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STUDYING HOST-MICROBIOTA MUTUALISM USING GNOTOBIOTIC DROSOPHILA

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SUMMARY

The complex interaction between the metazoan host and its commensal gut microbiota is one of the essential features of symbiosis in the animal kingdom. As there is a burgeoning interest to decipher the molecular dialogue that shapes host-microbiota mutualism, the use of gnotobiotic model organism becomes an imperative approach to unambiguously parse the specific contributions to such interaction from the microbiome. In this review, we focus on several remarkable gnotobiotic studies in *Drosophila* that functionally depicted how the gut microbes can alter host physiology and behaviour through transcriptomic regulation, hormonal control and diet modification. These results in concert illustrate that the gnotobiotic flies mono- or poly-associated with members of its gut microbiota deliver a versatile and powerful model that is amenable to different types of studies ranging from classic genetics to large-scale systems approaches.

INTRODUCTION

In 1883, Louis Pasteur expressed his wish to raise a "microbially deprived" young animal on "pure" food from birth, and postulated that "without any preconceived notion…. life under such condition… shall become impossible" (*Pasteur*, 1885). Nearly 30 years later, Eugene Wollman at the Pasteur institute in Paris successfully cultured the first germfree common blowflies (*Calliphora vomitoria*) and observed that except for certain minor growth delay, the adult flies appeared perfectly normal (*Wollman*, 1911). At first, Wollman's experiment seemed to have put an end to Pasteur's claim, yet in truth it was only the beginning. Throughout his productive career as a microbiologist, Wollman probably did not realize that his germfree blowflies spawned an entire field of animal physiology based on host-microbe interactions; and only when a germfree life was made possible, the concept of "gnotobiology" could spring to life. In the past century, Pasteur's musing on what life would be like without its resident microbes gradually transformed to a quest to understand how the eukaryotic hosts and their bacterial partners orchestrate the symphony of life, and how such interactions probably profoundly changed the course of our evolutionary history (*McFall-Ngai* et al., 2013).

Microbes occupy every possible ecological niche on earth. A set of particular niches comprise the various

internal epithelia of the metazoan hosts, who, through eons of evolution, have forged complex and intricate relationships with this rich and diverse microbial community, called the "microbiota" (*McFall-Ngai* et al., 2013; *Douglas*, 2014). A human host carries on his body far more microorganisms than his own cells, and these invisible dwellers constitute 1-3% of his body mass (*Human Microbiome Project*, 2012; *Sommer* and *Backhed*, 2013). The human gut alone harbours approximately 500 to 1,000 bacterial species (*Eckburg* et al., 2005), and represents the largest mucosal surface where the exchanges between the host and the microbiota take place. In the last decades, many studies together generated a systematic understanding of how the gut microbiota and its diverse gene repertoire, called the "microbiome", can configure the fitness parameters of the host; a healthy microbiota can expand the host's metabolic potential, fortify its immune system, promote healthy aging and even dictate its emotional and psychological well-being (reviewed in: *Grenham* et al., 2011; *Clemente* et al., 2012; *Sommer* and *Backhed*, 2013; *Kaiko* and *Stappenbeck*, 2014; and *Sharon* et al., 2014). However, as the community structure and activities of the gut microbiota are extremely sensitive to fluctuations in the environment, perturbations to the microbiota pose significant risks to the host (reviewed in: *O'Hara* and *Shanahan*, 2006; and *Gibson* et al., 2014). Subtle changes in host immunity, diet or xenobiotic concentration can disrupt the balance in the gut microbial community, which consequently compromises host fitness. In mammals, microbiome imbalance, or dysbiosis, positively correlates to the onset of obesity, diabetes, colon cancer (reviewed in: *Tremaroli* and *Backhed*, 2012; *Karlsson* et al., 2013; and *Irrazabal* et al., 2014) and human psychiatric disorders such as schizophrenia and autism (reviewed in *Fond* et al., 2014).

Currently, a large amount of research on host-microbiota mutualism employs vertebrate models, yet the high complexity of the microbial composition in the mammalian gut, the difficulty to culture most of these microbial species, and the cost of raising these animals in a strictly sterile environment pose a considerable obstacle. Therefore, to delve deeper into the molecular interplay between the host genome and the microbiome and the environmental contributions to such interplay, a more genetically tractable model organism with simpler and even defined microbiota is an attractive option. *Drosophila melanogaster* fits these criteria. First of all, the intestinal tract of the fruit fly is anatomically and physiologically similar to the mammalian gut (*Lemaitre* and *Miguel-Aliaga*, 2013), yet the microbial composition is rather simple: throughout the larval and adult life, the fly gut hosts five to twenty aero-tolerant commensal species, all of which are readily cultured in the laboratory (*Broderick* and *Lemaitre*, 2012; *Erkosar* et al., 2013). Two families of bacteria: Acetobacteraceae and Lactobacillaceae, dominate the community (*Chandler* et al., 2011; *Shin* et al., 2011; *Storelli* et al., 2011; *Wong* et al., 2011; *Ridley* et al., 2012; *Chaston* et al., 2014). However, the fly gut microbiota is transient in nature and requires constant replenishment, thus the community structure and bacterial load fluctuate highly as the flies develop and age (*Blum* et al., 2013; *Broderick* et al., 2014; *Erkosar* and *Leulier*, 2014). Such inconstancy makes it difficult to clearly pinpoint the bacterial genetic factors contributing to host physiology. Therefore, the use of gnotobiotic fly models, in combination with classic genetic approaches and next-generation sequencing, proves to

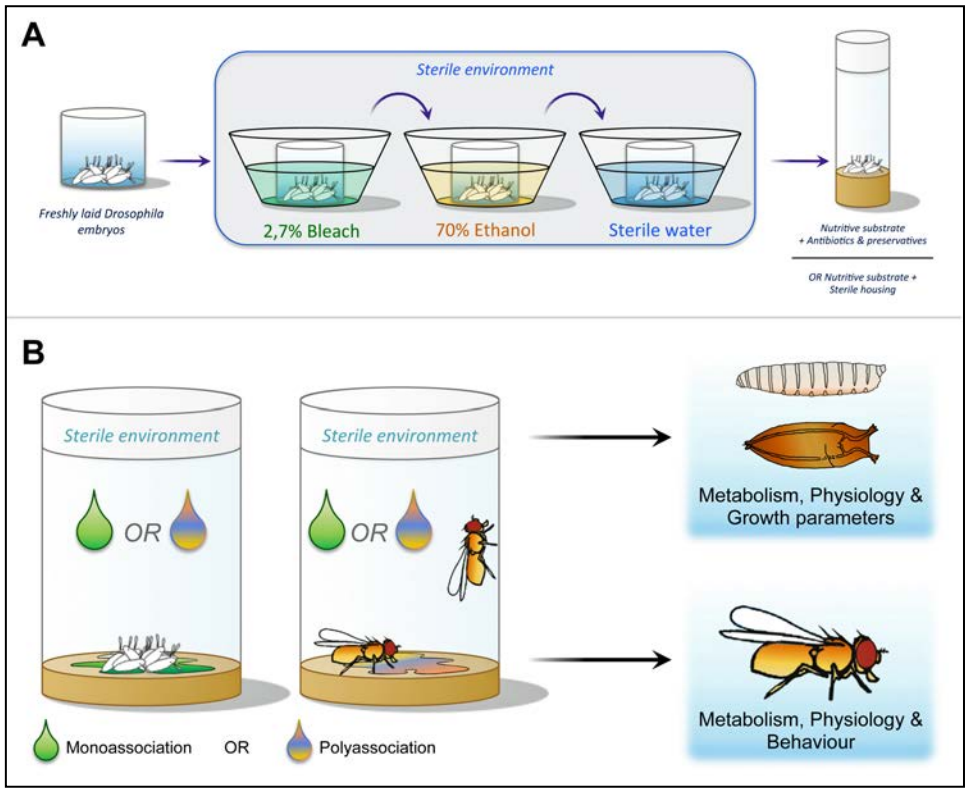

Figure 1: Building a gnotobiotic *Drosophila* model to study host-microbiota mutualism.
A. To obtain germfree flies, freshly laid eggs are harvested in large-scale and washed in succession with bleach, ethanol and sterile water. To maintain axeny, the dechorionated eggs are then grown in the presence of antibiotics and preservatives or in a sterile environment.
B. To study the specific contribution of the microbiome to the different aspects of host physiology, ex-axenic eggs or adults are mono-associated with a single gut commensal species (green drop) or poly-associated with a defined set of gut commensal bacteria (blue and yellow coloured drop). Such gnotobiotic flies have been used to study the impact of specific commensals on host juvenile growth, developmental timing, metabolic homeostasis and adult behaviour.

be the new and effective means to study intestinal mutualism with added advantage, because it enables the investigators to inoculate the germfree subjects with various bacterial strains of predefined quantity and composition – such of any member of the fly microbiota. In this setting, the researchers not only can rigorously monitor the phenotypic changes in different aspects of host physiology, but also can robustly correlate and even attribute particular changes to the specific functions from the microbiome, as the genomes of many gut microbiota species are being rapidly sequenced and annotated (*Human Microbiome Project*, 2012). Moreover, except for *Acetobacter*, which is mostly found in insects (*Crotti* et al., 2009; *Chouaia* et al., 2014), *Lactobacillus* species are commensal to mammals (*Reuter*, 2001; *Rastall*, 2004 ; *Walter* et al., 2011). Therefore, the results from such gnotobiotic fly studies can be readily translated to mammalian studies. *Drosophila* models were first used to dissect the genetic networks governing host/pathogen interaction

(*Buchon* et al., 2014). With the same approach, pioneering studies have shown promising results to identify and functionally characterize the genetic components of the molecular crosstalk between *Drosophila* and its commensal bacteria. In this review, we discuss the findings from the studies using gnotobiotic fly models to unravel the impact of the members of gut microbiota on host metabolism, physiology and behaviour (Figure 1).

THE MAKING OF THE GNOTOBIOTIC FLIES

As mentioned before, in the early 1910s, Eugene Wollman and his colleagues at the Pasteur institute were among the first to raise germfree animals such as common blowflies, tadpoles and guinea pigs. Wollman made the first germfree common blowflies by treating the egg surface with diluted hydrogen peroxide and raising the larvae on sterilized meat substrate (*Wollman*, 1911). Interestingly, Wollman observed that the germfree larvae reached normal body size, but at a slower rate. Moreover, these flies seemed slower in movement and less interested in foraging. Therefore, even though the "microbially deprived" life was indeed possible in a sterile environment, the difference between such a life and it's conventionally reared (CR) siblings was already observable to the naked eye. In the next few decades, *Drosophila melanogaster* was attaining a more and more prominent status as a model genetic organism. As a result, in the 1950s and '60s, different methods were developed to sterilize *Drosophila* eggs on a large-scale and keeping axenic fly stocks turned into a routine laboratory practice.

In 1969, Marion Bakula developed the first monoxenic *Drosophila* model by associating bleached fly eggs with either "native" or "foreign" bacteria strains (*E. coli*) (*Bakula*, 1969). In her study, only the "native" bacteria isolated from the fly gut persisted throughout larval development in the fly host, who pupariated at a slightly faster pace than the axenic controls. This is also the first gnotobiotic model to demonstrate that the essential mode of microbial transmission in fruit flies is through larvae ingestion of the contaminated chorion. Therefore, thorough dechorionation of the eggs can effectively render a fly stock germfree. In the next several decades, after trying different sterilizing agents such as antiformin and formalin (*Begg* and *Sang*, 1950) researchers found that the treatment with common household bleach (diluted sodium hypochloride solution) in combination with ethanol wash is the safe, simple, rapid and effective way to dechorionate the embryo and rid the surface of bacterial "contaminants". However, bleaching alone cannot eliminate intracellular endosymbionts such as *Wolbachia*, the most widespread insect symbiont whose relationship with the host ranges from parasitism to mutualism. Depending on the context, the presence of *Wolbachia* is known to affect reproductive success, enhance insulin signalling and boost host defence (*Ikeya* et al., 2009; *Gronke* et al., 2010; *Ringo* et al., 2011; *Hamilton* and *Perlman*, 2013). Therefore, to obtain a "true" germfree or gut-commensal specific phenotype unadulterated by *Wolbachia*, different laboratories have adopted various protocols to maintain germfree stocks, either by combining bleaching with rearing flies on food containing a mixture of antibiotics, or by one-time treatment of bleach and the subsequent maintenance of the flies in

a sterile environment (Figure 1A). Of note, bleaching and/or antibiotic treatment can lower fly viability and fecundity and have certain unintended negative cellular and systemic effects on the host (*Ridley* et al., 2013). Therefore, the studies using germfree flies mandate careful and thorough controls. In the following sections, we review a few seminal gnotobiotic *Drosophila* studies that have uncovered important molecular mechanisms governing host-microbiota interaction.

THE STUDY OF HOST PHYSIOLOGY USING A GNOTOBIOTIC FLY MODEL

A gnotobiotic fly model with classic genetics approach

That the germfree flies develop and grow at a slower pace is an old observation that has held true since Wollman's time. For example, in Baluka's monoxenic culture, the native bacterial isolates from the *Drosophila* gut: Stock 13, a *Brevibacterium* variant, accelerated pupariation compared to the axenic stock (*Bakula*, 1969). This observation has now been further characterized in greater detail. On a "standard" laboratory diet, the pupariation and adult eclosion rate of the axenic flies are delayed by one day compared to their CR siblings (*Shin* et al., 2011; *Wong* et al., 2014). However, this delay becomes striking when the axenic flies are presented with nutritive challenges. Particularly, when raised on a diet where the yeast content was below 0.1%, or was completely replaced by casamino acids, the germfree flies die (*Shin* et al., 2011). This observation suggests that an intact gut microbiota provides life-sustaining factors for the host experiencing severe nutritive duress. Next, when fed on a diet with low yeast content, germfree flies pupariate six days later than the CR flies (*Storelli* et al., 2011). Therefore, the gut microbioa can also override the developmental delay to potentiate growth in suboptimal nutritive environment. Importantly, these two studies also demonstrated that inoculating the axenic fly embryos with one or several defined gut commensal species, such as *Lactobacillus plantarum (L. plantarum)* or *Acetobacter pomorum (A. pomorum),* can recapitulate the growth benefits conferred by the entire gut microbiota. Moreover, only certain strains of *L. plantarum* sustain growth on a low-yeast diet: several other isolates from the fly origin were unable to promote host growth even though they could colonize the larval gut and the fly food just as efficiently as the beneficial strains (*Storelli* et al., 2011). This observation unequivocally illustrates that the gut microbiota promotes growth by not just serving as a food source, but through complex molecular and biochemical interactions with the host.

How then, does the gut microbiota promote host growth? First of all, like for many metazoan species, the source of the fly gut bacteria comes from contaminated food (*Broderick* and *Lemaitre*, 2012; *Erkosar* and *Leulier*, 2014), and naturally, some of the primary functions of the gut bacteria are to enhance digestion and expand the host's metabolic potential. The additional enzymatic activities from the bacterial origin help break down the specific nutritive substrates that are otherwise indigestible for the host, who

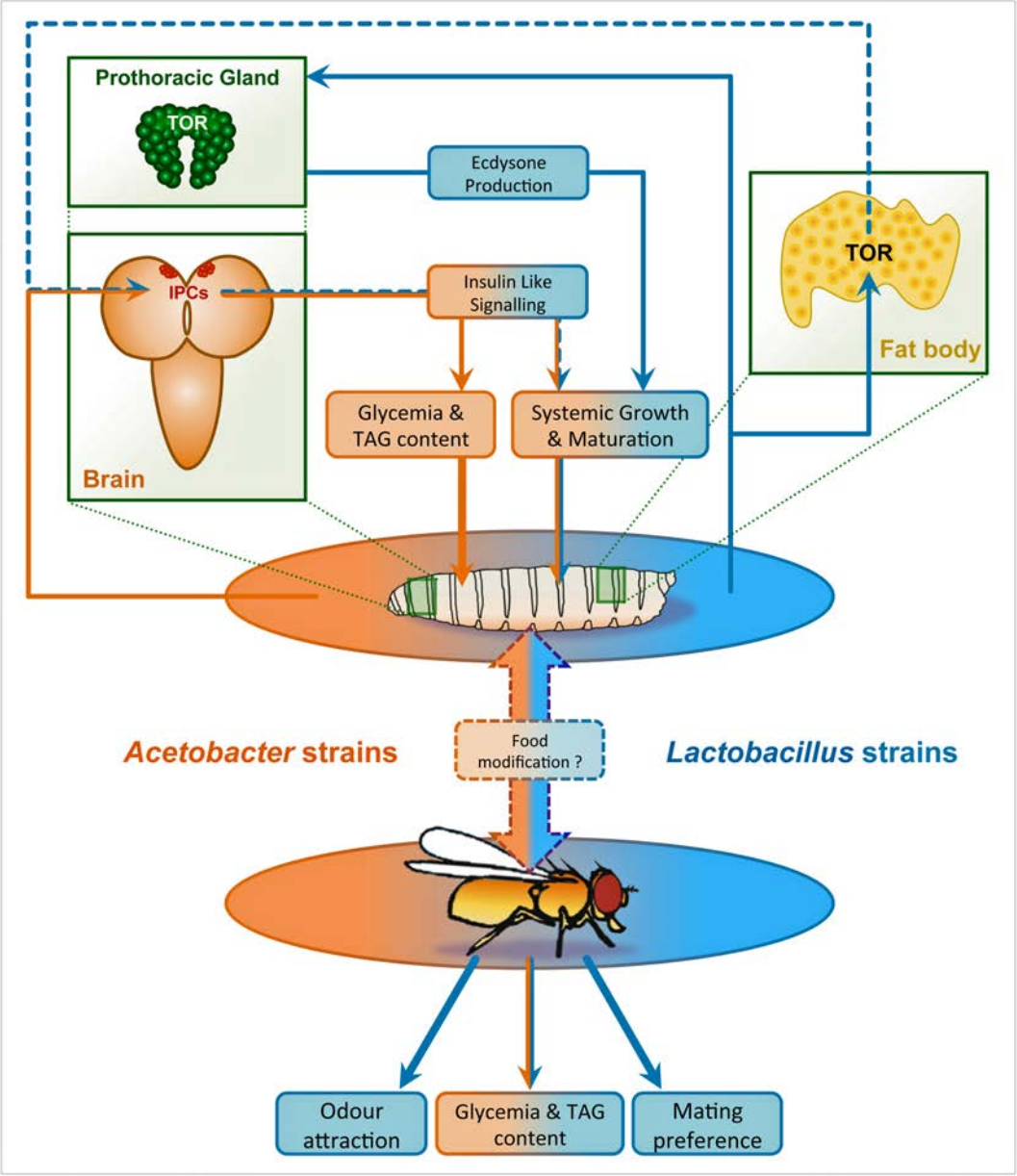

Figure 2: The host physiological and behavioural responses to the addition of different gut commensal strains.
Gnotobiotic studies have depicted the effect of *Acetobacter* and *Lactobacillus* strains on systemic growth, metabolic homeostasis and adult behaviour. Specifically, in the presence of nutritive challenge, *A. pomorum* (orange) regulates host insulin signalling in the insulin producing cells (IPCs) and thus promotes larval growth and maturation, whereas *L. plantarum* (blue) interacts with host TOR (target of rapamycin) pathway in the fat body and the prothoracic gland to control ecdysone production and affects insulin signalling directly or indirectly (dotted blue line). During the adult stage, both *Acetobacter* and *Lactobacillus* strains regulate host triacylglyceride (TAG) and circulating glucose levels, but only *Lactobacillus* strains have been shown to impact host behaviours such as mating preference and odour attraction to food. The effect of the gut commensals represented by *Acetobacter* and *Lactobacillus* can be direct or via modifying the nutritional substrates.

can in turn harvest energy from these food substrates and extract necessary metabolic building blocks for various biological processes (*Sommer* and *Backhed*, 2013). In addition, essential micronutrients derived from bacterial metabolism such as vitamins and short chain fatty acids directly fuel the host's metabolism (*Natarajan* and *Pluznick*, 2014). Indeed, two recent studies found that fortifying the food fed to the germfree flies with B vitamins phenocopies the effect of the presence of the gut bacteria to a large extent, indicating that the gut microbiota accomplishes metabolic sparing of the B vitamins for the host through a yet unknown mechanism (*Fridmann-Sirkis* et al., 2014; *Wong* et al., 2014).

However, the growth benefits from the gut microbiota are probably beyond vitamin B provision. To identify the microbial factors that can rescue host lethality on the casein diet, Shin et al. conducted a random mutagenesis in *A. pomorum* and isolated strains that restored ex-germfree larval survival on casamino acid diet but led to delayed pupariation when compared to animals mono-associated with the wild-type *A. pomorum*. Several such mutations affect Pyrroloquinoline quinone-dependant alcohol dehydrogenase (Pqq-adh), an enzyme involved in the ethanol respiratory chain, and whose end product is acetic acid. Although *Pqq-adh* mutant bacterial strains were impaired in their production of acetic acid, supplementation of casamino acid diet with acetic acid alone failed to rescue germfree larval lethality. However, concomitant association with *Pqq-adh* mutant *A. pomorum* strains and supplementation with acetic acid completely rescued larval developmental timing. Therefore, upon severe nutritive challenge, the addition of *A. pomorum* first and foremost restores the viability of the fly host, and then the intact activity of the bacterial ethanol respiratory chain promotes host growth and maturation. Based on this result, it is likely that the molecular mechanisms that sustain larval life and promote growth are separable.

Genetic factors from the host

What are the host factors responding to the beneficial growth promotion effect of the microbiota in the presence of nutritional challenges? The studies of *Shin* et al. (2011) and *Storelli* et al. (2011) demonstrate that the addition of *A. pomorum* or *L. plantarum* can accelerate growth and maturation by modulating host systemic hormonal signalling. In the Shin et al study, larvae mono-associated with the *Pqq-adh* mutant strain of *A. pomorum* survived to adulthood, but displayed metabolic features reminiscent of defective insulin/insulin like growth factor (IIS) signalling, such as low body weight, retarded growth, elevated haemolymph glucose and trehalose levels, and higher level of triacylglyeride (TAG), the main form of stored lipids. At the molecular level, in the fat body of the flies mono-associated with mutant *Pqq-adh A. pomorum* strains on the casamino diet, membrane activation of PI3K and cytoplasmic retention of dFOXO were abolished, and the expression of Insulin-Like peptides (Dilps) such as Dilp3 and 5 was reduced in the larval brain. Most importantly, the ectopic expression of Dilp2 largely rescued both the defective IIS phenotype and the molecular signatures associated with such defects in flies mono-associated with mutant strain of *A. pomorum*. Therefore, *A. pomorum*, partly via its *ppq-adh* activity, regulates IIS to maintain the host's metabolic homeostasis (Figure 2). Similarly, on a low-yeast diet, mono-

association with *L. plantarum* lowered the expression of insulin receptor, a negative readout of pathway activity, suggesting that the presence of *L. plantarum* also enhances insulin signalling (*Storelli* et al., 2011). Moreover, *L. plantarum* reduced the juvenile growth period through TOR signalling: dampening TOR activity in the fat body – the functional analogue of the mammalian liver – and the prothoracic gland compromised the *L. plantarum* growth promoting effect as measured by adult emergence (Figure 2). TOR is the host nutrient-sensitive signalling pathway devoted to balance organismal growth and maturation in a nutrient-dependent manner (*Hietakangas* and *Cohen*, 2009; *Danielsen* et al., 2013). In the developing larvae, TOR activity in the prothoracic gland directly controls ecdysone production, which in turn affects the parameters of systemic growth via IIS. As TOR responds to the circulating levels of different micronutrients in the haemolymph, such as branched-chain amino acids, *L. plantarum* may act upstream of TOR in several ways. First, *L. plantarum* can directly regulate TOR activity by making certain metabolites or other biochemical pathway intermediates or/and end products. Secondly, *L. plantarum* can either modify the diet or boost the host's digestive capacity to enhance nutrient assimilation, which then indirectly activates TOR pathway. Therefore, how *L. plantarum* promotes host juvenile growth is to yet be studied in detail.

Now two groups have demonstrated that specific strains from both *Acetobacter* and *Lactobacillus* families can promote juvenile growth upon nutritive challenge (*Shin* et al., 2011; *Storelli* et al., 2011). What effect does the combined action of *Acetobacter* and *Lactobacillus* have on the host? To study how these two commensal bacteria interact in the host and how such interactions impact adult host physiology, a study by Newell and Douglas compared differences in circulating glucose levels, TAG contents and adult body weight between axenic flies and ex-germfree flies associated with a single or different combinations of the five fly commensal species (*Newell* and *Douglas*, 2014). Specifically, using a set of defined microbiomes with up to five commensal species (*A. pomorum*, *A. tropicalis*, *L. plantarum*, *L. brevis*, and *L. fructivorans*), the authors inoculated the germfree flies with one or different combinations of these strains and found that all these combinations lowered circulating glucose concentrations in comparison to axenic flies. However, in terms of lowering host TAG levels, these different combinations of bacteria worked at different efficiency: the *Lactobacillus* species can lower TAG level moderately; *Acetobacter* did so more effectively than *Lactobacillus*, but not as effectively as the five species co-inoculation, which was the only treatment that recapitulated the benefits of the conventional commensal flora. Interestingly, one specific co-inoculation, with *Acetobacter tropicalis* and *Lactobacillus brevis*, was particularly potent in that it lowered host TAGs more than in animals poly-associated with the five commensal species. Other forms of co-associations with two bacterial representatives from the *Lactobacillus* and *Acetobacter* genera failed to reproduce the phenotype. These results indicate that *Acetobacter* and *Lactobacillus* strains can act in synergy, but not consistently. A plausible explanation for this puzzling phenomenon is that the content of the microbiome, rather than the taxonomic combination, determines the TAG content of the host (see below).

Gnotobiotic model with systems approach

Large-scale identification of bacterial genetic determinants

Using classic genetic analysis on a mono-association fly model, *Shin* et al. (2011) and *Storelli* et al. (2011) identified the entry points to further dissect the molecular dialogue between the host and the gut microbiota that alters host physiological traits. Gnotobiotic models are now deployed for large-scale search to fit the same purpose. In a metagenomic study, Chaston and colleagues first gathered a collection of 41 fully sequenced bacteria strains broadly encompassing different *Acetobacter* and *Lactobacillus* genus. Raised in mono-association with each of the 41 strains, the fly hosts showed a spectrum of different responses in terms of pupariation timing and adult TAG content. Based on the comparison of the amplitude of the mono-association effect on these two parameters, the authors undertook a metagenome-wide association study (MGWAS) that effectively correlates bacterial genetic determinants to the magnitude of changes in developmental timing and TAG content (*Chaston* et al., 2014). Remarkably, the MGWAS based on developmental timing first yielded clusters of genes operating in the cellular respiratory chain, including the PQQ enzyme that converts sugar and alcohol substrates to acetic acid. This result corroborates the finding from the transposon screen in *A. pomorum* by *Shin* et al. (2011). Interestingly, they recovered the *Pqq* mutant bacteria on a casein-only fly medium that causes lethality in germfree flies, whereas the MGWAS was conducted on standard laboratory fly food, where the developmental delay in germfree flies was subtle. At a glance, it is a bit surprising that both studies uncovered the same bacterial factor based on host developmental timing, a trait that varies drastically in the two experimental setups. It provocatively suggests there is a robust and canonical host interaction with bacterial ethanol respiratory chain products that cannot be masked by different host nutritional backgrounds. Such response has been shown to involve host insulin signalling. How such robust interaction is maintained in a different nutritional background is an extremely interesting topic to explore. Moreover, in the study by *Chaston* et al. (2014), the clusters of genes that correlated to lower host TAG content are known to regulate redox sensing and glucose oxidation, such as glucose dehydrogenase (GDH), gluconate-2 dehydrogenase (GnDH) and a single domain oxidoreductase (SDR). Importantly, introducing these candidate genes into selected *Acetobacter* strains that lack these enzymes conferred the ability to the bacteria to reduce host TAG level. Furthermore, the authors observed that ectopic expression of GDH and GnDH concomitantly lowered glucose content in the media where the gnotobiotic flies were raised. These results strongly suggest that the gut microbiota can modulate host lipid storage and nutritional homeostasis through altering the nutrient composition of the food. Another intriguing observation from the study is that the clustering of bacterial strains based on the effect on host developmental timing and TAG level is largely unrestricted to the taxonomic structure of the bacteria. Hence, the collective genetic composition of the gut microbiome once again proves to be a more faithful predictor of host response than taxonomic classification. Now looking back, the finding by *Chaston* et al. (2014) probably also partially explains why *Newell* and *Douglas* (2014) observed inconsistent TAG lowering ef-

fect in flies associated with different combinations of *Acetobacter* and *Lactobacillus* strains (see the previous section). Altogether, this particular study raises a few interesting issues. For example, Chaston and colleagues propose that by modifying the food, bacterial glucose metabolism impacts the adult host's capacity to store lipid (*Chaston* et al., 2014). Does this observation hold true in the developing larvae? The published studies seem to favour the likelihood, as *Shin* et al. (2011) unequivocally demonstrated that gnotobiotic larvae harbouring mutant *Pqq* mutant *Acetobacter* strain show higher circulating sugar and triglyceride as a result of the compromise in the host insulin signalling activity (Figure 2). If this is the case, do the bacteria directly elicit the host insulin response, or is such insulin response an indirect result of bacteria altering the glucose content of the food? These two possibilities are not mutually exclusive but require further detailed mechanistic studies that either tease them apart or meld them together. So far Chaston and colleagues were unable to rescue developmental delay by ectopically expressing the enzymes involved in glucose oxidation, but such negative outcome likely to imply that the interaction between host maturation and microbiota metabolism is more complex than we think.

The host's transcriptomic response to gnotobiotic association

The association with certain commensal species modulate host IIS and TOR signalling. What other kind of molecular changes take place in the host in the presence of the gut microbiota? To answer this question, several groups recently undertook microarray studies to compare the transcriptomic differences between the germfree flies and their conventionally reared siblings at different age, and demonstrated that in the fly gut, the presence of the microbiota significantly alters the expression of a core set of genes that control transcription, gut structure, immunity, metabolism, signalling and stress response (*Broderick* et al., 2014; *Guo* et al., 2014) and reviewed in *Erkosar* and *Leulier* (2014). Among these studies, Guo and colleagues extended the microarray finding and elegantly showed that in the aging fly gut, the transcription factor Foxo represses peptidoglycan recognition protein SC2 (PGRP-SC2), which subsequently leads to hyperactivation of Rel/NFkB activity that is responsible for an intestinal dysbiosis phenotype. In addition to these studies, another noteworthy microarray analysis using poly-associated gnotobiotic flies identified a short but focused list set of genes whose functions are enriched in digestion and primary metabolism. *Erkosar* et al. (2013) conducted the microarray study on exgermfree adult flies exposed to a defined set commensal bacterial strains (*A. pomorum, Commensalibacter intestini, Lactobacillus brevis and L. plantarum*). First, the polyassociation yielded certain genes that overlap with those found in the concomitant study by *Broderick* et al. (2014) using CR flies. Specifically, such polyassociation markedly up-regulates the expression of a set of digestive enzymes and other genes involved in primary metabolism. This result reflects the conventional notion that gut bacteria assist in host digestive functions to effectively extract nutrients and energy from food. Intriguingly still, half of the polyassociation upregulated genes identified by *Erkosar* et al. (2013) were also involved in response to intestinal infection, and the majority of these genes are directly or indirectly under the control of Relish, the *Drosophila* orthologue of the mammalian NFκB factor p105 (*Buchon* et al., 2009). This

result once again corroborates the study by *Broderick* et al. (2014), who also observed that more than half of the up-regulated genes in the CR fly gut changed expression pattern in *Relish* mutant flies. As Relish is essential to the interplay between host innate immunity and nutritional response, *Erkosar* et al. (2013) postulated the following scenario: the presence of commensal strains usually promotes the expression of a certain set of digestive enzymes and metabolic genes, but in the presence of an acute infection, a change in the host transcriptome is triggered, so that these microbiota-mediated metabolic genes are down-regulated to prepare for immune defence, and such change is mediated by Relish. Consistent with this hypothesis, the authors found that the expression patterns of several selected candidate genes such as *trypsin* and *Jonah* proteases are indeed down-regulated upon pathogen infection or in the genetic background where Relish activity is compromised. In summary, the finding by *Erkosar* et al. (2013) first largely recapitulates the host's transcriptomic response to gut-microbiota in CR flies, thus cementing the utility and relevance of the poly-association model. Furthermore, like in *Broderick* et al. (2014), the authors identified Relish as the central regulator of a transcriptional trade-off between metabolic response and immunity, and thus opened a new chapter for potential mechanistic studies of such switch. Altogether, the studies by *Erkosar* et al. (2013) and others were the first to demonstrate that the gut microbiota profoundly alters the host transcriptomic landscape, yet we know little how the bacteria mechanistically effect these changes. Secondly, these studies also provide an exhaustive list of genes that govern the host response to the gut microbiota. The functional studies of these candidates will immensely advance our understanding of the molecular basis of host-microbiota interaction. Furthermore, how are these host transcriptomic changes integrated into the known insulin and TOR signalling networks - as a response to the gut microbiota - to control systemic growth and metabolic homeostasis? Similarly, are these transcriptomic changes directly mediated by unknown bacterial factors, or through bacterial modification of the food substrate, or both? If both, what are the bacterial factors and how is the food modified? These are immediate questions that can be addressed with gnotobiotic models coupled to metabolomics and mutagenesis studies.

The gut microbiota impacts social behaviour

Throughout the long eukaryotic evolutionary history, many animal species abandoned the solitary life style for group living in highly developed social structures, in exchange for bodily protection, cooperative foraging and increased chances of mating and reproduction. As the long-time evolutionary partner of its eukaryotic host, it is not surprising that the symbiotic gut bacteria also evolved to control host individual and social behaviour, probably with the interest to maximize its transmission among the members of the society (*Montiel-Castro* et al., 2013). Through bidirectional signalling along the "microbiota-gut-vagus-brain axis", the activities of the gut microbiota can impact the activities of host neural circuitry and alter host foraging behaviour, stress and anxiety response and even the development of empathy (for

extensive reviews, see: *Cryan* and *Dinan*, 2012; *Montiel-Castro* et al., 2013; *Stilling* et al., 2014). Gnotobiotic flies have recently emerged to be a productive model to study social interactions. For example, fruit flies preferentially mate with partners fed on the same kind of diet, a phenomenon termed "positive assortive mating", which is readily lost in axenic flies. However, the gnotobiotic addition of *L. plantarum* restores such positive assortive mating, indicating that the gut microbiota may play a direct role in altering fly pheromone composition according to the host's dietary environment (*Sharon* et al., 2010). Besides mating preference, the presence of gut microbiota was also shown to determine how fruit flies are attracted by odours from different food substrates (*Venu* et al., 2014). In controlled learning experiments, Venu et al. presented the larvae subjects with three separate food choices: fresh laboratory food, food processed by axenic larvae, and food used by conventionally reared larvae. While the larvae and adult female subjects showed no preference between the fresh food and axenically processed food, they were strongly attracted to the food substrate where the CR larvae were raised. Furthermore, the same larvae subjects equally preferred food that has been used to raise larvae mono-associated with *Lactobacillus brevis* or *L. plantarum*. As an important control, the same larvae subject showed no preference to fly food containing only cultured *L. brevis*, indicating the interaction between *L. brevis* or *L. plantarum* with the fly larvae is imperative to generate the source for such social attraction. The nature of such source is unknown, but it can be a volatile compound produced by either the bacteria or the larvae when both are residing in the same niche (Figure 2). In the wild, fruit flies search of hospitable habitat with suitable food substrate for mating, egg laying and rearing larvae (*Durisko* and *Dukas*, 2013). The results from this study imply that the host interaction with the commensal *Lactobacillus* genus of the gut microbiota can manufacture compounds that serve as cues for the host's searching effort and decision-making. What are these compounds? Through what pathways and neurons do they act? What other aspects of fly behaviour do they affect? These are the questions that probably can also be answered with gnotobiotic studies.

CONCLUSION

In a recent essay, McFall-Ngai and colleagues commented that we humans are just "animals in a bacterial world" (*McFall-Ngai* et al., 2013). This pithy statement rightly illustrates the overwhelming number and the diversity of the microbes that we live with, yet we have only began to grasp how these seemingly humble dwellers can powerfully change our being throughout evolution. By enhancing the host's metabolic potential, the gut microbiota helps expand the host's ecological niche. By altering host behaviour, these bacteria probably also played their parts in shaping social hierarchies and caste systems in the animal kingdom. We still know very little about how the bacteria do it. However, by harnessing the power of the gnotobiotic flies, we have begun to systemically characterize how the gut microbiota potently elicits a myriad of host physiological responses and behavioural changes. Importantly, the gnotobiotic model, in combination with classic genetics and

large-scale next generation sequencing methods, grant us the unprecedented power of resolution to pinpoint the specific bacterial factors responsible a particular host phenotype. Only with such resolution, we can delve deeper into the mechanisms that govern host-microbiota interaction, and find answers to how these mechanisms evolved over time in different species. However, no matter how complex and unexpected these answers are, they never will deviate from the truth that Pasteur and Wollman prompted us to discover, that our genetic makeup is metagenomic, and our life story is indispensably, microbial.

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