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Caractérisation des mécanismes impliqués dans la promotion de croissance de la Drosophile par *Lactobacillus plantarum*

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Abstract

Intestinal microbiota can modulate virtually all aspects of their host physiology, and particularly, digestion and metabolism. However, the molecular mechanisms at play remain largely unknown. To tackle this question, we use a simple gnotobiotic model: *Drosophila* larvae monoassociated with one of its major natural symbiont, *Lactobacillus plantarum*. Previous work from our group showed that *L. plantarum* promotes the juvenile growth of larvae facing a nutritional stress, thereby dampening the deleterious effect of the nutrient deficiency on larval growth. This growth enhancement partially relies on the upregulation of intestinal proteases, as well as on the modulation of the host TOR pathway by the symbionts. My thesis work aimed at unraveling other host genetic mechanisms involved in the interaction between *Drosophila* and *L. plantarum* during growth. Our work showed that host natural genomic variations affect the fly physiologic response to *L. plantarum*. Furthermore, we unveiled a novel role of intestinal bacteria, revealing their ability to act as a genetic buffer to compensate the growth impairments due to the fly genetic background. In addition, Lp^{WJL} decreases the phenotypic variations in various host fitness traits (growth, organ size, timing to pupariation) and it also confers robustness to organ patterning. Finally, we showed that the TGF-β ligand, Dawdle plays an important regulatory role on digestive enzymes in a protein-deficient nutritional context, and that this regulation can be inhibitory or activating depending on the microbial environment.

Résumé long

Les animaux sont colonisés par une grande diversité de microbes, représentant le microbiote. Ces communautés de microorganismes ne se contentent pas de cohabiter avec leur hôte mais mettent en place des interactions complexes et finement régulées, modulant en profondeur la physiologie de l'hôte. En effet, le microbiote agit sur pratiquement tous les processus biologiques de l'hôte tels que l'immunité, le développement post-embryonnaire, le comportement, etc. En retour, l'hôte fourni « le gîte et le couvert » aux microorganismes le peuplant. La nutrition et le métabolisme, et par conséquent, la croissance, sont des aspects clés de la physiologie modulés par le microbiote intestinal. Cependant, les mécanismes sous-jacents restent encore à ce jour peu connus. Afin de comprendre les mécanismes d'interaction entre l'hôte et son microbiote au cours de la croissance, nous utilisons un model gnotobiotique simple : *Drosophila melanogaster*, communément appelée mouche à vinaigre, monoassociée à une de ses bactérie symbiotique naturelle : *Lactobacillus plantarum*. Le microbiote de la Drosophile présente l'avantage d'être bien plus simple que celui des mammifères, de plus, il affecte de nombreux aspects de la physiologie de la Drosophile : la longévité, les préférences sexuelles, l'immunité, le comportement social, le développement post-embryonnaire et la croissance. Notre équipe s'intéresse à ce dernier trait. Chez la Drosophile, la croissance a lieu exclusivement durant les stades larvaires. Nous avons montré que *L. plantarum* accélère la croissance de la larve de Drosophile en cas de carence en protéine. En effet, un déficit en protéines cause un retard de croissance chez les larves axéniques (dépourvues de microorganismes), qui est tempéré par l'association avec *L. plantarum*. Cette accélération de la croissance en condition de carence nutritionnelle supportée par *L. plantarum* nécessite l'activation des protéases digestives de l'hôte par la bactérie. L'activation des protéases par les bactéries permet d'améliorer l'assimilation des acides aminés à partir du milieu pauvre en protéine, conduisant ainsi à l'augmentation de l'activité de la voie TOR (Target Of Rapamycin) de l'hôte. En retour, la voie de signalisation TOR améliore la production d'insuline et d'ecdysone, deux hormones modulant la croissance de la Drosophile. L'objectif de cette thèse est d'étudier plus avant les mécanismes génétiques de l'hôte impliqués dans

l'interaction entre la Drosophile et *L. plantarum* pendant la croissance larvaire en contexte de carence alimentaire.

Dans le premier chapitre de cette thèse, nous avons évalué l'impact des variations génomiques sur l'amélioration de la croissance conférée par *L. plantarum*. Nos résultats montrent que le gain de croissance est un caractère quantitatif. *L. plantarum* supporte la croissance quelque soit le fond génétique des mouches, cependant, les variations génomiques telles que les SNPs (Single Nucleotide Polymorphism) affectent le degré d'efficacité du gain de croissance larvaire. En outre, au cours de notre étude nous avons découvert une nouvelle fonction du microbiote qui agit comme « tampon génétique », en compensant les défauts de croissance résultant du fond génétique des animaux. De plus, il réduit la variation phénotypique de certains traits comme la croissance, et la taille de certains organes. Par ailleurs, *L. plantarum* confère également une robustesse développementale au niveau de la structuration des organes comme l'aile.

Dans le second chapitre de cette thèse, nous nous sommes intéressés à Dawdle, un ligand de la voie du TGF- β, qui participe à la répression par le glucose en inhibant l'expression de diverses enzymes digestives (notamment les maltases et maltases) au niveau du l'intestin de mouches adultes. La boucle de régulation comprenant Mondo-Mlx-Dawdle assure la régulation de nombreux gènes impliqués dans la réponse aux sucres. Nous avons examiné le rôle de *dawdle* dans l'interaction Drosophile-*L. plantarum* au cours de la croissance sur milieu pauvre en protéine. Nous avons montré que la boucle de régulation incluant Mondo, Mlx and Dawdle semble jouer un rôle dans l'interaction. De plus, nous montrons que Dawdle a un rôle important dans la régulation des protéases digestives dans contexte nutritionnel de carence en protéine. Cet effet régulateur peut-être soit inhibiteur ou activateur selon l'environnement microbien.

Ainsi, nos travaux participent à la compréhension des mécanismes régulant le mutualisme nutritionnel. De plus, nos résultats montrent que des modèles simples comme le notre : le couple Drosophile-*L. plantarum* ont encore leur importance dans le monde de la biologie moderne, et ce, malgré les nombreuses avancées technologiques effectués au cours des deux décennies passées. Notre model a permis de révéler un nouveau rôle du microbiote dans des concepts fondamentaux tels que le la canalisation développementale et le tampon génétique.

Introduction

INTRODUCTION

1. Animals and microbes symbiosis

1.1. The endosymbiotic origin of life

Microbes are everywhere, from the deepest ocean waters, to the clouds. They arose and diversified long before the appearance of large multicellular organisms, thus, the world animals originated from what, was smothered in and shaped by microbes. All animal life has evolved among an ocean of microorganisms, and as a result, all animals and plants have established symbiotic relationships with microorganisms.

The term "symbiosis" was coined by the German botanist Heinrich Anton de Bary to describe "differently named organisms" living together, or the long-term association of two or more partners (Anton de Bary, Die Mycetozoen. Ein Beitrag zur Kenntnis der niedersten Thiere, 1859). One of the most striking examples illustrating the foremost importance of symbiosis is endosymbiosis, which gave rise to some organelles of eukaryotic cells (mitochondria, chloroplasts and basal bodies of flagella). The endosymbiosis, theory, or symbiogenesis was described by Lynn Margulis (named Lynn Sagan at the time she published her theory) in her seminal paper "On the origin of mitosing cells" published in 1967 in the *journal of theoretical biology* (Sagan, 1967). This theory posits that eukaryotic organelles were created when a prokaryotic cell was ingested by a larger heterotrophic anaerobic host cell (illustrated in Fig 1). This endosymbiosis became obligate: the fossil bacterium gave rise to the mitochondria thus changing the evolutionary course of the eukaryotic cells. This extreme case of symbiosis laid the foundation of virtually all life on earth.



Figure 1: *Endosymbiosis: Homage to Lynn Margulis*, a painting by Shoshanah Dubiner, February 13, 2012.

1.2. The different types of symbioses

Symbiosis literally means "living together". It is the interaction between at least two different organisms. Animals and microbes engage in different types of symbioses ranging from parasitism to mutualism resulting in a wide range of effects on both partners. Host-microbes interactions can be generally divided in three types of interactions depending on the outcome: parasitism, commensalism and mutualism (Casadevall and Pirofski, 2000; Hentschel et al., 2000), which we will detail thereafter.

1.2.1. Parasitism

Parasitism is defined as an interaction between a host and microorganisms where one of the two partners is advantaged while the other is harmed. This class of interaction is therefore detrimental to one of the partner and the parasite utilizes the host for its own benefit (Peterson, 1996). One classic example of parasitism is the interaction between

Agrobacterium tumefaciens and plants. *A. tumefaciens* is a soilborne gram negative bacteria. It is a biotrophic tumorigenic pathogen responsible for the crown gall disease (Zaenen et al., 1974; Bourras et al., 2015). The bacterium enters the plant through a wound site; once inside the plant it injects a part of its DNA in the form of a plasmid (plasmid Ti) into a plant cell. The plasmid integrates the plant genome and highjacks the activity of the cell to force it to proliferate and produce opines, molecules that are used by the bacteria but are of no use for the plant. In this system, the bacterium completely highjacks the plant metabolism to favor its own proliferation, to the detriment of the plant growth.

1.2.2. Commensalism

The classic definition of commensalism (*commensalis* meaning "share a table") depicts a type of interaction between two partners where one benefits from the association while the other is neither advantaged nor harmed (Hooper and Gordon, 2001). The definition of commensalism was first introduced to describe the use of food waste by second animals. The Remora fish, belonging to the family of Echeneidae, evolved a flat oval sucking disc on the top of its head, which is used to attach to the body of larger animals such as sharks or whales to gain easy access to food as it feeds on the leftovers of its hosts' meals. Another well-known example is the lifestyle of epiphytes plants such as Orchids that grow on the branches or trunk of other plants. They use the tree as physical support only and do not derive any nourishment nor harm them in any way (Benzing, 1990). The human microbiota (group of microorganisms associated to a host) was first viewed as commensal, as at the time it was thought that the gut microbes took advantage of their human hosts who provided shelter and food source, without influencing their host's physiology. It is only years later that human-associated microbes were found to profoundly impact their host physiology. For instance, intestinal bacteria participate in energy harvest through fermentation and absorption of undigested carbohydrates (Gill and Prasad, 2008).

1.2.3. Mutualism

Two organisms engaged in a mutualistic interaction when both draw benefits from the association. Many examples of mutualism exist in nature, most of which are nutrition-

driven. Mealybugs (*Planococcus citri*) are sap-feeding insects living in an obligate symbiosis with the endosymbiont *Tremblaya princeps*. *P. citri* offers shelter and food to *T. princeps*, and in return, the symbiont plays a critical role in providing metabolic products, such as essential amino acids, vitamins that the host can neither make on its own nor acquire from the plant sap, which is a nutrient-defective diet, (McCutcheon and von Dohlen, 2011). *T. princeps* has a very degenerate genome of 139 Kilo base pairs-long, one of the smallest existing genomes known. Several essential genes are missing, including genes related to translation, aminoacyl t-RNA synthesase for instance. Furthermore, genome sequencing of both *P. citri* and *T. princeps* failed to explain how certain essential amino acids were synthesized, as neither the host nor its symbiont could produce them. It turns out that *Tremblaya princeps* has acquired its own endosymbiont: *Moranella endobia* (von Dohlen et al., 2001) which synthetizes the essential amino acids missing in Tremblaya. Strikingly, the biosynthesis of tryptophan and threonine requires a patchwork of genes form both Moranella and Tremblaya (McCutcheon and von Dohlen, 2011). The mealybug is a rare case of tripartite symbiosis and the metabolic requirements of the three partners are fully entangled.

It is important to note that the outcome of the interaction between two organisms is not always unambiguous to categorize. While many symbionts provide clear-cut cases of mutualism, supplying their host with nutrients or defense means, the outcome of the interaction between several organisms can be difficult to categorized: it can vary depending on the nature of the host or the host physiological state. More particularly, many host-associated microorganisms combine mutualistic and pathogenic properties. For example, *Wolbachia*, a maternally transmitted bacterium that infects a great number of arthropod species, produces a remarkable diversity of phenotypes on the host. The best-known being the manipulation of the host's reproduction to increase its transmission to the next generations at the expense of the host's fertility, but *Wolbachia* can also protect its host from viral infection (Teixeira et al., 2008), thereby, benefiting the host. Symbiosis functional outcome depends on both the host and the microbes and fluctuates according to the context of the interaction.

1.3. Obligate versus facultative symbioses

The relationship between two organisms can be further characterized as obligate or facultative. In the case of a facultative symbiosis, each organism can live without the other. Ants and aphids can engage in such facultative symbiosis. They can live independently, but when they live in each other's vicinity, aphids produce a sugary fluid that the ants can live off, in return the ants protect the aphids from predators such as lady bugs. On the contrary, for organisms involved in an obligate symbiosis, the interaction is an absolute requirement for survival for one or both partners therefore they cannot live without each other. Namely, such symbiosis can be obligate for the microorganisms only, for the host only or for both. Many examples of obligate symbiosis can be found in insects. For instance, lower termites can thrive on a diet of wood, feeding on the lignocellulose, the principal cell wall component of woody plants, thanks to the presence of gut symbionts housed within a hindgut paunch (Brune, 2014). The termite symbionts are cellulolytic flagellates belonging to two eukaryotic phyla (the Parabasalia and the Preaxostyla). Once ingested, the wood particles are partially digested by enzymes secreted by the termite salivary glands, then they pass through the hindgut paunch, where the flagellates further break down the remaining polysaccharides including mannosidases, xylanases. The microbial fermentation products, mainly short-chain fatty acids, are then absorbed by the host (Brune, 2014).

1.4. Modes of transmission

The acquisition of microbes can be horizontal, where the microorganisms are acquired from the environment, or vertical, where the microorganisms are transmitted across generations from parents to progeny usually in a transovarian manner. Most insect orders acquire their symbiont vertically. For instance, *Spiroplasma*, an endosymbiont infecting *Drosophila*, is incorporated into the fly oocytes and thereby is transmitted into the embryo directly (Haselkorn, 2010). In the case of horizontal transmission, the organism is colonized anew at each generation. It is the case of the squid *E. scolopes* with its symbiont *Vibrio fisheri*. The colonization process will be detailed in a further section.

2. Impact of microbiota on evolution of multicellular organisms

The concept of biological individual or biological unit is now debated and many scientists argue that an individual should be considered as a "holobiont": the sum of the organisms composing it, meaning the individual (plant or animal) with all its associated microorganisms (Margulis, 1993). The sum of the genetic information from the host and its associated microbiota comprises the hologenome (Zilber-Rosenberg and Rosenberg, 2008). The hologenome theory of evolution, developed by Ilana Zilber-Rosenberg and Eugene Rosenberg in 2008, posits that the holobiont along with the hologenome acting as a consortium should be considered as a unit of selection in evolution, and that variations in the diverse microbial symbionts can have an important role in the adaptation and evolution of the holobiont (Zilber-Rosenberg and Rosenberg, 2008). According to Darwinian theory, nature selects the "fittest" individual to survive and pass on its genes to the next generation. The association between a host and its microbiota affects the fitness of both the host and the microbial partners in the holobiont environment. The genetic diversity of the host is enriched by the microbiome that responds faster to the changing environment-as bacterial genomes can mutate and adapt much faster than the host's- thus allowing the host to respond and adapt faster to environmental changes, hence improving natural selection success for the holobiont.

The holobiont theory arose from the study of the mechanisms of infection underlying bacteria-induced coral bleaching caused by the increase in seawater temperature (Kushmaro et al., 1996). They discovered that coral had become resistant to the bleaching agent *Vibrio shiloi* and they hypothesised that a dynamic relationship occurred between the coral host and symbiotic microorganisms upon different environmental conditions, and that corals could adapt quickly to the changing environment, by altering their population of symbiotic bacteria (Reshef et al., 2006). Changing their microbial partners allows the corals to adapt to changing environmental conditions more rapidly (days to weeks), than via mutation and selection, which would take many years (Reshef et al., 2006). Therefore, microbial partners can be instrumental to the evolutionary success of their host.

The microbiome (collective genomes of the microbiota) represents an enormous number of genes contributing to the host genetic potential. For instance, symbiotic relationships have expanded the limited metabolic network of most eukaryotes by providing several bacterial features such as methanogenesis, nitrogen assimilation, chemolithoautotrophy (Engel and Moran, 2013). Considering that the microbiome is an extension of the host genome, as stated by the holobiont theory of evolution, it makes sense that symbionts could be involved in speciation. Using the parasitoid wasp from the genus *Nasonia*, Bordenstein's group studied the impact of the microbiome on interspecific hybrid lethality (Brucker and Bordenstein, 2013). *N. vitripennis*/*N. giraulti* and *N. vitripennis*/*N. longiconis* hybrids are lethal. They found that bacterial abundance and diversity from the hybrids differed from the parental lines, and hybrid lethality was almost fully rescued by rearing the animals as germ-free. This study suggests that intestinal microbiota can play crucial roles in the determination of the viability of offspring, which is important for speciation. Behavioral reproductive isolation is another speciation barrier as it prevents mating between species because of differences in the courtship behavior or sexual attraction. Mating behavior can be altered by different gut microbiota composition. For example, isogenic *Drosophila melanogaster* individuals raised on different diets (Starch or molasses) are associated with different microbiota (Sharon et al., 2010). After only one generation, the difference in microbiota caused by the diet led to strong mating discrimination between the starch and molasses-raised *Drosophila*: "molasses-reared" flies preferred to mate with other "molasses-reared" animals whereas "starch-reared" flies preferred to mate with other "starch-reared flies. This mating preference is caused by the fly microbiota by changing the levels of cuticular hydrocarbon sex pheromones (Sharon et al., 2010).

Microbiota assists the host for nutritive resource exploitation and specificity by providing the host with many metabolic enzymes. Microbes associated to the host can create speciation events by providing their host with means to adapt to new foods; such adaption could lead to niche adaptation, thereby geographic isolation and, speciation. For instance, the weevil *Sitophilus* is ecologically isolated from its closest relatives because its symbiont, contained in specialized structures called the bacteriocytes, enables it to feed exclusively on cereal grains, as opposed to the rest of the

Rhynochophorinae subfamily that feeds on monocotyledon stems (reviewed in Brucker and Bordenstein 2012).

Wolbachia pipientis are cytoplasmic intracellular bacteria infecting a wide range of arthropods. They are transmitted vertically through the host eggs. They alter host biology in many ways, including reproduction. *Wolbachia* has developed different mechanisms to manipulate host reproduction: cytoplasmic incompatibility, parthenogenesis-induction, feminization, and male-killing (Stouthamer et al., 1999). Cytoplasmic incompatibility (CI) is the most frequently found *Wolbachia*-induced phenotype (Werren et al., 2008). This phenomenon causes the incompatibility between *Wolbachia*-infected males sperm and eggs from females that do not harbor the same *Wolbachia* strain, resulting in failure to form viable offspring. CI comprises two distinct features: 1) Sperm is modified by *Wolbachia* during spermatogenesis, and 2) The rescue of this modification in embryos infected with the same strain. *Wolbachia* is only transmitted by females, therefore, CI promotes *Wolbachia* spread. The incompatibility is due to the asynchrony of the male and female pronuclei during initial step of mitosis (Werren et al., 2008). Cytoplasmic incompatibility between diverging populations could drive the evolution of new species.

As we have previously seen, evolutionary success of the host is often associated to its capacity to extend its genetic potential. Genetic buffering mechanisms alter the relationship between phenotypic and genotypic variations concealing the consequences of genetic and environmental variation on phenotype (Rutherford, 2000). This mechanism can have important evolutionary consequences. We can wonder whether microbial environment could be involved in such buffering mechanisms, but this facet of microbiota function have only started to be investigated.

Microbiota has been completely ignored for centuries in the study of evolution, but we now know that symbionts have played an important role in driving their host evolution. However, the hologenome theory of evolution has to be taken with caution. Symbiotic bacteria have surely played crucial role in evolution, however, because of the versatile nature of microbiota, it is likely that selection does not act predominantly at the holobiont level (Douglas and Werren, 2016).

3. Impact of microbiota on host physiology and behavior

In the last decade, research on host-microbiota interactions has been very fruitful and special efforts have been focused in understanding how the microbes affect their host physiology. These studies showed that microbiota affects virtually every physiological process during the entire host's lifetime. A non-exhaustive list of traits impacted by the microbiota will be detailed in this chapter with the help of few specific examples.

3.1. Host Post embryonic development

Animal development is largely directed by its genome. However, microbes play a role in providing signals for multiple developmental steps. If the involvement of microbes in embryonic development has just begun to be investigated, the impact of symbiosis on the post-embryonic development (postnatal stages) has been well documented. One of the most convincing examples of microbial effect on animal development is the induction of settlement and metamorphosis of many marine invertebrates (Hadfield, 2011). Notably, the study of the association between the Hawaiian bobtail squid *Euprymna scolopes* (Figure 2A) and the bioluminescent bacterium *Vibrio fischeri* during squid post embryonic development has laid the foundations of the understanding of the influence of bacteria to host development. *E. scolopes* uses counter illumination as a means of defense against predators: when it hunts near the sea surface, the bioluminescence emitted from the light organ prevents the squid from casting a shadow and being detected by predators awaiting deeper in the ocean waters. The bioluminescence is provided by *Vibrio fischeri* contained in a specialized light organ. The interaction between the squid and *V. fischeri*, although highly specific, is not obligate for neither partners' survival or growth, however it is a requirement for the development of the light-emitting organ. The bacteria are not vertically transmitted, therefore the symbionts must be acquired anew by each juvenile host. Shortly after the youngling hosts emerge from the eggs, the association with the symbionts, which colonize the surrounding seawater, is established. The porous mantle cavity of the organ is colonized by pumping seawater during normal ventilation process. The mantle cavity includes ciliated epithelial appendages that will facilitate the bacterial inoculation (Figure 2B). *V.*

fischeri specifically aggregates within the mucus secreted by the epithelium, and then colonize host tissue. The establishment of the symbiont within the newly hatched squid will trigger profound remodeling of the mantle cavity to give rise to the adult complex light-emitting organ. Following the symbiont settlement, a daily rhythm of symbiosis will be established: 5% of the bacterial population will settle once and for all in the host, whereas 95% of the population is discarded at dawn and regrown at night when the light emission is required for the squid.

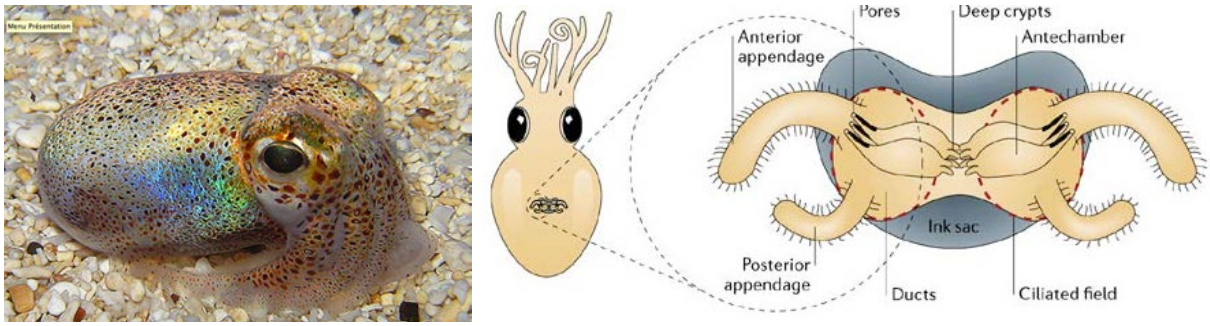


Figure 2: Structure of the light-emitting organ of *E. scolopes*

A) Photography of a Hawaiian bobtail squid *Euprymna scolopes*. (Source: S. Cohen ©) B) Structure of the juvenile light organ. A ventral view of the newly hatched squid reveals the nascent light organ in the centre of the mantle cavity (scale bar 0.5 mm). This picture was modifed from Cloud-Hansen et al., (2004).

3.2. Microbiota supports host immune system

Immunity is another key process affected by the microbiota. Indeed, the immune system function is to detect and counteract foreigners, mostly microorganisms. It is therefore anticipated that the microbiota interacts dynamically with the host immune system. Symbiotic microorganisms have co-evolved with the host and provide the host with several functions supporting immunity such as promoting immune homeostasis (immune resilience), immune response and protection against pathogen colonization. They do so by diverse mechanisms such as competition for limited nutrients or for niche space, direct killing or enhancement of the host immune response.

First, microbiota supports the development and the maturation of the immune system. A study suggests that in mammals, the expansion and maintenance of naïve CD4+ T cells in

the periphery is mediated by the intestinal microbiota (Dobber et al., 1992). In this study, the authors investigated whether the observed age-related changes in a subset of CD4+ T cells are caused by previous antigenic exposure. To address this question, they performed analyses on splenic CD4+ T cells isolated from mice in different gnotobiotic background, at both young and more advanced age. They found that the number of splenic CD4+ T cells isolated from conventionally reared (CR) mice was increased two-fold as compared to GF mice, and association of GF mice with CR-microbiota triggered the expansion of CD4 T cells. As for the maturation of the immune system, the gut-associated lymphoid tissues of mammals mature thanks to the presentation of peptidoglycan monomers presented by Gram-negative bacteria from the intestinal microbiota during their early establishment, through recognition by the innate receptor of epithelial cells, NOD1 (nucleotide-binding oligomerization domain containing 1) (Bouskra et al., 2008).

Furthermore, in addition to its role in development and maturation of the immune system, the microbiota also provides the host with an additional line of protection. Colonization resistance is the protection of the host from exogenous pathogens by the endogenous bacteria (*i.e*, the microbiota). Indeed, bacteria secrete a large amount of different molecules, including antibiotics and other antimicrobial molecules such as reactive oxygen species (Nitric oxide). For example, commensals can protect the host by direct killing of pathogens: Bifidobacteria secrete Short Chain Fatty Acids (SCFAs) such as acetate and they have been shown to protect the host from lethal infection with enterohaemorrhagic *Escherichia coli* (Fukuda et al., 2011). Bacteroidetes species also secrete SCFAs such as butyric or acetic acids which inhibit the growth of pathogenic *Salmonella* spp (Miller and Bohnhoff, 1963).

3.3. Microbiota affects the host behavior

It is anticipated that the host behavior, more particularly social and feeding activities, profoundly impacts the establishment and the regulation of microbiota. But in turn, the host-associated microbes can manipulate the host behavior (Ezenwa et al., 2012).

Recent research has brought increasing evidences revealing a surprising role for host-associated microbes in shaping behaviors in dramatic ways across animal taxa (reviewed in Ezenwa et al., 2012). In the past years, microbiota, and more particularly intestinal bacteria have been shown to affect mating, reproductive behavior, mood, anxiety, and satiety, etc.

The intestine is sometimes referred to as the "second brain" because of its numerous neuronal connections, and the bidirectional communication between the gut and the brain has long been recognized. The human digestive tract hosts trillions of microbial cells, and as Ed Yong wrote "there are more bacteria in our gut than stars in our galaxy" ("I contain multitudes, the microbes within us and a grander view of life", Ed Yong, 2016). Furthermore numerous studies have recently unveiled a role of intestinal microbes to affect cognitive functions such as anxiety or depression. Therefore, a new concept has emerged: the microbiota-gut-brain axis. Intestinal bacteria can directly affect the CNS functions via the regulation of endocrine signaling pathway such as YY peptide or glucagon-like peptide 1 (GLP1) pathways (Fetissov, 2017). For instance, GF rats have defects in brain regions controlling anxiety resulting in alteration of the dopaminergic turnover rate in brain upper structures. These animals exhibit an exacerbated response to stress (Crumeyrolle-Arias et al., 2014). Moreover, the influence of gut microbiota can go as far as affecting our mood: a medical trail demonstrated that consumption of a probiotic-containing yogurt composed of *Lactobacillus casei* significantly improved the mood of the participants whose mood was initially poor (Benton et al., 2007).

3.4. Microbiota affects metabolism and nutrition

Nutritional symbiosis is the major form of symbiosis and it is particularly widespread among insects. Insects have adapted to a tremendous range of ecological niches and often, the metabolic genes from the bacteria allow them to thrive on sub-optimal diets. One crucial mechanism that enabled herbivorous insects to cope with nutrient deprivation is the association with endosymbiotic bacteria. The pea aphid is one of the best-studied cases of nutrition-based endosymbiosis. The pea aphid, *Acyrthosiphon pisum*, is associated to its obligate symbiont *Buchnera aphidicola* housed in large

specialized adipocytes called the bacteriocytes. They are passed on through strict maternal transmission. The aphids feed on plant phloem sap, an imbalanced diet deficient in essential amino acids. To utilize plant sap as their sole food source, *A. pisum* fully relies on *Buchnera aphidicola*. The association is obligate for both partners as neither of them can survive in absence of the other. This symbiont has co-evolved with its aphid host throughout its evolution and as such, it exhibits a characteristic feature of long-term co-evolution: its genome has been significantly reduced (Shigenobu et al., 2000; Shigenobu and Wilson, 2011).

The viviparous tse tse fly has established obligate symbiosis with *Wigglesworthia*. A unique feature of the tsetse fly biology is the adenotrophic viviparity, in which the majority of the larval development occurs *in utero*. The developing larva supplies nutritious lipids and proteins through female accessory glands in a process reminiscent of mammalian lactation. In the absence of the symbiont the larval development is stunted and the progeny is aborted (Rio et al., 2012). *Wigglesworthia*'s genome encodes a high number of vitamins (biotin, thiazole, lipoic acid, FAD (riboflavin, B2), folate, pantothenate, thi- amine (B1), pyridoxine (B6), protoheme and nicotinamide), which are necessary for the fecundity and survival of its host (Akman et al., 2002; Rio et al., 2012). The obligate symbiosis established with *Wigglesworthia* drove the strict nutritional specificity of the tsetse fly feeding exclusively on the blood of vertebrate animals.

The bovine rumen is a case of nutritionally-driven obligate symbiosis in mammals. Bovines' rumen acts as a temperature-controlled anaerobic fermentor, which permits the host to harness the ability of bacteria to break down cellulose. In the rumen, bacterial enzymes convert the cellulose into glucose subunits, which are then fermented by different group of bacteria to synthetize short-chain fatty acids, which will be absorbed through the rumen wall and pass into the blood stream to fuel various tissues of the body (Russell and Rychlik, 2001).

In summary, a great majority of hosts derive nutritional benefits from symbiosis, as the gut symbionts maximize nutrient and energy harvest from complex food. The bacteria supply the host with additional enzymatic activities, enabling the host to utilize nutrients that it would otherwise not be capable of feeding on. Mutualistic relationships can drive the host dietary needs of both insects and vertebrates, like mammals.

Functional studies can be conducted in mammals to study host-microbiota-diet interaction thanks to the rearing of axenic (i,e, germ-free) and gnotobiotic (i.e, associated with a defined microbiota) animals. However, rearing axenic mammals is costly and inconvenient, moreover, unbiased mechanistic approaches are difficult to conduct. Therefore, there is still today a need of simpler model. Arthropods perfectly fulfill these demands, and among them, particularly, *Drosophila* proved to be a potent host model.

4. *Drosophila* as a host model to study symbiosis

The fruit fly, *Drosophila melanogaster*'s history as a model organism in biological research is rich and its relevance and value are unquestioned among scientists. The fruit fly has been widely used in laboratories for over a century, and has played a pivotal role in understanding and dissecting the molecular and cellular processes of evolution, development and physiology. We share 60% of our DNA with the fruit fly and 75% of human genes involved in diseases have a homolog in *Drosophila*. *Drosophila* presents high throughput screening aptitudes, they are inexpensive and easy to raise, they have a short life cycle (it takes approximately 10 days for a *Drosophila* to develop from egg to adult on optimal rearing condition) and high fecundity, so one can obtain very large numbers of individuals in a short window of time. In addition, *Drosophila* possesses an amazing and powerful genetic toolbox, making it one of the most genetically amenable model organisms. Therefore *Drosophila* stands out as an excellent model organism, and it does more particularly to study symbiosis.

Another interesting point is that fly physiology is not so different from that of mammals: In particular, fly and mammal gastrointestinal tracts are very similar in term of overall structure and functions. The digestive tract's primary role is the breakdown and absorption of nutrients. Underestimated for a long time, the gut is a very complex and sophisticated organ, and in addition of its role in digestion, it also provides one of the first defense lines against pathogens, and maintains intestinal homeostasis by exchanging neuronal as well as hormonal signals with other organs, such as the brain or

the fat-body (Lemaitre and Miguel-Aliaga, 2013). A gold standard for studying intestinal microbiota uses germ-free (GF) animals to demonstrate the importance of microbes in a defined process. A key advantage in using *Drosophila* as a host model is that it can easily be made and maintained as germ-free (GF) without the requirement of costly and complex equipments and facilities (Koyle et al., 2016). In addition of being easy to maintain GF, *Drosophila* can be easily raised as gnotobiotic (with defined microbial communities) (Ma et al., 2015).

Drosophila microbiota is environmentally acquired (horizontal transmission) as in the wild, *Drosophila melanogaster* feeds on the microorganisms (bacteria and yeasts) found on rotting fruits (Chandler et al., 2011; Téfit et al., 2017). With the exception of intracellular symbionts such as *Wolbachia*, *Drosophila* embryos are sterile but the eggshell protecting the embryo is contaminated with bacteria most likely acquired from parents' feces. As a consequence, newly hatched larvae are virtually germ-free. They acquire a microbiota within the first 24 hours of life, through ingestion of microorganisms seeded on the food by their parents' feces (Fig 3), and the acquired microbiota continuously changes its quantity and composition as a function of the fly's developmental stage and physiological well-being. However, the bacterial communities are not resident of the fly gut; instead, they are ingested, travel through the digestive tract and are eliminated. Hence, fly gut microbiota is transient but undergoes constant re-association cycles (Storelli et al., 2017, *in press*; Blum et al., 2013). *Drosophila* has a simple and easily manipulated intestinal microbiota. It is composed of far fewer species (1-30) than mammals (hundreds) and most of them are aerotolerant and therefore cultivable in laboratory, as opposed to mammals gut microbiota whose most species are anaerobic and difficult to culture in lab. *Drosophila*-associated bacterial species have a relatively low diversity (1-30 different species) and are taxonomically restricted. 85% of the bacteria composing the wild fly microbiota are represented by three or four bacterial families: *Enterobacteriaceae*, *Acetobacteraceae* (*Proteobacteria* phyla), *Lactobacillaceae* and *Enterococcaceae* (*Firmicutes* phyla) (Chandler et al., 2011; Erkosar et al., 2013; Broderick and Lemaitre, 2012). Laboratory-raised flies are dominated by species belonging to the *Acetobacter* or *Lactobacillus* genus. *Lactobacillus plantarum* and *Acetobacter pomorum* have been identified in most laboratory-reared fly stocks (Broderick and Lemaitre, 2012). The diet is the major factor driving *Drosophila*

microbiota composition. For example, lab flies raised on a diet containing simple sugars are dominated by *Acetobacter* genus, whereas if the diet is rich in starch as a carb source, *Lactobacillus* genus is the most represented.

Drosophila microbiota impacts various traits of its physiology (Figure 4) such as mating behavior (Sharon et al., 2010), lifespan (Brummel et al., 2004), immunity (Ryu et al., 2008), food choice behavior (Leitão-Gonçalves et al., 2017). Our group focuses on one aspect of fly physiology greatly impacted by its microbial partners: growth.

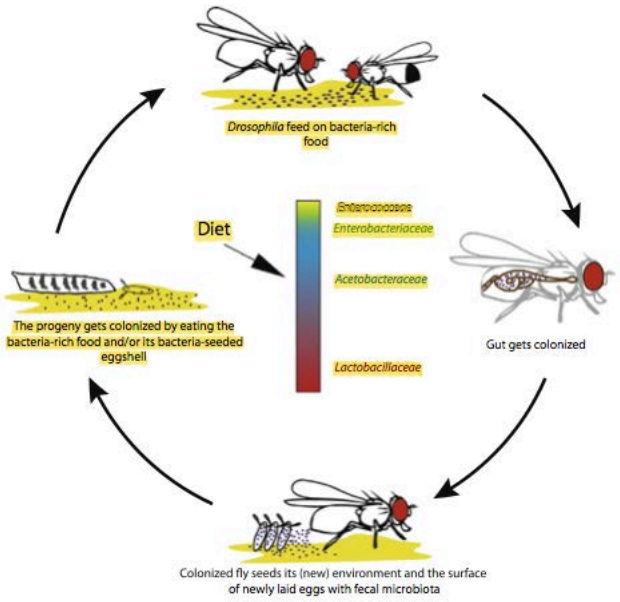


Figure 3: The establishment and the maintenance of *Drosophila* microbiota depends on the constant intake of microbes from the diet. The gut of newly emerging flies contains a very low number of microbes. Constant ingestion of bacteria-rich food allows the microbial colonization of the digestive tract and the maintenance of an intestinal microbiota. The parents' fecal microbiota is transmitted to other flies and to the progenies through the deposition of feces on the substratum on which they thrive. Female also ensure optimal transmission of their own microbiota by seeding the embryonic eggshell of their progenies, which is eaten by the hatching larvae and subsequently smeared onto the food substratum. The dominant bacterial families associated to *Drosophila* adults are color-coded according to their representative proportions. The type of diet the flies encounter can significantly alter the depicted proportions. Adult *Drosophila* drawings are modified from. (Figure and legend extracted from Erkosar and Leulier, 2014).

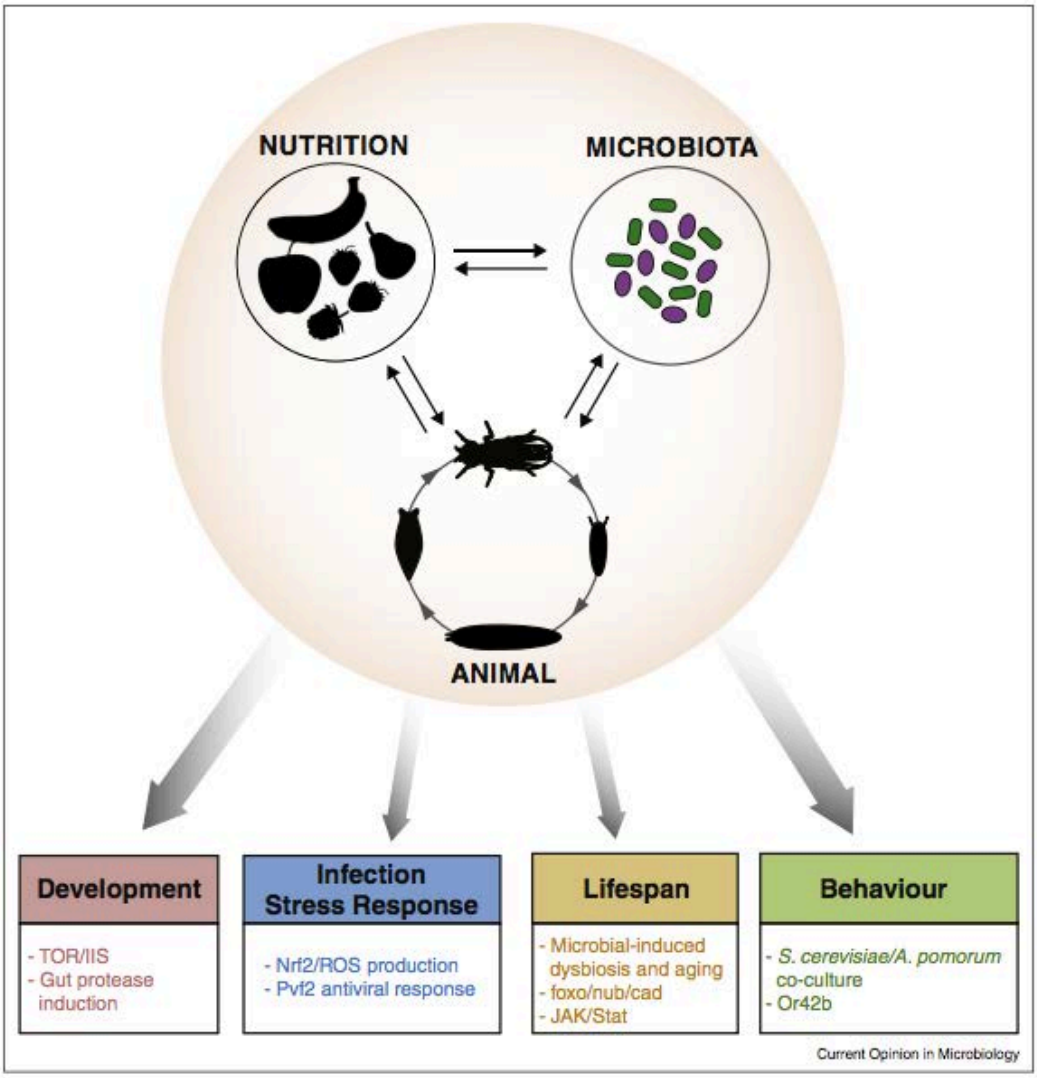


Figure 4 : Integrative view of the functional outcome of the relationships between *Drosophila melanogaster*, its microbiota and nutrition. *Drosophila*'s microbiota shapes its host biology throughout its life cycle by affecting its survival, behaviour, immunity and development through the activation of diverse hormonal and metabolic pathways. Figure extracted from (Martino et al., 2016).

5. *Drosophila* growth and metabolism

Juvenile growth, defined as an increase in size and weight before reaching adulthood (Efstratiadis, 1998), is a tightly controlled process, where nutrition and microbiota play an essential role. The genetic and molecular basis of growth control and regulation has been extensively studied in *Drosophila* (reviewed in Boulan et al., 2015; Strigini and Leulier, 2016). Mainly genetically encoded, developmental programs result in the

production of signaling molecules that define growth and size of each organ. Temporal and spatial distribution of cells, are controlled by signaling pathways such as Wnt, Hedgehog, TGF- β/Activin, Notch and EGF pathways. In parallel, growth is controlled systemically through endocrine regulation which further links the increase in body size with the metabolic state of the animal and nutrient availability. In addition, endocrine signals coordinate inter organ communication, which is essential to regulate metabolic homeostasis and system growth (Boulan et al., 2015). Cell and tissue growth rates are regulated by insulin-like peptides (ILPs) belonging to the Insulin/IGF-signalling (IIS), and developmental transitions are controlled by the steroid hormone ecdysone. Both cell size and cell proliferation, are regulated by target of rapamycin (TOR) signalling, which mediates the energy and amino acid sensing and couples growth cues to cellular metabolism (Wullschleger et al., 2006). In the FB, intracellular availability of amino acids is detected via TOR pathway, which then remotely triggers the release of dILPs from the Insulin Producing Cells (IPCs) in the brain (Colombani et al., 2003), while activation of TOR signalling in the prothoracic gland (PG) positively regulates ecdysone synthesis (Layalle et al., 2008). Ecdysone is secreted by the prothoracic gland (PG), an endocrine tissue that integrates multiple inputs to adjust the progression through the life cycle with developmental and environmental signals. During the late larval development, a sharp peak in the production of prothoracicotropic hormone (PTTH) by two pairs of brain neurons induces the production of ecdysone by the PG. Finally, insulin and target of rapamycin complex 1 (TORC1) signaling in the PG coordinate steroid hormone production with nutritional environment.
Furthermore, TGF-β/Activin signaling pathway has a dual function in controlling the ecdyson production by modulating both the PTTH and insulin signaling in PG (Gibbens et al., 2011). We will detail this pathway as we have studied it further in this thesis.

In *Drosophila*, the TGFβ signalling cascade is divided in two branches: the bone morphogenic protein (BMP) branch and the Activin-like branch. The Activin family is composed of the ligands Actβ, Dawdle (Daw) and Myoglianin (Myo), whereas the BMP family ligands are represented by Decapentaplegic (Dpp), Glass bottom boat and Screw. Both ligand families might use a common set of type II receptors, but pathway specificity achieved through activating different receptors: the type I receptors such as Baboon (babo) binds to Activins while either *thickveins* or *saxophone* are receptors for

fly BMPs. Downstream of receptor activation, signal transduction is mediated by the R-Smads, dSmad2 for Activin ligands or Mad for BMPs. Activin/TGFβ pathway plays a pivotal role in the regulation of developmental transitions of *Drosophila* (Gibbens et al., 2011). Moreover, over the past years, one particular ligand of the Activin/TGFβ signalling casacade, Dawdle (Daw) was revealed to play a very important role in many aspects of development and metabolism. The results of these studies are detailed in the following sections.

5.1. Developmental and immune functions of *dawdle*

Dawdle (Daw) is a TGF-β ligand functioning via the TGF- β/Activin signaling cascade through the type-I-receptor Baboon (Jensen et al., 2009). Daw was mainly studied in the context of development. Two studies (Parker et al., 2006; Serpe and O'Connor, 2006) reported a role for Daw in motoneuron axon guidance. Moreover, Daw acts redundantly with Actβ to regulate neuroblast proliferation within the developing brain of larvae (Zhu et al., 2008).

Daw is expressed in different adult tissues including muscles, midgut, fat body and phagocytes (Bai et al., 2013; Buchon et al., 2013; Chintapalli et al., 2007). Adult *Drosophila* TGF- β/Activin signaling plays an important role in wounding and infection. Daw is transcriptionally modulated upon wounding. Daw is activated by Gram-positive bacteria whereas it is repressed by Gram-negatives. Its role is to limit infection-induced melanization (Clark et al., 2011). In addition of its role in development and in immune response, Daw has critical functions in diverse facets of fly metabolism.

5.2. Metabolic functions of *dawdle*

First, Dawdle plays a role in insulin secretion. Bai et al. reported that Dawdle binds and is repressed by dFOXO in the muscle. Reduced Activin signaling within muscles improves performance and protein homeostasis in aged flies through control of autophagy by Smox, a Daw downstream target, thereby delaying functional aging in muscles. Furthermore, reducing *daw* in the muscles decreased DILP2 peptide secretion

from IPCs in the brain, resulting in a reduced peripheral insulin /IGF secretion. (Bai et al., 2013). In line with this study showing a role of Daw in the regulation of DILP2 release, another group demonstrated that *dawdle* mutants display an increase in triacylglycerol (TAG), glucose, and glycogen stores as well as an increase in circulating sugar concentration (Ghosh and O'Connor, 2014). To validate the implication of Daw in IIS, the authors found that the transcript levels of dILP1, 2, 3 and 5 were not affected in Daw mutants, however, dILP2 accumulated in the IPCs, which indicates that Daw affects IIS by positively regulating dILP2 release from the IPCs in the brain (Ghosh and O'Connor, 2014). In the same study, the authors found that in addition of deregulated insulin release, loss of Daw also caused a food-dependent larval lethality associated to the acidification of hemolymph pH. The decrease in pH was associated to the accumulation of intracellular metabolic acids, including a majority of intermediates of the tricarboxylic acid cycle, indicating a role of *daw* in the regulation of intracellular sugar and mitochondrial metabolism.

In another study, Chng et al. (2014), focused on the role of Daw in glucose repression in adult *Drosophila*. Adult flies fed on a glucose-rich diet show a repression of intestinal digestive enzymes such as carbohydrases, glycosyl-hydrolases or lipases, a phenomenon described as glucose repression. Chng et al. investigated the mechanism of glucose repression and demonstrated that in response to glucose feeding, Dawdle is secreted by the fat body and acts on the midgut to inhibit the expression of several digestive enzymes, such as amylases or maltases (Figure 5A) (Chng et al., 2014). Furthermore, *daw* is involved in a feed-forward gene regulatory loop along with Mondo-Mlx, two sugar-responsive transcription factors (Mattila et al., 2015). Mondo-Mlx controls the expression of the majority of genes responding to sugar. They are involved in nutrient digestion and transport, and amino acid (glutamine and serine), lipid and carbohydrate metabolism. This regulation functions through the downstream effectors Dawdle and Sugarbabe (Figure 5B).

To sum up, *Drosophila* growth is a very tightly regulated process influenced by both nutritional and environmental cues, but the importance of the microbial environment to fly growth has also recently been demonstrated (Strigini and Leulier, 2016) and will be detailed further.

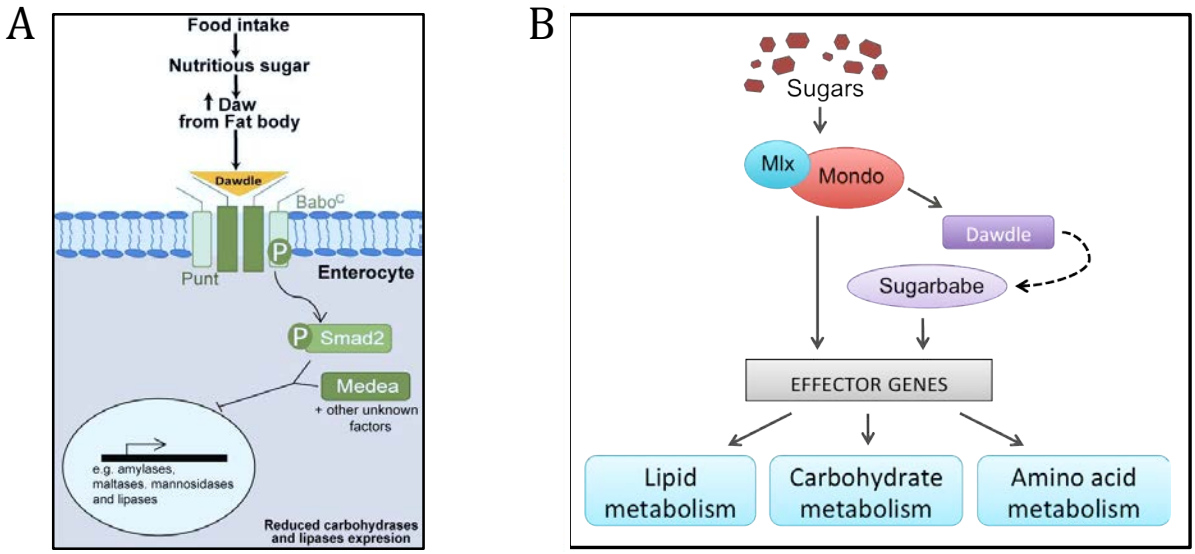


Figure 5: A) Model for repression of carbohydrases and lipases upon sugar sensing. Nutritious sugars consumption induces *daw* expression in the fat body. Secreted Daw then activates the canonical TGFβ/Activin signaling in the midgut through Babo^{C} and Punt receptors, leading to the activation of Smad2 and reduction of carbohydrase and lipase expression (Extracted from Chng et al., 2014)

B) Mondo-Mlx is a master regulator of a sugar-sensing regulatory network, including transcription factors Sugarbabe and TGF-beta/Activin ligand Dawdle (Adapted from Mattila et al., 2015).

6. *L. plantarum* affects Drosophila growth

Drosophila larval growth rate is intimately linked to the nutrient availability, and a lack of resources results in a decrease of the growth rate and subsequent developmental delay. Microbial context also participates in the regulation of the fly development. A study showed that *Acetobacter pomorum* promotes fly developmental rate, body size, energy metabolism, and intestinal stem cell activity through the pyrroloquinoline quitone-dependent alcohol dehydrogenase (PQQ-ADH) activity which modulates insulin/insulin-like growth factor signaling (IIS) (Shin et al., 2011). In parallel, Storelli et al. showed that upon protein scarcity, GF animals experience a developmental delay compared to their conventionally reared siblings, but *Lactobacillus plantarum*, a natural fly symbiont, promotes larval growth at the same extent as the entire microbiota does. This growth benefit is strain specific, as not all *L. plantarum* strains benefit growth. In

this study (Storelli et al., 2011) the strain *L. plantarum*^{WJL} (*Lp*^{WJL}) was used. *Lp*^{WJL} benefits growth through the regulation of systemic hormonal growth signaling modulated by the host TOR nutrient-sensing pathway: Storelli et al showed that reduced TOR activity achieved through fat body-specific expression of TSC1 and TSC2 (two negative regulators of the TOR pathway) resulted in the loss of *Lp*^{WJL}-conferred growth enhancement. Likewise, similar results were obtained by silencing expression of the TOR upstream activator *slimfast*, which encodes an amino acid transporter, in the FB. Our group further explored the mechanisms behind the growth benefit sustained by *L. plantarum* when flies face nutritive challenge (Erkosar et al., 2015). In this study, Erkosar et al. found that *L. plantarum* promotes the expression of intestinal peptidases partly through the PGRP-LE/Imd/Relish signaling cascade. This transcriptional regulation of proteases results in increased proteolytic activity leading to enhanced protein digestion and improved amino acid levels in the fly, thereby, triggering the activation of the fly TOR pathway and subsequent endocrine changes. This beneficial effect in protease expression is antagonized upon pathogenic infection showing a tradeoff between the beneficial response to intestinal bacteria and response to infection (Erkosar et al., 2015).

L. plantarum allows the fly to overcome nutritional challenges and grow better upon nutrient deprivation. However, the bacteria do not reside in the fly intestine. The majority of the ingested bacterial cells are inactivated when passing through the fly gut, at the level of the copper cells region, which is highly acidic. The few "survivors" are excreted in larval feces and seed the diet. This high fitness cost for the bacteria is compensated as *Drosophila* secretes maintenance factors necessary to sustain *L. plantarum* growth on the fly diet (Storelli et al., 2017, *in press*). GF larvae suffer severe growth impairments upon protein deficiency and *Lp*^{WJL} does not grow on the fly diet in absence of larvae. But when associated, *Lp*^{WJL} and Drosophila larvae sustain each other's growth upon nutritive conditions that are detrimental for them. Furthermore, the accelerated growth and subsequent earlier adult emergence on an imbalanced diet has no deleterious effect on adult *Drosophila*: adults emerging from *Lp*^{WJL}-monoassociated larvae are as fit as their GF siblings (Téfit and Leulier, 2017). *Lp*^{WJL} presence during larval development even resulted in a lifespan extension of nutritionally challenged adult males (Téfit and Leulier, 2017). To conclude, *Drosophila-L. plantarum* association is an elegant example of true nutritional mutualism (Storelli et al., 2017, *in press*).

L. plantarum is also a common member of mammalian gut microbiota. Our group investigated whether the growth benefit could be translated into mammals. Lp^{WJL} benefits juvenile growth of mice. Lp^{WJL} sustains both weight gain and longitudinal growth of infant mice on both standard and nutrient-deficient diets. Lp^{WJL} modulates the somatotropic axis activity to enhance systemic growth (Schwarzer et al., 2016).

7. Thesis objectives

Our group focuses on understanding the mechanistic of the interaction between *Drosophila melanogaster* and *Lactobacillus plantarum*. Before I started my thesis, our group had already shown that *L. plantarum*^{WJL} benefits the fly growth upon nutrient scarcity and enables the larvae to overcome nutrient deprivation. This growth promotion partly relies on the host TOR nutrient sensing pathway as well as on the upregulation of intestinal proteases. During my thesis, I endeavored to study the genetic basis of the interaction, focusing on the fly side.

In a first project, we explored how natural genomic variants affected the growth benefit supplied by *L. plantarum*^{WJL} (Lp^{WJL}) on a low-yeast diet by studying the growth effect of *L. plantarum*^{WJL} on the *Drosophila* Genetic Reference panel, a collection of extensively inbred fly lines derived from the wild. Then we performed genome wide association studies (GWAS) to identify the genetic variants associated to the optimal growth promotion provided by the intestinal microbiota. Finally we addressed the role of microbiota as a genetic buffering mechanism in the presence of nutritive stress. These questions are addressed in the first chapter of this thesis and a follow up study to which I am a co-author is presented in Annex 1.

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In a second project, we aimed at better understanding the mechanisms underlying the growth enhancement conferred by *L. plantarum*^{WJL} when flies face a nutritive stress. Our GWAS analysis uncovered the candidate gene *dawdle* as potentially involved in the molecular dialog between Lp^{WJL} and the fly during juvenile growth.

Daw is a TGF-β ligand that has recently been shown to be involved in different aspects of digestion and metabolism in *Drosophila* (see section "*Drosophila* growth"), and particularly it was shown to regulate multiple digestive enzymes (Chng et al., 2014) and promote the release of dILPs in the larvae (Ghosh and O'Connor, 2014). Our group published that the monoassociation with *L. plantarum*^{WJL} enhances the expression of digestive enzymes (proteases, lipases, and glycosyl-hydrolases) and that this upregulation is necessary to the growth benefit sustained by *L. plantarum*^{WJL} (Erkosar et al., 2015). Moreover, *L. plantarum*^{WJL} promotes growth through mTOR pathway and is associated with amplified insulin signaling activity. During my thesis, I investigated the role of *daw* in the molecular dialog between *Lp*^{WJL} and the fly during juvenile growth and studied how Dawdle metabolic functions behave in different microbial contexts. This is the focus of the second chapter of my thesis.

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Chapter 1

Host genetic bases of the interaction between *Drosophila melanogaster* and *L. plantarum* during juvenile growth

CHAPTER 1

Introduction

During the past decades there was a growing interest in the field of host/microbiota interactions, leading to many publications that demonstrated a role of microbiota, and more particularly intestinal microbiota, in affecting virtually every aspects of our physiology. Microbiota participates actively in our health. However, our knowledge of the mechanisms at play is still largely incomplete.

Our group published a few years ago that *L. plantarum*, a natural fly symbiont, promotes juvenile growth upon chronic protein deficiency as efficiently as the entire microbiota (Storelli et al., 2011). This growth promotion relies on activation of the fly TOR nutrient-sensing pathway in endocrine organs as well as on the upregulation of a set of the peptidase in the intestinal epithelium (Erkosar et al., 2015). However, the physiological response to Lp^{WJL} is a nutritive stress protective response and we assumed it is a complex polygenic trait. Therefore, we believe we have only started to unveil the genetic bases underlying the host/microbiota interactions during juvenile growth. To tackle this issue, we decided to assay how natural genomic variations affected the growth benefit sustained by Lp^{WJL}, and to conduct GWAS. Our objectives were two folds: 1) determine whether natural genomic variants impinge on the interaction between fly and its microbiota, and 2) identify the genetic variants associated to optimal growth promotion supplied by the intestinal microbiota.

Results

1) Natural genomic variants impact the growth benefit sustained by *L. plantarum^{WJL}*

Monoassociation with *L. plantarum^{WJL}* (*Lp^{WJL}*) promotes larval growth upon nutrient deprivation. Storelli *et al* and Erkosar et al. showed that *L. plantarum^{WJL}* is able to affect host gene expression and impact their physiology. The first goal of our study was to find out how naturally occurring genomic variations could impinge on the fly physiological response to *Lp^{WJL}* during juvenile growth. To tackle this question, we used the DGRP (*Drosophila melanogaster* Genetic Reference Panel) (Mackay et al., 2012), a collection of wild-derived fly lines generated by the Mackay lab and made available to the scientific community. These lines were established by capturing individual wild gravid females whose progeny was fully sibling-inbred for twenty generations to obtain virtual total homozygosity. To date, 205 lines are available. Their genome was fully sequenced and annotated with very high coverage, and many studies used the DGRP to study various quantitative traits such as ethanol sensitivity, sleeping patterns, aggressive behavior, starvation resistance, oxidative stress resistance, etc. All these data are gathered on one website to facilitate the access (http://dgrp2.gnets.ncsu.edu/). To start, we rendered 53 DGRP lines germ-free (GF) to get rid of all the microbes they carried. This process alone took us several months because we had to amplify the lines a lot as DGRP lines do not lay as well as standard lab lines such as *y,w* or canton-S. A few lines could not survive in absence of a microbiota therefore, we failed to establish them GF. For each line individually, we inoculated embryos with either PBS (GF condition) or 7 x 10^7 CFUs of *Lp^{WJL}* (monoassociated condition), and assayed the larval growth on a poor-protein diet. Our readout to assay larval growth was the larval length measured 7 days after egg deposition as described in Erkosar *et al*. For practical reasons, we were not able to test all the 53 lines at the same time because here again, we had to greatly amplify the GF DGRP lines in order to get enough individuals to proceed with larval length assay, moreover, the assay is time consuming, therefore, it took us more than a year to test all the lines 3 independent times. To see if there is experimental bias due to technical

issues, for each batch of DGRP tested, we included the *y,w* strain as a reference. *y,w* line is routinely used in our lab and we know very well how it responds to Lp^{WJL}, therefore, such a control enabled us to verify the experiment was not comprised by technical problem *e.g* differences in the quality of the fly diet, or the humidity of the incubator. Each DGRP line was tested three independent times to get a robust phenotype. As shown in the figure 1, for each line we measured the length of GF (panel A) and Lp^{WJL}-monoassociated larvae (panel B). To better visualize Lp^{WJL} effect, we calculated the fold-increase in larval length conferred by Lp^{WJL} as compared to GF condition (panel C). Fig 1A shows how genomic variants affected the capacity of the flies to grow on a low-protein diet and we observed important variation in the size among the DGRP lines. Therefore, GF DGRP flies grow at different pace when they face a nutritive stress. This result indicates that the DGRP lines, which are genetically very diverse, have different intrinsic growth capacities to overcome the nutritive challenge, and these differences in growth we observed are the result of the different genetic backgrounds. We next assayed the growth response to Lp^{WJL} on a low-protein diet (Fig1 B). We observed that, as for the GF condition, the length of monoassociated larvae varied among the DGRP lines, meaning that depending on their genetic background, monoassociated larvae do not grow at the same rate. From this result we could say that there might be variations in the degree of response to Lp^{WJL}: however, to better assess the growth enhancement specific to Lp^{WJL} presence, we calculated the fold increase in length conferred by Lp^{WJL} compared to the GF condition by dividing the average Lp^{WJL}-monoassociated length by the average GF length for each DGRP line we tested (Fig 1 C). First, we observed that Lp^{WJL} benefited growth in all the DGRP lines we tested. Indeed all calculated ratios are above 1: meaning that at the 7th day of larval development on the poor-yeast diet, all DGRP monoassociated larvae were longer than their GF siblings. None of the genetic variants carried by the DGRP we tested resulted in a complete loss of function of the growth benefit. However, it is of note that, although beneficial to all the lines, the intensity of the growth benefit provided by Lp^{WJL} was highly variable among the DGRP lines. This result indicates that the host genetic makeup is essential to determinate how the host will respond to the bacteria it carries. To conclude, our results show that natural genomic variations greatly affect the growth of *Drosophila* larvae on a low-protein diet, and they provide evidence that the host genetic background affects the host/microbiota interaction.

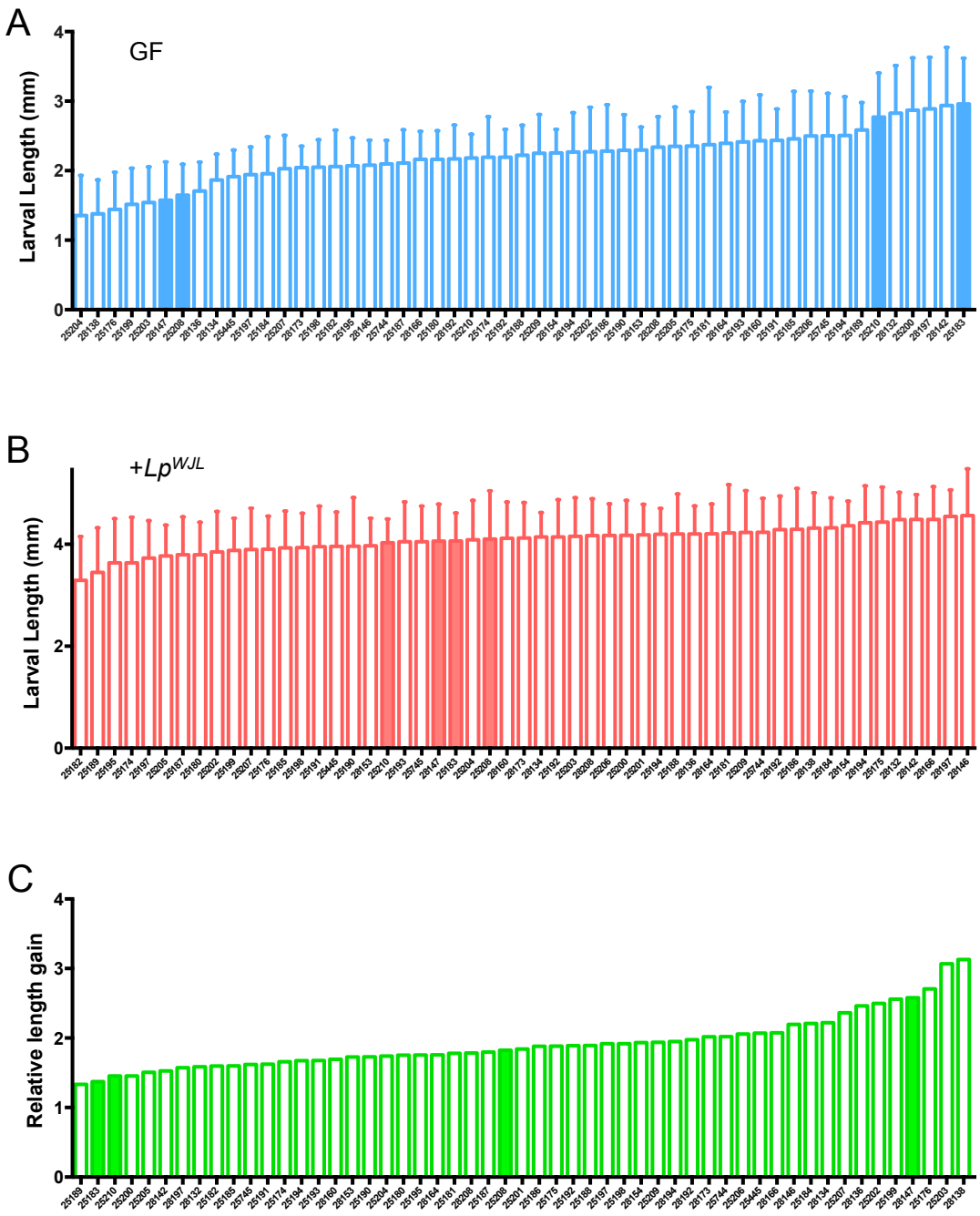


Figure 1: *L. plantarum*^{WJL} enhances juvenile growth of 53 DGRP lines upon protein deprivation.

A and B. Bar graphs showing the average larval length on day 7 after egg laying of 53 Germ-Free (A) or *Lp*^{WJL}-monoassociated (B) DGRP lines raised on low-protein diet. Standard deviation is plotted. Each bar represents the mean of samples containing between 10 and 40 viable larvae in each replicate, and 3 biological replicates were done per experiment, each experiment was repeated 2 to 3 times. The reference of each DGRP line is indicated below each bar. « Top » and « low » lines are color-filled.

C. Bar graph showing the relative length gain conferred by *L. plantarum*^{WJL}. Each bar represents the fold increase in larval length provided by *Lp*^{WJL} for each DGRP line compared to the GF condition. It is calculated by dividing the mean Lp^{WJL} length by the mean GF length for each line. The reference of each DGRP line is indicated below each bar. « Top » and « low » lines are color-filled.

2) Host genetic determinants of the physiologic response to *L. plantarum*^{WJL} monoassociation

Based on the larval-size ranking in different conditions, we conducted a Genome Wide Association studies (GWAS) to match the phenotypic variations we observed in the DGRP lines with the genomic diversity harbored by the DRGP flies. The GWAS was performed in collaboration with Bart Deplancke's group at EPFL in Lausanne. GWAS associates DNA variants with phenotypic traits by scanning thousands of genomic markers simultaneously in the form of SNPs (Single Nucleotide Polymorphisms) throughout the whole genome. Then, SNPs that demonstrate allele frequency differences between 2 lines situated at the opposite ends of the phenotypic range are identified. In spite of the small number of lines we tested, 53 out of 183 lines available at the time, the GWAS yielded a robust association of SNPs to our growth phenotypes. We generated 3 data sets: "GF", represents SNPs linked to genes associated to the variation in the intrinsic growth capabilities of the DGRP flies devoid of a microbiota; The "*Lp*^{WJL}-monoassociated" dataset represents the SNPs associated to the variation in the growth of animals possessing a microbiota. The 3rd data set, the "relative length gain" represents the SNPs linked to genes associated to the physiological response to *Lp*^{WJL} (i.e. in our case growth-promotion on low-protein diet). A total of 83 SNPs were uncovered from the three sets of GWAS, associated to 39 different genes (table 1). GWAS on the GF data set led to the identification of 23 SNPs associated to 12 different genes (table 2). The GWAS on the *Lp*^{WJL} -monoassociated data set (table 3) yielded to 40 SNPs associated to 28 different genes. The ratio GWAS (table 4) identified 20 SNPs associated to 12 different genes. It is interesting to note that in all 3 data sets, most of the significantly associated SNPs were located into introns, UTRs or intergenic regions, which suggests that modulation of the gene expression level could explain the phenotypic variations we observed in response to *Lp*^{WJL} monoassociation. Of note, we found very few overlap between the 3 gene lists. In addition and as expected, some of the SNPs yielded by the GWAS overlapped between the GF and the Ratio datasets.

Table 1: summary of the genetic variants identified by the GWAS

	Intergenic polymorphisms	Polymorphisms associated with coding genes	Coding Genes	Total SNP
GF	5	23	10	23
WJL	11	30	20	40
WJL/GF	0	21	13	20

Table 2: SNPs identified by the GWAS performed on the GF dataset.

Coordinate	MinorAllele	MajorAllele	RefAllele	MAF	MinorAlleleCount	MajorAlleleCount	SinglePval	Variant Class	Associated candidates
X_10326054_SNP	A	T	T	0,2453	13	40	5,27E-07	Intron/Downstream	CG32683:Intron/CG12640: Downstream
X_10326062_SNP	A	C	C	0,2453	13	40	5,27E-07		CG32683:Intron/CG12640: Downstream
X_10326075_SNP	T	A	A	0,22	11	39	5,73E-07		CG32683:Intron/CG12640: Downstream
X_10326094_SNP	T	G	G	0,2245	11	38	7,52E-07		CG32683:Intron/CG12640: Downstream
X_10326054_SNP	CTGTTG	C	C	0,283	15	38	2,34E-06		CG32683:Intron/CG12640: Downstream
X_1,214,976_SNP	T	C	C	0,07692	4	48	4,05E-06	Intron/Downstream	CG11398
X:16,714,419_SNP	A	C	C	0,1538	8	44	1,86E-05	Intergenic	Between CG34325 and CG13008
X_6,916,443_SNP	C	A	C	0,2143	9	33	1,58E-05	Intron	CG14431
X_6,916,468_SNP	A	G	G	0,1346	7	45	9,90E-06	Intron	CG14431|
2L_11294354_SNP	A	G	G	0,08163	4	45	4,96E-06	Intergenic	between CR43409 2L:11,290,632..11,291,077 and CR13721
2R_17656964_SNP	T	C	C	0,09804	5	46	7,01E-06	Intron	CCha-1R:2R:17,646,848..17,665,068
2R_17657540_INS	AACTGCTACCCTCTA	A	A	0,09804	5	46	5,34E-06	Intron	CCha-1R:2R:17,646,848..17,665,068
2R_17718701_SNP	T	A	T	0,2444	11	34	3,18E-07	Intron	CG13492
2R_17718742_SNP	C	T	C	0,2453	13	40	1,34E-06	Intron	CG13492
2R_17718760_SNP	G	A	G	0,25	13	39	1,61E-06	Intron	CG13492
2R:13,098,260 INS	CC	C	C	0,07692	4	48	5,24E-06	Intergenic	Betwenn Derailed 2 2R:13,055,246..13,079,539 and CG44341 2R:13,103,895..13,104,895
2R:.13,098,290_SNP	A	G	G	0,07692	4	48	5,24E-06	Intergenic	Betwenn Derailed 2 2R:13,055,246..13,079,539 and CG44341 2R:13,103,895..13,104,895
3L_11572688_SNP	T	A	A	0,09804	5	46	9,32E-07	Intergenic	CG43390: downstream/CG33270:Upstream
3L_11573225_SNP	G	C	C	0,08	4	46	5,92E-06	intergenic	CG43390: Upstream/CG43391:Downstream
3L_11574599_SNP	C	T	T	0,1064	5	42	8,34E-06	intergenic	CG33269:Downstream/CG43391: Upstream/ CG32086:Downstream
3L_11574652_SNP	G	A	A	0,14	7	43	5,25E-06	intergenic	CG33269:DOWNSTREAM/ CG43391:UPSTREAM/ CG32086:DOWNSTREAM|849]
3L_2437029_SNP	A	G	G	0,1373	7	44	1,71E-06	intron	CG42669
3R_4822536_SNP	C	T	T	0,28	14	36	7,45E-06	Synonymous coding	CG31100

Table 3: SNPs identified by the GWAS performed on the *Lp^{WJL}*-monoassociated dataset.

Coordinate	Minor Allele	Major Allele	RefAllele	MAF	MinorAlleleCount	MajorAlleleCount	SinglePval	Variant Class	GeneAnnotation
X_11,892,460_SNP	C	G	G	0,07692	4	48	2,83E-05	Intron	CG15738/ Sicily
X_19434886_SNP	A	G	G	0,2653	13	36	1,32E-05	Intron	CG32532
X_20474872_SNP	A	G	G	0,2642	14	39	1,29E-05	Intron	RunxA
X_20475001_DEL	C	CAC	CAC	0,1538	8	44	6,95E-06	Intron	RunxA
X_20475013_SNP	A	C	C	0,1569	8	43	8,25E-06	Intron	RunxA
X_7081445_SNP	T	C	C	0,1154	6	46	4,19E-06	Intron	CG9650
X_8390313_SNP	A	T	A	0,3409	15	29	4,90E-05	Intron	CG33181
2L_1029711_SNP	T	C	C	0,09804	5	46	1,51E-05	Intron	IA-2
2L_10721400_SNP	T	G	G	0,1154	6	46	2,23E-05	Intergenic	Between Lipase 2 2L:10,701,940..10,709,453 and CG6431 2L:10,724,203..10,728,740
2L_10721416_SNP	A	T	T	0,1176	6	45	2,60E-05	Intergenic	Between Lipase 2 2L:10,701,940..10,709,453 and CG6431 2L:10,724,203..10,728,740
2L_10721463_SNP	A	C	C	0,1224	6	43	1,80E-05	Intergenic	Between Lipase 2 2L:10,701,940..10,709,453 and CG6431 2L:10,724,203..10,728,740
2L_10721843_DEL	A	ATTAACATTATTATTAAATA	ATTAACATTATTATTAAATA	0,1395	6	37	1,42E-05	Intergenic	Between Lipase 2 2L:10,701,940..10,709,453 and CG6431 2L:10,724,203..10,728,740
2L_12439700_SNP	C	A	A	0,16	8	42	6,97E-06	3'UTR	Elf
2L_21303614_SNP	A	G	A	0,3125	15	33	1,32E-05	Intron	Mondo
2L_21303621_SNP	A	C	A	0,32	16	34	1,25E-05	Intron	Mondo
2L_3242011_SNP	T	A	A	0,3778	17	28	9,53E-06	Intergenic	No gene within 20KB on each side
2L_4021779_SNP	G	T	T	0,102	5	44	1,24E-05	Intergenic	Between CG43774: 2L:4,008,653..4,009,090 and Ed:2L:4,031,377..4,115,749
2L_4870441_SNP	T	C	C	0,1458	7	41	2,88E-06	Intron	pog
2L_6090501_SNP	T	C	C	0,16	8	42	1,93E-05	Intron	Kr-h1
2L_8819203_SNP	G	A	A	0,1154	6	46	5,87E-06	Intergenic	Between SoxN 2L:8,825,645..8,829,670 and CG42710 2L:8,781,799..8,782,500
2L_9143858_SNP	G	C	C	0,3636	16	28	1,84E-06	Intron	CG32982
2R_17186681_SNP	A	G	G	0,2264	12	41	1,96E-05	Intron	CG42672
2R_3971786_SNP	G	A	A	0,2174	10	36	3,29E-05	Intron/Upstream	sut1/slv
2R:13,602,028_SNP	C	A	A	0,1957	9	37	1,24E-05	Intergenic	Between CG43058 2R: 13,600,645..13,601,099 and CG34236 2R: 13,604,495..13,605,655
2R:13,602,588_SNP	G	A	A	0,1569	8	43	3,50E-06	Intergenic	Between CG43058 2R: 13,600,645..13,601,099 and CG34236 2R: 13,604,495..13,605,655
3L:11,467,276_SNP	C	T	T	0,1591	7	37	4,10E-06	Intergenic	Between CG6140 3L:11,463,570..11,465,864 and Vha16-3 3L:11,473,291..11,473,972
3L_14342706_SNP	A	G	A	0,07547	4	49	5,38E-06	Intron	Fz
3L_14460656_INS	G	GCG	G	0,3636	16	28	4,36E-06	Intron	bbg
3L_14904600_SNP	G	T	T	0,09434	5	48	1,05E-05	Intron	DCX-EMAP
3L_14905152_SNP	T	C	C	0,09615	5	47	1,35E-05	Intron	DCX-EMAP
3L_14905173_SNP	A	T	T	0,09804	5	46	1,73E-05	Intron	DCX-EMAP
3L_14923371_SNP	G	A	A	0,07547	4	49	3,06E-05	Intron	DCX-EMAP
3L_14923956_SNP	T	C	C	0,07547	4	49	3,06E-05	Intron	DCX-EMAP
3L_15541733_SNP	T	A	T	0,3725	19	32	1,97E-06	Intron	CrebA
3L:5,503,443_SNP	G	A	A	0,2292	11	37	2,77E-06	Intergenic	CG34342 3L:5,503,528..5,510,860 near Lkr: 3L:5,512,421..5,529,341
3L:5,506,702_SNP	A	T	T	0,16	8	42	2,56E-05	Intron	CG34342
3L:5,506,717_DEL	A	TG	TG	0,16	8	42	2,56E-05	Intron	CG34342
3L_7,127,783_SNP	T	C	C	0,2885	15	37	2,46E-05	Synonymous Coding	BBS1 |ctC/ctT|SYNONYMOUS_CODING CG8629|
3R_10376804_SNP	T	C	C	0,1321	7	46	6,60E-06	Intron	Pde-6
3R_5199699_SNP	G	A	A	0,2549	13	38	4,87E-06	Inron	Fps85D

Table 4: SNPs identified by the GWAS performed on the relative length gain dataset.

Coordinate	Minor Allele	Major Allele	Ref Allele	MAF	Single P value	R^2	Variant Class	Associated Candidates
X:4,989,087	G	A	G	0,4	9,25E-06	32,14%	Intron	rg
X:10,432,021	A	T	T	0,2453	2,76E-06	39,04%	Intron/Downstream	CG32683/CG12640
X:10,432,029	A	C	C	0,2453	2,76E-06	39,04%	Intron/Downstream	CG32683/CG12640
X:10,432,042	T	A	A	0,22	4,03E-06	29,32%	Intron/Downstream	CG32683/CG12640
X:10,432,061	T	G	G	0,2245	3,19E-06	29,07%	Intron/Downstream	CG32683/CG12640
X:10,432,164	CTGTTG	C	C	0,283	1,17E-05	29,80%	Intron/Downstream	CG32683/CG12640
2L:2809596	T	C	C	0,1837	4,45E-06	15,10%	Synonymous coding: silent substitution for codon for Leucine 589	daw
2R:9343451	G	C	C	0,1875	1,69E-06	14,68%	Intron	arr
2R:21,831,196	T	A	T	0,2444	4,53E-07	45,81%	Intron	CG13492
2R:21,831,237	C	T	C	0,2453	1,23E-06	46,46%	Intron	CG13492
2R:21,831,255	G	A	G	0,25	1,65E-06	45,56%	Intron	CG13492
3L:922730	G	T	T	0,2245	1,56E-06	11,14%	Intron	Glut1
3L:2437029	A	G	G	0,1373	1,23E-05	26,66%	Intron	CG42669 3L: 2,377,655..2,499,226
3L:6517813	G	T	T	0,4706	9,18E-06	27,37%	Intron	sfl
3L:9092134	C	T	T	0,2	3,76E-06	25,07%	3' UTR	bol
3L:10121196	A	G	G	0,08	7,77E-06	21,34%	Intron	dpr6
3L:10128548	A	T	T	0,1224	2,49E-05	33,06%	Intron	dpr6
3L:11574652	G	A	A	0,14	8,21E-06	35,58%	Intergenic	CG33269:Downstream/ CG43391:Upstream/ CG32086:Downstream
3L:15,333,778	G	T	T	0,3269	4,53E-06	23,70%	Intergenic	CR43247, lincRNA.566
3L:18051039	C	T	C	0,1176	1,22E-05	32,65%	Intron	Eip75B

3) Validation of the candidate genes revealed by the GWAS

To identify *Drosophila* genes that influence the host response to *L. plantarum* during growth, we focused on the SNPs associated with the "ratio", as this data set represents the genes associated to the growth enhancement conferred by Lp^{WJL} under protein scarcity. The ratio data set led to the identification of 20 SNPs (table 3), 2 of which overlapped with the SNPs identified in the GF data set, suggesting that these 2 SNPs may be not be specific to the response to Lp^{WJL}, and 10 SNPs were associated two the same gene. Therefore, in the end, 9 SNPs associated with 9 unique candidate genes were identified (Table 5). The candidates have either unknown functions or are associated with different biological processes such as immunity, chemosensory response, hormonal signaling and cellular signal transduction, regulation of translation. To investigate the role in Lp^{WJL}-mediated growth promotion of the 9 candidate genes (listed in table 2) uncovered by the GWAS, we interfere with their expression using the in-vivo RNA interference technology (RNAi) (Hannon, 2002). Specifically, we crossed a GAL4 "driver" line to a UAS-RNAi line targeting the gene of interest to induce the expression of a specific hairpin structure which silences the expression of the target gene using the RNA interference pathway. All UAS-RNAi lines were driven by a ubiquitous GAL4 line (*Daughterless*-GAL4) coupled to a temperature sensitive GAL80 ($Gal80^{ts}$). GAL80 is a repressor of the GAL4 protein. We used it to regulate the expression of the GAL4-driven RNAi constructs: At low temperature (18°C) $GAL80^{ts}$ has a high efficiency to inhibit GAL4 transcriptional activity, so no hairpin is expressed, whereas at 29°C, $GAL80^{ts}$ becomes inactive and GAL4-dependent expression occurs normally, in addition GAL4 affinity to UAS sequences is increased at 29°C *vs* 18°C, reinforcing this regulatory system. In order to minimize lethality caused by the candidates-knockdown, the GAL4 strength was dampened with $GAL80^{ts}$ by raising the flies at 25°C. As described previously (see paragraph 1.), we measured the length of GF and Lp^{WJL}-monoassociated larvae at the 7^{th} day after egg laying (AEL) on a low-protein diet for larvae with reduced expression of the candidate genes to assess whether the growth enhancement conferred by Lp^{WJL} was altered. As shown on figure 2, loss of function of the candidates impacted the growth of both GF and monoassociated animals. In particular, GF led to a very large variability in larval growth. One line, Eip75B, was fully lethal as GF, meaning that in absence of this gene, the presence of a microbiota is required for survival on a low-

protein content diet. For monoassociated larvae, the range of phenotype is lower. However, candidates' loss of function lines still benefited from the monoassociation as Lp^{WJL}-associated larvae were longer in all the lines. Figure 2, panel C shows the growth benefit (the relative length gain provided by Lp^{WJL}) normalized to the growth gain of the respective genetic control lines. Although we did not observe a complete loss of the growth improvement, the intensity of the growth benefit is highly impacted by the altered expression of the candidate gene. This result indicates that the candidates seem to play a role in the interaction between *Drosophila* larvae and *L. plantarum* during juvenile growth. It illustrates very well that the response to Lp^{WJL} is a quantitative trait, meaning that it results from the cumulative action of many genes as well as the environment; therefore it relies on a complex genetic regulation.

Table 5: Variants associated with the relative length gain conferred by *Lactobacillus plantarum*^{WJL}.

Variants	R^2	P-value	Molecular and cellular functions
dpr6	33.06%	2,94E-05	Immunoglobulin subtype 2, chemosensory perception
Eip75B	32.65%	1.22e-05	Nuclear hormone receptor, ecdysone response, antimicrobial humoral response
rg	32.14%	9.25e-06	PKA-binding, cone cell differentiation, mushroom body development, olfactory learning
sfl	27.37%	9.18e-06	heparan sulfate proteoglycans (HSPGs) biosynthesis/wg morphogen diffusion
bol	25,07%	3,76E-06	RNA binding protein. Role in spermatogenesis
CR43427	23,70%	4,53E-06	Unknown, lncRNA
daw	15,10%	4.45E-06	TGF-β ligand: growth; regulation of insulin secretion
arr	14,68%	1,69E-06	wnt protein binding/canonical wnt pathway
glut1	11,14%	1,56E-06	General glucose/sugar transporter

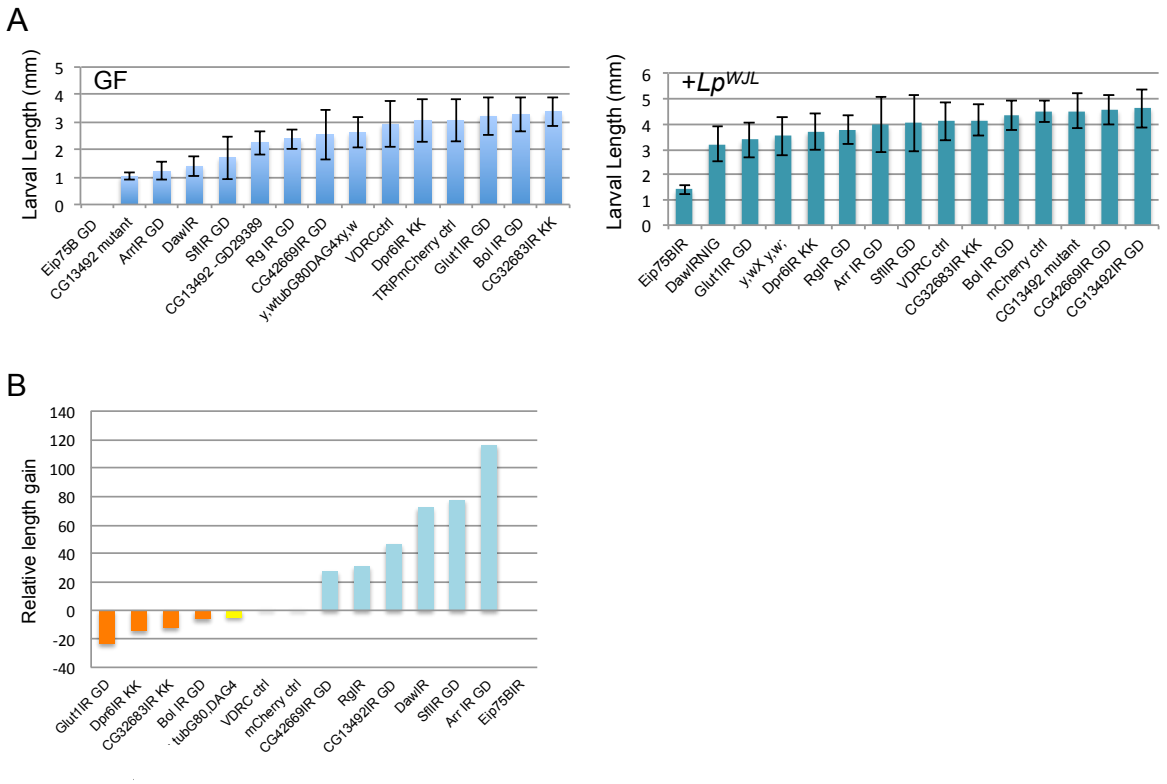


Figure 2: knockdown of the candidate genes leads to a spectrum of beneficial effect

A. Bar graphs showing the effect of RNAi knockdown on larval length on day 7 AEL. Each bar represents the average length from pooled 3-5 biological replicates from GF (top panel) or Lp (bottom panel) condition, with 15-40 larvae in each replicate.

Three different control knockdowns are used: one control fly strain recommended by VDRC for RNAi constructs obtained from VDRC, one control strain (targeted against mCherry) recommended by Harvard TRiP collection, and the y,w strain from Bloomington. All control and RNAi strains are crossed to y,w, tubulin>GAL^{80},DA>GAL4. "GD" refers to the VDRC RNAi GD collection. "KK" refers to the VDRC RNAi KK collection. For specific genotypes, refer to Material and Methods.

B. Bar graph illustrating the length gain provided by *Lp*^{WJL} for all RNAi knockdowns relative to their corresponding control line. Each bar represents the fold increase of the efficiency of *Lp*^{WJL} to promote growth of the knockdown (KD) line relative to that of the controls for KD lines. Orange bars are the lines for which the KD rendered *Lp*^{WJL} less efficient than for the control line while blue bars are the lines for which the KD improved the capacity of *Lp*^{WJL} to enhance growth compared to the control line. Blue bars represent the fold increase relative to the control line. Blue bars, represent the fold increase of size for lines that KD rendered *Lp*^{WJL} more efficient to promote growth compared to the control line.

4) *Lp*^{WJL} acts as a genetic buffer

In the course of our experiments, we assayed the effect of *L.p*^{WJL} on *Drosophila* juvenile growth in many different genetic contexts (the DGRP lines and the RNAi contexts). We showed that the genetic background highly impacts the intrinsic growth of the flies both in absence or presence of a microbiota and that it also affects the physiologic response to the intestinal bacteria. To better characterize how *Lp*^{WJL} affects larval growth, we next decided to explore the dynamics of the growth in presence and absence of *Lp*^{WJL} to determine how the growth benefit changes across larval development. We compared the growth profile in the presence and absence of *Lp*^{WJL} from DGRP lines situated at each end of the phenotypic range (Fig 1A). We selected 2 lines that respond very well to *Lp*^{WJL} upon protein scarcity, these lines are called "top responders" (lines #28147 and #25208), and 2 lines that showed a lower growth enhancement by *Lp*^{WJL}, meaning that the growth benefit is minimal, these lines are called "low responders" (lines #25183 and #25210). We inoculated GF embryos of the 2 "top responder" and two "low responder" DGRP lines with either *Lp*^{WJL} or PBS and we measured larval length every day from day 2 AEL to the day approximately half of the population had reached pupariation for both GF and monoassociated larvae (Fig 3). The growth curves showed that *Lp*^{WJL} started benefitting growth very early in the development: at day 2, monoassociated larvae were already longer than their GF siblings. When comparing the growth profile of larvae associated with *Lp*^{WJL} or not, we focused on the profile of the curves themselves. The pattern of length evolution is strikingly different between the top and low responders. For the "top" lines (Fig 3 A and B) *Lp*^{WJL}-associated larvae follow an almost linear growth phase with a high growth rate during the first 5-6 days AEL. Then, the growth rate drastically slows down and reaches a stationary phase. For the "low" lines however, the growth of *Lp*^{WJL}-associated larvae is more uniform, and although we can still distinguish these 2 phases, they are less obvious. The GF condition is more variable among the 4 DGRP lines. For the low responders (Fig 3 C and D), the GF growth is higher, hence the difference between the 2 conditions is lower than for the top lines. On the contrary, top lines, cope less well with the absence of microbiota upon nutritive challenge: GF larvae grow slowly, thus the difference is greater explaining the enhanced response to *Lp*^{WJL}. This is better demonstrated by the figure 3E where we pooled the growth dynamics data of the 2 top lines for either GF or *Lp*^{WJL} condition (the GF condition is the green curve

and *Lp*^{WJL} is in blue), and the 2 low responder lines (GF is in black and *Lp*^{WJL} is in red). Fig 3E shows that the top and low lines' growth differs by the GF condition (pval=1.82e-12), while the *Lp*^{WJL} -monoassociated curves are not significantly different (pval=0.5). To conclude, what mainly explains the differences we observed in the intensity of the growth benefit conferred by *Lp*^{WJL} in Top and Low responder DGRP lines is the growth profile of the GF larvae that is more or less retarded, the addition of *Lp*^{WJL} seems to buffer the differences between the lines.

Furthermore, we noticed that when the flies do not harbor a microbiota, *i.e.* when they are GF, the larval length is more variable among the different lines than when they are monoassociated, as we see by comparing panel A and B of Figure 1: GF DGRP larval length vary from 1,35 to 2,96 mm whereas *Lp*^{WJL}-monoassociated lengths range from 3,29 to 4,56 mm. However, although we still observed variability when animals were monoassociated with *Lp*^{WJL}, the variability was much lower. We could observe the same with the RNAi knockdown validation: GF lengths were highly variable, ranging from lethality to being only slightly smaller than the monoassociated ones, in contrast, *Lp*^{WJL} larvae showed decreased variations in length among the lines (Fig 2 A-B). A simple way to quantify this is to calculate the coefficient of variation (CV) of all larval lengths measured for each condition. For the GF condition, CV of the larval length is 27.82% while it is equal to 18.74 for the *Lp*^{WJL}-monoassociated condition (Fig 4). Therefore, the phenotypic variability in the GF is greater than in presence of *Lp*^{WJL}. Another manner of quantify this is to calculate the heritability estimate (H), which is a value indicating how much of the variation in a phenotypic trait among a population is the result of the genetic variability in this population. For the pooled GF DGRP larvae, our collaborators (B. Deplancke et al.) estimated H is 0,37 while it is 0,1 for the monoassociated condition; this indicates that genetic variability is more responsible for the phenotypic variation in absence of intestinal bacteria. Taken together, our results seem to suggest that in addition of increasing juvenile growth of larvae facing a nutritional stress, *Lp*^{WJL} also acts as a genetic buffer to compensate the growth impairments due to the fly genetic variations. *Lp*^{WJL} action thus results in a phenotypic homogeneity among a given population.

While I was embarking on the work presented in Chapter 2, Dr Dali Ma took over this part of the project and explored this buffering effect in detail and showed that when flies are facing a nutritional challenge, *Lp*^{WJL} decreased the variance of other traits than

growth, namely, timing to pupariation, adult body and organ (wings, eyes) size. In addition, Lp^{WJL} confers robustness in developmental programs upon nutritional stresses, as it has the ability to decrease the incidence of wing pattern defects such as ectopic vein tissue, missing margins, incomplete vein formation. Furthermore, she demonstrated that ROS activity is necessary for Lp^{WJL} to reduce variance in fitness traits and to suppress developmental patterning defects (Ma *et al*, manuscript in preparation, see in annex).

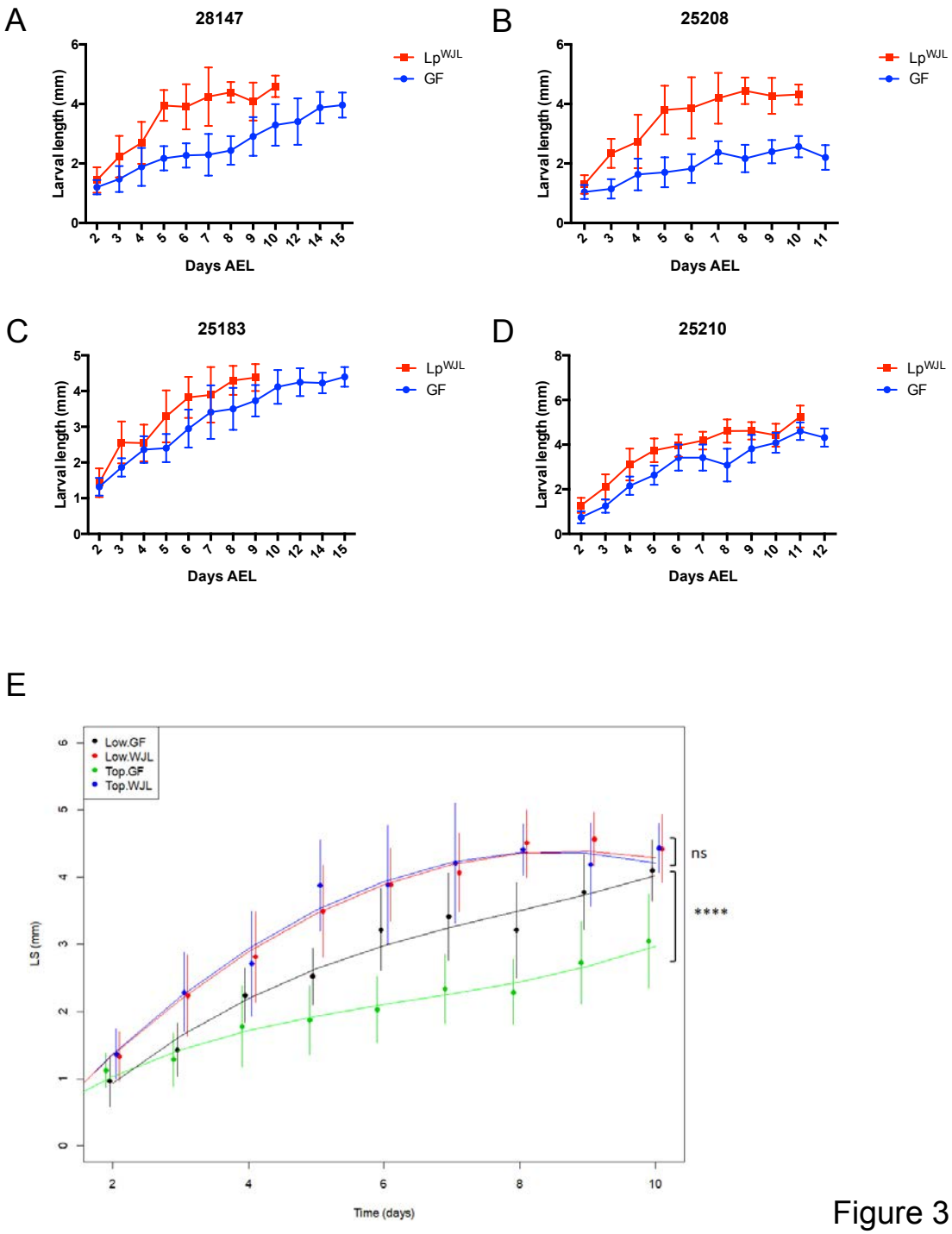


Figure 3

Figure 3: The different response to *Lp*^{WJL} of the DGRP lines is mainly explained by the growth of the GF condition.

Growth kinetics of "top" and "low" responder DGRP lines. The curves show the longitudinal larval length in mm along time AEL of 2 "top" (A, and B) and 2 "low" (D and E) DGRP lines raised on a poor-protein diet. Each dot represents the mean longitudinal body length of a pool of larvae for one day of the development. Blue lines represent GF condition and red lines represent *Lp*^{WJL} -monoassociated larvae. E) Growth models associated to the low responders, GF and *Lp*^{WJL}-monoassociated, respectively the black and red curve, and GF and *Lp*^{WJL}-monoassociated top responders, green and blue curve respectively. Dots represent the mean larval length for a given day of larval development. Vertical lines represent the SD and the curves are the fitted models. Cubic linear mixed effect models were performed for GF and for monoassociated data. For each we compared cubic linear regression with time as variable, to a model with time and being top or low as the variables. An Anova was performed to compare the two models. Being top or low makes a difference in GF condition ($p=1.82^{e-12}$), whereas it does not in monoassociated condition ($p=0.5$).

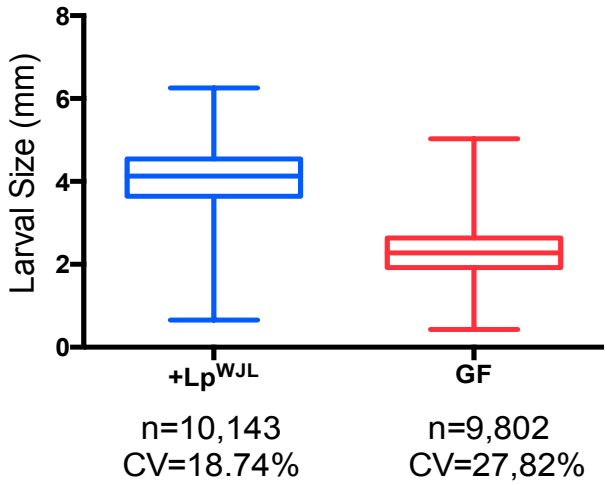


Figure 4: Larval length of all DGRP lines data pooled

Box plots showing the average longitudinal length of DGRP larvae in mm, all data are pooled for Lp^{WJL} (in blue) or GF (in red). (CV_{GF}=27.82%, CV_{LpWJL}=18.74%). The horizontal line represents the median value and the each end of the whiskers represents the minimal and maximal value.

Conclusion

Using a simple model of gnotobiotic fly (*Drosophila* monoassociated with *L. plantarum*^{WJL}), our group previously showed the upon protein deprivation, *Lp*^{WJL} has the capacity to promote *Drosophila* growth. Our work showed that natural genomic variations, in the form of SNPs, among a population greatly influence the physiologic response to the intestinal microbiota as well as to the environment. This strengthens the idea that the effect of one bacterial species on its host physiology is greatly dependent on the host genetic background. Our results report that the fly physiologic response to *Lp*^{WJL} is a quantitative trait, and therefore it is multigenic. We also showed that the intensity of the response to *Lp*^{WJL} is mainly dictated by the growth of GF animals, and that *Lp*^{WJL} is acting as a genetic buffer to compensate the growth impairments due to the fly genetic background. In addition, *Lp*^{WJL} decreases the phenotypic variations in various host fitness traits (growth, organ size, timing to pupariation) and it also confers robustness in organ patterning.

Discussion

In recent years, several groups including our own have characterized the impact of the gut microbiota on different aspects of the fly host physiology. We particularly focused on *Lactobacillus plantarum*^{WJL} (*Lp*^{WJL}), a natural fly member of intestinal microbiota, to understand the molecular basis of the nutritional mutualism between the two partners during chronic undernutrition. Findings from our group demonstrated that *Lp*^{WJL} promotes *Drosophila* growth upon protein deficiency (Storelli et al., 2011). However, the host genetic elements involved in the interaction are largely unknown. To tackle this question, we used the *Drosophila melanogaster* Genetic Reference Panel (DGRP) (Mackay et al., 2012) to assay how *Lp*^{WJL} impacted the growth of the flies with different genetic backgrounds. Our work first shows that in both the presence and absence of the microbiota, the among-line variations in growth are striking, therefore the natural genomic variations greatly influence the host's physiological response to the intestinal microbiota as well as to dietary content. This strengthens the idea that the effect of one bacterial species on its host physiology is greatly dependent on the host genetic background, and that the larval growth in the presence of *Lp*^{WJL} is a quantitative trait, and therefore it is multigenic. Secondly, we also show that the intensity of the response to *Lp*^{WJL} is mainly dictated by the growth difference among the GF animals, and that *Lp*^{WJL} is acting as a genetic buffer to compensate the growth impairments due to the fly genetic background. In addition, *Lp*^{WJL} decreases the phenotypic variations in various host fitness traits (growth, organ size, timing to pupariation) and it also confers robustness to organ patterning.

Natural genetic variations alter the response to microbiota

We initiated the study of the GF and mono-associated growth in 53 DGRP lines on a low-protein diet. First, we observed that the *Lp*^{WJL} benefit varied greatly among the DGRP lines, indicating that host genetic variations assert a substantial impact on the growth response of *Drosophila* to *Lp*^{WJL} upon protein deficiency. This result is congruent with that reported by Dobson et al. (2015) who, by assaying the levels of multiple metabolic readouts such as weight, protein, glucose and glycogen contents in the DGRP lines,

showed that the host genetic backgrounds directly alter the among-line variations of the microbiota-dependent nutritional traits. How the host genetic background shapes the intensity of its response to bacteria is a complex question. A simple answer is that the bacterial persistence in each host's nutritional niche is largely attributed to the host's immune-competence, digestive capacity, metabolic rate and even food-searching behavior. Variation in these traits is directly encoded in the host genome. For example, Chaston et al. (Chaston et al., 2015) demonstrated that the abundance of *Acetobacter tropicalis* is associated to SNPs in four different loci including genes encoding a cyclic AMP phosphodiesterase (*dunce*) and a sodium channel (*paralytic*). Similarly, we could verify the CFUs of Lp^{WJL} among the 53 tested DGRP lines and see if the bacteria abundance directly correlates to the growth enhancement in different hosts.

Despite the difference in growth among the DGRP lines, Lp^{WJL} benefits overall growth regardless of the fly genotype. Furthermore, our group showed that Lp^{WJL} also benefits juvenile growth in mice (Schwarzer et al., 2016), therefore, the beneficial effect of Lp^{WJL} is maintained across phylum. This suggests that the sustainment of host development and growth under suboptimal conditions by certain gut microbes, and such mutualistic symbiosis, has a deep evolutionary ancestry and broad ecological implications.

Through GWAS, we uncovered nine unique variants associated with the Lp^{WJL} growth benefits. In the process of validating the variants' function, we found no complete loss of growth promotion in the RNAi lines. This finding is consistent with the conclusion that animal growth in the presence of the gut microbiota is a multigenic trait, and each genomic variant probably contributes a small effect on the growth, therefore, knocking down only one single gene is not sufficient to abrogate the phenotype.

Furthermore, the GWAs analyses based on larval length in GF and monoassociated conditions yielded two completely different sets of genes with no overlap. Similarly, the GWAS study from Dobson et al. (Dobson et al., 2015) on nutritional inputs in absence and presence of microbiota also produced non-overlapping sets of variants. This shows that genetic programs at play in the presence or absence of microbiota are very different. Furthermore, our study and Dobson et al. uncovered variants in the same genes or paralogues with similar functions, despite the fact that our analysis is based on a singular trait: mono-associated larval growth, and theirs on five adult nutritional traits in the presence of a cocktails of five different bacteria. For example, *boule* (*bol*), *rugose*

(*rg*) were found in both studies. Boule is a transcriptional regulator required in spermatogenesis for entry into meiosis and spermatid differentiation. It also plays a role in mushroom body neuron as a negative regulator of axon pruning, a strategy used to selectively remove exuberant neuronal branches and connections in the immature nervous system to ensure the proper formation of functional circuitry (Low and Cheng, 2006). Rugose is involved in mushroom body development, neuromuscular junction, olfactory learning and short-term memory as well as eye photoreceptor cell development. Finally, we found *dpr6* and Dobson study found *dpr10*, these two defective proboscis extension response (Dpr) genes have similar functions. They belong to immunoglobulin superfamily and are involved in specificity of synaptic connection between neurons and target cells. They are associated to sensory perception of chemical stimulus, synapse organization and neuron projection. In summary, the candidate genes common to our study and that of Dobson et al. are mostly involved in neuronal development and functions, olfactory learning and memory, sensory perception. How these genes affect growth and nutritional phenotypes related to microbial input requires detailed studies on their own. Specially several studies support a role of microbiota in brain development (reviewed in Sampson and Mazmanian, 2015), and microbiota impacting olfaction and chemical stimulus perception implies a role in food searching behavior.

In addition to study the novel functions of individual genes from the GWAS through classic genetic approach, we can carry the analyses further to better understand to what extent each variant contributes to the growth response to bacteria. As previously described, most of the variants mapped to the introns, UTRs and intergenic regions, strongly suggesting that they affect transcription. For instance, we could start by assaying the expression level of the candidate genes uncovered by the GWAS, in presence or absence of Lp^{WJL} in the DGRP lines carrying different alleles of the same gene, and correlate the expression level to the growth benefit in different fly lines.

Lp^{WJL} acts as a genetic buffer

In the process of variant discovery, we found that Lp^{WJL} acts as a genetic buffer that conceals the effect of natural genomic variants or mutations in altering growth while

supporting optimal host growth upon challenging conditions. This result goes in line with the holobiont theory of evolution in that the bacterial association leads to an improved fitness for the host and help the host to adapt to changing environment (Zilber-Rosenberg and Rosenberg, 2008). Microbiome adds on genes to support the host phenotypes. Here we show a new mechanism, in addition of just providing additional genes, the microbiota also contributes to make the host fitter by masking genetic variability of the host. Association with Lp^{WJL} produces the fittest individuals and a more homogenous population. By ensuring a greater number of fit individuals, which grow and reach adulthood faster, reproductive success can be maximized in a challenging environment.

Lp^{WJL} also decreases phenotypic variations in fitness traits among a population compared to GF, and therefore it can be considered as a genetic buffering machinery. Buffering of genetic variations is a well-studied process. Genetic buffering mechanisms alter the relationship between phenotypic and genotypic variations by concealing the consequences of genetic variation of different phenotypes in specific environmental context, and thus bringing forth phenotypic robustness (Rutherford, 2000). Various mechanisms have been proposed so far to explain this process, such as the involvement of microRNAs (Posadas and Carthew, 2014; Cassidy et al., 2016), or the Hsp90 chaperone machinery that suppresses phenotypic variations by suppressing the mutagenic activity of transposons (Specchia et al., 2010). Finally, last year, a study showed that gut bacteria contribute to phenotypic stability as the elimination of gut bacteria uncovered mutation-specific phenotypes in embryogenesis and larval development (Elgart et al., 2016). Consistent with the findings of Elgart et al., our results demonstrate that the removal of microbiota (GF condition) actually "unmasks" the genetic variability of the host leading to large phenotypic heterogeneity in a population. Going further however, we also show that the presence of intestinal bacteria can correct developmental flaws such as wing pattern defects and bring robustness in organ patterning. Therefore our results provide a new role for microbiota in buffering mechanisms to ensure phenotypic robustness. Using a simple model of *Drosophila* monoassociated with *L. plantarum*^{WJL}, we identified a novel role of the gut microbiota in genetic buffering and developmental canalization. Such genetic buffering mediated by symbiotic bacteria should be further studied. Hitherto, only our work and one other study (Elgart et al., 2016) have revealed such a system. The precise mechanism and the

extent of microbiota-supported buffering effect should be investigated in the future, as well as the consequences of inter-bacteria interaction.

In the future, it would be of interest to investigate the buffering effect in the presence of a conventional and complex microbiota, and see if such phenomenon is widespread. We have already confirmed that the fly gut microbial communities derived from the wild also assert a buffering effect, but it is difficult to consistently control the composition and quantity of each bacterial species. Artificial polyassociation (association with multiple bacterial species at the same time) could be the used. This would enable to assess how the interactions among bacteria impact the genetic buffering.

Material and methods

Fly stocks and husbandry

Drosophila were reared at 25°C on a light-dark cycle (12h light, 12h dark) on a rich-protein diet consisting of 50g/L of inactivated yeast (Bio Springer, Springaline BA95/0-PW), 80g/L of cornmeal (Westhove, Farigel maize H1), 10g/L of agar (VWR, ref #20768.361), 5,2g/L of methylparaben sodium salt (Merck, ref #106756) and 4mL/L of 99% propionic acid (CARLO ERBA, ref #409553), and a mix of antibiotics (50 µg/mL ampicillin, 50 µg/mL kanamycin, 15 µg/mL erythromycin and 50 µg/mL tetracyclin).
We ordered the DGRP lines from Bloomington *Drosophila* Stock Center.
y,w flies were used as a reference strain.

Generation of axenic fly stocks

Embryos from CR flies were collected overnight on fruit juice-agar medium supplemented with fresh yeast paste. Under sterile conditions, embryos were then successively soaked for 2 minutes in bleach (to dechorionate the embryos), 70% ethanol and sterile water. These newly sterile embryos were then deposited on sterile rich-protein fly food supplemented with antibiotics and raised at 25°C. Once GF flies emerge, they were amplified on sterile rich-protein food added with antibiotics. The absence of contamination was confirmed by plating 100 µl of lysate of 5 adults or 3rd-instar larvae on both MRS and LB plates and incubated at 37°C for 48h followed by 7 days at RT.

Bacterial association

L. plantarum^{WJL} was cultured overnight in Man, Rogosa and Sharpe (MRS) liquid medium (Difco, #288110). OD was measured at 600 nm, the culture was diluted in 1X PBS at OD=0,5. 333 µl of this diluted bacterial solution was poured homogenously on the embryos and medium.
For each mono-association experiment, Lp^{WJL} (Ryu et al., 2008) was grown in Man, Rogosa and Sharpe (MRS) medium (Difco, ref. #288110) over-night at 37°C, and diluted

to O.D.=0.5 the next morning to inoculate 40 freshly laid eggs on each 55mm petri dish or standard 28mm tubes containing fly food of low yeast content. The inoculum corresponds to about $7x10^7$ CFUs. Equal volume of sterile PBS was spread on germ-free eggs for control.

Larval length measurements

Axenic flies were put in an egg laying chambers overnight to lay eggs on sterile poor-protein diet. Axenic embryos were collected the next morning, and pools of 40 embryos were transferred to new dishes of poor-protein food. The pools of embryos were then inoculated under sterile conditions with either 1x PBS or $1x10^7$ CFUs from an overnight culture of *L. plantarum*. At minimum, triplicates were done for each condition. The caps containing the inoculated embryos were incubated at 25°C. 7 days after inoculation, larvae were collected from the caps and heat-killed on a heater plate for 5 seconds in order to make them straighten and mounted between slide and coverslip in 80% glycerol diluted in 1x PBS. Pictures were taken using a leica M205 FA Stereomicrosope, magnification 7,8 with the software LAS. Length was measured with Image J.

GWAS validation

Drosophila mutants or RNAi lines corresponding to the candidate genes identified by the GWAS were ordered from Bloomington Drosophila Stock Center.

All RNAi lines were crossed to y,w; tubGAL80^{ts}, DA>GAL4. To minimize lethality, we dampend the GAL4 strength by leaving the genetic crosses at 25°C. The following fly strains were used: *y,w*, UAS-dpr-6-IR(P{KK112634}VIE-260B*)*, UAS-CG13492-IR, (w^{1118};P{GD14825}v29390)*,* UAS-daw-IR(NIG #16987R-1), UAS-sfl-IR (*w^{1118}*; P{GD2336}v5070), UAS-arr-IR (*w^{1118}*; P{GD2617}v4818), UAS-rg-IR(*w^{1118}*; P{GD8235}v17407), UAS-bol-IR(*w^{1118}*; P{GD10525}v21536), UAS-glut1-IR(*y^1 v^1*; P{TRiP.JF03060}attP2, Bloomington 28645), UAS-CG32683(P{KK112515}VIE-260B), UAS-CG42669-IR*(w^{1118}*;P{GD7292}v18081),UAS-Eip75B(*w^{1118}*; P{GD1434}v44851), UAS-mCherry-IR (*y^1 v^1*; P{CaryP}attP2), VDRC GD control (VDRC ID60000).

Statistical Analyses

GraphPad Prism software version 6.0f for Macintosh (GraphPad Software, La Jolla California USA,) was used to make the statistical analyses.

A dedicated R script has been used to compare the growth of “Top” and “Low” lines in each microbial context (GF or LpWJL). For each of these two conditions, two cubic linear mixed effect models (package *nlme*) have been fitted to the larval length measurement over 10 days. One model contained the Top-Low condition as factor and the other not. These two models were then compared with an anova (package *stats*).

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2nd Chapter

Manuscript in preparation

dawdle regulates digestive enzymes expression in a microbiota-dependent manner.

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CHAPTER 2

Introduction

Past work from our group showed that *L. plantarum*, a natural fly symbiont, promotes *Drosophila* growth upon chronic protein deficiency (Storelli et al., 2011). This growth benefit relies on the upregulation of host's digestive proteases (Erkosar et al., 2015) which results in altered amino acids homeostasis and redirection of host metabolism: ameliorated uptake of amino acids from the fly food enhances the activity of the fly TOR nutrient-sensing pathway in endocrine organs such as the prothoracic gland and the fat body, which results in an increase of systemic InR signaling and ecdysone production (Storelli et al., 2011). In order to further our understanding of the mechanisms underlying the growth enhancement conferred by Lp^{WJL}, we measured the larval length of GF and Lp^{WJL}-monoassociated individuals from 53 lines of the *Drosophila melanogaster* Genetic reference Panel (DGRP) (Mackay et al., 2012) raised on low-protein diet to assess how Lp^{WJL} affects their growth. DGRP is a library of naturally occurring polymorphisms; these lines harbor a rich collection of genomic variants and thus represents a great tool for association mapping to look for genomic elements correlated to a phenotype. To unveil the host genes affected by the monoassociation with Lp^{WJL}, we conducted GWAS on larval growth response to Lp^{WJL} of these DGRP lines (Chapter I).

The GWAS analysis identified 20 SNPs associated to 12 different genes. Among them, A *dawdle* (*daw*) variant was uncovered. The SNP associated to *daw* gene was a synonymous coding mutation- a silent substitution for leucine 589. Synonymous mutations do not change the protein sequence. However, this type of mutation can affect transcription, splicing, mRNA transport, timing of translation resulting in modification of protein conformation, and consequently affect the phenotype (Kimchi-Sarfaty et al., 2007). For instance, this study (Kimchi-Sarfaty et al., 2007) reported that synonymous mutation in *MDR1* (Multidrug Resistance1) gene affected the gene product: P-glycoprotein. The authors attributed the altered conformation of P-glycoprotein to the modification of the timing of co-translational folding of the protein. Other studies also

showed that synonymous SNPs are as likely as non-synonymous SNPs to be associated to the phenotype of interest (Chen et al., 2010).

Daw is a TGF-β ligand that has recently been shown to be involved in different aspects of digestion and metabolism in *Drosophila* (Bai et al., 2013; Chng et al., 2014; Ghosh and O'Connor, 2014; Mattila et al., 2015). Feeding adult flies with a glucose-rich diet represses the gene expression of intestinal enzymes such as glycosyl-hydrolases or lipases, a phenomenon described as glucose repression. Chng et al. investigated the mechanistic basis of glucose repression and demonstrated that in response to glucose feeding, Dawdle is secreted by the fat body and acts on the midgut to inhibit the expression of several digestive enzymes, such as amylases or maltases (Chng et al., 2014). Another study showed that, Dawdle positively regulates insulin secretion in larvae by promoting the release of dILPs (*Drosophila* Insulin Like Peptides) from the IPCs (Insulin Producing cells) in the brain (Ghost & O'Connor, 2014). Furthermore, *daw* is involved in a feed-forward gene regulatory loop along with Mondo-Mlx, two sugar-responsive transcription factors. Mondo-Mlx controls the expression of the majority of genes responding to sugar. They are involved in nutrient digestion and transport, and amino acid (glutamine and serine), lipid and carbohydrate metabolism. This regulation functions through the downstream effectors Dawdle and Sugarbabe (Mattila et al., 2015).

Our group previously identified that the monoassociation with *L. plantarum* enhances the expression of digestive enzymes (proteases, lipases, and glycosyl-hydrolases) and such upregulation at least partially accounts for the growth benefit sustained by *L. plantarum* (Erkosar et al., 2014, 2015). Moreover, *L. plantarum* promotes growth through mTOR pathway and is associated with amplified insulin signaling activity. We hypothesized that *daw* could be involved in Lp^{WJL}-dependent growth support upon protein deficiency through the regulation of digestive enzymes. Therefore, during my thesis, I investigated the role of *daw* in the molecular dialog between Lp^{WJL} and the fly during juvenile growth.

Results

1) *dawdle* knockdown alters the growth enhancement conferred by *L. plantarum*^{WJL}.

To find out whether *daw* played a role in the functional interaction between *L. plantarum*^{WJL} and *Drosophila* growth, we first tested the effect of *daw* knockdown (KD) on larval development upon protein deficiency. To address this question, we took advantage of the RNA interference (RNAi) technology. We knocked down *daw* using a ubiquitous driver and measured larval length 7 days AEL (After Egg Laying). On our first attempt to test the effect on *daw*-KD on larval growth, we used a strong ubiquitous driver: *daughterless*-GAL4. However, this strong expression of *daw* RNAi construct turned out to be lethal as previously reported for *daw* alleles (Ghosh and O'Connor, 2014). For that reason, the rest of the experiments with *daw*-KD were conducted using a ubiquitous GAL4 driver (*daughterless*-GAL4) coupled to a temperature sensitive GAL80 (*tubulin*-GAL80^{ts}). GAL80 is a repressor of the GAL4 protein. We used it to regulate the expression of the GAL4-driven RNAi constructs: At low temperature (18°C) GAL80^{ts} has a high efficiency to inhibit GAL4 transcriptional activity, so no hairpin is expressed, whereas at 29°C, GAL80^{ts} becomes inactive and GAL4-dependent expression occurs normally. In order to minimize lethality caused by the candidates-knockdown, the GAL4 strength was dampened with GAL80^{ts} by raising the flies at 25°C. When the larvae were raised at 25°C, the tubulin-GAL80^{ts} is still partially active to repress the expression of GAL4, which consequently dampens the RNAi efficiency. Efficiency of the knockdown (*tubulin*-GAL80ts; *daughterless*-GAL4 > UAS-*daw*^{IR}) is showed in figure S1. We found that reducing *daw* levels of larvae raised on low-protein diet triggered a systemic growth delay (Fig 1 A), as both GF and *Lp*^{WJL}-monoassociated larvae were smaller than control conditions, suggesting that *daw* plays a crucial role in systemic growth. To better see the effect on growth of *Lp*^{WJL}, we plotted the relative length gain sustained by *Lp*^{WJL} (Fig 1 B) showing that the ratio of mono-associated larval length to the GF larval length is higher for *daw*-knocked-down animals, meaning that although larvae are smaller, GF larvae suffered more from the reduced level of *daw*. One possible explanation for the increased ratio could be that GF larvae are stalling at an earlier developmental stage where *Lp*^{WJL} is more efficient to promote growth, which would explain the greater ratio. To rule out this possibility, we collected size-matched GF larvae for *daw*-KD and controls, and measured

the corresponding monoassociated counterparts. We found that in the GF larvae of the same length, the sibling *daw*-KD monoassociated larvae were longer (Fig 1C), leading to an increased relative length gain (Fig 1 D). These results indicate that *daw*-knockdown in larvae leads to a greater growth benefit mediated by *L. plantarum*^{WJL}.

To confirm that *dawdle* knockdown is indeed the cause of the observed phenotype, we tested two other RNAi lines but they did not work in our hands: no larval growth phenotype, and we failed to see the knockdown by RT-qPCR. Therefore we decided to test the growth capacity of *dawdle* mutants. We used the *daw*^{aac1} (referred as *daw*^{1}) line, which is a null mutant line (Gesualdi and Haerry, 2007). This line was obtained by P-element excision. It deletes 2.5 kb including the second promoter and most of the coding sequence. The second line we used, *daw*^{ext11} (referred as *daw*^{11}) was obtained by imprecise excision of a transposable element (Strain #13221, Japan) inserted in the first intron of the *daw* gene, 2.5 kb downstream of the transcription start of the Alp23B-RA (Flybase) (Serpe and O'Connor, 2006). It lacks 1.8 kb of the *daw* gene sequence, including the start codon. We crossed the two lines to obtain transheterozygous mutant animals *daw*^{1}/*daw*^{11} as others have done (Ghosh and O'Connor, 2014). Unfortunately, *daw* null mutation resulted in a very high larval lethality on our low-protein diet, as did the DaGal4 > *daw*-KD. Only a few larvae survived and we were unable to collect enough individuals to reliably assess larval growth. However, these mutants were used to test another phenotype (see below).

After showing that knocking down *daw* altered the growth response to *Lp*^{WJL}, we tested how the overexpression of *daw* impacted larval growth in presence and absence of *Lp*^{WJL}. We raised larvae on low protein diet with or without *Lp*^{WJL} and assayed the larval length on day 7 AEL (Fig 1 E-J). Our results with the overexpression were irreproducible from one experiment to another. Regarding the larval length, overexpressing *daw* was very deleterious to the systemic larval growth: both GF and *Lp*^{WJL}-monoassociated larvae were much smaller than the control animals (Fig 1E, G and I), and high larval lethality was observed. We could not conclude on the effect of *Lp*^{WJL} on larval growth when *daw* was overexpressed as we obtained different results each time we repeated the experiment (Fig 1F, H, J). However, Overexpressing *dawdle* was consistently very deleterious for the larval growth, even though we dampened the effect of the GAL4 with GAL80 by raising the flies at 25°C or lower.

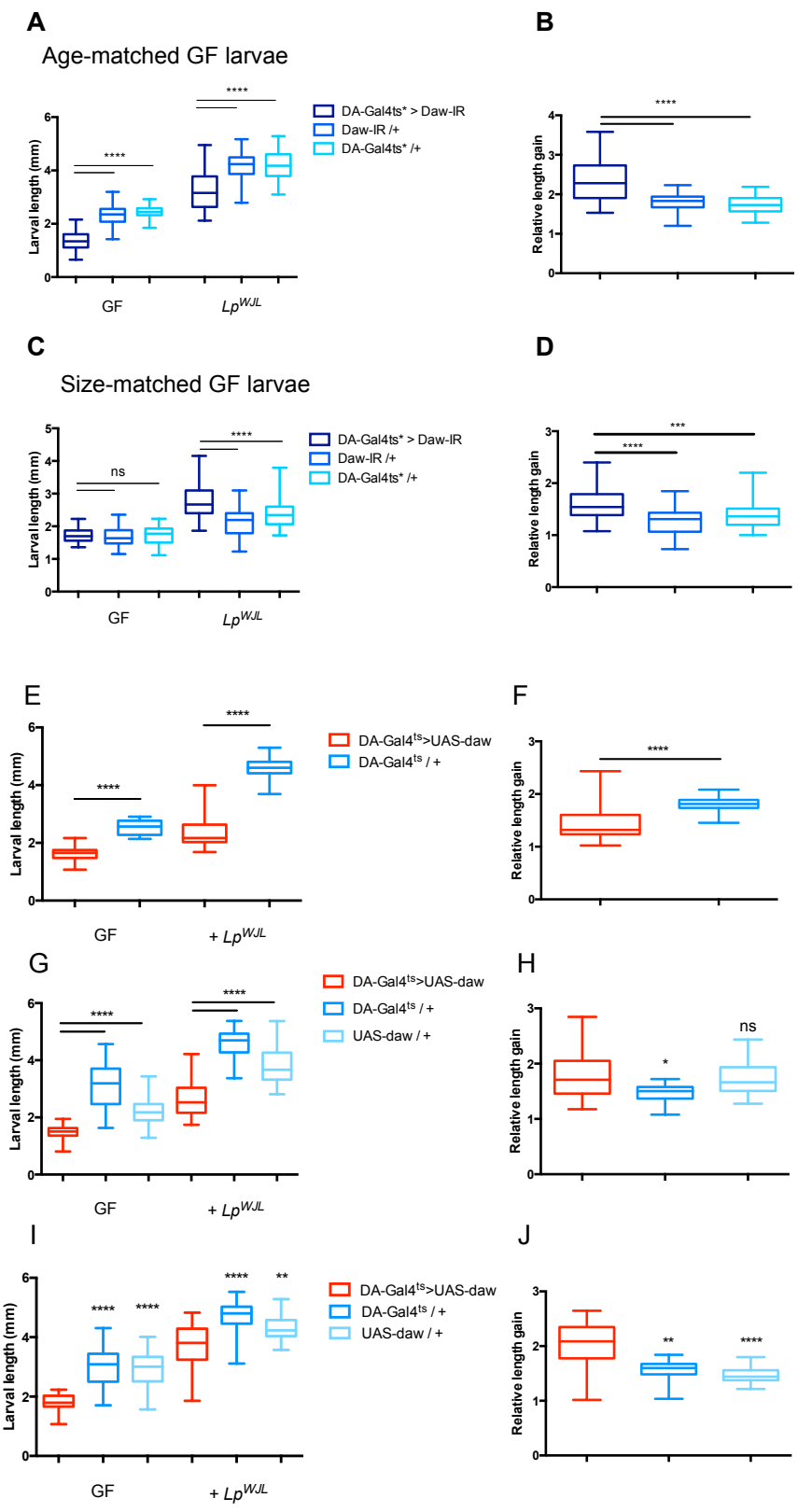


Figure 1: The growth boost provided by *L. plantarum*^{WJL} is enhanced when *dawdle* is knocked-down

A) and C) Box plots show the longitudinal larval length in mm for *dawdle* knockdown (dark blue), and its controls (lighter blue shades). Each box plot represents the average length from a pool of larvae (n>20). A) larvae are age-matched at day 7 AEL, B) larvae are size-matched as GF (n > 25).

C) and D) box plots represent the relative length gain conferred by *Lp*^{WJL}.

E, G, I) Box plots show larval size (n > 20) measured 7 days AEL on low-protein diet for GF and *Lp*^{WJL}-monoassociated larvae overexpressing dawdle (*DA-GAL4*^{ts} > *UAS-daw*) and control larvae (*DA-GAL4*^{ts} / + and *UAS-daw* / +) for the three repeats.

F, H, J) Box plot represent the relative length gain provided by *Lp*^{WJL} for larvae overexpressing *dawdle* (*DA-GAL4*^{ts} > *UAS-daw*) and control larvae (*DA-GAL4*^{ts} / + and *UAS-daw* / +).

2) *dawdle* knockdown in the fat body affects the growth enhancement conferred by *L. plantarum*^{WJL}

We showed that ubiquitous *daw*-knockdown aggravated growth delay in absence of *Lp*^{WJL}. To better understand this phenotype, we looked at tissue-specific knockdowns. In larvae, *daw* is highly expressed in the fat body, in the gut and in muscles (Bai et al., 2013; Chintapalli et al., 2007). Moreover, Chng et al. reported that Daw secreted from the fat body acts on the midgut cells to inhibit the expression of various digestive enzymes. Hence, we decided to study the effect of muscles, midgut (Fig 2A and B) and fat body (Fig 2C and D) specific *daw*-knockdown on larval growth, using respectively, 24B-GAL4, mex-GAL4 and Lpp-GAL4 drivers. Muscle-specific knockdown of *daw* induced very high larval lethality therefore we were not able to conduct the experiment. When knocked down in the midgut, *daw* had no effect on longitudinal larval length (Fig 2 A and B). However, when we knocked down *daw* specifically in the FB, GF *daw*-KD animals had no length difference with the controls, but we observed a slight increase in the larval length of monoassociated animals (Fig 2 C) resulting in an increased relative length gain (Fig 2D). Taken together, these results suggest that fat body is an important source of *daw* for the ameliorated growth benefit conferred by *Lp*^{WJL}. However, fat body specific knockdown of *daw* does not fully recapitulates the ubiquitous knockdown, therefore, if fat body is an important source of *daw* to explain our larval length phenotype, other tissues may be involved. Of note, for both fat body and gut-specific RNAi, the systemic growth delay observed with a ubiquitous KD was lost. Therefore, it is likely that *daw* produced by another tissue plays an important role in larval growth. According to the previously reported role of Daw in muscles on secretion of dILPs in adult flies (Bai et al., 2013), the source of Daw involved in larval growth can be muscle, but the high lethality as a result of daw KD in muscle prevented us from further assessing the larval muscle involvement.

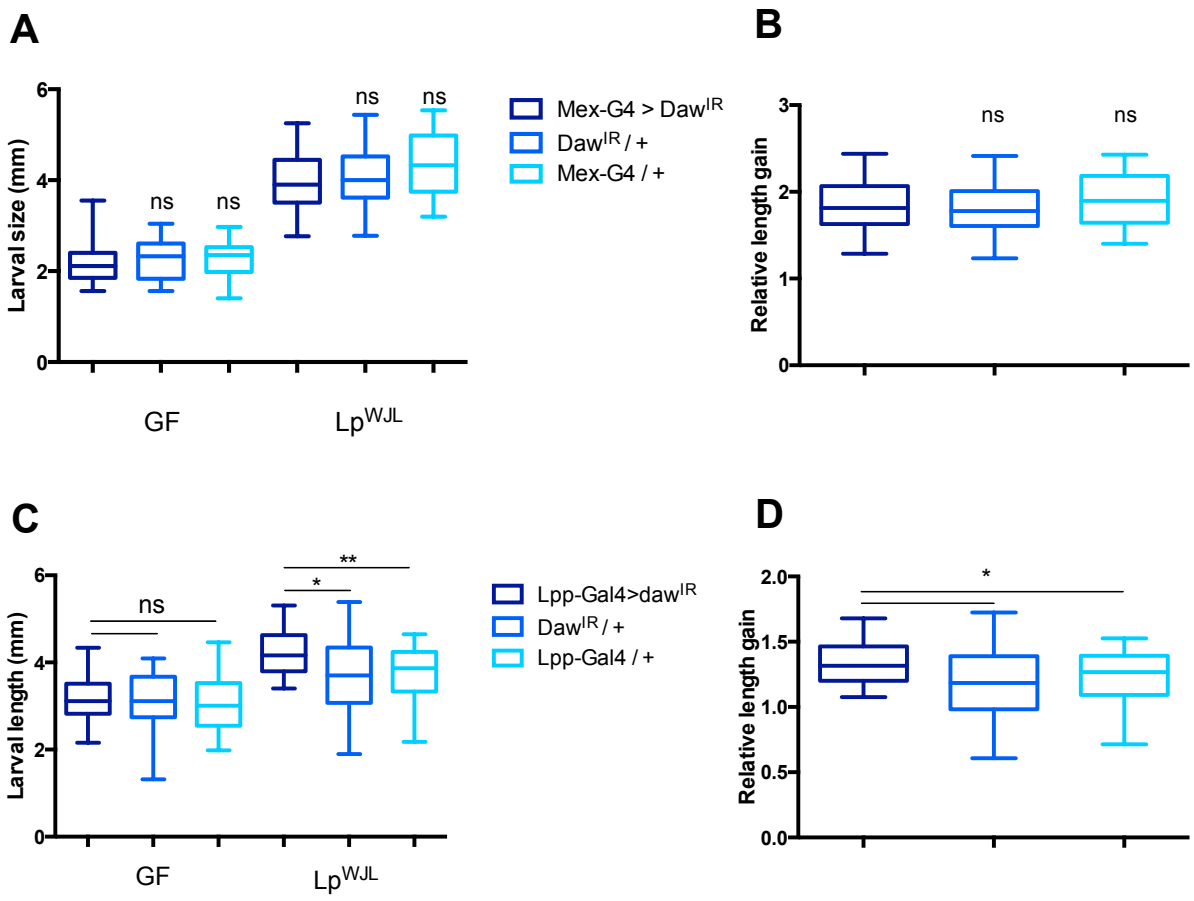


Figure 2: The growth boost provided by *L. plantarum*^{WJL} is enhanced when *dawdle* is knocked-down in the fat body

A) Box plots show larval size (n > 20) measured 7 days AEL on low-protein diet for GF and *Lp*^{WJL}-monoassociated larvae *dawdle* knockdown (*mex-GAL4* > *UAS-daw-IR*) and control larvae (*mex-GAL4* / + and *UAS-daw-IR* / +).

B) Box plots show the relative length gain conferred by *Lp*^{WJL} for the following genotypes: *mex-GAL4* > *UAS-daw-IR*, *mex-GAL4* / + and *UAS-daw-IR* / +.

C) Box plots show longitudinal larval size (n > 20) measured 7 days AEL on low-protein diet for GF and *Lp*^{WJL}-monoassociated larvae *dawdle* knockdown (*lpp-GAL4* > *UAS-daw-IR*) and control larvae (*lpp-GAL4* / + and *UAS-daw-IR* / +).

D) Box plots show the relative length gain conferred by *Lp*^{WJL} for the following genotypes: *lpp-GAL4* > *UAS-daw-IR* and control larvae *lpp-GAL4* / + and *UAS-daw-IR* / +.

Stars indicates statistical significance from Kruskal-Wallis tests *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3) *mondo* and *mlx* knockdowns phenocopy *daw* knockdown

Mattila *et al*, reports that the sugar-responsive transcription factors *mondo* and *mlx* regulate *daw* expression upon feeding on high-sucrose diet (Mattila et al., 2015). This signaling pathway involving *daw* is key to many aspects of metabolism. Interestingly, *mondo* was uncovered by the GWAS from the monoassociated dataset (see table 3 from chapter 1), indicating that it may be important for larval systemic growth. Indeed, in absence of Mondo or Mlx (Mondo's binding partner), flies show a slight growth delay on a standard medium, and on high-glucose diet, they are unable to grow and die as larvae (Havula et al., 2013). Mondo, along with its binding partner Mlx, controls genes involved in glycolysis and *de novo* lipogenesis and are therefore important for optimal larval growth (Mattila et al., 2015). This publication prompted us to wonder whether these two genes were also involved in Lp^{WJL} -conferred growth benefit.

We investigated the effect of the ubiquitous *mondo*-knockdown on larval growth raised on a low-protein diet, in presence and absence of Lp^{WJL} (Fig 3A) by measuring larval length 7 days AEL on low-protein diet. We observed that GF larvae were smaller when *mondo* was knocked down. However, unlike GF, $mondo^{IR}$-monoassociated larvae grew as well as the control larvae (Fig 3A), resulting in an increased length gain provided by Lp^{WJL} as demonstrated by the higher relative length gain (Fig 3B). Therefore, *mondo*-KD resulted in a stronger growth benefit conferred by Lp^{WJL}. We then tested how *mlx* complete loss of function affected larval growth (Fig 3C and D) using a line carrying a null mutant allele, mlx^{1} (Havula et al., 2013), which deletes the entire region of *mlx* gene as well as the beginning of the C-terminal part of CG3368, a neighboring gene. We raised GF and Lp^{WJL}-monoassociated mlx^{1} larvae on low-protein diet and measured larval length 7 days AEL. In absence of *mlx*, GF larvae suffered from a growth delay (Fig 3C, left panel) that was restored upon monoassociation with Lp^{WJL} (Fig 3 C, right panel). To conclude, like daw^{IR} and $mondo^{IR}$, *mlx* loss of function resulted in an increased growth benefit by Lp^{WJL} (Fig 3 D).

Mattila et al. reported that *sugarbabe* (*sug*) is a downstream effector of the Activin signaling, and the last effector of the pathway involving *daw* and controlled by Mondo-Mlx. To assess whether the increased growth gain observed in presence of Lp^{WJL} depends on *sug*, we tested whether *sugarbabe* (*sug*) mutant larvae led to the same phenotype as *mondo*, *mlx* and *daw* loss of function, i.e. an increased relative length gain

provided by *Lp*^{WJL}. We crossed two *sugarbabe* deletion mutant lines: *sug*$^{17\Delta}$ and *sug*^{def} (Mattila et al. 2015) to obtain a transheterozygous mutant for *sugarbabe* (*sug*$^{17\Delta}$/ *sug*^{def}). We quantified the length of *sug* mutant larvae after 7 days AEL of development on low-protein diet (Fig 3E). Regarding larval length, *sug*$^{17\Delta}$/ *sug*^{def} GF larvae were smaller than controls, however, the length difference was only significant for one of the two control lines (Fig 3E). Monoassociated larvae were also smaller in *sug* mutant animals (Fig 3E). The growth response to *Lp*^{WJL} was not different in *sug* mutant larvae as the relative length gains were not significantly different (Fig 3F). Thus, the enhanced growth benefit seems to be *sug*-independent. Collectively, our results show that the loss of function of *mondo*, *mlx*, *daw,* which are part of the same signaling pathway, leads to more growth enhancement conferred by *Lp*^{WJL}. This makes sense because *mondo* or *mlx* loss of functions result in a decreased level of *dawdle* (Mattila et al., 2015). However, the *Lp*^{WJL}-related growth phenotype of *sug* transheterozyote does not resemble that of *mondo*, *mlx* or *daw*, and therefore is probably independent of *sugarbabe*.

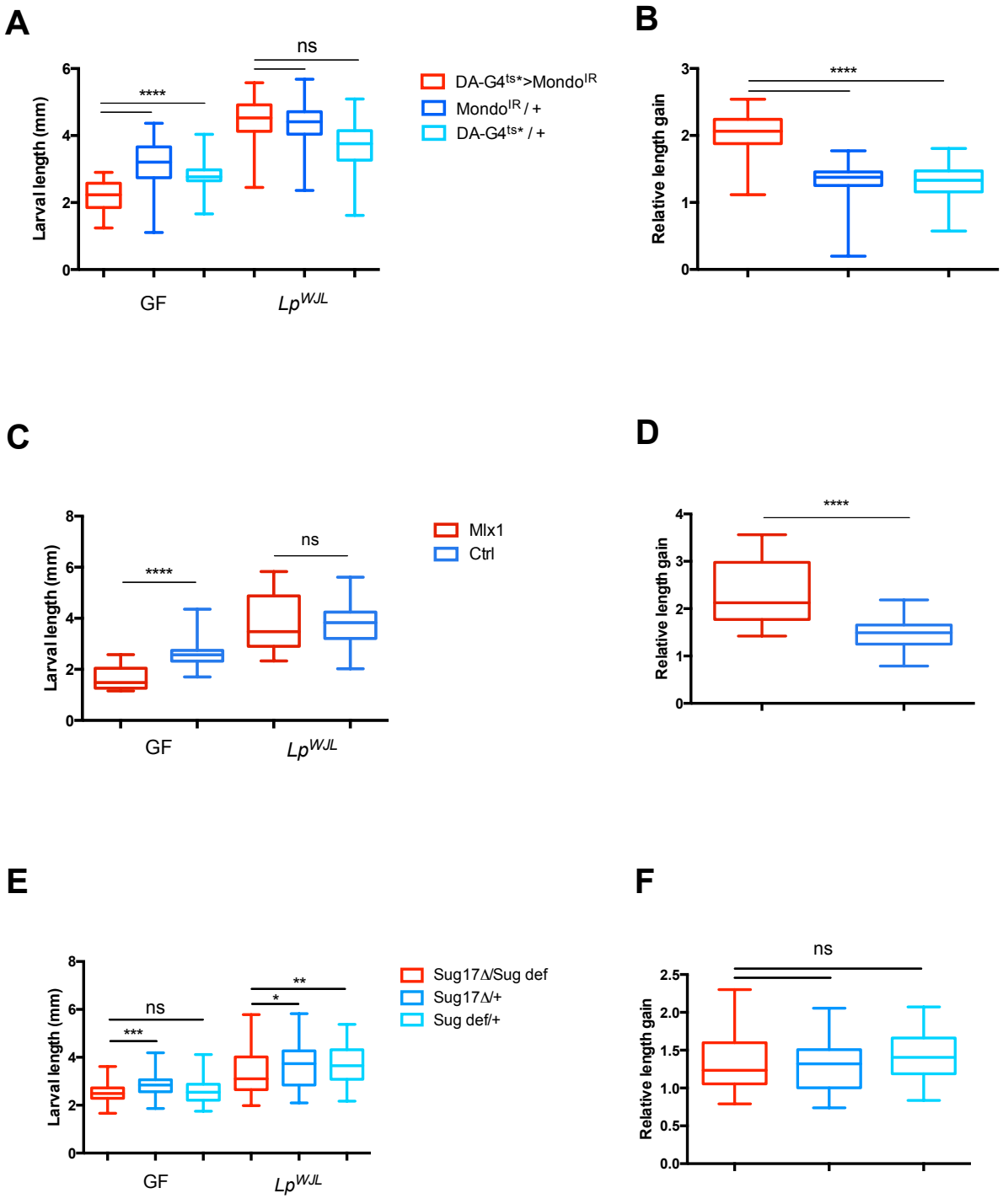

A
Larval length (mm)
ns
****
DA-G4ts*>MondoIR
MondoIR / +
DA-G4ts* / +
GF
LpWJL
B
Relative length gain
****
C
Larval length (mm)
****
ns
Mlx1
Ctrl
GF
LpWJL
D
Relative length gain
****
E
Larval length (mm)
ns
***
**
*
Sug17Δ/Sug def
Sug17Δ/+
Sug def/+
GF
LpWJL
F
Relative length gain
ns

Figure 3 : *mondo* and *mlx* knockdowns phenocopy *daw* knockdown

A) Box plots represent the larval length (n > 40) measured 7 days AEL on low-protein diet for GF and Lp^{WJL}-monoassociated larvae for *mondo* knockdown (*DA-GAL4^{ts}* > *UAS-mondo-IR*) and control larvae (*DA-GAL4^{ts}* / + and *UAS-mondo-IR* / +).

B) Box plot represent the relative length gain provided by Lp^{WJL} for *mondo*-knockdown larvae (*DA-GAL4^{ts}* > *UAS-mondo-IR*) and control larvae (*DA-GAL4^{ts}* / + and *UAS-mondo-IR* / +).

C) Box plots represent the larval length (n > 20) measured 7 days AEL on low-protein diet for GF and Lp^{WJL}-monoassociated larvae for *mlx^{1}* mutant (*mlx1*) and control larvae (Ctrl).

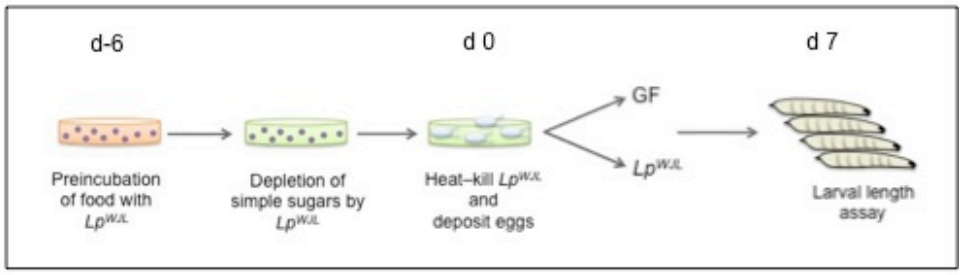
D) Box plot represent the relative length gain provided by Lp^{WJL} for *mlx* mutant larvae (*mlx^{1}*) and control larvae (Ctrl).

Stars indicates statistical significance from Kruskal-Wallis tests *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

4) The growth defect of GF *daw*-KD larvae is not caused by sugar toxicity

mondo, *mlx* and *dawdle* knockdown or loss of function leads to a growth defect in GF animals, and such defect is compensated by the monoassociation with Lp^{WJL}. Mattila *et al* reported that the Mondo-Mlx complex controls the majority of sugar responsive genes acting through the downstream effectors Dawdle and Sugarbabe. As a consequence animals deficient for these genes are more sensitive to sugars, which become lethal for these animals due to an important increase of circulating sugar concentration. Interestingly, our group showed that Lp^{WJL} possesses the ability to rescue sugar toxicity in flies by consuming a part of the sugars present in the medium (Storelli et al. unpublished data). Gilles Storelli assayed the effect of increasing sucrose concentrations on pupariation timing and showed that gradual increase of sucrose in the diet results in lethality for GF larvae while Lp^{WJL}-monoassociated larvae were barely affected (Figure S2 A). Moreover, metabolomics studies showed that Lp^{WJL} totally depletes simple sugars from the fly diet, which may account for the observed rescuing the sucrose-induced lethality in GF animals (Fig S2 B). Hence, we hypothesized that the aggravated growth defect observed in GF *daw*-KD larvae could be caused by the intolerance to sugars present in the fly diet, and Lp^{WJL} rescues this toxicity. To test this hypothesis, we studied the larval length of *daw*-KD animals raised on the low-protein diet preincubated with Lp^{WJL} (or PBS as a control) (experimental procedure is explained in Figure 4 A). If the hypothesis holds true, we expected that the *daw*-KD GF larvae raised on a medium depleted from sugars by Lp^{WJL} would be longer at day 7 AEL than their counterparts raised on the medium preincubated with PBS because Lp^{WJL} would have depleted the diet from sugars, which *daw*-KD animals are highly sensitive to (Ghosh and O'Connor, 2014). However, we did not detect a difference in the length of *daw*-KD larvae raised on the medium preincubated with PBS or with Lp^{WJL} (Fig 4 B). Monoassociated larvae were in fact smaller when the diet was preincubated with the bacteria compared to larvae raised on the diet preincubated with PBS. One possible explanation to this result could be that during the preincubation, the bacteria utilized most of the sugars from the medium, therefore, the fresh bacteria added on the eggs deposited grew less well and could not be as beneficial. To conclude, these results indicate that the reduced larval growth phenotype of *daw*-KD GF larvae detected on our low nutritional condition is not the result of an enhanced sugar toxicity of these animals.

A



B

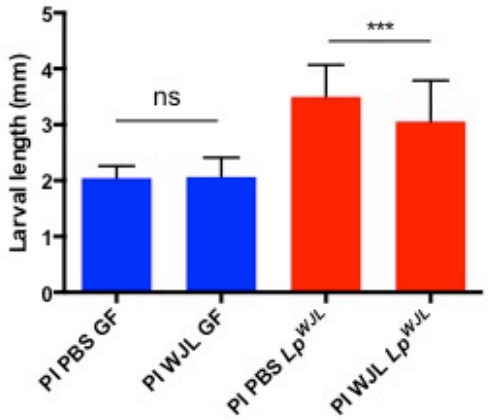


Figure 4: sugar toxicity does not explain the growth delay of *daw*-KD larvae

A) Scheme of the experimental set up for the sugar toxicity assay: low-protein diet is preincubated with Lp^{WJL} (PI WJL) or PBS (PI PBS, serves as control) during 6 days to deplete the medium from simple sugars. The day of the inoculation (d0), the preincubated fly food caps are heated for 30 minutes at 65°C to heat-kill the bacteria. Feshly laid embryos are deposited on the caps and freshly inoculated with either PBS (GF) or Lp^{WJL}.

B) Graph bars show the mean larval length at day 7 AEL (n > 30) of GF or Lp^{WJL}-monoassociated larvae raised on a low-protein diet preincubated with PBS (respectively, PI PBS GF and PI PBS WJL) or Lp^{WJL} (PI WJL GF and PI WJL Lp^{WJL}).

5) *daw* expression is downregulated upon monoassociation with *L. plantarum*^{WJL}

So far we have studied the larval growth phenotype in different *daw*-KD contexts. Next we wondered whether the expression of *daw* could be modulated by the presence of *Lp*^{WJL}: as the SNP associated to *daw* gene was a synonymous mutation, it may impact *daw* transcript level and/or stability. Therefore, we wondered if *daw* mRNA levels are modified in different microbial contexts. We studied *daw* mRNA levels in *y,w* whole larvae by RT-qPCR. We collected size-matched larvae raised on low-protein diet in the presence or absence of *Lp*^{WJL} at different time points of the larval development (Fig 5) corresponding to three different sizes: "Size 1" corresponds to L2 larvae, "size 2" corresponds to L2/L3 transition and "size 3" to mid L3 stage (for the corresponding size range, see table S1). *Lp*^{WJL}-monoassociated and GF larvae were collected respectively on day 2.5 for size 1, day 7 and 4 for "size 2", and day 10 and 7 for "size 3". We observed that *daw* mRNA levels are decreased upon monoassociation with *Lp*^{WJL} in size-matched larvae at mid-larval development (size 2) periods corresponding to L2/L3 transition stages (Fig 5A). However, in age-matched larvae collected at day 7 AEL, no differential expression of *daw* was observed between the GF and monoassociated condition (Fig 5B). Next we wondered whether the transcriptional regulation of *daw* upon exposure to *Lp*^{WJL} was tissue specific. To tackle this question, we isolated RNA from different larval tissues: the guts, the fat bodies and the carcasses of size-matched larvae at size 2. We found no significant difference in the level of expression of *daw* (Fig 5 C, D, E). The downregulation we observed in whole larvae is either not tissue-specific, or we were not able to detect it. However, we noticed that, although not significant, there was a slight tendency of *daw* downregulation in *Lp*^{WJL}-mono-associated larvae in each tested tissue (Fig 5 B, C, D). This result indicates that the downregulation is probably not occurring in one particular tissue, and that the downregulation we observed with whole larvae is a collective effect of several tissues.

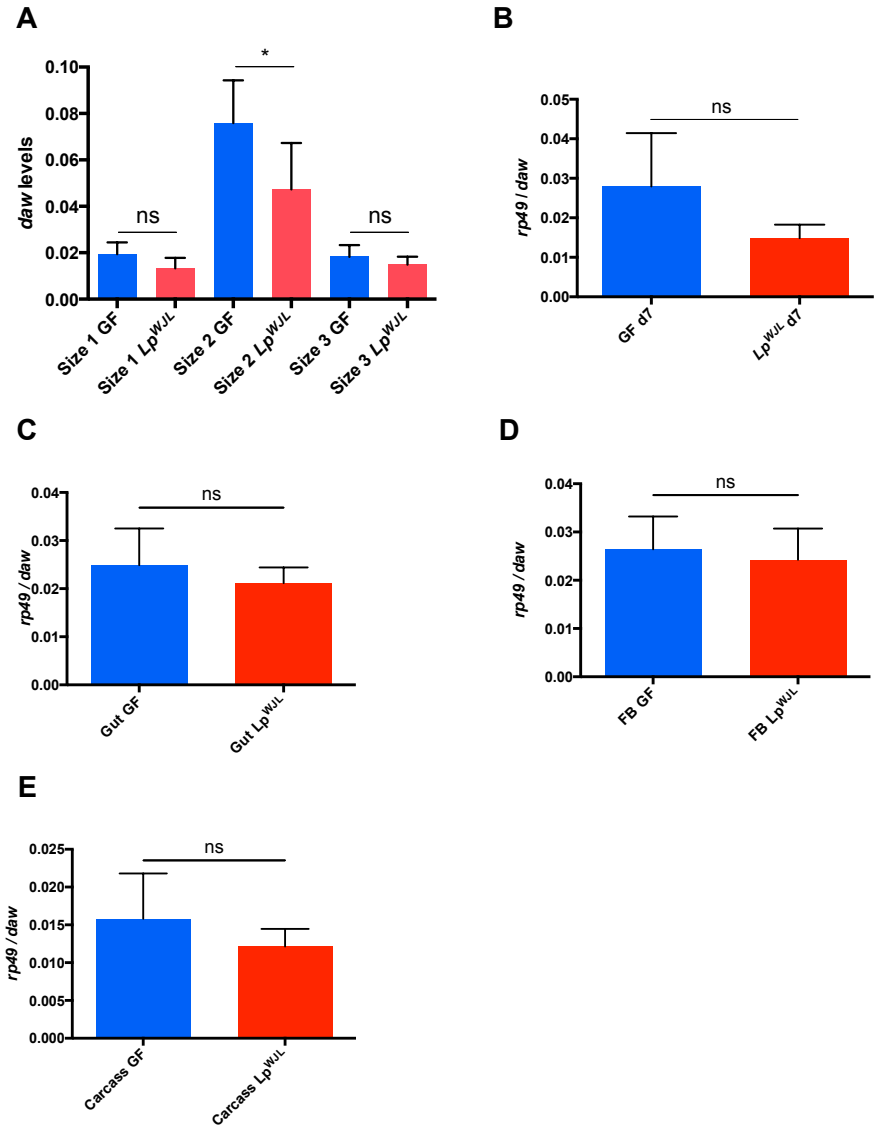


Figure 5: *daw* is downregulated upon *Lp*^{WJL} -monoassociation during larval development

A) Bar graphs show mean ± SD of *daw* transcripts levels relative to transcripts levels of the housekeeping gene *rp49* analyzed by RT-qPCR. GF and *Lp*^{WJL}-monoassociated larvae were size-matched at "size 1", "size 2 " and "size 3". Asterisks represent statistical significance with GF samples.

B) Bar graphs show mean ± SD of *daw* levels relative to levels of the housekeeping gene *rp49*. GF and *Lp*^{WJL}-monoassociated larvae were collected 7 days AEL. Asterisks represent statistical significance with GF samples.

C-E) Bar graphs show mean ± SD of *daw* levels relative to levels of the housekeeping gene *rp49*, for cDNA from pools of 15 guts (C), fat bodies (D) and carcasses (E) from GF or *Lp*^{WJL}-monoassociated larvae dissected at "size 2".

Stars indicate statistical significance from Mann and Whitney tests *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

6) *dawdle* regulates digestive enzymes expression on low-protein diet

We previously found that *daw* and *mondo* knockdowns and *mlx* mutants led to a similar growth phenotype: we observed an increased length gain conferred by Lp^{WJL}, indicating that the bacteria are more efficient to promote growth in the mutant context. This phenotype is mainly caused by the fact that GF larvae suffer from an important growth defect that is compensated by Lp^{WJL}. Chng et al. reported that in adult *Drosophila*, *dawdle* from the fat body acts on enterocytes to inhibit the expression of many digestive enzymes, particularly amylases and maltases. Mattila *et al* reported that Mondo-Mlx controls the vast majority of sugar-regulated genes involved in many aspects of digestion and metabolism (nutrient digestion and transport, lipid, carbohydrates, amino acid metabolism) and maintains the metabolic homeostasis through the downstream effector *dawdle*. Furthermore, we have found that Lp^{WJL} monoassociation triggers the upregulation of several host intestinal proteases as well as other digestive enzymes such as lipases and glycosyl-hydrolases (Erkosar *et al*, 2014), we thus hypothesized that *daw* could modulate the expression of proteases during growth in a microbial dependent manner. We therefore investigated the expression level of a number of proteases known to be upregulated by the presence of Lp^{WJL} (*jon66cii*, *jon66ci*, *jon44e*, *CG18179*) (Erkosar et al., 2015; Matos et al., 2017) in *daw* knocked-down and control larvae to assess whether *daw* regulates the expression of proteases and if so, whether the regulation is affected by the presence of the bacteria.

We first tested the expression level of *jon66cii*, the most induced protease upon Lp^{WJL} association (Erkosar *et al* 2015) in size-matched larvae at size 1 and size 2 (Fig 6). In presence of Lp^{WJL} at size 1, *daw*-KD larvae expressed the same proteases level as the controls. In size 2 and age-matched conditions, in presence of Lp^{WJL}, *jon66cii* was less expressed in *daw*-KD individuals (Fig 6B and C), meaning that in presence of Lp^{WJL}, Daw promotes the expression of *jon66cii*. We found the opposite that what has been reported so far for other enzymes (maltases and amylases) (Chng et al., 2014). However, in the GF condition, which is a new context that had never been tested before regarding *daw,* we observed that *jon66cii* is upregulated in *daw*-KD larvae compared to the control larvae, for both size-matched, size 1 and 2 (Fig 6A and B), and age-matched (day 7 AEL) individuals (Fig 6C). This result suggests that Dawdle represses the expression of this protease in absence of bacteria. Therefore our results suggest Daw somehow regulates

jon66cii expression in opposite manner depending on the microbiota status. In GF animals, our results unveil the role of *daw* to repress proteases, on our low-protein diet whereas upon monoassociation with Lp^{WJL}, *daw* promotes the expression of proteases.

Next, to verify whether *daw* regulated other gut proteases, we assayed the level of *jon66ci* (Fig 7A), *CG18179* (Fig 7B), *jon44e* (Fig 7C). This time, we focused on size 2 larvae, as it was the condition that exhibited the most striking phenotype with *jon66cii*. In GF larvae, *daw*-KD had no effect on *jon66ci* expression compared to the control larvae (Fig 7 A) whereas in presence of Lp^{WJL}, *daw*-KD led to a decrease of *jon66ci* expression level. Regarding *CG18179*, while control animals expressed almost no *CG18179*, *daw*-KD GF larvae had high expression level of the enzyme. When raised in presence of Lp^{WJL}, *daw*-KD and control animals exhibited no difference in the expression level of *CG18179*. For *Jon44e, daw*-KD larvae expressed less *jon44e* than the control line in both GF and monoassociated conditions. Our results therefore indicate that *daw* influences the expression of a number of digestive proteases in larvae but this influence is clearly impacted by the microbial environment of larvae (i.e. GF or Lp^{WJL} associated).

Previous work from our group, showed that the growth promotion conferred by Lp^{WJL} can be partially attributed to the upregulation of of host proteases by the bacteria (Erkosar et al., 2015). As demonstrated above, ubiquitous yet partial knock-down of daw in larvae resulted in augmented growth benefit expressed as larval length ratio (Figure lB), despite the fact that both GF and Lp^{WJL} mono-associated larvae were smaller than their corresponding controls. Therefore, we nonetheless expected that *daw*-KD larvae would show higher protease expression when monoassociated with Lp^{WJL} compared with control animals, which could account for the augmented growth enhancement. To our surprise, we observed extremely perplexing mode of regulation of protease expression by Daw that is in fact uncoupled to bacteria growth benefit. Specifically, in GF larvae lacking Daw, the expression of different proteases seemed to be de-repressed, but GF larvae were severely delayed in growth (Figure 1A and Figure 6B). In Lp^{WJL} monoassociated larvae lacking Daw, the proteases failed to be upregulated yet the growth promotion effect is enhanced (Figure 1B and Figure 6B). These results strongly suggest that the upregulation of proteases is not strictly correlated to Lp^{WJL} growth benefit, and Dawdle-dependent regulation of proteases failed to explain the *daw*-KD growth phenotype. For that reason, we decided to assay how digestive enzymes other than proteases behaved in *daw*-KD larvae. We studied the level of expression of

amy-p and *malA1*, two enzymes downregulated by *daw* in adult flies upon sugar feeding (Chng et al., 2014). GF size-matched larvae expressed a higher level of *amy-p* when *daw* was knocked-down (Fig 7D) meaning that Daw inhibits *amy-p* in larvae without the presence of the gut microbiota. For monoassociated individuals where *daw* was knocked-down, although there was a tendency to express more *amy-p*, it was not statistically significant. As for *malA1* we observed the same: GF size-matched larvae expressed more of the enzyme when *daw* was knocked-down (Fig 7E) while there was no difference for monoassociated larvae. Here again we observed a differential regulation of enzymes by *daw* depending on the microbiota status. So far, all published results regarding Dawdle control of enzymes were conducted in conventional flies with an intact microbiota. In this context, both Chng et al 2014, Mattila et al 2015 show a role of *daw* in regulating enzymes upon sugar feeding. Our results also show that *daw* regulates the expression of digestive enzymes on fly food which contains a low amount of protein and very low quantity of simple sugars; however our results reveal another layer of regulatory complexity by Daw that is tightly coupled to the microbial context of the host. Despite the interest of this novel result, as we already mentioned above, the regulation of digestive enzymes by *daw* in absence or presence of Lp^{WJL} failed to explain why *daw*-KD animals benefit more from the monoassociation with Lp^{WJL}. Therefore, the effect of *daw* on growth and on digestive enzymes regulation seems disconnected.

As the *daw* RNAi experiments yielded complex and unexpected results, we wished to examine protease expression in *dawdle* mutants. As explained in the first section of this chapter, we crossed two lines to obtain the transheterozygous mutant animals daw^{1}/daw^{11} (Ghosh and O'Connor, 2014). In agreement with report by Ghosh and O'Connor, *daw* null mutant larvae showed a high larval lethality on our standard low-protein diet, probably due to an important accumulation of acidic metabolites in the hemolymph causing an internal acidosis (Ghost and O'Connor 2014). Most of fly diets, including ours, contains propionic acid as a mold inhibitor, but it has been demonstrated that *daw* mutants are highly vulnerable to propionic acid. We first tried to raise *daw* transheterozygotic larvae on our conventional diet, but larval lethality was too high, and we could recover very few individuals. As a consequence, we decided to remove the propionic acid from our diet as reported by Gosh and O'connor. We also raised daw^{1}/daw^{11} transheterozygote and control lines (daw^{1}/+, daw^{11}/+ flies) on a rich-

protein diet as our low-protein medium was also too harsh for the survival of *daw* mutants. Removing the propionic acid greatly increases the chance of contamination, consequently we had to intensify our efforts to work under sterile conditions when separating the transheterozygous from the heterozygous animals. On the rich-protein diet, larvae develop about twice as fast as on the low-protein diet, therefore we collected larvae early in the development, at day 1.5 and 3 AEL to overcome the larval lethality of *daw* mutants. Removal of propionic acid indeed allowed us to recover more animals. We then performed RT-qPCR on *daw* mutants and control larvae in the presence and absence of Lp^{WJL} and studied the expression level of *jon66cii*, *jon66ci* and *CG18179* (respectively, Fig 8 A, B and C) (Erkosar *et al*, 2015). Our results show that *jon66cii*, *jon66ci* and *CG18179* expression in GF larvae was higher in absence of *daw*, while in monoassociated animals the level was not affected. Therefore, *daw* represses the three proteases expression in absence of bacteria, but not in presence of Lp^{WJL}. This confirms the results we obtained for *jon66cii* with *dawdle* RNAi size 1 larvae: in absence of bacteria, Dawdle represses proteases expression while it does not in presence of Lp^{WJL}. The results we obtained with *daw* mutants and the RNAi line were consistent for early developmental time points. Yet, at day 3, *daw* loss of function did not affect the proteases level (Fig 8 D-F), contradicting the phenotype obtained with the RNAi line. However, the caveat of the experiment is that *dawdle* mutants had to be raised on a protein-rich standard laboratory diet to improve viability, whereas *daw*-KD animals were raised on the low-protein diet. The different diet could explain the discrepancies observed between the mutant and the RNAi experiments.

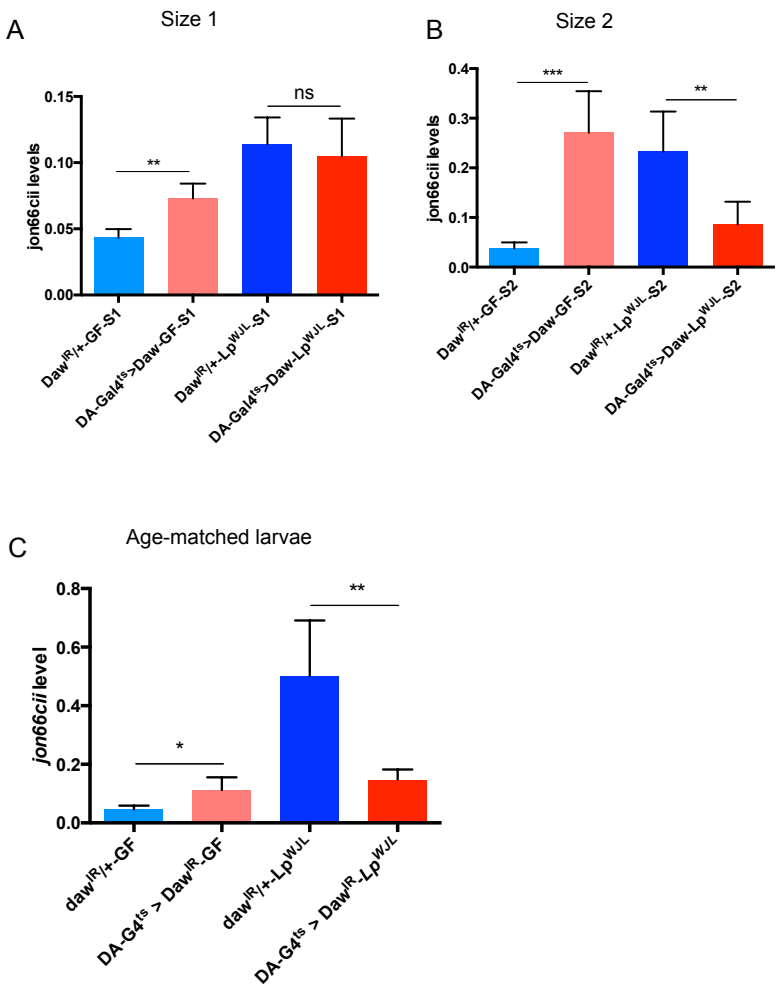


Figure 6 : *daw* regulates *jon66cii* expression on low-protein diet

Bar graphs show mean ± SD of *jon66cii* transcripts levels relative to transcripts levels of the housekeeping gene *rp49* analyzed by RT-qPCR. GF and *Lp*^{WJL}-monoassociated *daw*-KD (*DA-GAL4ts > daw*^{IR}) and control (*daw-IR / +*) larvae raised on low-protein diet were size-matched at "size 1" (A), "size 2" (B) or age-matched at day 7 AEL (C).

Stars indicate statistical significance from Mann and Whitney tests *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

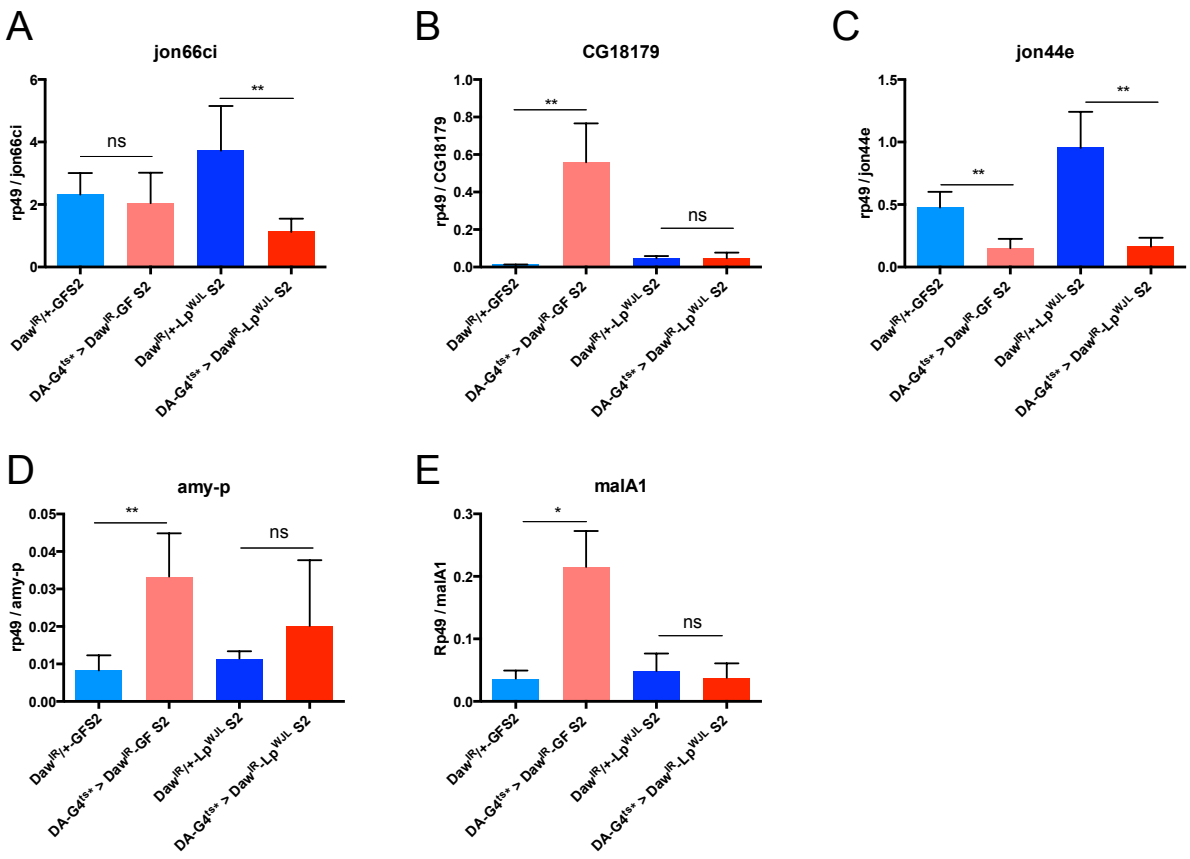


Figure 7 : *daw* regulates the expression of a plethora of enzymes

Bar graphs show mean ± SD of *jon66ci* (A), *CG18179* (B), *jon44e* (C), *amy-p* (D) and *malA1* (E) transcripts levels relative to transcripts levels of the housekeeping gene *rp49* analyzed by RT-qPCR. GF and Lp^{WJL}-monoassociated *daw*-KD (*DA-GAL4ts > daw*^{IR}) and control (*daw-IR* / +) larvae raised on low-protein diet were size-matched at "size 2".

Stars indicate statistical significance from Mann and Whitney tests *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

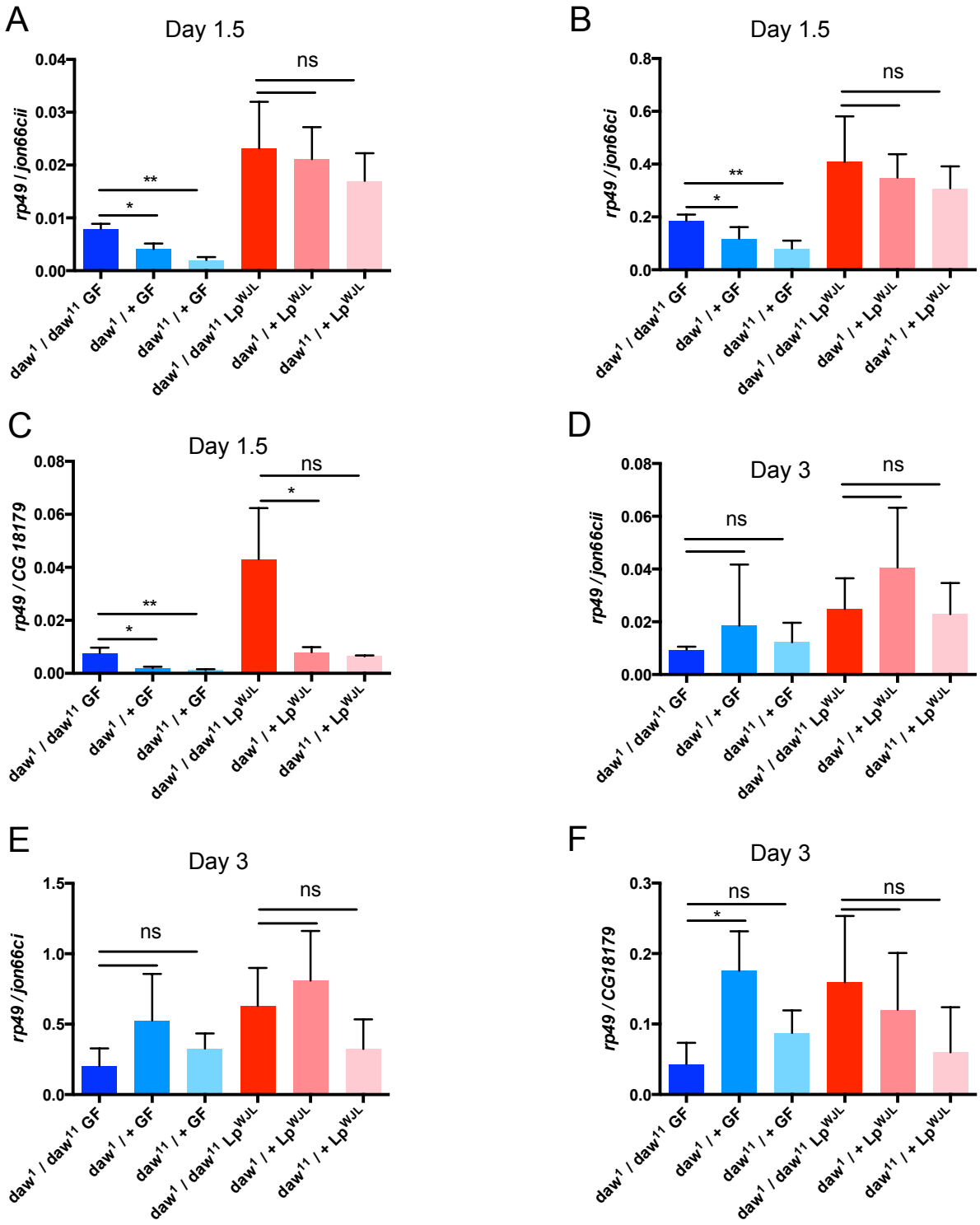

A
Day 1.5
rp49 / jon66cii
0.04
0.03
0.02
0.01
0.00
ns
**
*
daw1 / daw11 GF
daw1 / + GF
daw11 / + GF
daw1 / daw11 LpWJL
daw1 / + LpWJL
daw11 / + LpWJL
B
Day 1.5
rp49 / jon66ci
0.8
0.6
0.4
0.2
0.0
ns
**
*
C
Day 1.5
rp49 / CG 18179
0.08
0.06
0.04
0.02
0.00
ns
*
**
*
D
Day 3
rp49 / jon66cii
0.08
0.06
0.04
0.02
0.00
ns
ns
E
Day 3
rp49 / jon66ci
1.5
1.0
0.5
0.0
ns
ns
F
Day 3
rp49 / CG18179
0.3
0.2
0.1
0.0
ns
*
ns

Figure 8 : *dawdle* null mutants phenocopy *daw*-KD at early developmental stages

A-C) Bar graphs show mean ± SD of *jon66cii* (A), *jon66ci* (B), *CG18179* (C) transcripts levels relative to transcripts levels of the housekeeping gene *rp49* analyzed by RT-qPCR. GF and Lp^{WJL}-monoassociated *daw* null mutants *(daw^{1}/daw^{11})* and controls (daw^{1} / + and daw^{11} / +) whole larvae were raised on rich-protein diet and collected at day 1.5.

D-F) Bar graphs show mean ± SD of *jon66cii* (D), *jon66ci* (E), *CG18179* (F) transcripts levels relative to transcripts levels of the housekeeping gene *rp49* analyzed by RT-qPCR. GF and Lp^{WJL}-monoassociated *daw* null mutants *(daw^{1}/daw^{11})* and controls (daw^{1} / + and daw^{11} / +) whole larvae were raised on rich-protein diet and collected at day 3.

7) Proteases regulation by *mlx*

Mattila *et al* showed that high sugar feeding in larvae induced wide-spread transcriptomic changes in digestive enzymes involved in glycolysis and lipogenesis that are regulated by a upstream regulatory loop comprising Mondo, Mlx, Daw and Sug (Mattila et al., 2015). We therefore questioned if and how the regulatory network also differentially regulated digestive proteases in the presence or absence of an intestinal microbiota. We performed RT-qPCR on mlx^{1} mutant and control larvae to look at the expression level of *jon66cii*, the most upregulated protease by Lp^{WJL}. *mlx* loss of function had no impact on *jon66cii* transcription level, neither in GF nor in Lp^{WJL}-monoassociated condition (Fig 9 A and B). Hence the *daw* regulation of *jon66cii* is *mlx*- independent in our system.

We also wanted to verify whether the regulatory network controlled by Mondo-Mlx also operates in our low protein diet, and if and how microbiota impacts it. We first tested how *mlx* expression level was modulated by the monoassociation. We quantified *mlx* expression level in size-matched (size 2) wild-type larvae. Our results show that unlike *daw, mlx* expression level was not affected by the presence of the bacteria on low or rich-protein diet (Fig 9 C and D). It has been reported previously that *daw* expression is induced by Mondo-Mlx upon sugar feeding (Mattila *et al* 2015). Therefore, we wanted to verify whether this regulation was maintained on our diet, which contains a low sugar concentration. We investigated the expression level of *daw* in mlx^{1} mutant animals. When larvae were fed on low-protein diet, despite a strong tendency, we observed no significant decrease in the expression level of *daw* in presence or absence of Lp^{WJL} (Fig 9 E). When larvae were raised on rich-protein diet we observed a significant downregulation of *daw* expression level in *mlx* mutant larvae, both in presence and absence of the bacteria (Fig 9 F). This confirms the earlier findings by Mattila *et al* (2015) on the induction of *daw* by Mlx, however, our results unveil a novel aspect of this regulation as we show that it might be diet-dependent. Daw is regulated by Mondo-Mlx on rich-protein diet, but not on low-protein diet therefore, we could think that high proteins or amino acids quantity is required for Mondo-Mlx complex to regulate *daw*, as it is the case for sugar.

To conclude, mutations and/or knock-downs in *dawdle*, *mondo* and *mlx* resulted in similar growth phenotype, namely, alteration of the intensity of the growth benefit

conferred by *Lp*^{WJL}. Therefore, *mondo*, *mlx* and *daw* seem to act in the same pathway to regulate *Lp*^{WJL}-mediated growth benefit, whereas proteases regulation is a disconnected phenotype as in our setup, the regulation of digestive enzymes by *daw* is *mlx*-independent. This prompts us to assume that Mondo/Mlx-Daw regulatory cassette reported in Mattila et al. may be a specific transcriptomic response to high sugar diet, and that *daw* is probably is involved in other regulatory loops still to be discovered.

We found a case of growth benefit conferred by *Lp*^{WJL} independent from protease upregulation, which contradicts what we thought to be paradigm in the biology of *Drosophila*- *Lp*^{WJL} interaction during the fly growth. In order to attempt understanding what is going on at the transcriptional level in *daw*-KD animals, we are currently carrying out RNA-sequencing on *daw*-KD and control larvae raised on low-protein diet in GF and upon *Lp*^{WJL} association.

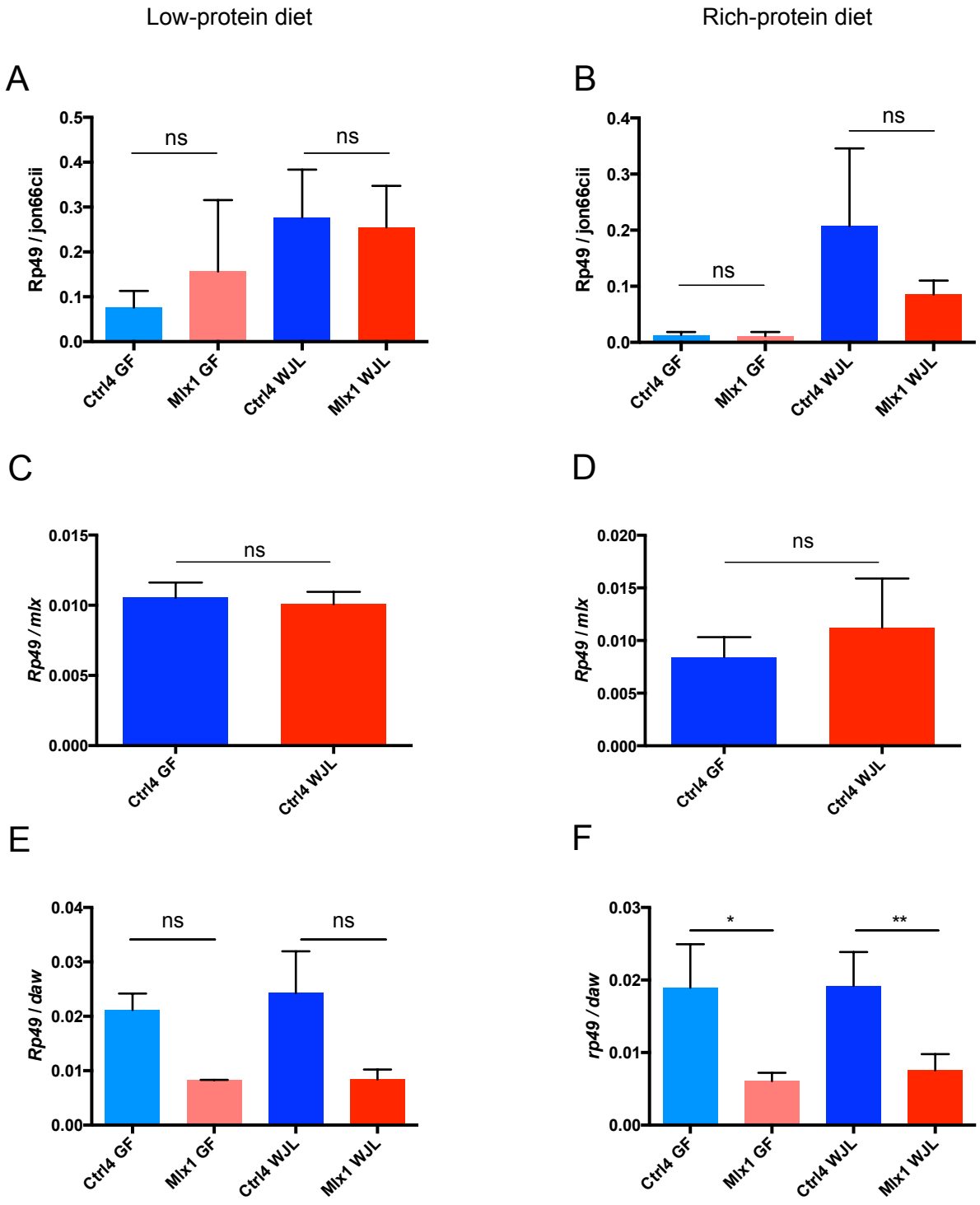

Low-protein diet
Rich-protein diet
A
B
C
D
E
F
Rp49 / jon66cii
Rp49 / mlx
Rp49 / daw
rp49 / daw
ns
*
**
Ctrl4 GF
Mlx1 GF
Ctrl4 WJL
Mlx1 WJL

Figure 9: *mlx* does not regulate proteases expression on low-protein diet

A-B) Bar graphs show mean ± SD of *jon66cii* transcripts levels relative to transcripts levels of the housekeeping gene *rp49* analyzed by RT-qPCR. GF and Lp^{WJL}-monoassociated *mlx* null mutants *(*mlx^{1}) and controls (*Ctrl4*) whole larvae were raised on low-protein (A) or rich-protein (B) diet and collected at day 7 and 4 respectively.

C-D) Bar graphs show mean ± SD of *daw* transcripts levels relative to transcripts levels of the housekeeping gene *rp49* analyzed by RT-qPCR. GF and Lp^{WJL}-monoassociated *mlx* null mutants *(*mlx^{1}) and controls (*Ctrl4*) whole larvae were raised on low-protein (A) or rich-protein (B) diet and collected at day 7 and 4 respectively.

E-F) Bar graphs show mean ± SD of *mlx* transcripts levels relative to transcripts levels of the housekeeping gene *rp49* analyzed by RT-qPCR. GF and Lp^{WJL}-monoassociated in controls (*Ctrl4*) whole larvae were raised on low-protein (A) or rich-protein (B) diet and collected at day 7 and 4 respectively.

Discussion

L. plantarum^{WJL} promotes *Drosophila* growth upon protein deficiency (Storelli et al., 2011) and this growth enhancement relies on the upregulation of host proteases (Erkosar et al., 2015) resulting in increased amino acids (AA) uptake from the fly diet. The increase of AA uptake, in turn enhances the host TOR (Target Of Rapamycin) nutrient-sensing pathway leading to an increase of insulin and ecdysone production. In order to further our understanding of the host genetic basis of Drosophila- Lp^{WJL} interaction during fly growth, we investigated the role of *daw* as part of the molecular dialog between Lp^{WJL} and the fly during juvenile growth, since a synonymous variant was discovered in the GWAS analysis of the DGRP collection.

We showed first that ubiquitous *daw*-KD results in an altered growth benefit mediated by Lp^{WJL} on a low-protein diet. Larval growth in general, with or without Lp^{WJL}, was stalled with ubiquitous *daw*-KD. This result resonates with the previous reports depicting the pleiotropic function of Daw in development, neuronal and organ patterning and metabolic adaptation. In the attempt to identify the specific tissue that produces Daw responsible for the growth phenotype, we found that fat body-specific *daw*-KD results in enhanced growth only in monoassociated larvae. Therefore, the fat body (FB) is one of the important tissues for this phenotype. However, the systemic growth defect was not observed upon FB-specific KD. This prompts us to think that another tissue would be an important source of Daw. When we tried to knockdown *daw* in the muscles, very high larval lethality was observed. Therefore, in the future we should reexamine the role of Daw in muscles by controlling the GAL4 strength through temperature shifts to overcome lethality. To do so, we should first build a muscle-GAL4 coupled to a temperature sensitive GAL80 in order to permit the modulation of the strength of the KD.

Next we investigated the influence of *daw* on the expression of digestive enzymes. Previous work in our lab indicates that a specific set of digestive enzymes is transcriptionally regulated by Lp^{WJL} association during chronic undernutrition. In this study we found that *daw* also controls the expression levels of these digestive proteases in both GF and mono-associated context and in both standard and poor nutritional

environments. However, the novelty of our results are several. First, we discovered that *daw* expression itself responds to the bacteria presence as it peaks and ebbs through the developmental time course (Figure 2A). Secondly, depending on the microbial context (GF or Lp^{WJL}-monoassociated), Dawdle asserts opposite regulatory functions on specific proteases. Furthermore, the studies of Dawdle's role in metabolic adaptation were mostly based on a sugar over-feeding regimen, and focused on the regulation by Daw on various amylases, maltases and lipases (Chng et al., 2014). Our study is the first to uncover that *daw* also controls the expression of proteases on a diet that barely contains simple sugars. Therefore the role of Dawdle in regulating digestive and metabolic enzymes is more complex and broader than expected, and we show also that this is microbe-dependent regulation. In the future, we should consider analyzing Dawdle and proteases protein quantity to verify whether the change of transcription affects the protein level. Indeed, transcription and translation levels are not always regulated accordingly for a same gene (Frochaux et al., unpublished data). Proteolytic activity should also be measured in dissected guts from WT and *daw*-KD animals, in presence or absence of Lp^{WJL}.

To verify the RNAi phenotype on *daw*-dependent proteases regulation, we tested the expression levels of the same proteases in *dawdle* mutants. During early development, the effect in *daw* mutants confirmed *daw*-KD phenotype: *jon66cii* was upregulated in GF *daw*-KD larvae whereas knockdown had no effect on *jon66cii* expression on monoassociated larvae. However at later time points, *daw* animals showed no difference with controls for the expression of digestive enzymes. One possible explanation to the discrepancy observed between the mutants and the knockdown is the possibility of a compensation mechanism set up to buffer the deleterious effect of the mutation. This type of mechanisms has been demonstrated in several models: zebrafish (Rossi et al., 2015), mouse (De Souza et al., 2006) and plants (Majláth et al., 2011). These studies reveal phenotypic differences between gene knockdown and knockout/mutations. For example, Rossi et al; compared *egfl7* (an endothelial extracellular matrix gene) knockdown with morpholino strategy, a technology similar to RNAi, to *egfl7* mutants in Zebrafish. *egfl7* knockdown resulted in severe vascular defects whereas *egfl7* mutants exhibited no obvious phenotypes (Rossi et al., 2015). They ruled out residual protein function in the mutants and effect of off-targets in the knockdown, and found a set of

proteins and genes upregulated in the mutants but not in the knocked-down animals indicating the presence of a compensatory network to buffer the deleterious effects of *egfl7* mutation. Therefore, this hypothesis should be tested in our set up. We should investigate the presence of such compensatory mechanisms, which could be done by comparing transcriptomes and proteomes of *daw*-KD and *daw* mutants.

Mattila et al. (Mattila et al., 2015) showed that the Mondo/Mlx transcription complex regulates *daw* expression on a high-sugar diet, and together with Dawdle they activate the downstream target Sugarbabe. We showed that on a low protein diet, with or without bacteria, the regulation of proteases is Mlx-independent. This leads us to think that the regulatory loop discovered by Mattila et al. might be specific to high sugar feeding, and that the proteases regulation by *daw* in our experimental set up is still to be unveiled. The next logical step is to investigate what is upstream of Dawdle in the context of proteases regulation on low-protein diet. In the future large-scale screens can be employed to discover the upstream regulation of *daw*. Candidate approaches should also be considered. One study demonstrated that the forkhead transcription factor dFOXO, a downstream effector of insulin signaling, represses *daw* in genetically engineered long-lived flies (Bai et al., 2013). Albeit this is a very different experimental context, it would be interesting to assess whether in our system dFOXO regulates *daw* transcription. Another possible gene of interest is GATAe, a Zinc-finger transcription factor which might be involved in the microbiota-dependent digestive functions (Erkosar et al., 2014). In a transcriptomic analysis of adult flies polyassociated with a cocktail of 5 commensal bacteria, Erkosar et al. reported that one third of the genes whose expression was increased by the association with were GATAe-dependent. Daw is not a known target of GATAe, but it is interesting to see if GATAe can co-regulate protease expression with Daw, and hence functionally act like Mondo/Mlx in our specific nutritional and microbial context.

Furthermore, we found that *sugarbabe* mutants exhibited no growth alteration. Therefore, *dawdle* effect on larval growth seems to be *sugarbabe*-independent. However, we have not tested the involvement of *sugarbabe* in digestive enzyme regulation and this should be assessed in the future. In mlx^{1} mutant, the larval growth phenotype related to Lp^{WJL} resembles *daw*-KD, yet the protease expression was unaffected. Therefore there is phenotypic uncoupling between growth regulation and protease

expression. Similar findings may be expected for *sugarbabe*. To answer that question, we should measure the expression level of proteases in *sugarbabe* mutant larvae.

Erkosar et al showed that the growth promotion conferred by Lp^{WJL} upon protein deficiency partly relies on the upregulation of a set of digestive proteases (Erkosar et al., 2015). We report that Lp^{WJL} enhances growth of *daw*-KD larvae while these proteases failed to be up-regulated. Moreover, we showed proteases are also upregulated on a normal protein rich diet, where the GF larvae grow as fast as mono-associated larvae (personal laboratory communication). Our results therefore suggest that the role of proteases upregulation in Lp^{WJL} growth benefit might not be as essential as previously demonstrated, and their role should be reexamined to verify whether the upregulation really is necessary for Lp^{WJL}-mediated growth, or if it is a mere readout of the monoassociation. Even though Lp^{WJL} seems dispensable for growth and maturation on a normal and protein rich diet, so far we cannot rule out that such association still confers fitness advantages at a molecular and physiological scale. Further analyses on animals raised on high-protein diet should be performed. For example, we should analyze the metabolic states of theses animals in presence and absence of Lp^{WJL}.

We found a case of growth benefit conferred by Lp^{WJL} independent from protease upregulation, which contradicts what we thought to be paradigm in the biology of Drosophila- Lp^{WJL} interaction during the fly growth. In order to attempt understanding what is going on at the transcriptional level in *daw*-KD animals, we have carried out RNA-sequencing of *daw*-KD and control larvae. RNA sequencing has been performed on size-matched larvae ("size 2") raised on low-protein diets in presence and absence of Lp^{WJL}. The analysis of the transcriptome is on going.

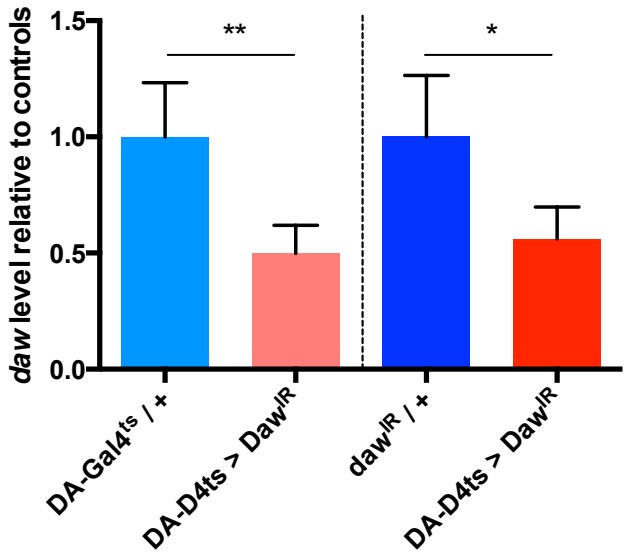


Figure S1: Control of the efficiency of *daw* knockdown

Bar graphs show the relative fold change of *dawdle* expression level of daw^{IR} line relative to the control lines raised on standard laboratory diet. Transcripts levels were normalized by quantifying the transcriptional level of the housekeeping gene *rp49*. Asterisks represent the statistical difference of the daw^{IR} line with the control line using Mann and Whitney tests *p < 0.05, **p < 0.01.

Sample	Size (mm)
« Size 1 »	1.2 - 1.3
« Size 2 »	2.2 - 2.5
« Size 3 »	3.8 - 4

Table S1: size range of the larvae sampled for the qPCR

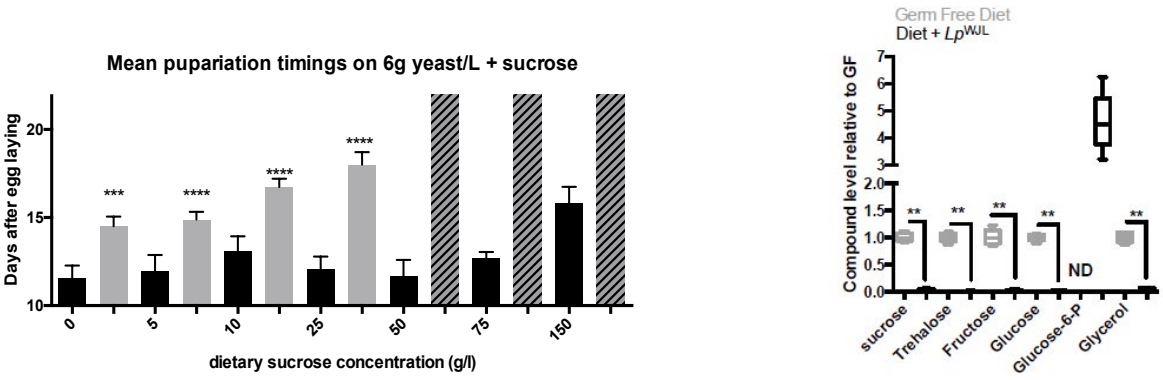


Figure S2 : *L. plantarum* protects *Drosophila* larvae from sugar toxicity by depleting sugars from the diet

A) Bar graphs show the mean time of pupae emergence expressed in days after egg-laying (AEL) of GF (grey bars) or Lp^{WJL}-monoassociated (black bars) larvae raised on low-protein diet supplemented with different quantity of sucrose

B) Box plots show the level of metabolites present in the fly diet inoculated for 3 days with only PBS (grey boxes) or Lp^{WJL} (black boxes). n = 5 samples per condition. Metabolites not detected in one condition (sample falling below compound detection) are marked ND (not detected). Asterisks illustrate statistical significance between conditions: **p < 0.01, ***p < 0.001, ****p < 0.0001.

These graphs were extracted from Storelli et al. unpublished data.

Material and methods

Fly stocks and husbandry

We ordered the DGRP lines from Bloomington Drosophila Stock Center.

y,w flies were used as a reference strain.

Drosophila were reared at 25°C on a light-dark cycle (12h light, 12h dark) on a rich-protein diet consisting of 50g/L of inactivated yeast (Bio Springer, Springaline BA95/0-PW), 80g/L of cornmeal (Westhove, Farigel maize H1), 10g/L of agar (VWR, ref #20768.361), 5,2g/L of methylparaben sodium salt (Merck, ref #106756) and 4mL/L of 99% propionic acid (CARLO ERBA, ref #409553), and a mix of antibiotics (50 µg/mL ampicillin, 50 µg/mL kanamycin, 15 µg/mL erythromycin and 50 µg/mL tetracyclin).

Generation of axenic fly stocks

Embryos from CR flies were collected overnight on fruit juice-agar medium supplemented with fresh yeast paste. Under sterile conditions, embryos were then successively soaked for 2 minutes in bleach (to dechorionate the embryos), 70% ethanol and sterile water. These newly sterile embryos were then deposited on sterile rich-protein fly food supplemented with antibiotics and raised at 25°C. Once GF flies emerge, they were amplified on sterile rich-protein food added with antibiotics. The absence of contamination was confirmed by plating 100 µl of lysate of 5 adults or 3rd-instar larvae on both MRS and LB plates and incubated at 37°C for 48h followed by 7 days at RT.

Bacterial association

L. plantarum^{WJL} was cultured overnight in Man, Rogosa and Sharpe (MRS) liquid medium (Difco, #288110). OD was measured at 600 nm, the culture was diluted in 1X PBS at OD=0,5. 333 µl of this diluted bacterial solution was poured homogenously on the embryos and medium.

For each mono-association experiment, *Lp*^{WJL} (Ryu et al., 2008) was grown in Man, Rogosa and Sharpe (MRS) medium (Difco, ref. #288110) over-night at 37°C, and diluted to O.D.=0.5 the next morning to inoculate 40 freshly laid eggs on each 55mm petri dish

or standard 28mm tubes containing fly food of low yeast content. The inoculum corresponds to about $7x10^7$ CFUs. Equal volume of sterile PBS was spread on germ-free eggs for control.

Larval length measurements

Axenic flies were put in an egg laying chambers overnight to lay eggs on sterile poor-protein diet. Axenic embryos were collected the next morning, and pools of 40 embryos were transferred to new dishes of poor-protein food. The pools of embryos were then inoculated under sterile conditions with either 1x PBS or $1x10^7$ CFUs from an overnight culture of *L. plantarum*. At minimum, triplicates were done for each condition. The caps containing the inoculated embryos were incubated at 25°C. 7 days after inoculation, larvae were collected from the caps and heat-killed on a heater plate for 5 seconds in order to make them straighten and mounted between slide and coverslip in 80% glycerol diluted in 1x PBS. Pictures were taken using a leica M205 FA Stereomicrosope, magnification 7,8 with the software LAS. Length was measured with Image J.

RNA extraction and RT-qPCR

GF and Lp-monoassociated larvae were collected either size or age matched. 15 larvae were picked and transferred into a crushing tube containing 0,75-1 mm of glass micro-beads, all liquid was removed and the tube was snap-frozen in liquid nitrogen. Larvae were lysed in the RA1 buffer using a Precellys24 Tissue homogenizer (Bertin instruments). RNA was isolated using the Nucleospin RNA extraction kit (Macherey Nagel. RNA was quantified on Nanodrop2000. Reverse transcription was performed using the SuperScript II RT Kit (Invitrogen) and random primers (Invitrogen) using 500 ng of RNA per sample. Quantitative PCR was performed on a Biorad CFX96 apparatus (Biorad) using SYBR GreenER qPCR Supermix (Invitrogen), cDNA (dilution of the reverse transcription products) and gene specific primer sets (available upon request). Melting curves of the detected amplicons were analysed to ensure specific and unique amplification. PCR efficiency was calculated using serial dilution of cDNA. We used the DDCt method for data analysis and *rp49* was the reference gene. Results were expressed as a relative value of DCtrp49/DCtgene ratios (fold induction). A total of 3 to 5 biological

replicates were used for all experiments in order to ensure representativity and the statistical significance.

Fly strains

daw^{aac1} is a null mutant line obtained by P-element excision (Gesualdi & Haerry, 2007). The mutation deletes 2.5 kb including the second promoter and most of the coding sequence.

daw^{ext11}: was obtained by imprecise excision of a transposable element (Strain #13221, Japan) inserted in the first intron of the *daw* gene, 2.5 kb downstream of the transcription start of the Alp23B-RA (Flybase) (Serpe & O'Connor, 2006). It lacks 1.8 kb of the *daw* gene sequence, including the start codon.

mlx^{1} mutant line was made by Havula et al 2013 by imprecise P-element (P(XP)*bigmax*^{d07258}) excision (Havula et al., 2013). *mlx*^{1} lacks the entire coding region of mlx ad well as 17 C-terminal amino acids of the neighboring gene *CG3368*. The control line (Ctrl4) is the line from which the P-element had been excised precisely, leaving *mlx* intact.

Sug$^{17\Delta}$ line was established by deletion of parts of the coding region as well as the promoter of the *sug*-RA isoform using CRISPR/Cas9 (Mattila et al., 2015). *sug*^{def} is a deficiency line obtained by FLP-induced recombination (Df(2R)Exel7123).

Statistical Analysis

GraphPad Prism software version 6.0f for Macintosh (GraphPad Software, La Jolla California USA,) was used to make the statistical analyses.

A dedicated R script has been used to compare the growth of "Top" and "Low" lines in each microbial context (GF or LpWJL). For each of these two conditions, two cubic linear mixed effect models (package *nlme*) have been fitted to the larval length measurement over 10 days. One model contained the Top-Low condition as factor and the other not. These two models were then compared with an anova (package *stats*).

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General conclusion and perspectives

General conclusion and perspectives

Animals are colonized by a wide variety of microorganisms collectively referred to as microbiota. The microbial communities and the host construct complex reciprocal interactions affecting their physiology and lifestyle (Clemente et al., 2012; McFall-Ngai et al., 2013). Symbiotic bacteria can modulate virtually all aspects of their host physiology such as immune homeostasis (Pickard et al., 2017), postembryonic development (Montgomery and McFall-Ngai, 1994), and behavior (Sharon et al., 2010). In return for nutritional benefit, the host provides bacteria with shelter and food supply. Nutrition and metabolism, and consequently, growth are key facets of host physiology shaped by microbiota. In the past years, a wealth of studies has demonstrated how symbiotic bacteria affect nutrition, but the underlying host mechanisms are still largely unknown. To further our understanding of the mechanisms of interaction between host and microbiota in the context of nutrition and growth, we used a simple model: *Drosophila melanogaster* monoassociated with one of its natural commensal: *Lactobacillus plantarum*. Drosophila microbiota supports many aspects of fly physiology such as lifespan (Brummel et al., 2004), mating preferences (Sharon et al., 2010), immunity (Teixeira et al., 2008), social behavior (Venu et al., 2014), and post-embryonic development (Shin et al., 2011; Storelli et al., 2011). Our research group focuses on this latest trait, growth. *Lactobacillus plantarum* has been shown to promote the growth of Drosophila larvae upon nutritional challenge: when flies are raised on low-protein diet, they experience developmental delay and slowed growth rate. These defects are rescued by the monoassociation with one single bacterial species: *L. plantarum*^{WJL} (*Lp*^{WJL}) in a strain-specific manner. This monoassociation recapitulates the effect of the entire intestinal microbiota (Storelli et al., 2011). This growth enhancement relies partly on the upregulation of host proteases (Erkosar et al., 2015) resulting in increased amino acids (AA) uptake from the fly diet. The increase of AA uptake, in turn enhances the host TOR (Target Of Rapamycin) nutrient-sensing pathway leading to an increase of insulin and ecdysone production. The scope of this thesis was to further investigate the host

mechanisms involved in the interaction between Drosophila and *L. plantarum*^{WJL} during larval growth upon nutritional challenge.

In the first chapter of this thesis, we addressed the impact of genomic variations on the growth benefit conferred by *Lp*^{WJL}. We showed that growth related to microbial effect is a multigenic and quantitative trait, *Lp*^{WJL} benefits growth regardless the fly genetic background, but genomic variations such as single nucleotide polymorphism (SNPs), impinges upon the efficiency of the growth promotion. This shows that microbiota and host functions are tightly entangled.
Furthermore, in the course of this study, we unveiled a novel function of microbiota to act as a genetic buffer, reducing the deleterious consequences of fly genomic variation on the phenotypic outcome of different fly fitness traits during growth. Besides, *Lp*^{WJL} also confers developmental robustness in organ patterning. When the host faces a nutritional challenge, the gut microbiota accelerates growth and provides phenotypic stability and homogeneity. The masking of genotypic variability suggests a pivotal evolutionary role played by the microbiota. *L. plantarum* is among the main commensal of the fly (Chandler et al., 2011), however, it does not reside in the fly gut but is constantly re-associated through larval feeding (Storelli et al., *in press*). Despite not being stably associated to the fly, *Lp*^{WJL} drives genetic buffering, a general and broad mechanism. Future studies should systematically assess if such buffering is a general function of symbionts and identify the host genetic variants that are buffered by the gut microbiome.

In the second part of the thesis, we studied Dawdle, a TGF-β ligand that participates in glucose repression by dampening the expression of various digestive enzymes (mostly, amylases and maltases) in the midgut when adult flies are fed nutritious sugars. Here we examined the role of *dawdle* in the Drosophila-*L. plantarum*^{WJL} interaction during growth upon protein scarcity. We found that the regulatory loop involving Mondo-Mlx and Dawdle seems to also play a role in the interaction. Moreover, we revealed that Dawdle plays an important regulatory role on digestive enzymes in the protein-deficient nutritional context, and that this regulation can be inhibitory or activating depending on the microbial context. Therefore, the novelty of our results lies in the discovery that the

microbial environment dictates the regulatory mode of Dawdle on these digestive enzymes.

So far, we thought that protease upregulation was a prerequisite of the growth promotion. However, we reported a case where Lp^{WJL} promotes growth without enhancing the host digestive proteases. Our results demonstrate that the mechanisms behind the growth benefit are more complex than bacteria enhancing proteases, increasing AA availability and thereby providing more energy to the fly. Therefore, what we thought explained most of Lp^{WJL} benefits is probably just the tip of the iceberg. In the future, we will investigate the other mechanisms intervening in the growth promotion conferred by Lp^{WJL}.

Altogether, our results demonstrates that simple models as ours, Drosophila monoassociated with *L. plantarum*^{WJL}, still have their parts to play in modern biology despite all the recent technologies advances made during the last decade. Our mono-association model can be further exploited to understand the very fundamental concepts such as genetic buffering and developmental canalization. We show that simple phenotypes such as bacteria sustaining host nutrition may have very complex mechanisms and regulation. Our study therefore strengthens the idea that bacteria and animals have co-evolved side by side and as a consequence, bacteria have developed mechanisms to robustly benefit the host and contribute to its ecological success. Such interaction also benefits the bacteria as the host provides them home, food and a safe shelter to replicate. Besides our results put forward that the effect of microbiota adds a layer of complexity in regulatory mechanism, as demonstrated with our study of dawdle-dependent regulation of digestive enzymes. Therefore to have a complete understanding of biological systems, the implication of microbiota should be investigated more systematically.

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Annexes

Commensal bacteria act as a broad genetic buffer that maintains phenotypic homogeneity and developmental robustness in *Drosophila*

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Abstract

Biological robustness is a recurrent theme in the grand scheme of evolution. To preserve genetic diversity, different buffering mechanisms have evolved to maintain constant and invariant phenotypic outcome despite environmental stress. However, when stress overwhelms the buffering mechanism, the previously unexpressed genetic variants manifest as visible phenotypes that can be exploited by natural selection. In the process of identifying host genetic variants interacting with the gut microbiota to reap growth benefit under nutritional stress, we unearthed a function of a *Drosophila* gut commensal bacteria as a novel buffering engine that reduces variation in different host fitness traits during development and that confers robustness in organ patterning. Our findings therefore suggest that the gut microbiota asserts an evolutionary impact on the host, and by masking the effect of cryptic genetic variants, such buffering by the microbiota may compound the explanation of "missing heritability" in many contemporary association studies.

The diversity of life on Earth is a testament of the evolutionary triumph of microbial symbioses, which encompasses a broad spectrum of complex interactions between the hosts and their resident microbes, ranging from debilitating parasitism to obligate mutualism [1-4]. Among these interactions, certain form of mutualism are considered as a major driving force of evolution[5]. For instance, some insect obligate endosymbionts can manipulate the host's reproductive outcome, and ultimately alter its evolutionary trajectory [6,7]. On the other hand, the gut microbiota frequently engages their host in nutritional mutualism that optimizes its various physiological parameters during its lifetime; so far only a few studies have demonstrated the evolutionary implications of the host's association with the gut microbiota [8]. The dearth of such studies is partly attributed to the volatile nature of nutritional mutualism, since the complex interactions between host factors and environmental inputs can often render the association persistent or transient [9,10].

The horizontally acquired gut bacteria in *Drosophila* perfectly represent such nutritional mutualists. They can help the hosts adapt to different nutritional environment and consequently improve their fitness [11]. Specifically, we and others have found that axenic, or "germ-free" (GF) larvae grow at similar rate on a standard laboratory diet as their conventionally raised, or gnotobiotic siblings associated with one or multiple gut bacteria strains. However, when fed on a food substrate containing either low yeast or inadequate amino acid content, GF larvae manifest significant growth delay or high lethality[12,13]. Interestingly, such growth defects are rescued in gnotobiotic flies that are mono-associated with selected strains of gut commensals. One such commensal strain is *Lactobacillus planturum*^{WJL} (Lp^{WJL}), which has been shown to enhance gut protease expression, amino-acid assimilation and to promote growth in a chronically stressful nutritional context[14].

To study the host genetic contribution to Lp^{WJL}-mediated growth during chronic undernutrition, we initiated a genome-wide association study (GWAS) using the Drosophila Genetic Reference Panel (DGRP) for variant discovery. We rendered 53 DGRP lines axenic and mono-associated them individually with Lp^{WJL} during embryogenesis and compared larval growth on day 7 after egg lay (AEL). First, we found that in the 53 DGRP lines, the derived heritability estimate (H) for GF larval growth is

37%, while H of the Lp^{WJL} -associated population is only 10% (Figure 1A, supplementary table 1). This indicates that genetic variants induce more pronounced developmental variation in the absence of the gut bacteria. Moreover, the coefficient of variation (CV) of larval length was greater in the GF population despite their overall smaller average size (Figure 1B). Secondly, based on the ranking of the larval growth with or without Lp^{WJL} (Fig.1A, Table S1), the GWAS yielded nine variants in genes with unknown function or associated with different biological processes such as immunity, chemosensory response, hormonal signalling and cellular signal transduction, etc. (Table 1, Figure S1 A and B). In the process of studying the candidate genes' contribution to Lp^{WJL} -mediated growth through RNA interference (RNAi), we observed that knocking down different candidates led to large variability in germ-free larval growth, ranging from full lethality to subtle growth delay; but such variability is diminished when Lp^{WJL} is present in the niche, resulting in no obvious " loss of function" of the growth benefit conferred by Lp^{WJL} in any particular RNAi cross (Figure S1 C and D). Together, these results strongly suggest that during chronic nutritional stress, the gut bacteria assert a robust buffering effect that restricts the variation in larval body size, independent of host genetic background. To better illustrate the "buffering effect" on larval growth, we plotted the average GF larval length of each DGRP line against its Lp^{WJL} -associated counterpart, and calculated the linear regression coefficient. If genetic background instead of buffering predominantly impacts growth, then this coefficient should be close to 1. We however observed that the derived coefficients for both the DGRP and the RNAi validation studies are effectively close to zero (0,15 and 0,14 respectively; Figure 1C and D). This strongly suggests that the presence of Lp^{WJL} buffers the underlying genetic variation, steering the animals to attain a similar growth target independent of genotype. These observations prompted us to hypothesize that in the presence of nutritional stress, the gut microbiota, represented by Lp^{WJL}, robustly confers phenotypic homogeneity to growth traits in a genetically diverse population; conversely, the effects of genetic variation are "unmasked" when the flies are devoid of their gut microbiota.

Different genetic buffering mechanisms have evolved to maintain phenotypic stability when organisms encounter various genetic and/or environmental perturbations[15-17]. Two classic examples of such buffering mechanisms have been extensively studied: a heat-shock protein chaperone, Hsp90, and certain microRNAs[18,19]. We wondered if the

gut bacteria also qualify as such a buffer. In the 53 DGRP lines, the reduced variation in larval size is apparent in the mono-associated population, and monoassociation reduces variability in the expression of specific set of genes in different biological pathways (need to phrase this better). However, such reduced variability was observed in inbred, homozygous strains .We wished to confirm that such buffering indeed operates in a population where genetic variants can act. Therefore, based on the GF growth profile, we selected four DGRP strains: two from each end of the phenotypic extremes (Figure 1A, green label), and set up diallelic crosses in six different ways (Figure S2A). We then collected the GF and mono-associated larvae from the F_2 progeny, measured the larval length in three consecutive days after a defined period of egg laying, and repeated the experiment three times in a five-months interval. In these particular sets of experiments, we opted to supplement the GF flies with 33% more yeast in the media (8g/L vs. 6g/L) for two reasons: first, we wished to exclude that the Lp^{WJL} might act purely as a" food source": if increasing yeast content would also reduce the variability in growth to the same extent as the gut bacteria, then the buffering effect may be mundanely attributed to improved caloric intake instead of live molecular or cellular dialogue between the host and its microbiota. Second, with the low-yeast diet, GF larvae are significantly delayed in growth, such that the mono-associated larvae of the same age are likely to be more mature, and therefore potentially more developmentally constrained to exhibit size variation. Since feeding the GF larvae with more yeast accelerates growth, we can thus first assess if Lp^{WJL} acts purely as food, and simultaneously minimize the size and stage difference between the GF and their mono-associated siblings when comparing overall growth variation.

We found that regardless of when the experiment was conducted, the CV values of the F_2 larvae tend to separate into two distinct groups driven by Lp presence (Figure 2A), When pooled, the F_2 Lp^{WJL} monoassociated larvae were slightly longer on average, but their GF siblings showed greater variability in size (Figure S2B). In addition, we found that the CV values derived from the different crosses decrease with increasing larval size, and that the GF CV values tend to be higher regardless of yeast content or larval age (Figure 2A,). To compare the overall variation between larvae that are matched in size, and therefore developmental stage, we then arbitrarily selected experiments where the averages of the GF and mono-associated larvae fell into a particular size range (between

3-4mm) (Figure 2A, red bracket), pooled the larval measurements, and calculated the size and variation of this group. We found that despite the fact that the GF larvae were fed with more yeast, they still display greater variation (Figure 2B) compared to their size-matched Lp^{WJL} siblings. We conclude that the buffering from Lp^{WJL} cannot be attributed to bacteria acting solely as a pure "food source". Instead, buffering appears to be driven by nutrition-independent biological processes that require commensal bacteria.

During chronic undernutrition, mono-association with Lp^{WJL} sustains optimal growth rate as effectively as the entire gut microbiota. We thus wondered if the observed buffering capability is also an Lp^{WJL}—specific attribute, or a general function of the gut microbiota. To address this question, we rendered a population of wild flies collected in a nearby garden germ-free, and re-associated them with their own fecal microbial community. In three out of four repeats, we found that GF larval growth varies more than that of their size-matched siblings that have ingested fecal microbiota mixed in the food (Figure S2C and data not shown). Furthermore, the average CV values and variances derived from individual food caps were significantly higher in the GF population (Figure S2D and E), strongly suggesting that the gut microbiota derived from the wild flies also asserts a buffering effect in growth in nature. However, since the wild-derived microbiota did not consistently buffer the larval growth, probably due to our inability to precisely control the quantity and composition of the fecal microbiota fed to the flies, we reconsidered the mono-association model for further studies.

Larval growth is a single fitness trait, which may or may not reflect adult performance, especially when fed on a poor yeast diet, more axenic larvae die before reaching adulthood compared to mono-associated ones (data not shown). If such observed increase in variation indeed reflects the organism's attempt to release its genetic potential in response to a stressful environment, then the increased variation is only meaningful when it persists in the surviving population with reproductive potential. We therefore examined the variations in pupariation timing and adult emergence in the F_2 crosses. First, the variances derived from calculating the average days of pupariation and eclosion were significantly higher in the GF larvae (Figure 2C,E). From tube to tube containing an equal number of larvae, the variance of puparation and eclosion derived

from each tube were also greater in the GF samples (Figure 2D,F). Therefore, the buffering effect not only reduces inter-individual variation, but also operates between populations. Lastly, GF adults were slightly shorter than the mono-associated flies (Figure 3A). Moreover, the size of representative organs, expressed as area of the eye and the wing, was also lower, yet there was significantly greater variation in GF body and organ size (Figure 3B and C). These observations prompted us to examine the wing/body-length allometry in Lp^{WJL} and GF flies. We found no difference in the allometric slopes, but the individual GF values are more dispersed along the slope (Figure S3); when taken as a ratio, greater variation in the wing/body-length trait was again observed in the GF flies (Figure 3D).

So far, we have shown that association with Lp^{WJL} reduces the variance of the mean in different fitness traits, indicating that Lp^{WJL} confers phenotypic stability when a host population encounters nutritional stress. In nature, genetic buffering systems have been shown to act in similar ways. For example, inactivating Hsp90 in different organisms increases variation in organ size, such as in *Arabidopsis thaliana* and the Mexican cavefish, *Astyanax mexicanus*, [20-22]. Consistent with our findings, a previous study by Elgart et al. showed that the progeny of axenic flies of different genetic backgrounds express certain mutant phenotypes in a much more exaggerated fashion than their parents [23]. Moreover, compromising the genetic buffering by Hsp90 can lead to morphological aberrations that are normally masked [21]. In line with these previous findings, we came across another unexpected observation while measuring the body and organ sizes of individual flies: a significant fraction of the GF F_2 progeny exhibited aberrant wing patterns such as missing margins, incomplete vein formations and ectopic vein tissue, etc ... (Figure 3E, F). The incidence of wing anomalies in GF adults differed according the genotype and females were more affected than males (Figure S3C). In contrast, the most discernable "defect" in their Lp^{WJL} associated siblings was subtle and barely visible (Figure S3D). Indeed, gross patterning anomalies were absent in the Lp^{WJL} associated F_2 population and in the parental homozygous strains that yield viable adults on the same low-protein diet (data not shown). Organ patterning is a robust process. For example, as a consequence of complex interactions between the fly 's genetic makeup and the fluctuating environmental conditions, changes in nutrition, humidity, temperature and crowding can alter the final fly adult body and wing size; yet the

patterning of the wing is virtually invariant and reproducible[24], unless preexisting "silent" mutations start to manifest themselves as visible phenotypic outputs[21,25]. The aberrant patterning events in the GF flies strongly suggest that Lp^{WJL} 's presence has effectively masked the contribution of genetic variants that impose such wing defects. Thus, in flies devoid of their microbiota, we essentially witnessed a breach of the canalization process during developmental patterning. All these observations have a commonality in that they point to the fact that Lp^{WJL} functionally resembles a genetic canalization engine for the host, which effectively masks the contribution of genetic variants in neutral conditions and provides phenotypic homogeneity and developmental stabilization.

The wing patterning anomalies in the GF F_2 progeny are highly reminiscent of what has been reported in a recent study, in which the blocking of ROS activity through antioxidant feeding led to wing pattern and regeneration defects [26]. We therefore repeated the DGRP F_2 cross experiment but now with the additional condition where we mixed the antioxidant molecule N-acetylcysteine (NAC) with the low-protein diet and fed it to the monoassociated flies. Interestingly, the buffering capacity of Lp^{WJL} was substantially diminished. Specifically, variation in larval size (Figure 4A), developmental timing (Figure 4B, Figure S4A) and adult emergence (Figure 4C, S4B) was increased in NAC fed larvae monoassociated with Lp^{WJL} to a level similar to or even higher than that in the GF larvae. Furthermore, we again observed wing patterning anomalies in NAC-fed, monoassociated flies (Figure 4D). Importantly, NAC feeding did not compromise Lp^{WJL} growth in the niche where larvae are present (Figure 4E). Our results therefore indicate that blocking ROS activity suppresses the genetic buffering effect mediated by the bacteria. ROS signaling constitutes an ancient signaling circuitry that has pleotropic functions in regulating stress response, regeneration and metabolism and our results suggest that ROS activity is a cornerstone of the molecular program that underlies genetic buffering. Interestingly, gut microbiome function is intimately linked to ROS metabolism: acute Lp feeding stimulates the dNox-dependent production of reactive oxygen species (ROS) in the larval enterocytes, and subsequently increases the gene expression in Nfr2-mediated cytoprotection program [27,28]. Future studies therefore demand further exploration into how ROS metabolism can be integrated into the

molecular dialogue between the host and its enteric microbiome to maintain robustness in developmental programs in different environments.

With the challenge of constant nutritional stress, the gut microbiota accelerates growth and provides phenotypic stability simultaneously. Having a group of developmentally homogeneous organisms probably reflects an adaptive advantage to a particular nutritional stress, in that it is desirable to retain more surviving individuals of similar growth trajectory in that population. Our mono-association model facilitates strict control over the environmental variables and the microbial input, and in doing so we unveiled the gut microbiota is a broad genetic buffer, which may be a universal feature of beneficial microbes. In the future, it will be interesting to analyze how interactions of different microbes can compromise or enhance such buffering effect in response to the environment. Lastly, our study indicates that the presence of gut microbiota can safeguard the multicellular host's genetic potential, and probably contributes to solving the long-standing enigma of incomplete penetrance and expressivity in classical genetics and may account partly for the "missing heritability" problem in genome-wide association studies.

Material and Methods

- **Fly stocks and genetic crosses**

  Drosophila were kept at 25°C in a Panasonic Mir425 incubator with 12/12 hrs dark/light cycles. Routine stocks were kept on standard laboratory diet (see below « media preparation and NAC treatment ») The 53 DGRP lines were obtained from Bloomington Drosophila Stock Center.

  Field-collected flies were trapped with rotten tomatoes in a garden in Solaize (France) and reared on a medium without chemical preservatives to minimize the modification to their gut microbiota. One liter of media contains 15g inactivated yeast, 25g sucrose (Sigma Aldrich, ref. #84100), 80g cornmeal and 10g agar.

To generate DGRP F_2s, four DGRP lines were selected for setting up diallele crosses: 25210 (RAL-859), 25183(RAL-335) were the "large" larvae as germ-free, and 25208(RAL-820) and 28147(RAL-158) were the "small" larvae as germ-free (see figure legend Figure S2A).

All RNAi lines were crossed to y,w; tubGAL80^{ts}, DA>GAL4. To minimize lethality, we dampend the GAL4 strength by leaving the genetic crosses at 25°C. The following fly strains were used: *y,w*, UAS-dpr-6-IR(P{KK112634}VIE-260B*)*, UAS-CG13492-IR, (w^{1118};P{GD14825}v29390)*,* UAS-daw-IR(NIG #16987R-1), UAS-sfl-IR (*w^{1118}*; P{GD2336}v5070), UAS-arr-IR (*w^{1118}*; P{GD2617}v4818), UAS-rg-IR(*w^{1118}*; P{GD8235}v17407), UAS-bol-IR(*w^{1118}*; P{GD10525}v21536), UAS-glut1-IR(*y^{1} v^{1}*; P{TRiP.JF03060}attP2, Bloomington 28645), UAS-CG32683(P{KK112515}VIE-260B), UAS-CG42669-IR*(w^{1118}*;P{GD7292}v18081),UAS-Eip75B(*w^{1118}*; P{GD1434}v44851), UAS-mCherry-IR (*y^{1} v^{1}*; P{CaryP}attP2), VDRC GD control (VDRC ID60000).

- **The making and maintenance of germ-free flies**

Axenic flies were generated by dechorinating embryos with 50% household bleach for five minutes; eggs were then washed in successive 70% ethanol and sterile distilled water for three minutes each. After washing, eggs were transferred to tubes containing standard diet and a cocktail of antibiotics containing 50µg/mL ampicillin, 50µg/mL kanamycin, 15µg/mL erythromycin, 50µg/mL tetracyclin. Axeny was routinely verified by plating larvae and adult lysates on LB and MRS plates

- **Media preparation and NAC treatment**

Standard laboratory fly food consists of 50g/L inactivated yeast (Springline™), 80g/L cornmeal, 7.14g agar, 5.12g/L Moldex (Sigma M-50109) and 0.4% propionic acid. Where applicable, experiments comparing variations in larval size, developmental timing, adult emergence were performed on diet with 6g or 8g inactivated yeast per liter of media

while keeping the same concentrations of other ingredients. Where approporiate, 1.7g of N-Acetylcystein (SigmaA7250-25g) was added to the low-protein diet.

- **Larval Length Measurement**

All live *Drosophila* larvae were collected from each nutritive cap containing low yeast diet by temporary immersion in sterile PBS, killed with a short heat treatment (5 sec at 90°C), transferred on a microscopy slide, mounted 80% glycerol/PBS. The images were taken with Leica stereomicroscope M205FA and individual larvae was measured using ImageJ software [29]. For each DGRP strain and each cross and/or condition, at least three biological replicates were generated.

- **Developmental timing and Adult emergence**

Developmental timing of and adult emergence individuals were quantified by counting the number of individual appearing every 24 hours until the last individual pupa/adult emerges. Each individual animal is assigned to the number that corresponds to the day it appeared, and the population mean and variance were calculated based on the cumulative numbers.

- **Adult measurements**

2-3 days old adult flies were anesthetized with CO_2 and immersed in 70% ethanol for individual body and its corresponding organ (wing and eye) was imaged under a Leica M205 stereomicroscope. Specifically, the adult body length was measured from the top of the head to the tip of the abdomen. The eye area was measured by manually tracing the circumference of both eyes. The wings were gently nipped at the base of the hinge and imaged, and the area was measured tracing the edge of the wing. All images were taken measured using ImageJ software [29].

- **Bacteria culture and mono-association**

For each mono-association experiment, Lp^{WJL} (Ryu et al., 2008) was grown in Man, Rogosa and Sharpe (MRS) medium (Difco, ref. #288110) over-night at 37°C, and diluted to O.D.=0.5 the next morning to inoculate 40 freshly laid eggs on each 55mm petri dish or standard 28mm tubes containing fly food of low yeast content. The inoculum corresponds to about $7x10^7$ CFUs. Equal volume of sterile PBS was spread on germ-free eggs for control.

To contaminate the garden-collected flies with their own microbiota, eggs were dechorionated and directly seeded on these food caps. Sterile PBS was used to wash the side of the bottles where the adult wild flies were raised to recover more fecal content, and 300 ul of the wash was inoculated to the dechorionated eggs. For GF control, 300 ul of sterile PBS was used to inoculate the dechorionated eggs.

- **Bacteria niche load**

Five to six 24 hour old germ-free larvae were collected from the low-protein diet food cap and transferred to a microtube containing 400ul of low-protein diet, and inoculated with 50ul of Lp^{WJL} of 0.5 O.D.. On the day of harvest, ~0,75-1mm glass microbeads and 900 μl PBS were added to each microtube and the entire content of the tube was homogenized with the Precellys 24 tissue homogenizer (Bertin Technologies). Lysates dilutions (in PBS) are plated on MRS agar with Easyspiral automatic plater (Intersciences). The MRS agar plates were incubated for 24h at 37°C. The CFU/ml count was calculated based on the readings by the automatic colony counter Scan1200 (Intersciences)

- GWAS (EPFL)

- **Statistical Analysis**

GraphPad Prism software version 6.0f for Macintosh (GraphPad Software, La Jolla California USA, www.graphpad.com) was used to compare GF and *L.p.*^{WJL}-associated conditions for larval length, developmental timing, adult emergence, allometry and linear regression analysis for the buffering effect. For small samples with less than 10 data points, nonparametric analysis was conducted. R-studio was used to conduct Levene's test and multivariate analyses. For all experiments, the p-values were reported on the corresponding figure panels only when inferior to 0.05.

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Figures

Table 1. Variants associated with the growth benefits conferred by *Lactobacillus plantarum* (Lp^{WJL})

Variants	R^2	P-value	Molecular and cellular functions
CG13492	46.46%	1.23e-06	Unknown
CG32683	39,04%	2.76e-06	Unknown
CG33269	35.58%	8.21e-06	Unknown
dpr6	33.06%	2,94e-05	Immunoglobulin subtype 2, chemosensory perception
Eip75B	32.65%	1.22e-05	Nuclear hormone receptor, ecdysone response, antimicrobial humoral response
rg	32.14%	9.25e-06	PKA-binding, cone cell differentiation, mushroom body development, olfactory learning
sfl	27.37%	9.18e-06	heparan sulfate proteoglycans (HSPGs) biosynthesis/wg morphogen diffusion
CR42669	26.66%	1.23e-05	Unknown
bol	25,07%	3,76E-06	RNA binding protein. Role in spermatogenesis
CR43427	23,70%	4,53E-06	Unknown, lncRNA
daw	15,10%	4.45E-06	TGF-β ligand: growth; regulation of insulin secretion
arr	14,68%	1,69E-06	wnt protein binding/canonical wnt pathway
glut1	11,14%	1,56E-06	General glucose/sugar transporter

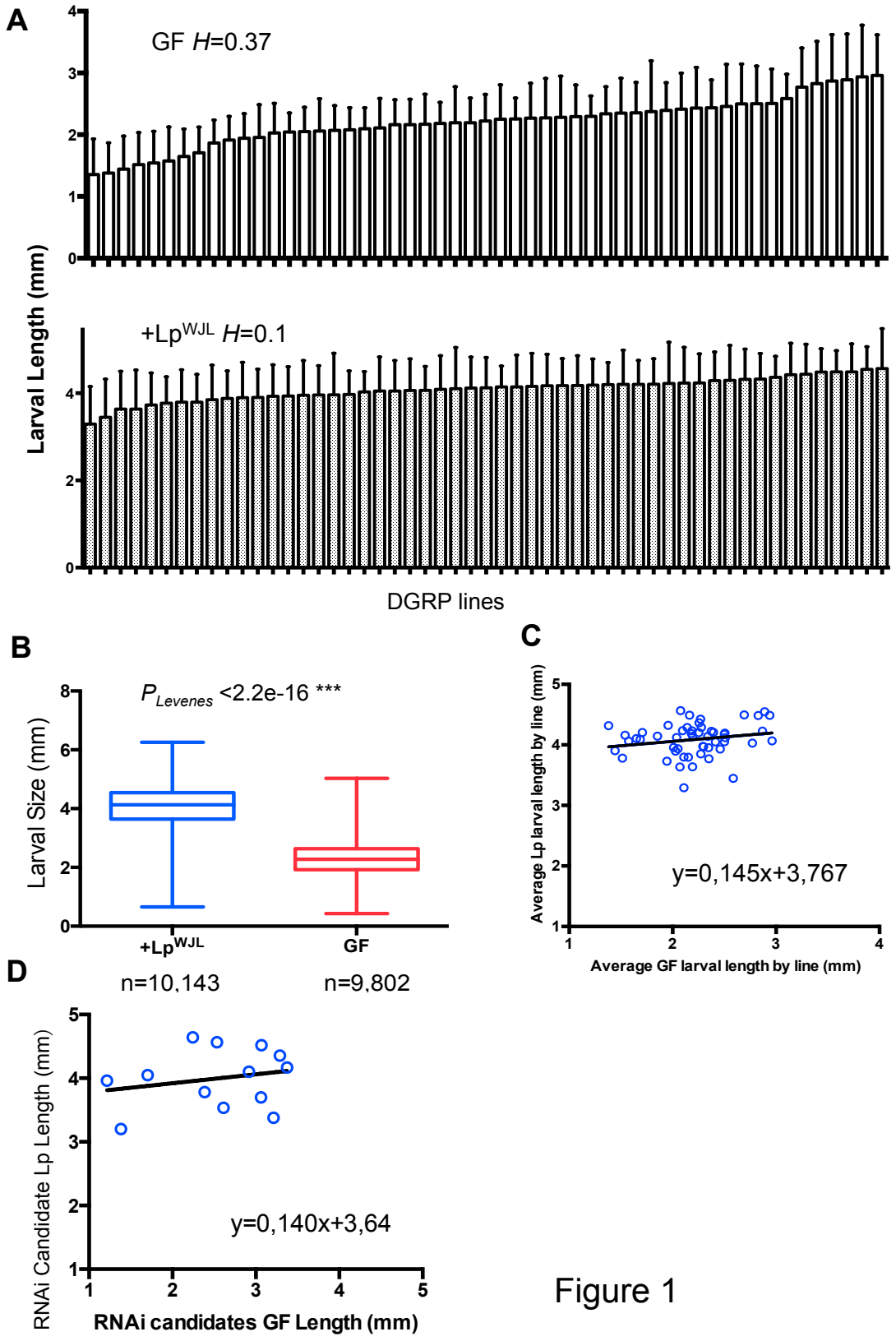

A
GF H=0.37
+Lp^WJL H=0.1
Larval Length (mm)
DGRP lines
B
P_Levenes <2.2e-16 ***
Larval Size (mm)
+Lp^WJL
GF
n=10,143
n=9,802
C
Average Lp larval length by line (mm)
Average GF larval length by line (mm)
y=0,145x+3,767
D
RNAi Candidate Lp Length (mm)
RNAi candidates GF Length (mm)
y=0,140x+3,64

Figure 1

Figure 1. Monoassociation with Lp^{WJL} buffers variations in larval lengths in different genetic background.

A). Bar graphs showing the average larval length on Day 7 AEL (after egg lay) for each of the 53 DGRP lines (Mean ± SD). Each bar represents the average from pooled biological replicates containing all viable larvae from all experimental repeats. For each line, there are between 10-40 viable larvae in each replicate, 3 biological replicates each experiment, and 2 to 3 repeats of the experiments. Top panel: germ-free (GF), bottom panel: mono-associated (+Lp^{WJL}). The green bars denote the "top" and "low" DGRP lines that were selected for setting up the F2 crosses (see Figure S2 for crossing schemes).Note the heritability estimate (H) in the GF population is higher than in the mono-associated population.

B). Box and whisker plot showing average larval length derived from pooled GF (red) or Lp^{WJL}- (blue) mono-associated DGRP lines (CV_{GF}= 27.82 %, CV_{Lp}= 18.74%)

C). Scatter plot showing the linear correlation between GF and monoassociated larval length, indicating that Lp buffers the growth difference in GF larvae in the DGRP populations. Each data point represents intercept of the GF length and its corresponding mono-associated length at Day 7 for each DGRP line (*Null hypothesis : slope=1. P<0.0001 the null hypothesis is therefore rejected* A linear standard curve with an unconstrained slope was used to fit the data)

D). Scatter plot showing that Lp also buffers growth difference in different RNAi knockdown experiments for each of the candidate genes. Each data point represents intercept of the GF length and its corresponding mono-associated length at Day 7 for RNAi knockdown experiment. (*Null hypothesis : Slope =1. P=0.0008 , the null hypothesis is therefore rejected).* That data points are fit into an unconstraint model. For specific genotypes, refer to Figure S1 and Material and Method.

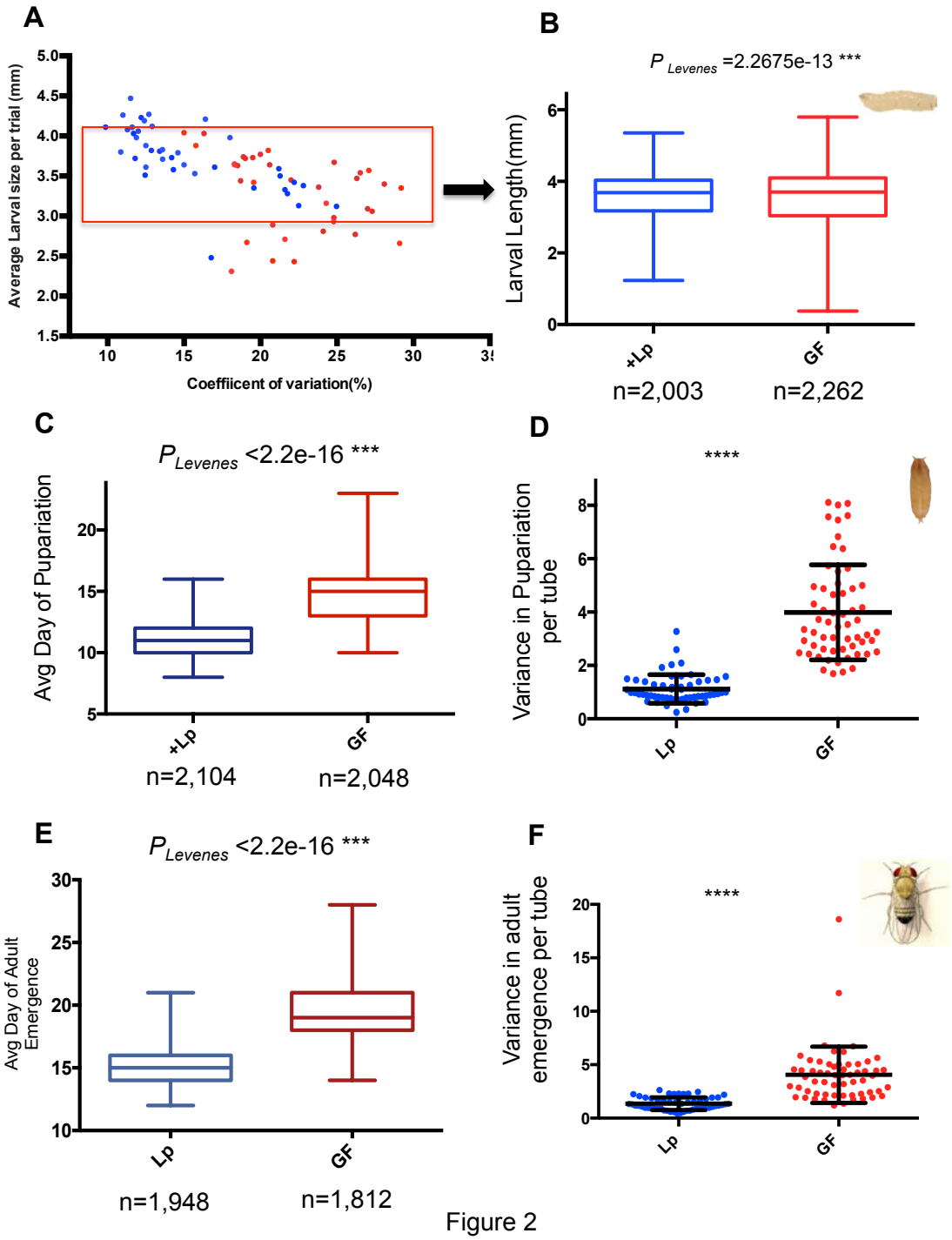

A
Average Larval size per trial (mm)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
10
15
20
25
30
Coeffiicent of variation(%)
B
P Levenes =2.2675e-13 ***
Larval Length(mm)
6
4
2
0
+Lp
GF
n=2,003
n=2,262
C
P Levenes <2.2e-16 ***
Avg Day of Pupariation
20
15
10
5
+Lp
GF
n=2,104
n=2,048
D
****
Variance in Pupariation per tube
8
6
4
2
0
Lp
GF
E
P Levenes <2.2e-16 ***
Avg Day of Adult Emergence
30
25
20
15
10
Lp
GF
n=1,948
n=1,812
F
****
Variance in adult emergence per tube
20
15
10
5
0
Lp
GF

Figure 2

Figure 2. In the DGRP F2 crosses, Lp^{WJL} asserts a robust buffering effect on variations in different fitness traits during development.

A). A scatter plot showing how coefficient of variation (CV) changes as a function of larval length, and how such change differs in the DGRP F2 GF (red) and mono-associated (blue) populations. Each data point represents the intercept of a CV value for its corresponding average larval length in a particular cross.The factors affecting variants in this plot are: larval age* (P=0.053), bacteria presence***(P=3.02e-06), larval length (P=8.27e-15***). The red bracket indicates the arbitrarily selected experiments where the average larval length for each cross falls between 3mm and 4mm.

B). The F2 average larval lengths from the pooled experiments in Figure 2A. While the average size is perfectly matched (GF Avg Length=3.522mm, Lp^{WJL} Avg Length= 3.582mm, P=0.857 ^{ns}), the GF population has higher variant than the Lp^{WJL} mono-associated population (Var_{GF}=0.642, CV_{GF}=22.8%, Var_{Lp}=0.427, CV_{Lp}=18.3%)

C). A Box and Whisker graph comparing the variance for average day of puparation in the F2 GF and monoassociated populations. (Difference in mean P<0.0001***, Var_{GF}= 2.42, Var_{Lp}=1.22).

D). Dot plot comparing the variances for pupariation from each tube containing approximately 40 larvae. The average variance per tube for the GF population=3.99; the average variance per tube for the Lp associated population =1.12. Var_{Lp}=0.54 , Var_{GF}=1.76. Note these are the "variance of variances")

E). A Box and Whisker graph comparing the variance for average day of adult emergence in the F2 GF and monoassociated populations (Difference in mean P<0.0001***, Var_{Lp}=1.84, Var_{GF}=5.27)

F). Dot plot comparing the variances for adult emergence from each tube containing approximately 40 larvae (V Difference in mean P<0.0001***),. Note these are the "variance of variances") The average variance per tube for the GF population=4.06; the average variance per tube for the Lp associated population =1.34. For 'variance of the variances" Var_{Lp}=1.33 , Var_{GF}=4.2.

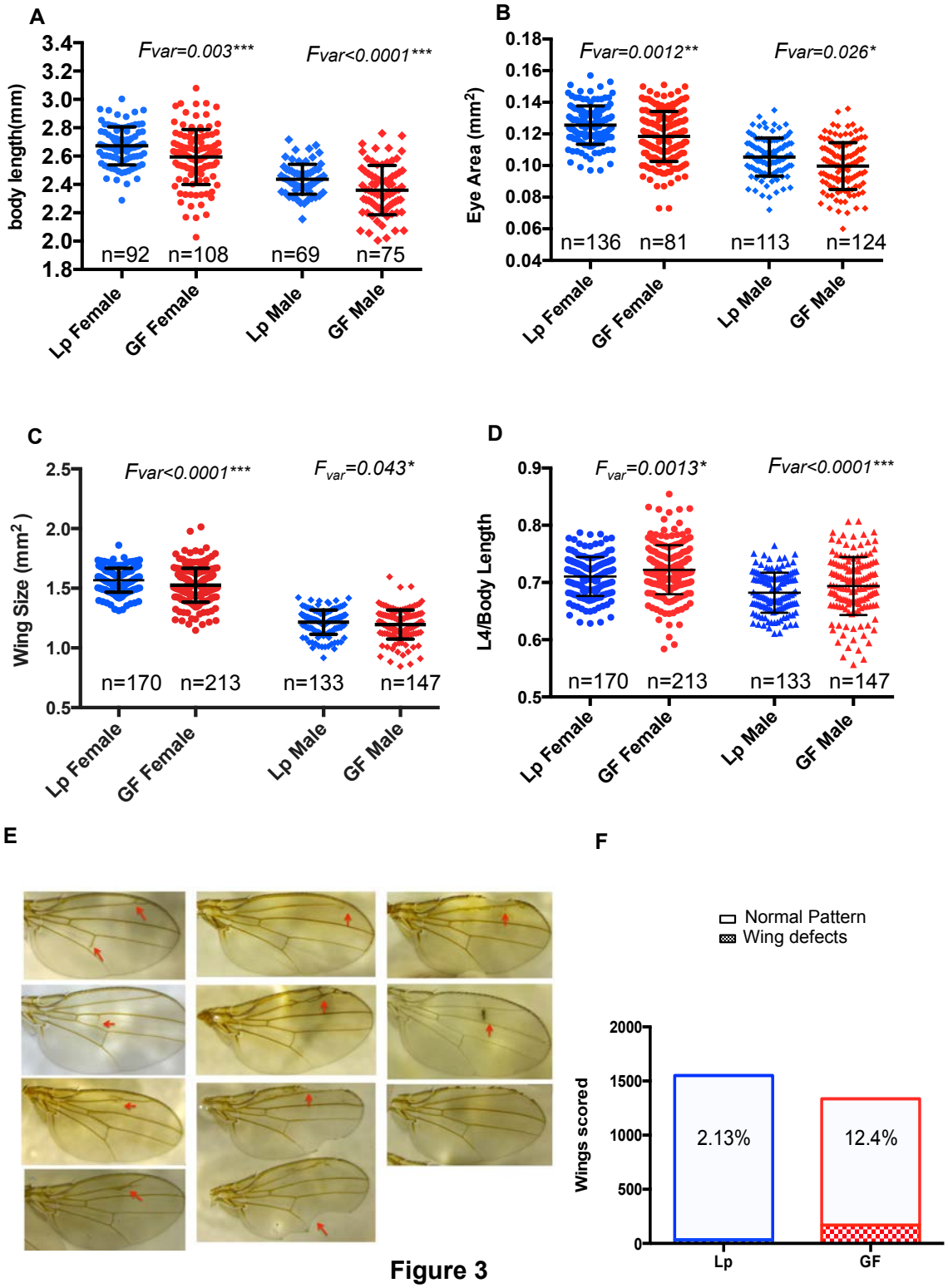

A
body length(mm)
Fvar=0.003***
Fvar<0.0001***
n=92
n=108
n=69
n=75
Lp Female
GF Female
Lp Male
GF Male
B
Eye Area (mm2)
Fvar=0.0012**
Fvar=0.026*
n=136
n=81
n=113
n=124
C
Wing Size (mm2)
Fvar<0.0001***
Fvar=0.043*
n=170
n=213
n=133
n=147
D
L4/Body Length
Fvar=0.0013*
Fvar<0.0001***
E
F
Normal Pattern
Wing defects
Wings scored
2.13%
12.4%
Lp
GF

Figure 3

Figure 3. In the adult DGRP F2 progeny, monoassociation with Lp^{WJL} reduces variations in body and organ size, and masks wing pattern abnormalities.

In both male (lozenge) and female (circle) adults, the variances in body size (**A**, the difference in mean body length: for females, P=0.0009***, for males, P=0.0015**), eye area (**B**, the difference in mean eye area, for females P<0.0001***; for males, P=0.0013**), and wing area (**C**. the difference in mean wing area for females, P=0.0010, *** for males, P=0.124, ns) are greater in the GF population than in mono-associated population. **D**). The length of L4 vein in the wing is used as a proxy of the wing length. In the accumulated ratios of wing length over body length, the variances are greater in the GF flies(The difference in average L4/ body length, for females P<0.0028**; for males, P=0.02*). The adult data sets presented in A, B, C and D take on normal distribution by D'Agostino & Pearson omnibus normality test, F variances are therefore calculated and compared

E). A compilation of representative images illustrating wing patterning anomalies in the axenic DGRP F2 progeny, indicated by red arrows. The number of such patterning anomalies are compiled together for GF and monoassociated flies (χ^2 test, P<0.0001***), and the incidence of the defects are indicated inside each bar

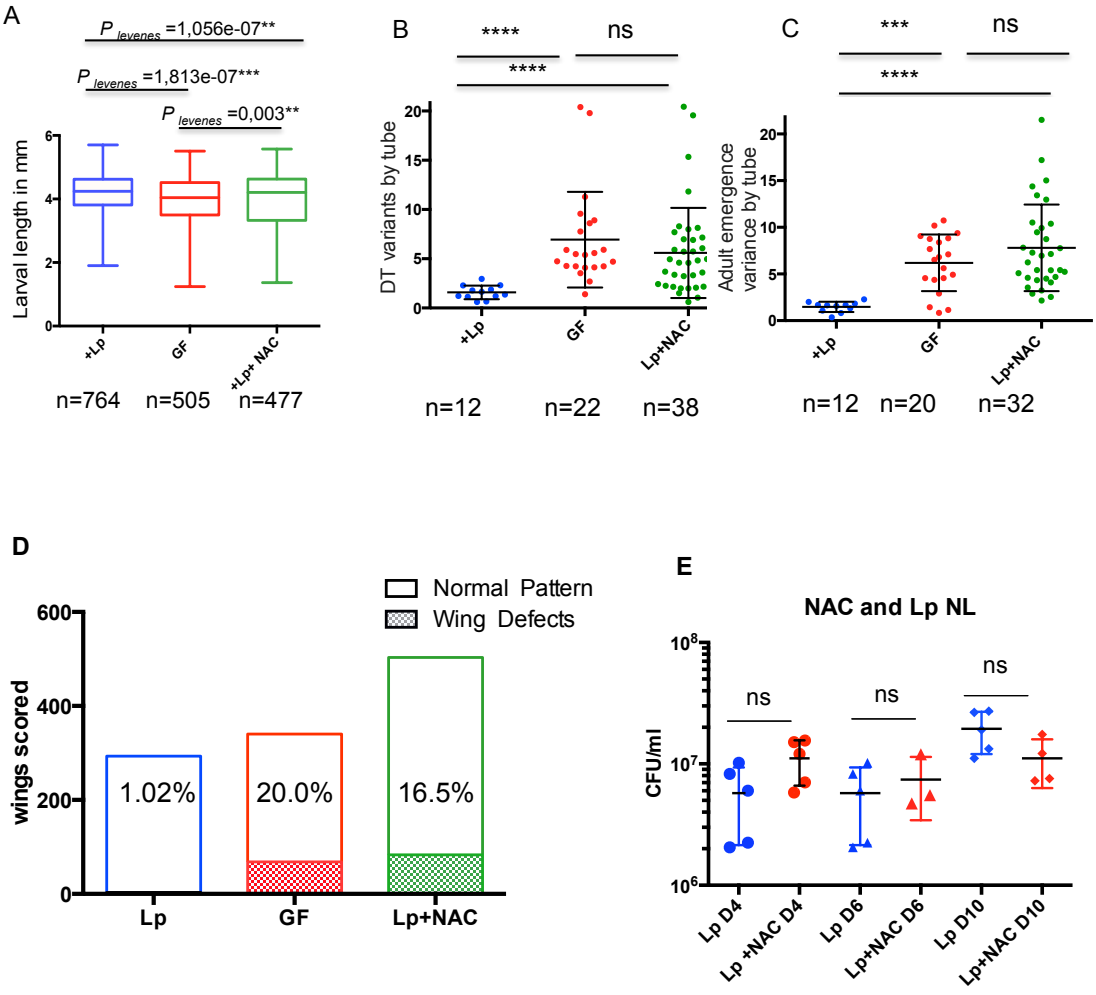

A
P levenes =1,056e-07**
P levenes =1,813e-07***
P levenes =0,003**
Larval length in mm
+Lp
GF
+Lp+ NAC
n=764
n=505
n=477
B
****
ns
****
DT variants by tube
+Lp
GF
Lp+NAC
n=12
n=22
n=38
C
***
ns
****
Adult emergence variance by tube
+Lp
GF
Lp+NAC
n=12
n=20
n=32
D
Normal Pattern
Wing Defects
wings scored
1.02%
20.0%
16.5%
Lp
GF
Lp+NAC
E
NAC and Lp NL
CFU/ml
ns
ns
ns
Lp D4
Lp +NAC D4
Lp D6
Lp+NAC D6
Lp D10
Lp+NAC D10

Figure 4

Figure 4. Blocking ROS by N-acetylcystein (NAC) compromises the Lp buffering capacity, without affecting bacteria growth

In the DGRP F_2 progeny, feeding Lp^{WJL}mono-associated animals with food supplemented with 10mM NAC increases the variances in size-matched larvae (A). Average Lp larval size: 4.08mm; average GF larval size: 3.83mm; average Lp+NAC larval size: 3.94mm. There is no size difference between GF and NAC treated flies associated with Lp^{WJL}, p=0.064. CV_{Lp} =15.8%, CV_{GF}= 20.8%; CV_{Lp+NAC}=24.0%. NAC treatement to the Lp-associated animlas also increases the variances of puparation in each tube (B, n=) and in variances of adult emergence by tube (C). Morphological defects in the wings are also significantly increased in NAC-treated mono-associated adults (D), χ^2 test, P<0.0001***) Red : GF ; Blue : Lp, Green : Lp+NAC. E). Bacteria niche load (NL) evolution (« Niche » is defined as the substrate with both larvae and Lp present) during the course of fly development with Lp^{WJL} with or without NAC treatment(Day 4, Day 6 and Day 10).

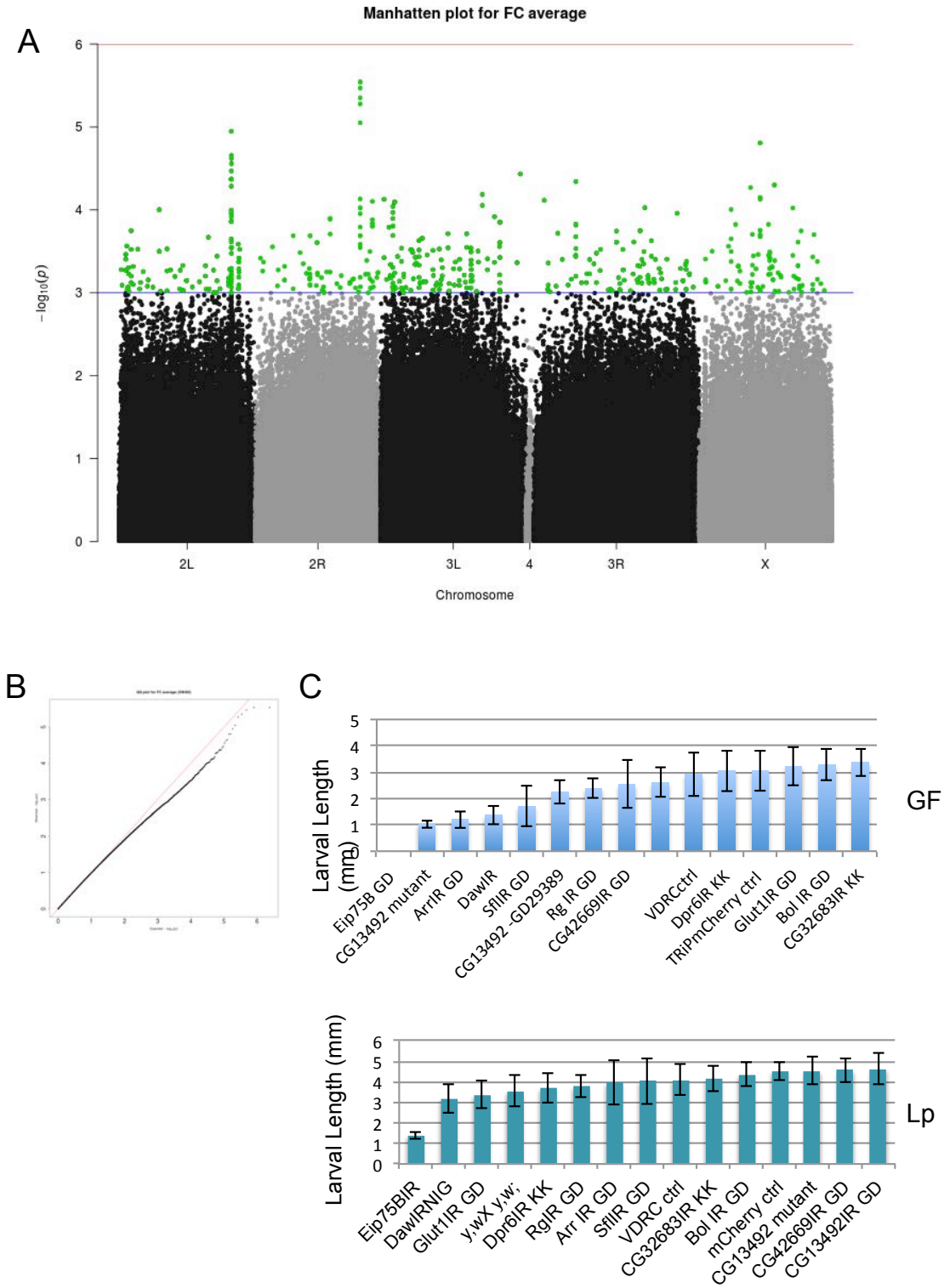

A
Manhatten plot for FC average
−log10(p)
Chromosome
2L
2R
3L
4
3R
X
B
C
Larval Length (mm)
GF
Eip75B GD
CG13492 mutant
ArrIR GD
DawIR
SflIR GD
CG13492 -GD29389
Rg IR GD
CG42669IR GD
VDRCctrl
Dpr6IR KK
TRiPmCherry ctrl
Glut1IR GD
Bol IR GD
CG32683IR KK
Larval Length (mm)
Lp
Eip75BIR
DawIRNIG
Glut1IR GD
y,wX y,w;
Dpr6IR KK
RgIR GD
Arr IR GD
SflIR GD
VDRC ctrl
CG32683IR KK
Bol IR GD
mCherry ctrl
CG13492 mutant
CG42669IR GD
CG13492IR GD

Figure S1

Figure S1.

A). Mahattan plot (from EPFL)

B). QQ plot (EPFL)

C) and D). Bar graphs illustrating the effect of RNAi knockdown on larval length on day 7 AEL. Each bar represents the average length from pooled 3-5 biological replicates from either condition, with 15-40 larvae in each replicate. C: GF. D: Lp^{WJL}. Three different control knockdowns are used: one control fly strain recommended by VDRC for RNAi constructs obtained from VDRC, one control strain (against mCherry) recommended by Harvard TRiP collection, and the y,w strain from Bloomington. All control and RNAi strains are crossed to y,w, tubulin>GAL^{80},DA>GAL4. "GD" refers to the VDRC RNAi GD collection. "KK" refers to the VDRC RNAi KK collection. For specific genotypes, refer to Material and Methods.

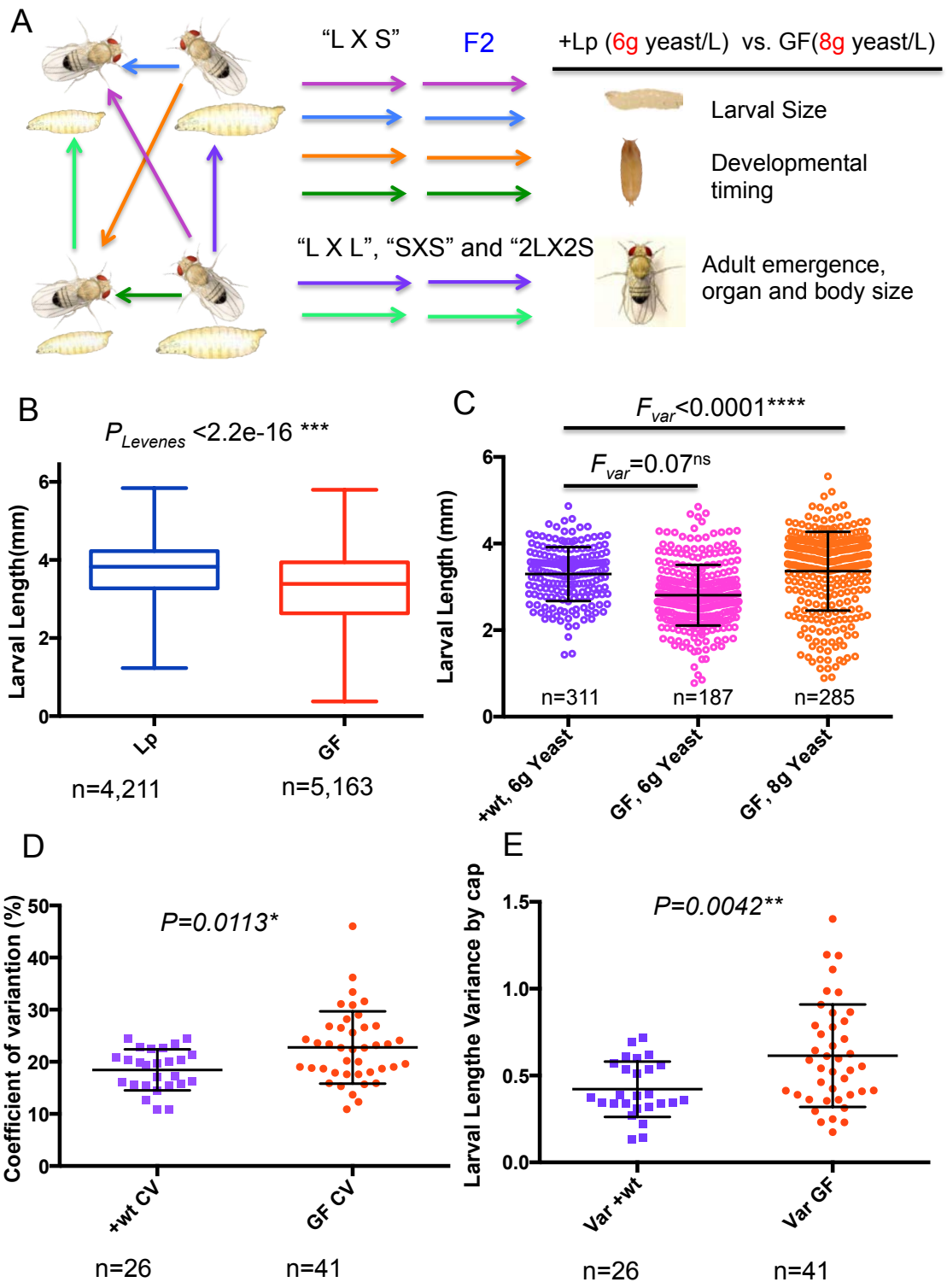

A
"L X S"
F2
+Lp (6g yeast/L) vs. GF(8g yeast/L)
Larval Size
Developmental timing
"L X L", "SXS" and "2LX2S"
Adult emergence, organ and body size
B
$P_{Levenes}$ <2.2e-16 ***
Larval Length(mm)
Lp
GF
n=4,211
n=5,163
C
F_{var}<0.0001****
F_{var}=0.07ns
Larval Length (mm)
n=311
n=187
n=285
+wt, 6g Yeast
GF, 6g Yeast
GF, 8g Yeast
D
P=0.0113*
Coefficient of variantion (%)
+wt CV
GF CV
n=26
n=41
E
P=0.0042**
Larval Lengthe Variance by cap
Var +wt
Var GF
n=26
n=41

Figure S2

Figure S2

A)**.** A diagram illustrating DGRP crosses to generate the F2 generation for studying variability in larval size, pupuration and adult emergence. 25210 (RAL-859), 25183(RAL-335) are the "large" (L) larvae as germ-free, and 25208(RAL-820) and 28147(RAL-158) are the "small" larvae as germ-free (S). Seven possible crosses are set up: 25210X25183 ("LXL"), 25208X28147("SXS"), 25210X25208, 25183X25208, 25210X28147,25183X28147 are the four "LXS" crosses, and 25183, 25210 X25208, 28147 is the "2L X 2S" cross.

B). Box and Whisker graph illustrating the average length and standard deviation from pooled GF(red) and mono-associated DGRP F2 larvae, pooled from all the crosses in all three different repeats (Average GF larval length: 3.29mm; average Lp mono-associated larval length: 3.71mm; CV_{GF}=24.9%, CV_{Lp}=19.5%).

C). One representative experiment showing that re-associating the field-collected flies tends to buffer the variability in body length in size-matched larvae. Purple dots represent body length from wild larvae grown on media contaminated with their untreated parents' fecal matter. Average GF larval length grown on 6g/L yeast media: 2.81mm; average GF larval length grown on 8g/L yeast media: 3.36mm: average re-associated larval length ("+wt"):3.07 mm; P= 0.338. CV_{GF} (6g/L, pink dots)= 24.9% , CV_{GF} (8g/L, orange dots)= 27.0%, CV_{Lp} (purple dots)= 18.9%/

The compiled CV values (D.) and variances (E.) from each cap containing 40∼50 field-collected larvae. The average CV and variance are lower in the population reassociated with its own microbiota (purple dots) than in the GF population (orange dots)

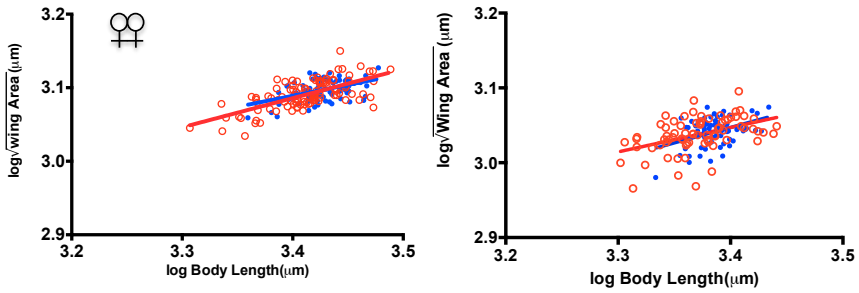


Figure S3

Figure S3

Scatter plots illustrating the allometric relationship between wing area and body size in female (A) and male (B) DGRP F2 adults. Red open circles: GF, blue filled circles: Lp^{WJL}. Each line represents the allometric slope of the data points shown by the same color. Either in males or females, there is no difference in allometric slope between the GF and mono-associated population. For GF females, Y_{GF} = 0.3963*X + 1.738, 95%C.I.= 0.3117 to 0.4810; for Lp^{WJL} females, Y_{Lp} = 0.2978*X + 2.076, 95%C.I.= 0,1785 to 0,4172, P=0.203, n.s ; for GF males,
Y_{GF} = 0.3261*X + 1.939, 95%C.I.= 0.1725 to 0.4796 ; for Lp^{WJL}males, Y_{Lp}= 0.4141*X + 1.639, 95% C.I. =0.1842 to 0.6439, P=0.55, ns.

The incidence of wing patterning defects separated by F_2 genotype is illustrated in C. The Axis denotes of the percentage of wings with aberrerant patterning as represented in Figure 3E. . In panel D, an image of a wing of an Lp^{WJL} adult is shown, as a representation of the most visible "defect" ever observed in mono-associated adults. Red arrow points to the subtle vein tissue thickening. We included these as « defects » in LpWJL F2 population in the analysis in Figure 3E, 3F, S3C, and 4D.

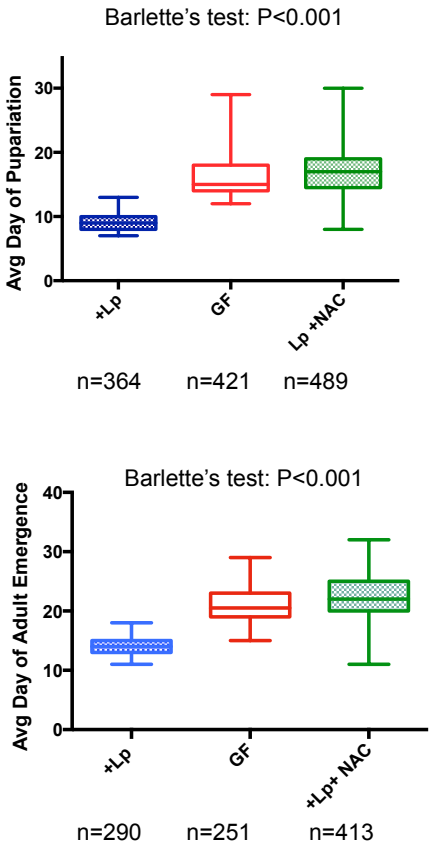


Figure S4

Figure S4

NAC addition increases the variance in average day of pupartion (A.) and adult emergence (B.) in the DGRP F2 population. The average day to become a pupa for Lp^{WJL} mono-associated larva: Day 8.9 (Var=2.13), for a GF larva: Day 16.1 (Var=8.27), for a NAC-treated, mono-associated larva: Day 16.8 (Var=8.36). The average day for an Lp^{WJL} mono-associated adult to emerge is: Day14.1 (Var=2.08), for a GF adult: Day 21 (Var= 8.3) and for a NAC-treated, mono-associated adult: Day 21.7 (Var=11.3)