenate sample in TE buffer was diluted with 200 µL of UltraPure distilled water (Invitrogen) and sonicated to get uniform solution. Samples were centrifuged at 2,000 rpm for 15 sec. to precipitate insoluble material. 100 µL of the sample were filtered through pre-washed Pall Nanosep® 3K Omega centrifugal device (MWCO 3KDa, Sigma-Aldrich) for 15 min. at 14,000 rpm, 7°C. The filtrate was dried on Speed Vac. The dry sample was reconstituted in 300 µL of UltraPure water and loaded onto pre-washed Dionex OnGuard® IIH 1cc cartridge. The flow through and 2x1 mL elution with Ultrapure water was collected in the same tube and lyophilized.

Monosaccharide analysis was done using Dionex CarboPac™ PA1 column (4 x 250 mm) with PA1 guard column (4 x 50 mm). Flow rate 1 mL/min. Pulsed amperometric detection with gold electrode. The elution gradient was as follows: 0 min - 20 min, 19mM sodium hydroxide; 20 min. - 50 min., 0 mM - 212.5 mM sodium acetate gradient with 19 mM sodium hydroxide; 50 min. - 65 min., 212.5 mM sodium acetate with 19 mM sodium hydroxide; 65 min. - 68 min., 212.5 mM - 0 mM sodium acetate with 19 mM sodium hydroxide; 68 min. - 85 min., 19 mM sodium hydroxide

Trehalose, arabinose, galactose, glucose, mannose, xylose, fructose, ribose, sucrose and xylulose were used as standards. The monosaccharides were assigned based on the retention time and quantified using Chromeleon™ 6.8 chromatography data system software. In Figure 10g – h, measurements of trehalose levels were performed following the same isolation procedure and subsequently processed using a Trehalose Assay Kit (Megazyme) according to the manufacturer's instructions.

For experiments treating flies with trehalose, arabinose or ribose, groups of conventional, axenic, or axenic flies previously treated with Xi* were administered with trehalose, arabinose, or ribose (10 mg/mL, Sigma) through application to the fly media (40 µL) for every day for 3 days before testing.

Statistical Analysis

All statistical analysis was performed using Prism Software (GraphPad, version 7). Sample size was based on data from pilot experiments and experimenters were not blinded as almost all data acquisition and analysis was automated. To analyze two sets of data following a normal distribution, we used an unpaired two-sided Student's t-test. If the data did not follow a normal distribution, a non-parametric two-sided Mann-Whitney *U* test was used. Comparisons among 3 or more data sets and comparisons with more than one variant were analyzed using One-way ANOVA or Two-way ANOVA, respectively. If statistical significance was identified for the variables tested, then a Dunn's post-hoc test was performed. ANOVAs on normally distributed data were followed by a Bonferroni post-hoc test to determine significant differences between genotypes. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. Bar graphs are presented as mean values +/- S.E.M.

Chapter 4

HOST PATHWAYS MEDIATING MICROBIAL MODULATION OF LOCOMOTION

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A modified version of this chapter along with Chapter 3 was submitted in "A gut microbial factor modulates locomotor behavior in *Drosophila*."

4.1 INTRODUCTION

The microbiota is an important regulator of host physiology, multiple aspects of which directly or indirectly alter locomotion. Microbial factors, such as xylose isomerase (Xi), may signal through these host pathways to subsequently alter motor behavior. For example, a reduction in host speed is also found in instances of ill health and could indicate recruitment of immune pathways involved in sickness behavior[1]. The gut microbiota also regulates host metabolism; therefore, increased locomotion upon removal of the gut microbiota may suggest a starvation-like state regulated by similar neuromodulators[2]. In order to investigate the host pathways involved, we broadly examined the importance of development, immunity, feeding behavior, and neuromodulators in mediating microbial effects on motor behavior.

4.2 POST-ECLOSION MICROBIAL SIGNALS DECREASE HOST LOCOMOTION

To investigate whether the effects of microbial exposure are dependent on host developmental stage, we mono-colonized flies at 3 – 5 days post-eclosion (Fig. 1a), a time point in which the development of the gastrointestinal tract and remodeling of the nervous system are complete[3–5]. Colonization with *L. brevis* in fully developed female flies decreases locomotor speed and average walking bout length to levels similar in flies treated immediately following eclosion (Fig. 1b – e). Changes in locomotion are likely independent of bacterial effects on host development, as conventionally-reared flies treated after eclosion with broad spectrum antibiotics exhibit similar walking speeds to animals born under axenic conditions (Fig. 1f). Administration of antibiotics increases fly locomotion in two different wild-type lines (Fig. 1g). Furthermore, colonization with *L. brevis*, but not *L. plantarum*, after the removal of antibiotics reduces locomotor behavior to levels similar to conventional flies (Fig. 1h – m). From these data, we conclude that locomotion is modulated by select bacterial species of the *Drosophila* microbiome and is mediated by active signaling, rather than developmental influences.

4.3 INTERACTION BETWEEN LOCOMOTOR PHENOTYPES AND OTHER ASPECTS OF HOST PHYSIOLOGY

Gut bacteria secrete molecular products that regulate multiple aspects of host physiology, including immunity and feeding behavior[6,7]. As colonization with *L. brevis* and treatment with either cell-free supernatant (CFS) or Xi from *L. brevis* reduced host locomotion in flies (Chapter 3), we subsequently used this bacterium and its products to examine the host pathways involved. Changes in mass can alter motor behavior; however, we did not find any significant differences in weight between *L.b* CFS-treated flies and axenic or *L.p* CFS-treated groups (Fig. 2a). Total protein levels are also similar between these groups (Fig. 2b). While the number of excreta was increased in axenic flies, it did not alter upon colonization with either *L. brevis* or *L. plantarum* (Fig. 3). We next explored the involvement of immunity and feeding behavior in microbial-mediated locomotion. Depletion of the microbiome in Immune Deficiency (IMD) and Toll knockout flies using antibiotics results in similar increases in walking speed compared to wild-type flies (Fig. 4a – b). There are no differences in the expression of anti-microbial peptides or the dual oxidase gene, *Duox*, in *L.b* CFS-treated axenic flies (Fig. 4c). Moreover, while food intake may be influenced by bacterial species and can inhibit locomotor behavior[7–9], there is no significant change in the amount of food ingested by *L.b* CFS-treated flies compared to controls (Fig. 4d – e). Although *L.b* CFS did not cause changes in antimicrobial peptides or food intake, xylose isomerase (Xi) could alter aspects of host health that contribute to changes in locomotion. While *L.b* CFS did increase the number of apoptotic cells similar to previous reports[10], no changes in survival or intestinal cellular apoptosis occur at the time of motor testing in Xi*-treated flies compared to controls (Fig. 5a – b). However, administration of Xi* throughout life did lead to an overall reduction in survival compared to axenic flies starting at day 42, suggesting Xi negatively affects host health at later time points (Fig. 5a).

The microbiota is known to affect circadian oscillations of host metabolites and transcription of certain genes in the epithelium[11,12]. Although recent work has revealed conflicting findings on changes in sleep in mice and *Drosophila*[13,14], alterations in sleep:wake behavior could contribute to our previous results. Axenic female flies do exhibit a significant reduction in total sleep compared to conventional and *L. brevis* mono-colonized flies during the light phase (Fig.

6a). However, Xi*-treatment did not significantly alter sleep in axenic flies (Fig. 6b), indicating that other microbial factor(s) could contribute to changes in sleep:wake behavior in female flies.

4.4 OCTOPAMINE SIGNALING MEDIATES XYLOSE ISOMERASE-INDUCED CHANGES IN LOCOMOTION

Specific neuronal pathways regulate complex behaviors in animals[2,15–17], and can be modulated by peripheral inputs, including intestinal and circulating carbohydrate levels[18,19]. To explore the involvement of various neuronal subsets in bacterial-induced motor behavior, we used the thermosensitive cation channel *Drosophila* TRPA1 (dTRPA1) to activate neuronal populations previously implicated in locomotion[15,20] via a repertoire of GAL4-driver lines. In combination with *UAS-dTrpA1* at the activity-inducing temperature (27˚C), we observed that activation of only two GAL4 lines that both label octopaminergic neurons, tyrosine decarboxylase (Tdc2) and tyramine beta-hydroxylase (Tβh), override *L. brevis* modulation of locomotion (Fig. 7a and 8). Accordingly, activation of Tdc-expressing cells abrogated the effects of Xi*-treatment and differences between conventional and antibiotic-treated groups (Fig. 7b – c and 9). The ability of *L. brevis* to decrease locomotion, however, is not changed by the activation of dopaminergic, serotoninergic, GABAergic, or cholinergic neurons (Fig 7a and 8e – h). The administration of octopamine to conventional, Xi*-, or *L.b* CFS-treated flies increases host walking speed to levels similar to that of axenic flies (Fig. 7d – e and 10a). Further, *Tdc2* and *Tβh* transcript levels are reduced in RNA extracted from the heads of Xi*- and *L.b* CFS-treated flies (Fig. 10b – c). As Tdc and Tβh are important for octopamine synthesis, these results further link octopamine to Xi-induced locomotor effects. Octopamine and tyramine are involved in multiple aspects of host physiology, including metabolism and behavior, and display opposite roles in regulating certain motor behaviors[21–29]. While administration of tyramine did not influence walking speed in Xi* and *L.b* CFS conditions (Fig. 7e and 10d), antibiotic-treated flies carrying a null allele for Tdc ($Tdc2^{RO54}$) no longer display differences in locomotion upon supplementation with Xi* (Fig. 7f), suggesting an indirect role for tyramine. Limiting the expression of a transgene for diphtheria toxin (*DTI*) to octopaminergic and tyraminergic neurons outside of the ventral nerve cord[24,30] results in equivalent speeds between antibiotic- and Xi*-treated flies (Fig. 10e), implicating the involvement of neurons in the supraesophageal and the subesophageal zones in microbial effects on motor behavior. Octopamine

signaling is necessary for locomotor changes, as axenic flies administered with mianserin, an octopamine receptor antagonist, and antibiotic-treated flies carrying a null allele for Tβh ($T\beta H^{M18}$) or expressing Tβh RNAi no longer respond to Xi* or *L.b* CFS treatment (Fig. 7g and 10f – h). Similar results are also found under conventional conditions compared to antibiotic-treated groups (Fig. 10i – k). Collectively, we conclude that defined products of the microbiome, and specifically Xi, negatively regulate octopamine signaling to control *Drosophila* locomotion (Fig. 10l).

4.5 CONCLUSION

The microbiome influences neurodevelopment, regulates behavior, and contributes to various neurologic and neuropsychiatric disorders. Herein, we demonstrate that gut bacteria modulate locomotion in female *Drosophila*. The depletion of the gut microbiota increases host exploratory behavior, and the commensal bacterium *L. brevis* is sufficient to regulate locomotion. In addition, we establish that xylose isomerase from *L. brevis* corrects the locomotor phenotypes of axenic flies, a process that is mediated by trehalose and octopamine signaling in the host. However, further work is needed to identify the exact neurons and neuronal mechanisms involved, including potential changes in firing patterns. It would also be important to clarify the sex-specific aspects of these microbial effects on locomotion[14]. It is intriguing that germ-free mice display hyperactivity similar to axenic *Drosophila*, and specific bacteria have been shown to decrease locomotor activity in mice[13,31,32], although the neuronal pathways implicated in mammalian systems have yet to be identified. The mammalian counterpart of octopamine, noradrenaline, modulates locomotion[2,33,34], potentially implicating adrenergic circuitry as a conserved pathway that is co-opted by the microbiome in flies and mammals. In addition to motor behavior, octopamine signaling is linked to sugar metabolism, and trehalose serves as a major energy source for *Drosophila*[21,35]. Xylose isomerase may therefore facilitate adrenergic regulation of host physiology through orchestrating metabolic homeostasis, such as via altering internal energy storage, although additional work is needed to define how the microbiome mediates interactions between sugar metabolism and octopamine signaling. The link between metabolic state and locomotion suggests that peripheral influences on metabolism, including the microbiota, may signal via neuronal pathways that modify physical activity.

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4.6 FIGURES

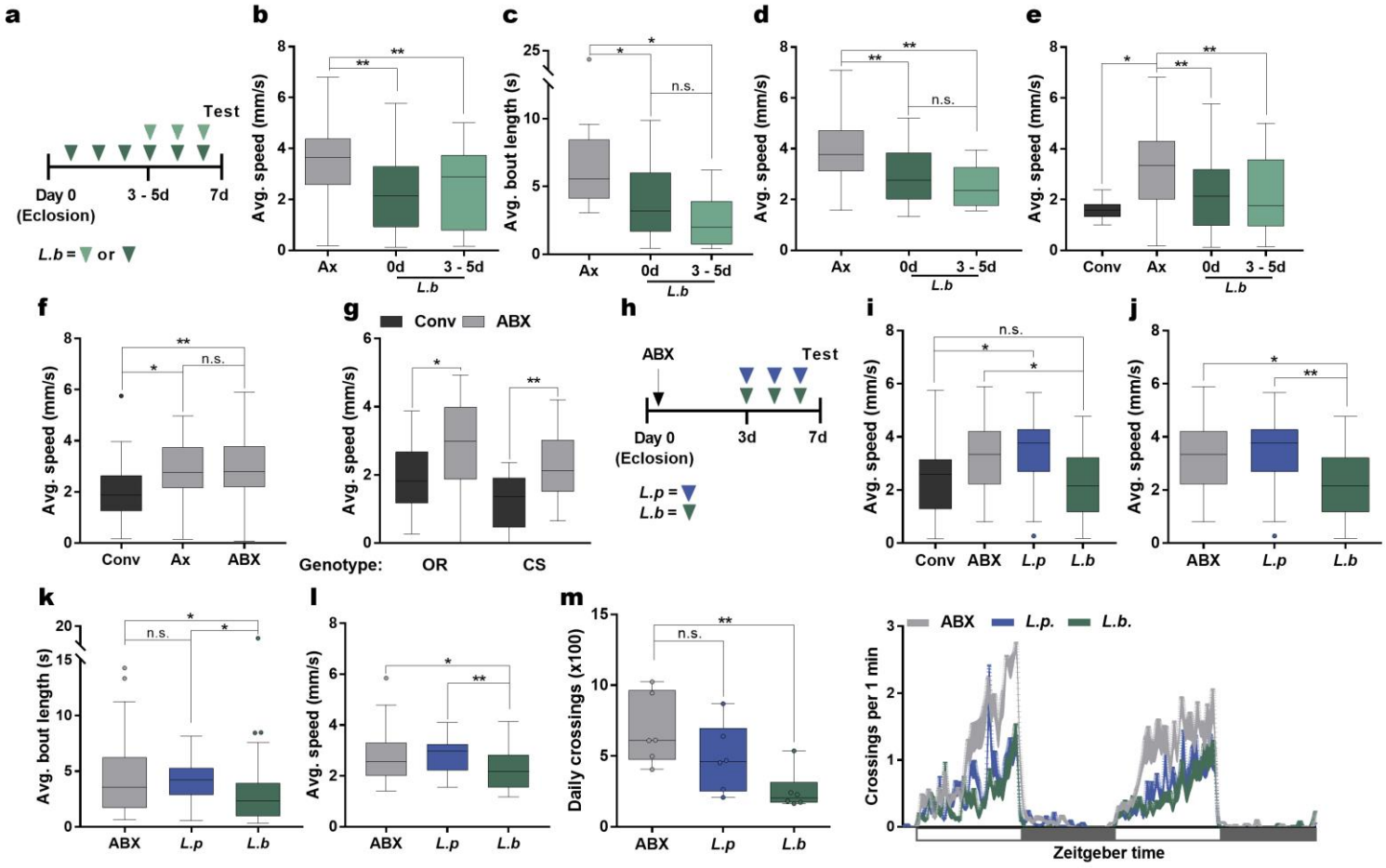

Figure 1. Post-eclosion microbial signals decrease host locomotion.

a, Experimental design (b – e) in which Ax flies were associated with *L.b* either directly after (day 0, dark green arrows) or 3 – 5 days (light green arrows) following eclosion. b – d, Average speed (b), average bout length (c), and average speed during walking bouts (d) of Ax and flies mono-associated with *L.b* at either day 0 or day 3 – 5. n = 46/condition. e, Average speed of Conv, Ax, and flies mono-associated with *L.b* at either day 0 or day 3 – 5. n = 46/condition f, Average speed of Conv, Ax, and Conv flies treated with antibiotics for 3 days after eclosion (ABX). n = 30/condition. g, Average speed of Oregon^{R} (OR) and Canton S (CS) Conv flies and Conv flies treated with antibiotics for 3 days after eclosion (ABX). n = 15/condition. h, Experimental design (i – m) in which conventionally-reared flies were treated with antibiotics (ABX, black arrow) for 3 days following eclosion. All flies were subsequently placed on irradiated media either without supplementation (ABX) or associated with *L.p* (blue arrows) or *L.b* (green arrows) for the 3 days

prior to testing. i, Average speed of Conv and Conv flies treated with antibiotics (ABX) for 3 days, after which flies were either left naïve or colonized with *L.p* or *L.b*. Conv, n = 25; ABX, n = 29; *L.p*, n = 24; *L.b*, n = 35. j – l, Average speed (j), average bout length (k), and average speed during walking bouts (l) calculated for ABX, *L.p*-, and *L.b*-associated flies. n = 36/condition. m, Daily activity of ABX, *L.p* and, *L.b* groups (virgin female Oregon^{R} flies) over a 2-day light-dark cycle period each lasting 12 hrs., starting at time 0. White boxes represent lights on and gray boxes represent lights off. n = 6/condition. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$, ** $P < 0.01$. Kruskal-Wallis and Dunn's (b – f and i – m) or Mann-Whitney *U* (g) post-hoc test was applied for statistical analysis. Data are representative of at least 2 independent trials for each experiment.

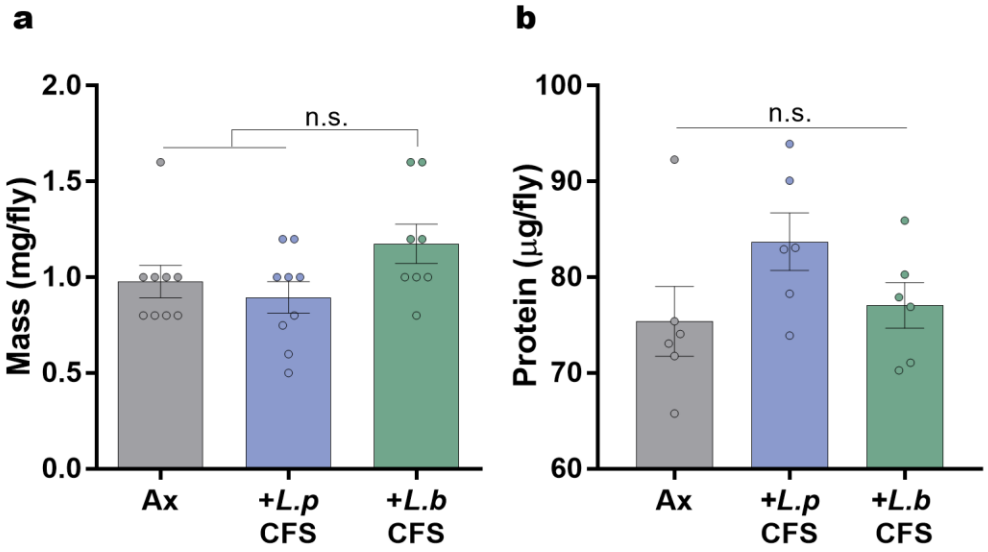

Figure 2. Mass and protein levels are not significantly altered by treatment with microbial metabolites.

a, Mass of Ax and *L.p* CFS- or *L.b* CFS-treated Ax flies. Error bars represent mean +/- S.E.M. Ax, n = 9; *L.p* CFS, n = 9; *L.b* CFS, n = 8. b, Protein levels in whole Ax and *L.p* CFS- or *L.b* CFS-treated Ax flies. n = 6 samples/condition. Error bars represent mean +/- S.E.M. Kruskal-Wallis and Dunn's post-hoc test was applied for statistical analysis. Data are representative of at least 2 independent trials for each experiment.

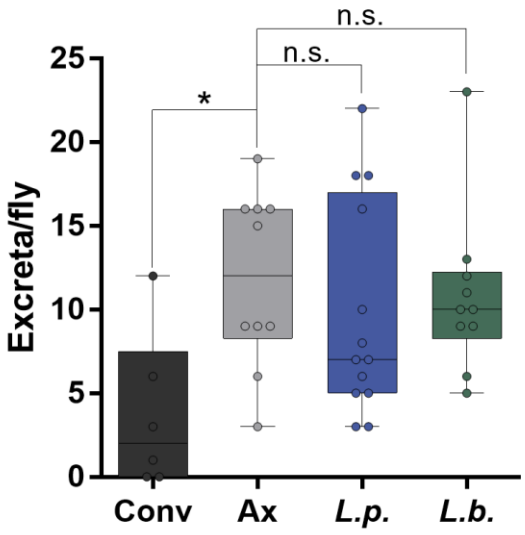

Figure 3. Colonization with *L. plantarum* or *L. brevis* alone does not alter excretion number.

Number of excreta per fly for Conv, Ax, and *L.p* or *L.b* colonized flies. Conv, n = 6; Ax, n = 10; *L.p*, n = 13; *L.b*, n = 10. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$. Kruskal-Wallis and Dunn's post-hoc test was applied for statistical analysis. Data are representative of at least 2 independent trials for each experiment.

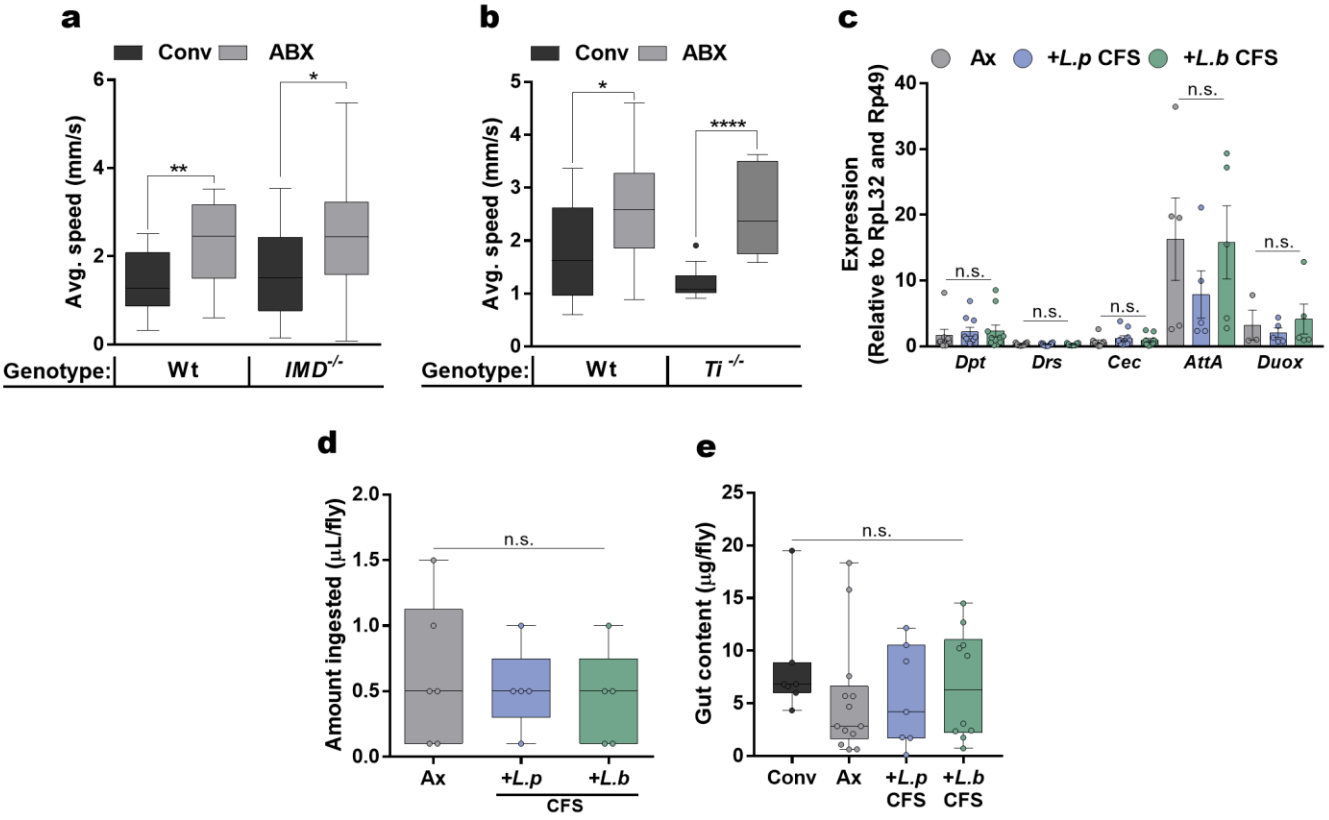

Figure 4. The role of food intake, anti-microbial peptides, as well as the Immune Deficiency (IMD) and Toll pathways in locomotor phenotypes.

a, Average speed of wild-type background (Oregon^{R}, Wt) and *Imd-/-* flies placed on either media alone or media supplemented with antibiotics (ABX) following eclosion. n = 24/condition. b, Average speed of wild-type background (Canton S, Wt) and *Ti-/-* flies placed on either media alone or media supplemented with antibiotics (ABX) following eclosion. n = 18/condition. c, qRT-PCR of immune-related transcripts in Ax and Ax *L.p*- or *L.b*-CFS treated flies. Error bars represent mean +/- S.E.M. n = 6 samples/condition. d, Amount ingested by Ax and Ax *L.p*- or *L.b*-CFS treated flies over 10 trials during MAFE assay. n = 6/condition. e, Intestinal content measured through supplementing the diet of Conv, Ax, and *L.p*- or *L.b*-CFS treated Ax flies with blue food dye. Conv, n = 7; Ax, n = 13; *L.p* CFS, n = 7; *L.b* CFS, n = 10. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$, ** $P < 0.01$, **** P <

0.0001. Mann-Whitney *U* (a – b), One-way ANOVA and Bonferroni (c), or Kruskal-Wallis and Dunn's (d – e) post-hoc test was applied for statistical analysis. Data are representative of at least 2 independent trials for each experiment. *Dpt*, Diptericin; *Drs*, Drosomycin; *Cec*, Cecropin; *AttA*, Attacin-A; *Duox*, Dual Oxidase.

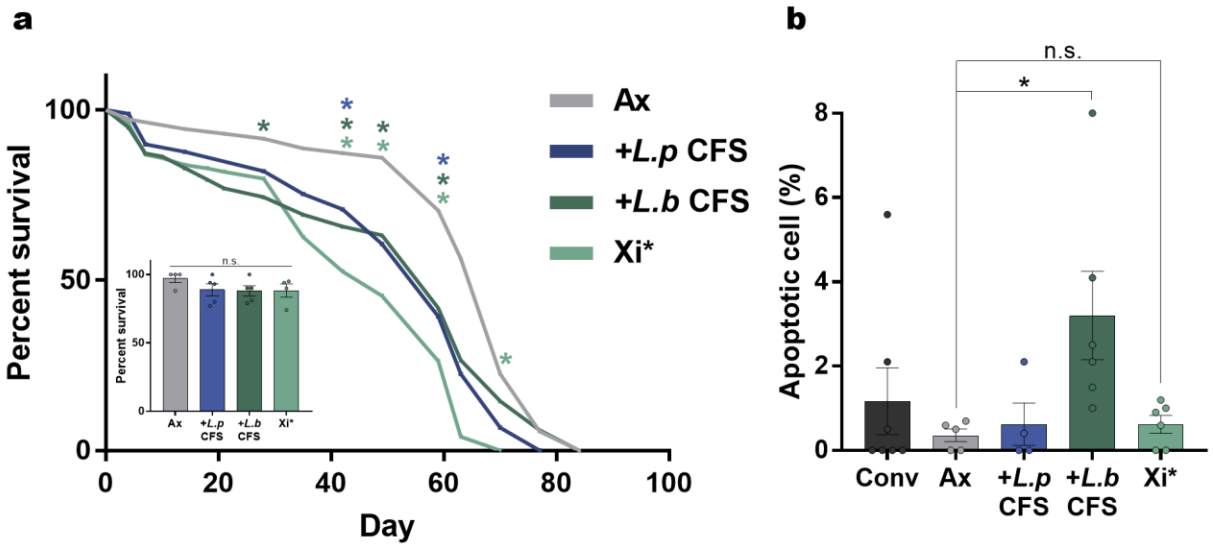

Figure 5. Lifespan and percentage of intestinal apoptotic cells under various microbial conditions.

a, Lifespan measurements for Ax and Ax treated with *L.p* CFS, *L.b* CFS, or Xi*. Asterisks above represent significance at the time point measured by Kruskal-Wallis and Dunn's post-hoc test. Inset image shows survival at day 7. Ax, n = 4 groups; *L.p* CFS, n = 5 groups; *L.b* CFS, n = 5 groups; Xi*, n = 4 groups. b, Percentage of apopotic cells in the intestine of Conv, Ax, and Ax treated with *L.p* CFS, *L.b* CFS, or Xi*. Error bars represent mean +/- S.E.M. Conv, n = 7; Ax, n = 5; *L.p* CFS, n = 4; *L.b* CFS, n = 6; Xi*, n = 6. * $P < 0.05$. Kruskal-Wallis and Dunn's post-hoc test was applied for statistical analysis. Data are representative of at least 2 independent trials for each experiment.

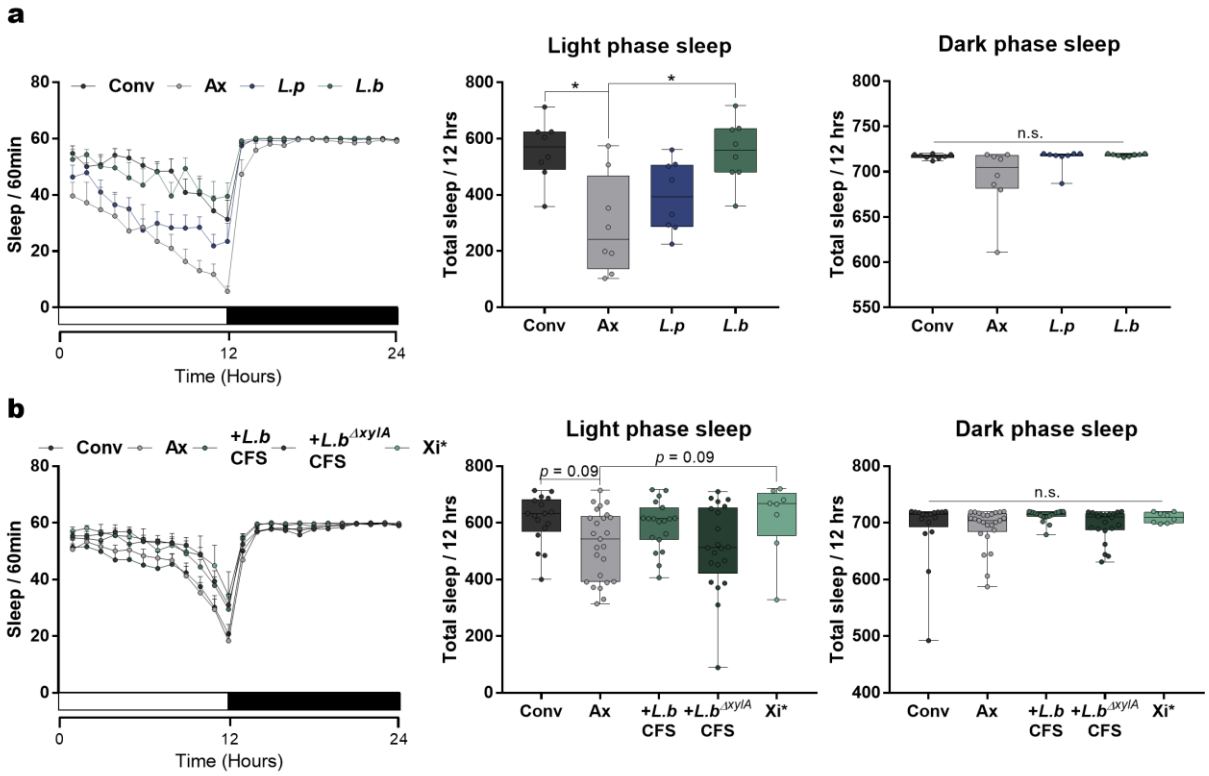

Figure 6. Sleep analysis for mono-colonized flies and flies administered with bacteria factors.

a, 24-hour sleep profiles of Conv, Ax, *L.p*-, and *L.b*-colonized virgin female Oregon^{R} flies with the number of sleep bouts in 60 min. time window and total sleep in the light or dark phase. n = 8/condition b, 24-hour sleep profiles of Conv, Ax, *L.b* CFS-, *L.b*$^{\Delta xylA}$ CFS-, and Xi* treated Ax virgin female Oregon^{R} flies with the number of sleep bouts in 60 min. time window and total sleep in the light or dark phase. Conv, n = 17; Ax, n = 25; *L.b* CFS-, n = 19; *L.b*$^{\Delta xylA}$ CFS-, n = 21; Xi*, n = 8. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$. Kruskal-Wallis and Dunn's post-hoc test for multiple comparisons was applied for statistical analysis. Data are representative of at least 2 independent trials for each experiment.

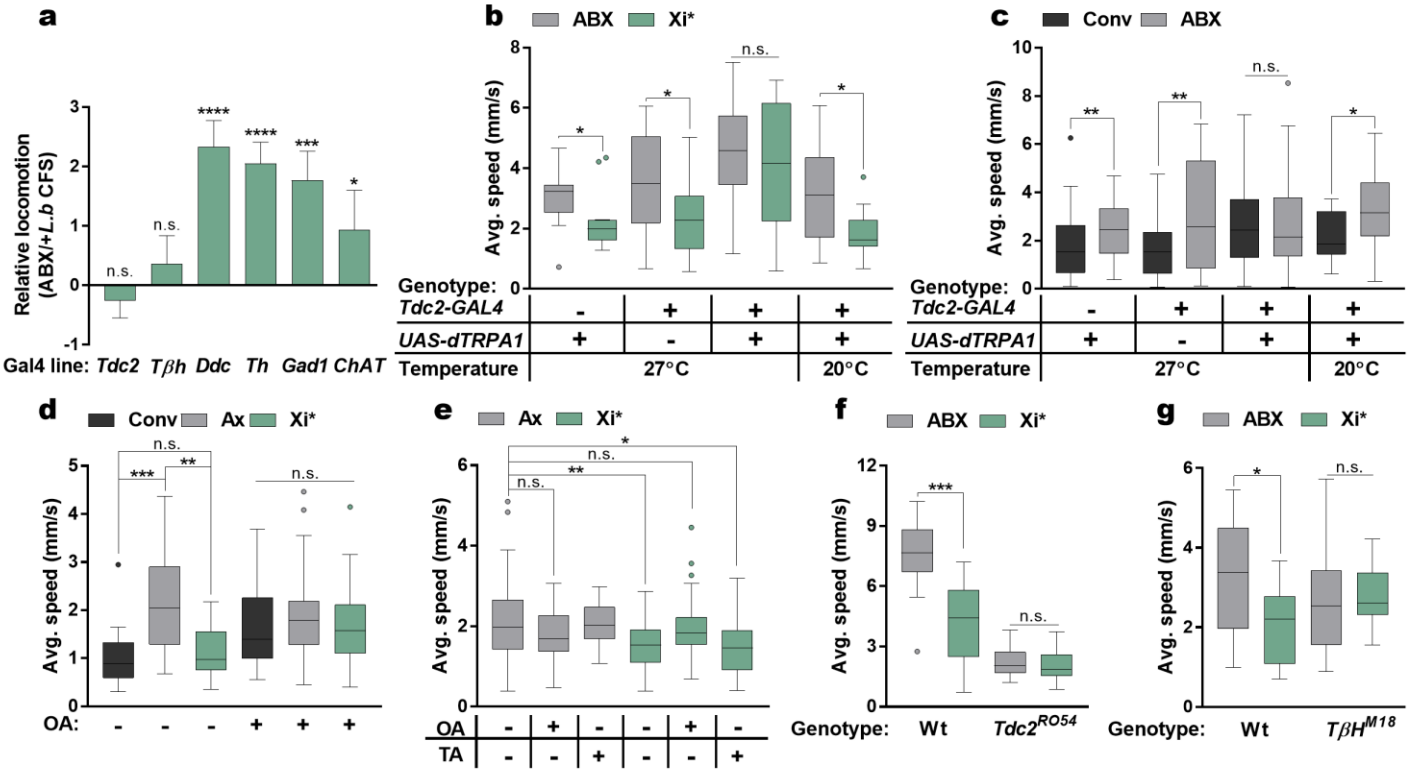

Figure 7. Octopamine mediates xylose isomerase-induced changes in locomotion.

a, Difference in average speed between flies previously treated with antibiotics and subsequently left untreated (ABX) or administered with *L.b* CFS calculated for each GAL4 line crossed with *UAS-dTRPA1* tested at 27˚C (for further details and graphs of each GAL4 line, see Fig. 8). Asterisks above bars represent statistical significance (Two-way ANOVA, Mann-Whitney *U* post-hoc tests) between untreated and *L.b* CFS-treated flies within each GAL4 line. Error bars represent mean +/- S.E.M. b, Average speed of flies previously treated with antibiotics and subsequently left untreated (ABX) or administered with *L.b* CFS with and without thermogenetic activation of the *Tdc2-GAL4* line. GAL4: ABX, n = 15; Xi*, n = 12; UAS: ABX, n = 23; Xi*, n = 23; GAL4>UAS (27°C): ABX, n = 14; Xi*, n = 12; GAL4>UAS (20°C): ABX, n = 12; Xi*, n = 15. c, Average speed of Conv flies and flies previously treated with antibiotics and subsequently left untreated (ABX) with and without thermogenetic activation of the *Tdc2-GAL4* line. GAL4: Conv, n = 57; ABX, n = 30; UAS: Conv, n = 59; ABX, n = 39; GAL4>UAS (27°C): Conv, n = 59; ABX, n = 39; GAL4>UAS (20°C): Conv, n = 15; ABX, n = 18. d, Average speed of Conv, Ax, and Xi*-treated Ax flies left untreated or supplemented with octopamine (OA, 10 mg/mL) daily for 3 days.

Conv, n = 13; Ax, n = 33; Xi*, n = 21; Conv+OA, n = 29; Ax+OA, n = 27; Xi*+OA, n = 32. Kruskal-Wallis and Dunn's post-hoc test for multiple comparisons was applied for statistical analysis. e, Average speed of Ax flies and Xi*-treated Ax flies either left untreated or supplemented with octopamine (OA, 10 mg/mL) or tyramine (TA, 10 mg/mL) daily for 3 days after the removal of Xi*. Ax, n = 58; Ax+OA, n = 13; Ax+TA, n = 10; Xi*, n = 54; Xi*+OA, n = 46; Xi*+TA, n = 27. Kruskal-Wallis and Dunn's post-hoc test for multiple comparisons was applied for statistical analysis. f, Average speed of wild-type background (w+, Wt) and Tdc2 null mutants ($Tdc2^{RO54}$) after treatment with antibiotics for 3 days following eclosion. All flies were subsequently placed on irradiated media without supplementation (ABX) or treated with Xi* daily for 3 days. Wt ABX, n = 12; Wt Xi*, n = 17; Tdc^{RO54} ABX, n = 19; Tdc^{RO54} Xi*, n = 17. Mann-Whitney *U* post-hoc test was applied for statistical analysis. g, Average speed of wild-type background (Canton-S, Wt) and Tβh null mutants ($T\beta H^{M18}$) after treatment with antibiotics for 3 days following eclosion. All flies were subsequently placed on irradiated media without supplementation (ABX) or treated with Xi* daily for 3 days. Wt ABX, n = 15; Wt Xi*, n = 15; $T\beta H^{M18}$ ABX, n = 11; $T\beta H^{M18}$ Xi*, n = 12. Mann-Whitney *U* post-hoc test was applied for statistical analysis. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. For experiments containing two independent variables (a – c), Two-way ANOVA was applied, and statistical significance was assessed. Mann-Whitney *U* post-hoc tests were subsequently performed. Data are representative of at least 2 independent trials for each experiment. *Tdc*, Tyrosine decarboxylase; *Tβh*, Tyramine beta-hydroxlyase; *Ddc*, DOPA decarboxylase; *Th*, Tyrosine hydroxylase; *Gad1*, Glutamate decarboxylase 1; *ChAT*, Choline acetyltransferase.

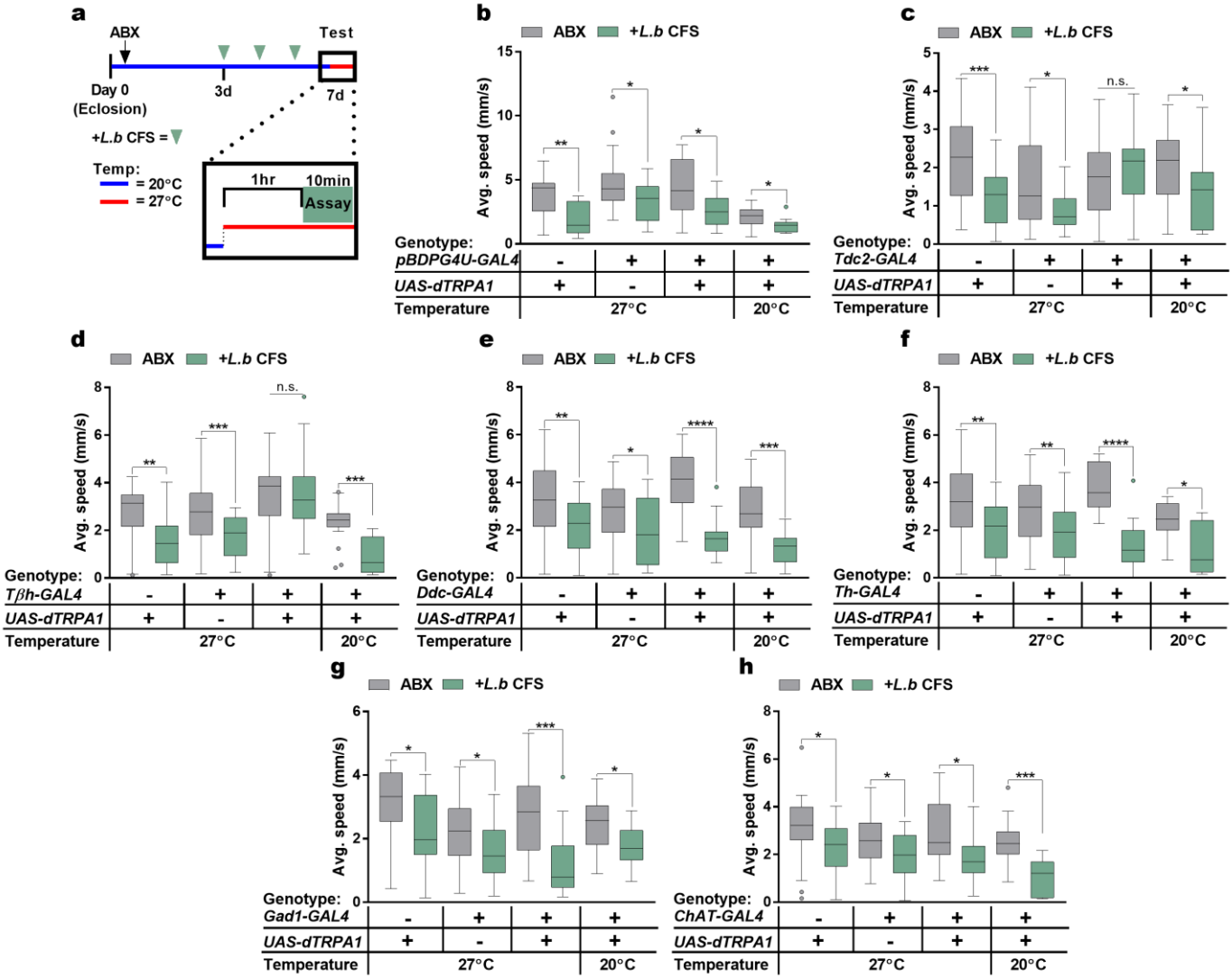

Figure 8. Thermogenetic activation of neuromodulator-GAL4 lines.

a, Experimental design in which Conv flies (Canton-S) were treated with antibiotics (ABX, black arrow) for 3 days following eclosion. All flies were subsequently placed on irradiated media either without supplementation or treated with *L.b* CFS (green arrows) for 3 days. 1 hr. prior to and during testing flies were either exposed to 27˚C (red line) to facilitate thermogenetic activation or kept at 20˚C (blue line). b – h, Average speed of flies previously treated with antibiotics and subsequently left untreated (ABX) or administered with *L.b* CFS for 3 days prior to testing. n = 24/condition. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from

bottom to top. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Two-way ANOVA was applied to test for the effect of two independent variables, and statistical significance was assessed for both variables. Mann-Whitney *U* post hoc tests were subsequently performed. Data are representative of at least 2 independent trials for each experiment.

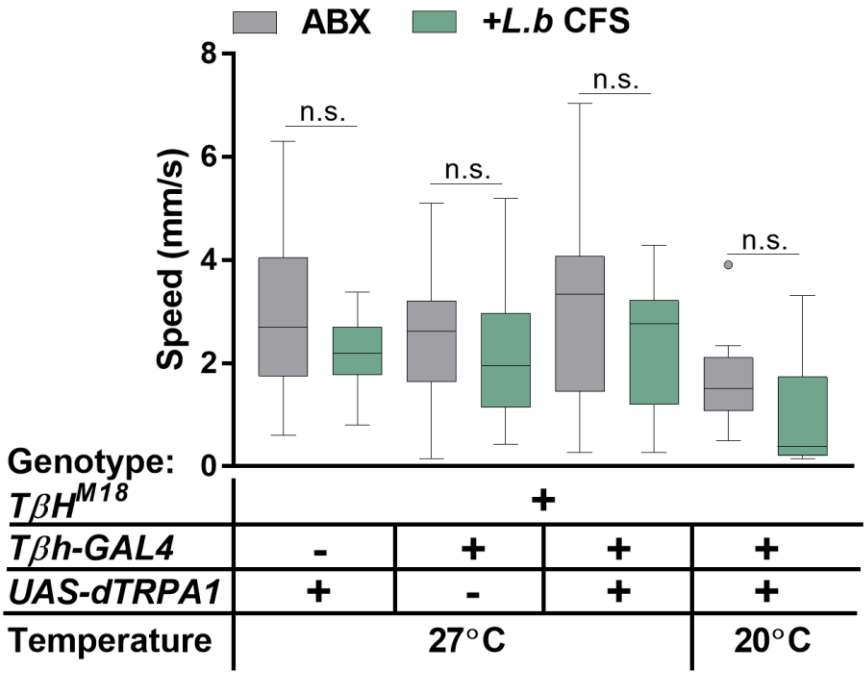

Figure 9. Activation of octopaminergic neurons in flies carrying a null allele for Tβh (*TβH*^{M18}).

Average speed of flies previously treated with antibiotics and subsequently left untreated (ABX) or administered with *L.b* CFS for 3 days prior to testing. n = 24/condition. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. Two-way ANOVA was applied to test for the effect of two independent variables, and statistical significance was assessed for both variables. Mann-Whitney *U* post hoc tests were subsequently performed. Data are representative of at least 2 independent trials.

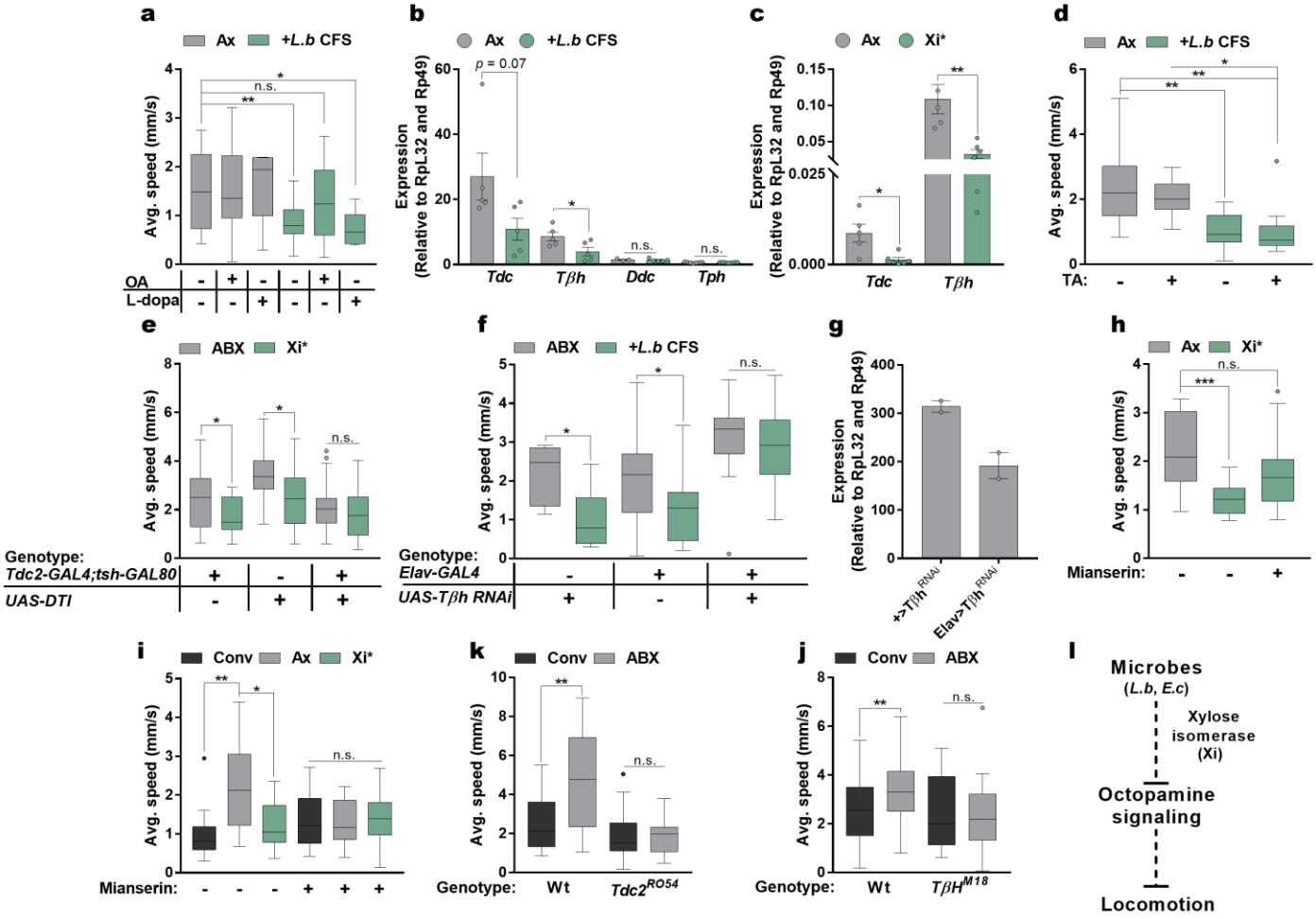

Figure 10. Octopamine mediates *L. brevis*- and xylose isomerase-induced changes in locomotion.

a, Average speed of Ax and *L.b* CFS-treated Ax flies left untreated or supplemented with octopamine (OA, 10 mg/mL) or L-dopa (1 mg/mL) for 3 days. n = 36/condition. b, qRT-PCR for transcripts from heads of Ax and *L.b* CFS-treated Ax flies. Error bars represent mean +/- S.E.M. n = 6 samples/condition. c, qRT-PCR for transcripts from heads of Ax or Ax Xi*-treated flies. Error bars represent mean +/- S.E.M. Ax, n = 5 samples; Xi*, n = 6 samples. d, Average speed of Ax and *L.b* CFS-treated Ax flies left untreated or supplemented with tyramine (TA, 10 mg/mL) for 3 days. n = 12/condition. e, Average speed of control lines and flies expressing *DTI* in octopaminergic and tyraminergic neurons outside of the ventral nerve cord. All flies were previously treated with antibiotics and subsequently left untreated (ABX) or administered with Xi* for 3 days prior to testing. n = 24/condition. f, Average speed of control lines and flies expressing Tβh RNAi in all neurons. All flies were previously treated with antibiotics and

subsequently left untreated (ABX) or administered with *L.b* CFS for 3 days prior to testing. n = 12/condition. g, Tβh mRNA measured from heads of flies previously treated with antibiotics. Error bars represent range. n = 2 samples/condition. h, Average speed of Ax and Xi*-treated Ax flies left untreated or supplemented with mianserin (2 mg/mL) for 3 days. n =14/condition. i, Average speed of Conv, Ax, and Xi*-treated Ax flies left untreated or supplemented with mianserin (2 mg/mL) for 3 days. n = 30/condition. k, Average speed of wild-type background (w+, Wt) and Tdc2 null mutants ($Tdc2^{RO54}$) either left untreated or after treatment with antibiotics for 3 days following eclosion. Wt Conv, n = 13; Wt ABX, n = 21; Tdc^{RO54} Conv, n = 28; Tdc^{RO54} ABX, n = 34. Mann-Whitney *U* post-hoc test was applied for statistical analysis. g, Average speed of wild-type background (Canton-S, Wt) and Tβh null mutants ($T\beta H^{M18}$) either left untreated or after treatment with antibiotics for 3 days following eclosion. Wt Conv, n = 38; Wt ABX, n = 42; $T\beta H^{M18}$ Conv, n = 26; $T\beta H^{M18}$ ABX, n = 33. Mann-Whitney *U* post-hoc test was applied for statistical analysis. l, Model of bacterial modulation of host locomotion. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Kruskal-Wallis and Dunn's (a, d, h – i), unpaired Student's t-test (b – c and g), or Mann-Whitney U (e – f and j – k) post-hoc test was applied for statistical analysis. Data are representative of at least 2 independent trials for each experiment. *Tdc*, Tyrosine decarboxylase; *Tβh*, Tyramine beta-hydroxlyase; *Ddc*, DOPA decarboxylase; *Tph*, Tryptophan hydroxylase.

4.7 METHODS

Fly Stocks and Rearing

We obtained Canton-S (#64349), *Imd-/-* (#55711), *Ti-/-* (#30652), *UAS-dTrpA1* (#26264), *Tdc2-GAL4* (#52243), *Tβh-GAL4* (#48332), *Th-GAL4* (#8488), *Ddc-GAL4* (#7009), *Gad1-GAL4* (#51630), *ChAT-GAL4* (#60317), *Elav-GAL4* (#46655), *UAS-TβhRNAi* (#27667), *UAS-DTI* (#25039), and *pBDPG4U-GAL4* (#68384) lines from Bloomington *Drosophila* Stock Center at Indiana University. Other fly stocks used were Oregon^{R} (kindly provided by A. A. Aravin and K. Fejes Tóth), *TβH^{M18}* (kindly provided by M. H. Dickinson)[36], *Tdc2^{R054}*, and *tsh-GAL80* (both kindly provided by D. J. Anderson)[37,38]. To minimize the effect of genetic background on behaviors, mutant fly lines were outcrossed for at least three generations onto a wild-type background.

Flies were cultured at 25˚C and 60% humidity on a 12-hr. light:12-hr. dark cycle and kept in vials containing fresh fly media made at California Institute of Technology consisting of cornmeal, yeast, molasses, agar, and p-hydroxy-benzoic acid methyl ester. Other dietary compositions used were created through altering this standard diet or the Nutri-Fly "German Food" Formula (Genesee Scientific) and were calculated using previously published nutritional data[39]. Axenic flies were generated using standard methods[10,40–42]. Briefly, embryos from conventional flies were washed in bleach, ethanol, and sterile PBS before being cultivated on fresh irradiated media[40]. Axenic stocks were maintained through the application of an irradiated diet supplemented with antibiotics (500 μg/ml ampicillin, Putney; 50 μg/ml tetracycline, Sigma; 200 μg/ml rifamycin, Sigma) for at least one generation. For experiments, virgin female flies were collected shortly after eclosion and placed at random into vials (10 – 15 flies per vial) containing irradiated media without antibiotics. Vials were changed every 3 – 4 days using sterile methods. The antibiotic supplemented diet was applied to conventional flies shortly after eclosion to generate antibiotic-treated (ABX) flies. Both antibiotic-treated and axenic flies were tested for contaminants through plating animal lysates on Man, Rogosa, and Sharpe (MRS, BD Biosciences); Mannitol (25 g/L Mannitol, Sigma; 5 g/L Yeast extract, BD Biosciences; 3 g/L Peptone, BD Biosciences); and Luria-Bertani (LB, BD Biosciences) nutrient agar plates.

Bacterial Supernatant Preparations

Cell-free supernatants (CFS) of specified bacterial strains were harvested from bacterial cultures (OD600 = 1.0) by centrifuging at 13,000 x g for 10 min. and subsequent filtration through a 0.22-µm sterile filter (Millipore). CFS was dialyzed in MilliQ water with a 3.5 kDa membrane (Thermo Scientific) overnight at 4˚C to generate *L.b* CFS and *L.p* CFS samples. Each of these treatments were supplied daily through application to the fly media (40 µL) for 6 days following eclosion.

Production of His-tagged proteins (Xi* and Ai*)

An expression plasmid for the production of His-tagged xylose isomerase from L.b, here termed as Xi*, was constructed by amplification of its gene and cloning the resulting PCR product in the pQE30 cloning vector (Qiagen) using SLIC ligation. The following primer sequences were used for the construct: 5'-CGCATCACCATCACCATCACGGATCTTACTTGCTCAACGTATCGATGATGTAA-3' and 5'-GGGGTACCGAGCTCGCATGCGGATCATGACTGAAGAATACTGGAAAGGC-3'. Conformation of the resulting plasmid was verified and transformed into E. coli (Turbo, NEB). This strain was then grown in LB containing ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL) with shaking at 220 rpm at 37˚C for 1 hr. before the addition of 0.1 mM IPTG. After 4 hrs. of shaking at 220 rpm at 37˚C, cells were pelleted and lysed using lysozyme (Sigma) and bead beating with matrix B beads (MP Biomedicals) for 45 sec. Supernatant was collected after centrifugation and the Xi* protein purified through metal affinity purification under native conditions using HisPur™ Ni-NTA Spin Columns (Thermo Scientific). Protein purification was verified through western blot using an Anti-6X His tag® antibody (Abcam) and quantified using a Pierce BCA Protein Assay kit (Thermo Scientific) after which protein was stored at -20˚C. Expression and purification of His-tagged L-arabinose isomerase from *L.b*, here termed as Ai*, was performed under the exact same conditions and the following primer sequences were used for the construct: 5'-GGGGTACCGAGCTCGCATGCGGATCATGTTATCAGTTCCAGATTATGAATTTTGG-3' and 5'-CGCATCACCATCACCATCACGGATCCTTACTTGATGAACGCCTTTGTCAT-3'.

Drug treatments

Axenic flies were either left untreated or administered with *L.b* CFS or Xi* for 3 days after eclosion. After switching to new irradiated fly media, groups of axenic flies were treated through application to the fly media (40 µL) with octopamine (OA, 10 mg/mL, Sigma), tyramine (TA, 10 mg/mL, Sigma), L-dopa (1 mg/mL, Sigma), or mianserin (2 mg/mL) every day for 3 days before testing, similar to previously published methods[16,17,22].

Locomotion Assays

Locomotor behavior was assayed through three previously established methods: the Drosophila Activity Monitoring System (DAMS, Trikinetics)[43,44], video-assisted tracking[45–47], and gait analysis[48].

Activity measurements

7-day-old individual female flies were cooled on ice for 1 min. and transferred into individual vials (25 x 95 mm) containing standard irradiated media. Tubes were then inserted and secured into Drosophila activity monitors (DAMS, Trikinetics) and kept in a fly incubator held at 25˚C. Flies were allowed to acclimate to the new environment for 1 day before testing and midline crossing was sampled every minute. Average daily activity was calculated from the 2 days tested and actograms were generated using ActogramJ[44]. Sleep was defined as a 5 min. bout of inactivity as previously described[49].

Video-assisted tracking

Individual female flies were cooled on ice for 1-2 min. before being introduced under sterile conditions into autoclaved arenas (3.5 cm diameter wells), which allowed free movement but restricted flight. After a 1 hr. acclimation period, arenas were placed onto a light box and recorded from above for a period of 10 min. at 30 frames per sec. All testing took place between ZT 0 and ZT 3 (ZT, Zeitgeber time; lights are turned on at ZT 0 and turned off at ZT 12) and both acclimation and testing occurred at 25˚C unless otherwise stated. Videos were processed using Ethovision software or the Caltech FlyTracker (http://www.vision.caltech.edu/Tools/FlyTracker/).

Bout analysis was performed using custom python scripts (available upon request). The velocity curve was smoothed from the acquired video at 30 frames per sec. using a 15 sec. moving average window. A minimum walking speed of 0.25 mm/s was given below which flies were moving but not walking ('pause bouts') and above which they were designated as walking ('walking bouts'). Lengths were measured as time between bout onset and offset.

Feeding Assays

Female flies were collected at the same time as described for Locomotor Assays. Flies were transferred regularly onto fresh food until day 7, upon which the flies were starved for 2 hrs. and subsequently transferred for 30 min. to an irradiated standard fly media dyed with FD&C Blue no. 1 (Sigma) at a final concentration of 0.5 g dye per 100 g food. Flies were allowed to feed on the food (3-4 biological replicates and 7 flies per replicate) at 25˚C after which they were decapitated and their bodies collected. Each replicate was homogenized in 150 µL of PBS/0.05% Triton X-100 and centrifuged at 5,000 x g for 1 min. to remove debris. Absorbance for all groups was measured together at 630 nm and the amount of food consumed was estimated from a standard curve of the same dye solution. The MAFE assay was performed as described previously[34,50]. Briefly, individual flies were introduced into a 200 µL pipette tip, which was cut to expose the proboscis. Flies were first water satiated and presented with 100 mM sucrose delivered in a fine graduated capillary (VWR). After flies were unresponsive to 10 food stimuli, the assay was terminated and the total volume of food was calculated.

Measurement of life span

Adult female flies were transferred under sterile conditions to irradiated fly media every 4 – 5 days. Survival in 3 or more independent cohorts containing 15 – 25 flies each was monitored over time.

Apoptosis assay

Midguts from 7-day-old female flies were dissected in PBS containing 0.1% Triton X-100 and the apoptosis assay was performed as previously described[10,41]. The percentage of apoptotic

cells was determined by dividing the number of apoptotic cells by the total number of cells in each section and multiplying by 100.

RNA isolation and quantitative real-time PCR

Heads (20 flies per sample) or decapitated bodies (5 flies per sample) were dissected on ice and immediately processed using an ArcturusTM PicoPureTM RNA isolation kit (Applied Biosystems) or a standard TRIzolTM-Chloroform protocol (ThermoFisher). 1 μg of RNA was reverse transcribed using iScript cDNA Synthesis Kit, according to manufacturer's protocol (Bio-Rad) and diluted to 10 ng/μl based on the input concentration of total RNA.

Previously published primer pairs were used to target immune-related gene transcripts[10,51]. Other primer sequences used include *Tdc* (F: GGTCTGCCGGACCACTTTC, R: CACTCCGATGCGGAAGTCTG), *Tβh* (F: GCTTATCCGACACAAAGCTGC, R: GAAAGCATTCTGCAAGTGGAA), *Ddc* (F: TGGGATGAGCACACCATCTTG, R: GTAGAAGGGAATCAAACCCTCG), *Tph* (F: TGTTTTCGCCCAAGGATTCGT, R: CACCAGGTTTATGTCATGCTTCT). All primers were synthesized by Integrated DNA Technologies. Real-time PCR for the house-keeping genes *Rp49* and *RpL32* were used to ensure that input RNA was equal among all samples. Real-time PCR was performed on cDNA using an ABI PRISM 7900 HT system (ThermoFisher) according to the manufacturer's instructions.

Statistical Analysis

All statistical analysis was performed using Prism Software (GraphPad, version 7). Sample size was based on data from pilot experiments and experimenters were not blinded as almost all data acquisition and analysis was automated. To analyze two sets of data following a normal distribution, we used an unpaired two-sided Student's t-test. If the data did not follow a normal distribution, a non-parametric two-sided Mann-Whitney *U* test was used. Comparisons among 3 or more data sets and comparisons with more than one variant were analyzed using One-way ANOVA or Two-way ANOVA, respectively. If statistical significance was identified for the variables tested, then a Dunn's post-hoc test was performed. ANOVAs on normally distributed data were followed by a Bonferroni post-hoc test to determine significant differences between genotypes. Boxplots: lower and upper whiskers represent 1.5 interquartile range of the lower and

upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile, from bottom to top. Bar graphs are presented as mean values +/- S.E.M.

Chapter 5

EXAMINING CONSERVATION OF MICROBIAL EFFECTS ON LOCOMOTION

Schretter, C. E. and S. K. Mazmanian.

This chapter was used as preliminary data for “Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson’s Disease.”

5.1 INTRODUCTION

The nervous system and behavior of mammals are also influenced by their gut microbiome[1–4]. Similar to our experiments in *D. melanogaster*, mice that lack a microbiota (germ-free, GF) exhibit increased speed compared to conventional or specific pathogen free (SPF) controls[5]. Further, GF mice exhibit elevated locomotion under both western and low-fat diets[6]. Colonization with specific bacterial species can also decrease locomotor behavior in mice[7]. In addition to behavior, higher striatal turnover of norepinephrine, dopamine, and serotonin are found in GF animals compared to their SPF counterparts[5]. Many of these studies thereby suggest that the microbial-dependent locomotor phenotypes found in *Drosophila* could be conserved in mice. However, differences in their development and the stability of the microbiota between the two organisms could lead to divergent effects on host behavior and mediation through dissimilar neuronal pathways.

5.2 MICROBIAL MODULATION OF MOTOR-RELATED BRAIN REGIONS AND BEHAVIOR IN MICE

As previous research identified changes in locomotion depending on microbial status, we examined SPF, GF, and antibiotic-treated SPF mice (ABX) in an open field test. While GF mice exhibited increased speed compared to SPF mice, ABX animals displayed lower speeds than GF (Fig. 1). Similar decreases in locomotion have been reported in C57BL/6 mice upon antibiotic treatment; however, this appears to differ based on the antibiotics used and host genetics[8,9]. Due to this difference in locomotion between SPF and GF animals, we next explored if there were any changes in neuronal populations related to motor behavior, including the substantia nigra pars compacta (SNc) and the striatum. Compared to SPF mice, GF animals had a 60% and 35.8% reduction in the number of cells positive for a marker of dopaminergic neurons, tyrosine hydroxylase (TH), and total neurons in the SNc, respectively (Fig. 2 – 3). Bacterial colonization of GF mice (EX-GF) increased the number of TH-positive cells to levels similar to SPF mice (Fig. 4). In the striatum, GF mice exhibit a decrease in the optical density of TH staining in the dorsomedial region, which is a target for projections from the SNc (Fig. 5).

A dramatic loss of dopaminergic neurons in the SNc as well as a resulting loss of dopamine in the striatum has been implicated in the cognitive motor deficits seen in Parkinson's Disease (PD)[10]. Due to microbial involvement in motor behavior and enteric contributions to the progression of PD[11,12], we examined the potential role of the gut microbiota in a mouse model of PD. Epidemiological studies have correlated pesticide exposure with the development of certain cases of PD[13,14]. Rotenone is common pesticide that inhibits mitochondrial Complex I and produces a loss of striatal dopaminergic terminals followed by progressive degeneration of dopaminergic neurons in the SNc[15–17]. While rotenone has traditionally been given systemically, oral treatment in mice results in specific nigrostriatal dopaminergic neurodegeneration, motor deficits, and the up-regulation of α-synuclein in the surviving dopaminergic neurons[18,19]. Moreover, oral treatment prompts α-synuclein inclusions to form in the ENS and follow a retrograde progression to the brain, as well as colonic motility deficits[18–20]. Due to these connections and our previous data, we employed this model of PD to examine if removal of the gut microbiota alters its progression. There were no significant changes in weight across the groups, and rotenone-treated SPF and GF animals exhibited no differences in speed (Fig. 6 – 8).

Specific populations of neurons in the myenteric plexus of the ENS, such as dopaminergic and cholinergic neurons, have been linked to the regulation of gut motility and PD[12]. While we found no differences in the total number of neurons in the proximal small intestine, GF mice display significantly lower numbers of TH-positive cells in this region (Fig. 9a – b). Additionally, mRNA expression of choline actyltransferase (ChAT), an enzyme involved in acetylcholine synthesis, is reduced in the proximal small intestine of GF animals (Fig. 9c). This data indicates that the gut microbiota may affect these neurotransmitters in the gastrointestinal tract, which have been implicated in the pathogenesis of PD.

5.3 CONCLUSION

Herein, we found that mice lacking a microbiota exhibit increased locomotor behavior along with decreases in TH-positive neurons in the SNc and TH staining in the dorsomedial striatum. TH-positive cells and ChAT expression is also reduced in the proximal small intestine of GF mice. Further, administration of rotenone appeared to diminish behavioral differences between GF and SPF animals. While the gut microbiota may play a role in locomotor behavior as well as

related regions and disorders in mammals, more research needs to be performed in order to evaluate these changes in the context of our previous results in *Drosophila*.

Additional recent research has shown that the microbiota contributes to the progression of certain mouse models of PD. In the rotenone model, changes in the microbiota precede intestinal and motor deficits[21]. Mice overexpressing wild-type human α-synuclein under the Thy1 promoter develop many features of sporadic PD, including: progressive changes in dopamine release and striatal content, α-synuclein pathology, deficits in motor and non-motor functions, inflammation, in addition to biochemical and molecular alterations similar to those observed in PD[17,22,23]. Research conducted in the Mazmanian laboratory after our experiments found that the microbiota is required for motor deficits in this mouse model of PD[24]. This work further suggests that the gut microbiota contributes to the progression of certain cases of PD, which may indicate its involvement in mammalian motor behavior during steady-state conditions. However, there are conflicting results upon antibiotic-treatment in addition to developmental and ecological differences between *Drosophila* and mice. These discrepancies between the model organisms support the need for more in-depth experiments evaluating if microbial modulation of locomotion remains consistent across phylogenies and if comparable pathways are utilized.

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5.4 FIGURES

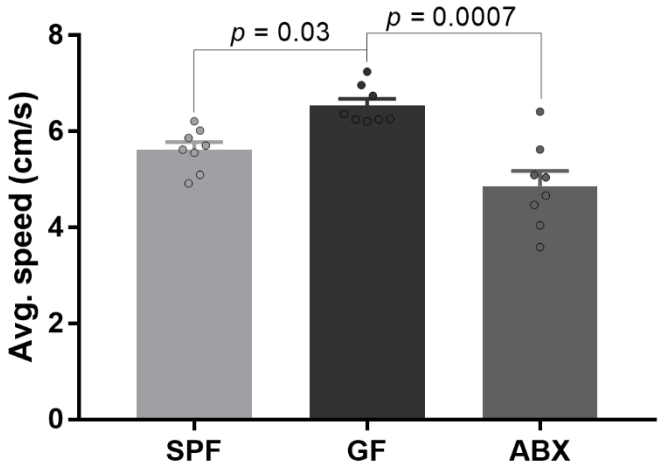

Figure 1. GF mice display increased speed compared to SPF and antibiotic-treated mice.

4-month-old SPF, GF, and antibiotic-treated SPF (ABX) mice were recorded over 10 min. and average speed was calculated by Ethovision software. n = 8/condition. Kruskal-Wallis and Dunn's post-hoc test was applied for statistical analysis.

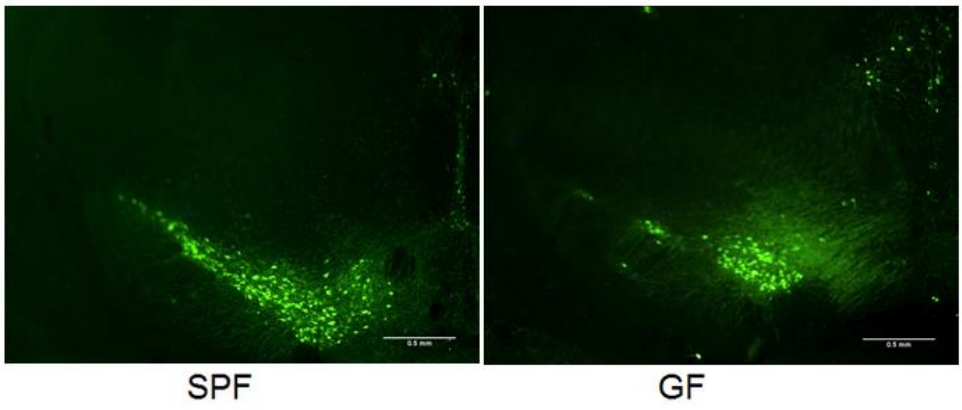

Figure 2. GF mice have reduced TH+ cells in the SNc compared to SPF controls.

Representative images at 4x of TH+ (green) staining in the SNc of SPF (6-month-old female) and GF (6-month-old female) C57Bl/6 mice.

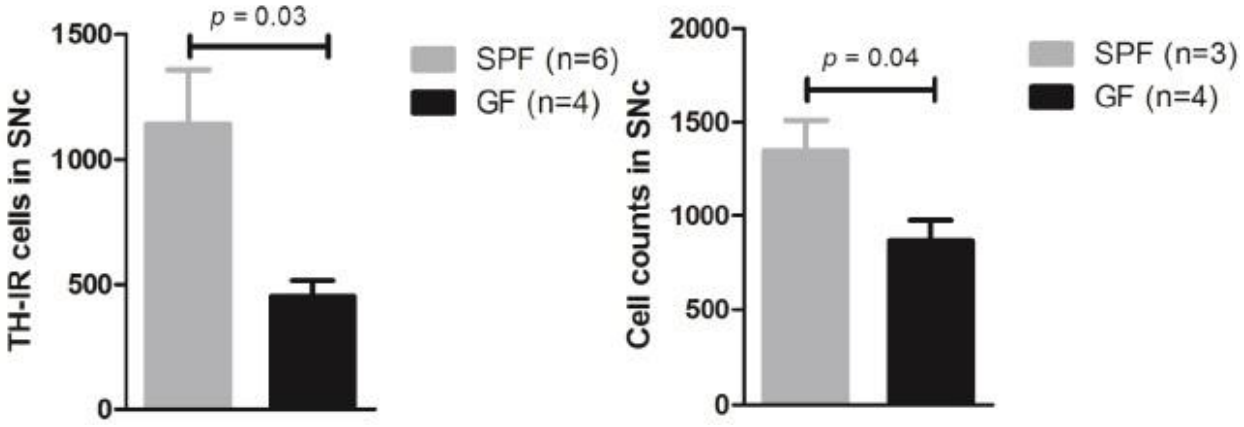

Figure 3. Lower TH+ cells and cell counts in the SNc of GF mice compared to SPF controls.

Brains from 3 – 6 month-old female SPF (black, n=4) and GF (gray, n=7) were stained using anti-TH and goat anti-rabbit Alexa Fluor 488 antibodies. Images were taken using a fluorescent microscope and analyzed using Image-J. Cell counts were performed after Nissl staining and were subsequently analyzed with Image-J. There were no significant differences between counts taken from 3-month versus 6-month-old animals in GF or SPF animals (data not shown). A two-tailed t-test test was applied for statistical analysis.

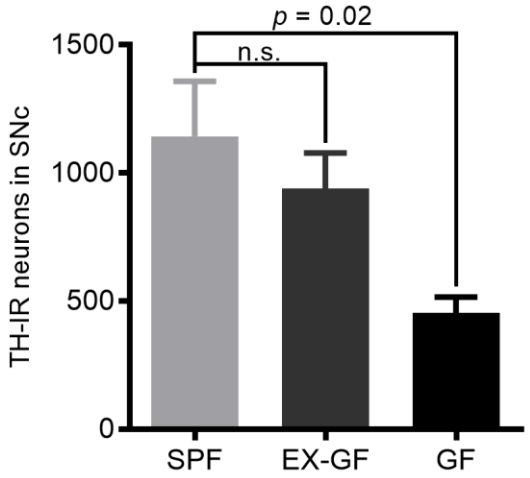

Figure 4. Reduced TH+ cell counts are reversed through colonization.

Brains from 4-month-old female SPF (n=6), EX-GF (n=4), and GF (n=4) were stained using anti-TH and goat anti-rabbit Alexa Fluor 488 antibodies. Images were taken using a fluorescent microscope and counts were performed by a blinded experimenter using Image-J. Kruskal-Wallis and Dunn's post-hoc test was applied for statistical analysis.

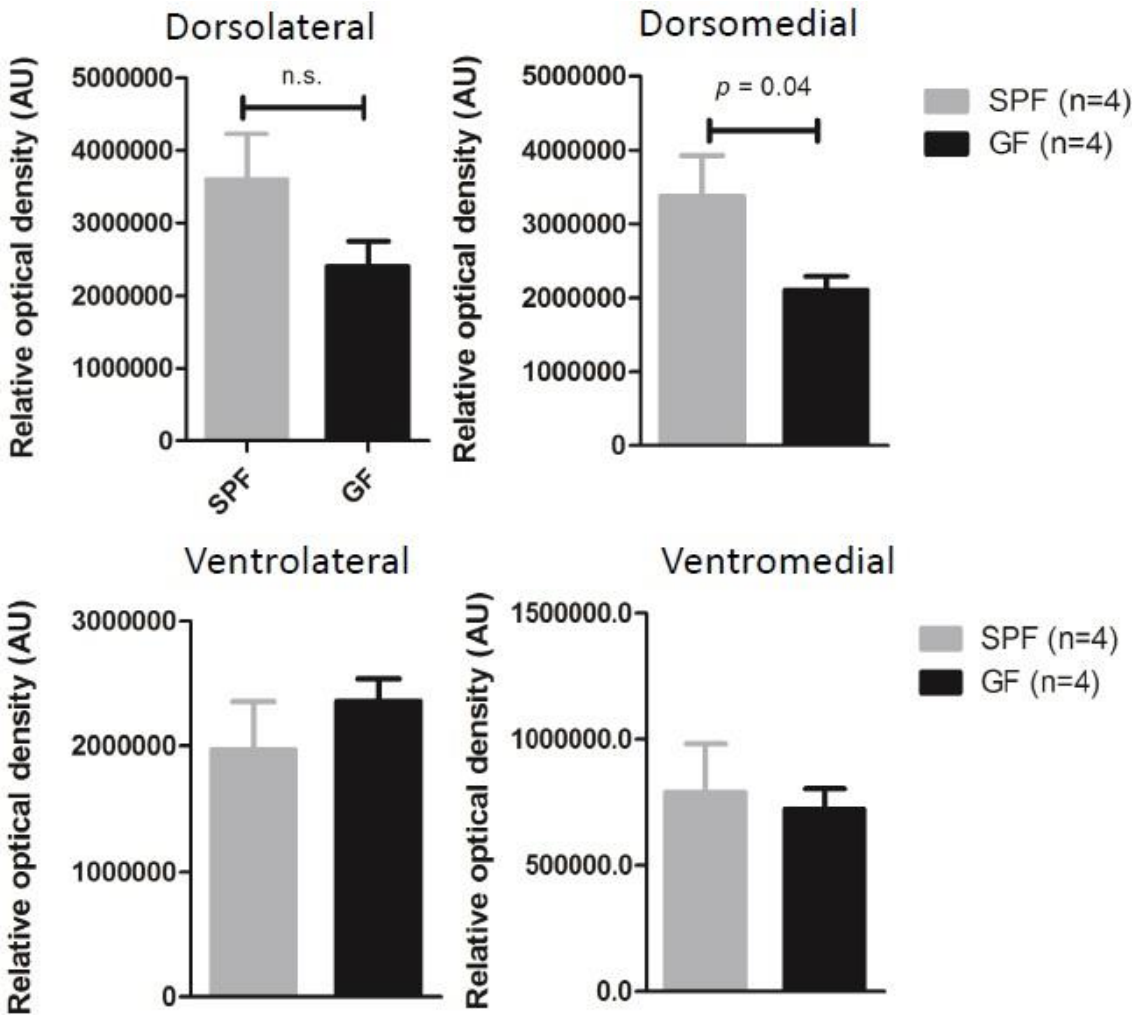

Figure 5. TH+ optical density in the dorsomedial striatum of GF mice is decreased compared to SPF animals.

Analysis was performed on slices from 4-month-old male SPF (gray, n=4) and GF (black, n=4) using Image-J. No differences were observed in the ventral striatum. A two-tailed t-test was applied for statistical analysis.

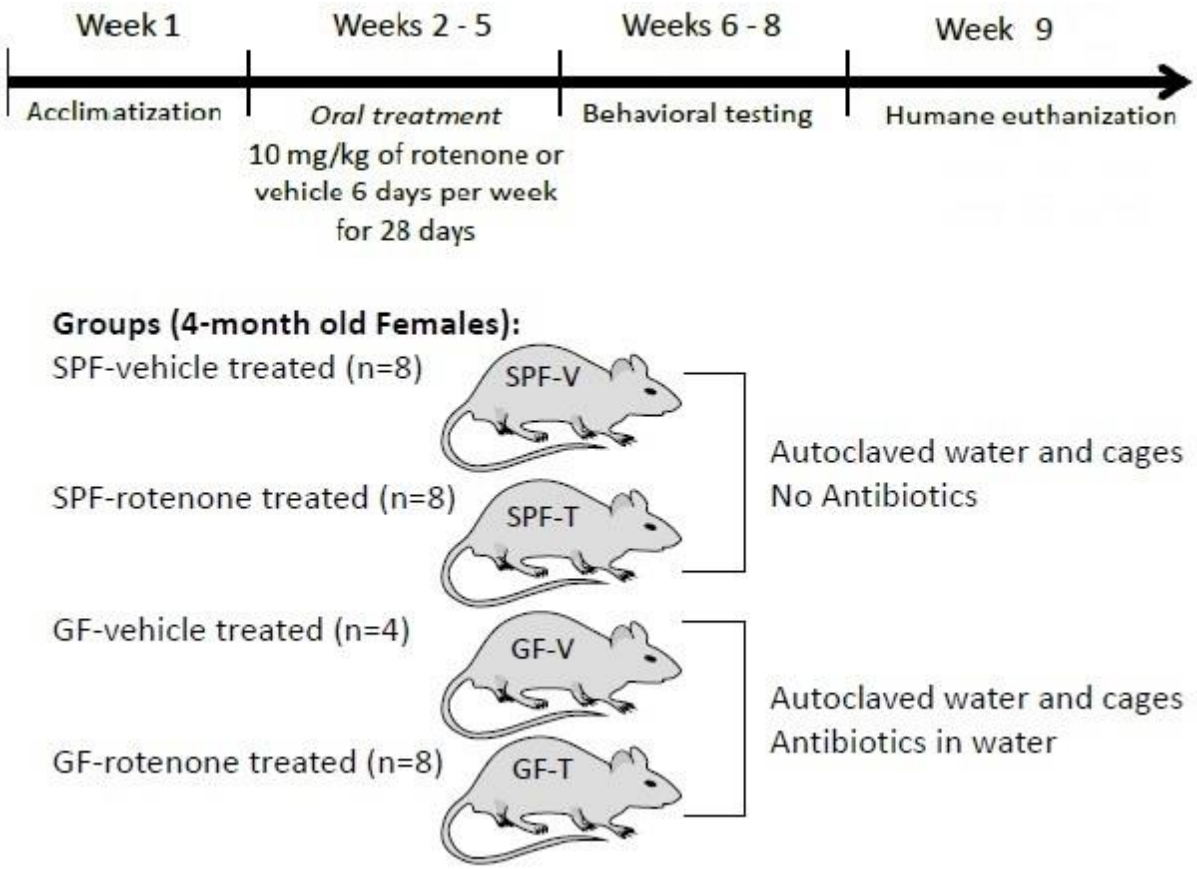

Figure 6. Experimental design for examining microbial involvement in a mouse model of Parkinson's.

4-month-old female specific pathogen free (SPF) or germ free (GF) mice were orally treated via gavage with rotenone (10 mg/kg) for 28 days under sterile conditions. Following treatment, open field tests were performed.

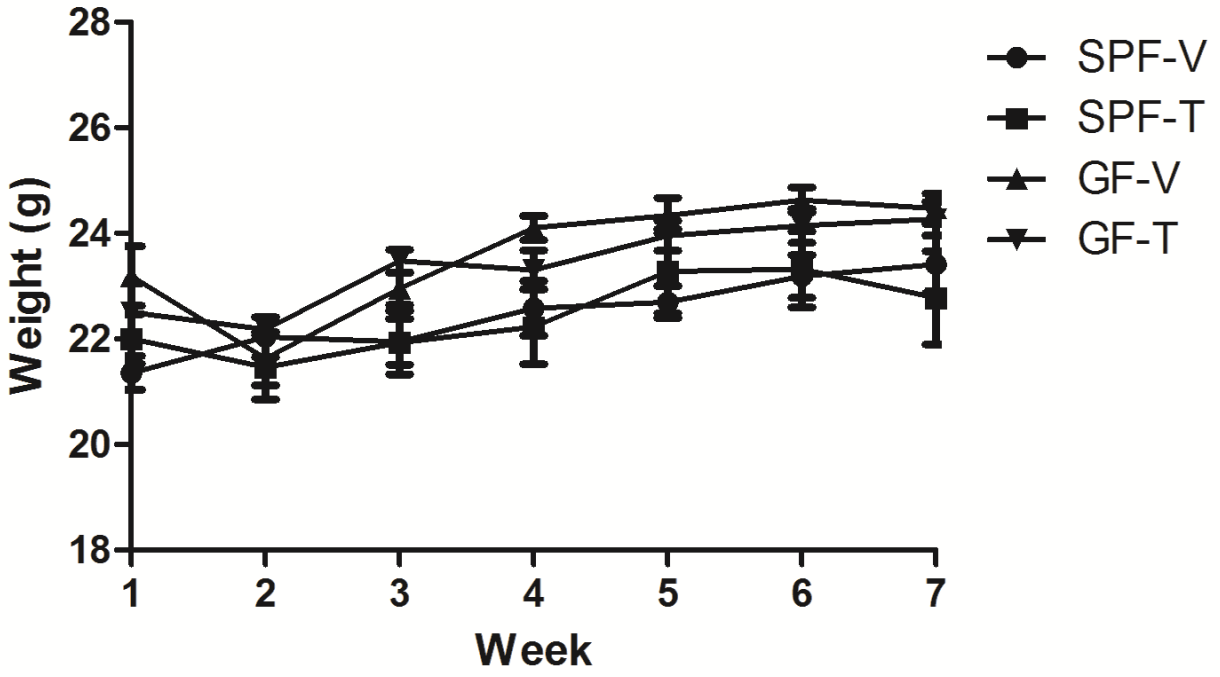

Figure 7. Weight of mice over the course of the experiment.

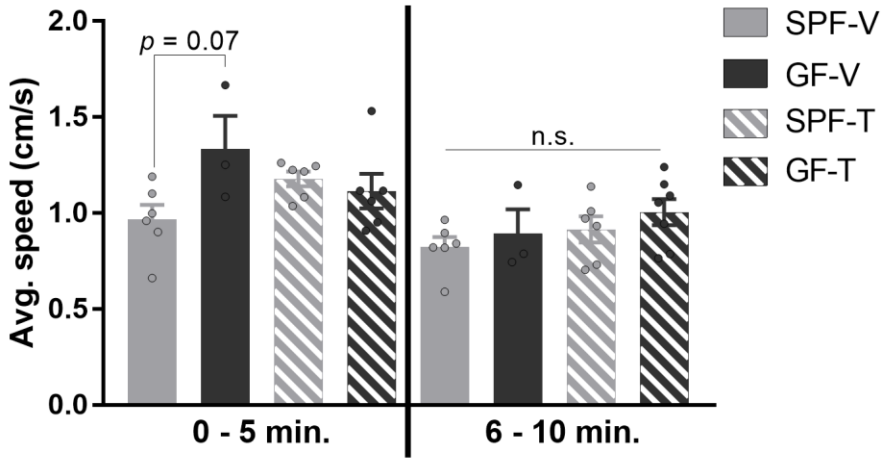

Figure 8. General locomotion in mice with varying microbial status and rotenone treatment.

Naïve and rotenone-treated SPF and GF mice were recorded for 10 min. and average velocity was calculated by Ethovision software. SPF vehicle-treated (SPF-V), n = 6; GF vehicle-treated (GF-V), n = 3; SPF rotenone-treated (SPF-T), n = 6; GF rotenone-treated (GF-T), n = 7. Kruskal-Wallis and Dunn's post-hoc test was applied for statistical analysis.

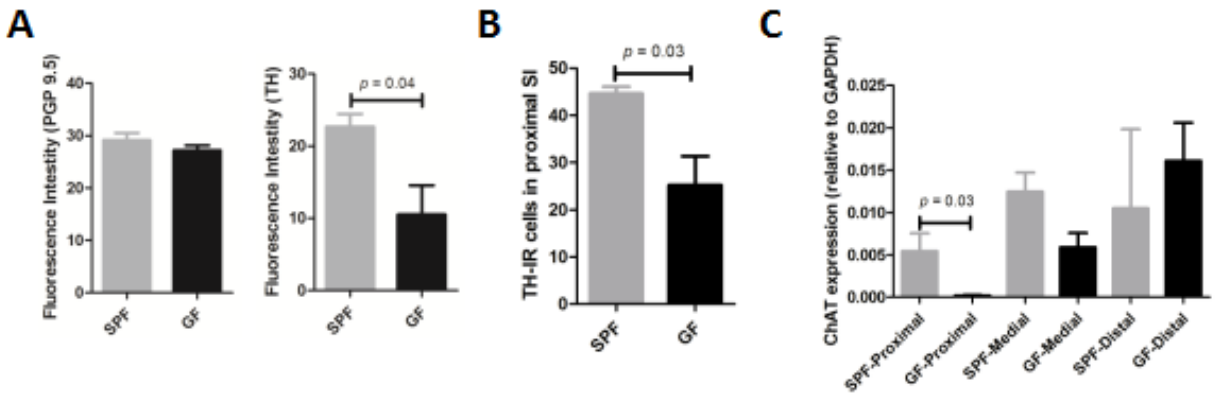

Figure 9. Staining for TH and ChAT expression is decreased in the proximal small intestine of GF mice.

A – B, Small intestine of 3-month-old male SPF (gray, n = 3) and GF (black, n = 3) mice stained with anti-TH or PGP 9.5. Fluorescence intensity and cell counts were measured using Image J. C, Whole small intestinal tissue from 3-month-old male SPF (gray) and GF (black) animals (n = 3-4 for proximal and medial; n = 2 for distal) were dissected, luminal contents flushed, and mRNA extracted using the Qiagen RNeasy mini kit. Expression was tested in triplicates and normalized using the housekeeping gene, GAPDH. A two-tailed t-test was applied for statistical analysis.

5.5 METHODS

Animals

C57BL/6 mice were purchased from Taconic Farms. 3- to 6-month-old age- and sex-matched mice were used. All mice were group housed (2–5 mice per cage) with a 13 hr. light/11 hr. dark cycle (lights on at 06:00) at 21–23 °C and 45% relative humidity within a range of 30–70% in ventilated cages. All procedures were performed in accordance with the guidelines and approved protocols from the Institutional Animal Care and Use Committee at the California Institute of Technology.

Immunohistochemistry

Brains were placed in 4% PFA for 48 hr. and subsequently sectioned at 50 μm in cold PBS using a vibratome. Sections were stained using anti-TH (1:400) and goat anti-rabbit Alexa Fluor 488 (1:400) antibodies. Images were taken using a fluorescent microscope and analyzed using Image-J. Nissl staining was performed on corresponding slices and subsequent analysis was performed with Image-J.

For staining in the small intestine, the gastrointestinal tract was dissected and luminal contents flushed with 4% PFA and OCT and subsequently frozen on dry ice. Sections were taken using a cryostat and stained with anti-TH (1:400) or PGP 9.5 (1:400). Fluorescence intensity and cell counts were measured using Image J.

RNA extraction and qRT-PCR

RNA extraction was based on the manufacturer's protocol (Trizol, Life Technologies). Before reverse transcription, RNA was treated with DNase I to eliminate genomic DNA contamination. 1 μg RNA from each sample was reverse transcribed by using the iScript cDNA synthesis kit (Bio-Rad). The gene expression in the small intestine was measured using Power SYBR Green PCR master mix (Life Technologies). Gene expression was normalized using the housekeeping gene, GAPDH.

Open Field Testing

Mice were placed in a novel behavior testing room in groups of 4. Animals were then individually placed in plastic open top arenas (50 X 50 X 30cm) and allowed to freely move over a 10-min. period. Video was captured using an overhead mounted video camera. Ethovision software was used to generate trajectory maps and analyze average velocity.

Chapter 6

CONCLUSION

In the wild, *D. melanogaster* is associated with a range of environments with different nutritional compositions[1,2]. Therefore, there is a need to maintain homeostasis under changing external conditions and to quickly identify beneficial situations. As the microbiota both acts as a food source and increases the amount of readily available nutrients[3,4], it could buffer against large changes in nutritional composition and indicate a favorable environment. Specific species within the microbiota significantly alter host metabolism[5–8], thus helping to maintain homeostasis within an individual. Given the close ties between metabolism and certain behaviors, including locomotion, we hypothesized that the microbiota also contributes to this aspect of host physiology.

In this work, we found that removal of the microbiota in female *Drosophila* results in hyperactivity compared to conventional counterparts, independent of developmental influences. Starvation is also reported to cause similar increases in fly locomotion[9]. Mono-colonization with select bacteria, including *Lactobacillus brevis*, restored host speed and daily activity to conventional levels and exhibited similar effects under more complex microbial conditions. The addition of a microbial-derived enzyme, xylose isomerase (Xi), was also sufficient to increase host speed and daily activity in axenic flies. Furthermore, we found that octopamine signaling is involved in mediating microbial effects on locomotion, which is similarly implicated in starvation-induced hyperactivity[9–11]. These overlapping phenotypes and pathways suggest that the removal of the microbiota may result in a starvation-like state reversed by certain bacterial species and their products, including Xi. However, a more in-depth analysis of the neuronal circuits involved in our phenotypes is needed to facilitate this comparison.

As male flies did not exhibit the same degree of changes in host speed upon removal of the microbiota or mono-colonization with *L. brevis*, these effects on host locomotion appear sex-specific. A previous report also found that increased levels of trehalose in axenic flies is sex-specific[8]. Further, sexually dimorphic locomotor behavior was previously linked to trehalose and insulin signaling[12,13]. Future work should examine potential ties between sex-specific microbial effects on locomotion and the differences in metabolic requirements and neuronal circuitry between males and females.

Microbial effects on locomotion may also be conserved as our work and that of others similarly reported hyperactivity in germ-free mice[14,15]. However, as antibiotic-treated mice

displayed decreased locomotion, there are likely distinct differences in the microbial effects and the underlying pathways mediating changes in locomotion in *Drosophila* and mice. It would be interesting to examine if the same bacterial species and products identified in *Drosophila* are able to alter locomotion in mice. However, researchers should carefully consider the ecological and developmental dissimilarities between the two model organisms.

The work presented in this thesis demonstrates that the microbiota acts as a source of stimuli that can alter host behavior. We have identified a novel role for Xi in modulating host locomotion and octopamine signaling in mediating microbial-induced changes in motor behavior. Additionally, our data lays the groundwork for future analysis into the mechanisms that underlie microbiota-related changes in locomotion. As we did not pinpoint the exact host molecular and neuronal pathways by which Xi signals, this is one important avenue for future research. The behavioral results from ablating Tdc-expressing neuronal populations within the central brain suggest their involvement; yet, central pattern generators within the ventral nerve cord could still contribute. Furthermore, parallel pathways by which Xi and the microbiota influence host locomotion may also exist. It is important to note that while we did not see any significant changes in the carbohydrates tested in the diet upon Xi* treatment, Xi could still act on carbohydrate ratios within the diet or the intestine of the fly.

As select bacterial species and their products restore locomotion to conventional levels, our work also demonstrates the importance of examining contributions from individual components of the microbiota. The microbiome differs by laboratory, individual, temperature, and genotype; therefore, this reductionist approach helps to minimize variability due to changes in the microbial community. As with most experimental designs, there are limitations to these types of experiments that can be complemented by additional work. For example, results from mono-association experiments may not directly correspond with effects under conventional conditions where there is increased microbial complexity. Combining experiments with mono-colonization or single metabolites with those using whole microbial communities provides insight into both the relative contributions of species and the natural environment. Furthermore, this thesis illustrates the importance of considering the whole organism, including its microbial counterparts, in behavioral analysis. In Brummel et al. (2002), the authors state: "*Drosophila* geneticists have paid relatively little attention to the bacterial flora within fly stocks, yet such flora may increase

phenotypic variation.” The tools for rigorous examinations of microbial communities and neuronal circuits have rapidly progressed, enabling studies revealing both a deeper understanding of the stimuli emanating from the microbiota and the corresponding host pathways mediating their potential behavioral and physiological effects.

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