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Experimental Microbiome:  
Investigating how host and gut microbiota  
evolve together

Relatore  
Dott.ssa Maria Elena Martino  
Correlatore  
Dott.ssa Marialaura Gallo

Laureando  
Nicola Gabardi  
Matricola n.  
1181322

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## ABSTRACT

Symbiotic bacteria significantly contribute to the host physiology, improving the health of humans and animals through different ways. For this reason, the study of animal-microbe symbiosis has become increasingly important. However, given the complexity of the interactions between metazoans and their microbial communities, understanding the mechanisms governing such relationships remains a challenge. In this light, the use of experimental animal models is of great help. Specifically, the use of the fruit fly *Drosophila melanogaster* coupled with its microbiota has proven to be a powerful animal model. *Lactobacillus* represents one of the most important bacterial genera of the animal and human microbiota. In particular, it has been demonstrated that *L. plantarum* (*Lp*) promotes *Drosophila* larval growth in a strain-specific manner. Additionally, a recent study showed that ***Lp* is able to improve its symbiotic benefit by adapting to the host diet** rather than to the host, which represents the predominant driving force in the evolution of animal-microbiota symbiosis. It is now necessary to understand if and how the host can shape the evolution of its gut microbes, and, specifically, whether it affects bacterial evolutionary rate. To this aim, we set up two parallel experimental microbiota evolution studies starting from a mid-growth promoting *Lp* strain (*Lp*<sup>NIZO2877</sup>), that only differed on the presence of the animal host. During this experiment, the phenotypic and genotypic evolution of *Lp*<sup>NIZO2877</sup> have been evaluated in both setups. No substantial differences were identified in the evolution of *L. plantarum* between the two experimental settings, further demonstrating that the diet exerts a prevailing influence on the evolution of the microbiota. This indicates that the host seems not to affect, at least at early stages, microbial adaptation and evolution. Further studies are thus needed to investigate whether the animal host has any effect on the evolution of its microbiota at later stages.

*“It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is most adaptable to change.”*

C. Darwin

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# 1. INTRODUCTION

## 1.1 The microbiota

### 1.1.1 Microorganisms as evolutionary partners

*Symbiosis* is a word of Greek origin, meaning *living together*. Biologists have been using it since 1879, thanks to de Bary, to describe different organisms, often of completely different phyla, that consort more or less closely (*Lewin, 1982*). Despite the definition of symbiosis has been debated for over 130 years, nowadays it is widely accepted that symbiotic relationships include *mutualism* (when both the host and the symbiont benefit from the interaction), *commensalism* (when the association is advantageous to one partner and does not affect the other) and *parasitism* (when the symbiont benefits and the host suffers damage) (*Martin and Schwab, 2013*).

In the last 20 years the study of symbiosis has become increasingly important, also thanks to the new sequencing technologies. Historically, the phenotype of the major macrobiotic groups (animals, plants and fungi) has been considered as the unique result of their own genotype, and microorganisms were seen only as cause of disease or as agents responsible for recycling. However, it is essential to consider that many microbes live in association with their animal partners, with which they forge alliances (*McFall-Ngai, 2008*). These microbes constitute the microbiota, the assemblage of microorganisms present in a defined environment. The term microbiome refers instead to the entire habitat, including the microorganisms, their genomes and the surrounding environmental conditions, though many researchers in the field limit the definition of microbiome to the collections of genes and genomes of members of microbiota. However, it would be more correct to assign the latter explanation to the word metagenome (*Marchesi and Ravel, 2015*).

Microbiota includes beneficial microbes that can help us and microbes that can hurt us. In the gut for example, many bacteria are symbionts and contribute to the host physiology through different ways, that range from optimizing digestion and absorption, to limiting pathogen invasion, to triggering developmental programs. At the same time, microbiota benefits from symbiosis, living in a nutrient rich environment. For these reasons, the beneficial host-bacteria interactions are a clear example of mutualism and should be considered an important step in the evolution (*Fraune and Bosch, 2010*).

The study of symbiosis began in 1684 with the observations of Antonie van Leeuwenhoek, but only recently scientific research has started to show the importance of mutualism, thanks to the advances in molecular technology, which allowed to reach an extremely detailed analysis of such symbiotic partnerships (*McFall-Ngai, 2008*). This is why understanding the genetic basis of host/microbe mutualism has become a new frontier of biology (*Ruby, 2008*).

### **1.1.2 The hologenome concept of evolution**

The intimate relationship between animals or plants and microorganisms described above leads to the concept of holobiont. This term was first introduced in 1992 to describe a host with its primary symbiont, but subsequently extended to all of its symbiotic microorganisms, including viruses. The second definition is certainly more appropriate since the prefix *-holo* derives from the Greek word *holos*, which means *whole*. As a result, the holobiont contains an enormous diversity of genetic material, the hologenome, therefore defined as the sum of the genetic information of the host and its symbiotic microorganisms. The hologenome concept places importance on the huge microbial diversity, much of which is being uncovered in recent years with molecular techniques. By comparing the number of unique genes encoded by the host and those encoded by the microbiome it is possible to understand the importance of this aspect. For example, the human genome contains about 23000 genes, while that of its microbiota more than nine million, corresponding to a ratio of bacterial to human genes of 390:1 (*Rosenberg and Ziller-Rosenberg, 2014*). As a consequence of the above considerations, the holobiont can be considered as a distinct biological entity from an



anatomical, metabolic, immunologic, developmental and even evolutionary point of view. The fact that natural selection can operate on the holobiont leads to the hologenome theory of evolution (*Rosenberg and Ziller-Rosenberg, 2018*). It explains that genetic variation occurs not only through genetic changes during sexual reproduction, chromosomes rearrangements, epigenetic changes and mutations in the host, but also with three additional processes: microbial amplification or contraction (this process is similar to host's gene duplication, but faster and more responsive to the environment), acquisition of novel strains from the environment (this mechanism introduces novel genetic material into holobiont) and horizontal gene transfer (*Gilbert and Tauber, 2012*). However, not all the scientific community accepts the hologenome concept of evolution, which is particularly debated: for example, some argue that this theory considers only the beneficial interactions between host and microbiota and not the antagonistic ones (*Douglas and Werren, 2016*). Anyway, the concept of hologenome is an intellectual effort that combines elements from different branches of biology in order to obtain a new way to understand evolution that is simultaneously exciting, confusing and challenging, resulting from the vast amount of microbial ecology data that has become available in the era of high-throughput sequencing (*Morris, 2018*).

### **1.1.3 The gut microbiota**

As mentioned, higher organisms live in constant association with microbes, with which they forge alliances. Taking the human body as an example, microorganisms are present in different sites of our body and the composition of the microbiota changes depending on the considered location. Moreover, the microbiota is highly variable from person to person, even if family members tend to harbor more similar microorganisms than unrelated individuals (*Spor et al., 2011*). Host-microbe interactions occur primarily at mucosal surfaces, the largest of which is the gastrointestinal tract (*Shanahan, 2012*). This means that most of microbes are found in the intestinal lumen and the remaining is mainly dispersed among the skin, the oral cavity and the genital mucosae. Overall a normal human being hosts a mass of bacteria equal to about 1.2 kg, comparable to the weight of the liver (*Bocci, 1992*). As a result, we can consider the intestinal microbiota

as a real organ within another organ, with its own metabolic activity. The localization of microorganisms in the gastrointestinal tract reflects natural selection at both the microbial and the host levels. In fact, selection acts by promoting the cooperation within this complex ecosystem. Hydrochloric acid, bile and pancreatic secretions prevent the colonization of the stomach and proximal small intestine by most bacteria. Then the density of bacteria constantly increases in the subsequent parts of the intestine: in fact, colon-residing bacteria achieve the highest cell densities recording for any ecosystem, consisting of  $10^{11}$ - $10^{12}$  cells per gram of colonic material (*O'Hara and Shanahan, 2006*). The human gut microbiota is formed by a very large microbial community, consisting of about 1000 or more different bacterial species (*Sansonetti and Medzhitov, 2009*).

Microbial colonization of the infant gut is a fundamental process that plays a key role in promoting short- and long- term benefits on human health: disruptions during the complex development of the gut microbiota have been shown to increase the disease susceptibility during life. The idea that fetuses are sterile thanks to the placental barrier and that microbial colonization of the newborns start during and after birth is widely accepted, although some studies have detected the presence of bacteria (without any indication of infection) in placenta tissue, umbilical cord blood, amniotic fluid and fetal membranes (*Rodriguez et al., 2015*). This observation therefore suggests that microbial exposure may start before delivery, allowing the colonization of the fetus with early pioneers derived from the maternal microbiota. In this case the colonization depends on prenatal, neonatal and postnatal factors (*Milani et al., 2017*). However, the main drivers of the microbial colonization of the infant intestine are (*Rodriguez et al., 2015, Milani et al., 2017*):

- mode of delivery: this is one of the most important factors that influence the early gut microbiota composition. Vaginal delivered infants are colonized by maternal vaginal and fecal bacteria, including in particular the genera *Lactobacillus* and *Bifidobacterium*. In contrast, infants born via C-section are not directly exposed to maternal microbes: they are therefore colonized by environmental microorganisms (i.e.: hospital staff) and show a reduced complexity of the gut microbiota;
- mode of feeding: this way of colonization plays a critical role in constituting the gut microbiota. Indeed, breast-fed infants are exposed to the milk microbiota,

which has been reported to contain more than 700 species of bacteria, many of which belong to the genera *Staphylococcus* and *Streptococcus*;

- environmental factors (family lifestyle and geographical location): it has been shown that the gut microbiota is also influenced by the family members and close relatives (i.e.: siblings have higher proportion of *Bifidobacterium* spp. than single infant). Moreover, geographical location may impact on the microbiota: different populations have distinct diets and cultural practices;
- host genetics: it seems that even the genetic of the host is involved in determining the composition of the microbiota. In fact, there are higher levels of microbial similarity in genetically identical twins than fraternal twins and unrelated controls.

Generally speaking, the microbiota of newborns initially consists mainly of bacteria belonging to the phyla Actinobacteria and Proteobacteria; subsequently bacteria belonging to the phyla Bacteroidetes and Firmicutes prevail. These last phyla are the most important also in the adult gut microbiota, while Actinobacteria is the next most abundant phylum, mainly represented by the genus *Bifidobacterium*. If the microbiota of adults is characterized by stability, that of the elderly has been shown to have great inter-individual variability; this could be related to a decline in general health (*Rodriguez et al., 2015*). However, long-term dietary habits are known to be involved in modulating the composition of the microbiota: for example, children that live in rural areas of Africa, who ingest high amounts of plant derived polysaccharides, show low levels of Firmicutes and high levels of Bacteroidetes (some of which, like *Prevotella* e *Xylanibacter*, are involved in degrading cellulose and xylans), compared with Italian children, who had high levels of Enterobacteriaceae (*Tremaroli and Bäckhed, 2012*).

As a result of what has been said, the microbial community that colonizes the gut affects many aspects of health. This is why one of the biggest challenges in this field is to understand the role of the microbiota in maintaining good health. Indeed, the overall state of the microbial community, in terms of its distribution, composition and metabolic outputs, outlines the balance of benefit and harm for the host (*Flint et al., 2012*). Probably the primary aspect in this context is the role of the microbiota in energy harvesting: carbohydrates are the most important source of energy, but many complex polysaccharides (i.e.: cellulose, inulin, resistant starch, xylans) cannot be degraded by

human enzymes. However, these carbohydrates are fermented in the colon by the resident microflora, from which it produces short-chain fatty acids (SCFAs) (*Tremaroli and Bäckhed, 2012*). SCFAs are saturated aliphatic organic acids composed of one to eight carbon atoms of which acetate (C2), propionate (C3) and butyrate (C4) are the most abundant (>95%). Bacteria belonging to the phylum Bacteroidetes mainly produce acetate and propionate, while those belonging to the phylum Firmicutes have butyrate as their primary metabolic end product. When absorbed by the host, a large part of SCFAs is used as a source of energy (in humans SCFAs provide 10% of the daily caloric requirement). SCFAs are also involved in the regulation of lipid and glucose metabolism thanks to the interaction with two G protein-coupled receptors, Ffar2 and Ffar3. Moreover, SCFAs regulate the balance between fatty acids synthesis, fatty acid oxidation and lipolysis in the body, are implicated in gluconeogenesis and reduce plasma concentration of cholesterol (*des Besten et al., 2013*). However, this is just one example of how the gut microbiota affects the physiology of the host, but many ways in which it occurs are still unknown.

#### **1.1.4 Probiotics**

The first to introduce the concept that intestinal microflora can play a beneficial role was Elie Metchnikoff. In his book *Prolongation of Life. Optimistic studies*, published in 1907, he proposed that senility is due to the accumulation of toxins produced by putrefactive flora resident in the gut, lethal in the long time (*Malago et al., 2014*). Moreover, he suggested that it is possible to prevent the proliferation of these harmful bacteria by replacing them with useful ones. He developed this assumption by observing that Caucasian shepherds, great consumers of fermented milk, had a longer average life than the inhabitants of Paris and the Americans. This observation leads to the concept of probiotic, a Greek term meaning *for life (pro-bios)*. The definition of probiotic currently accepted is that proposed by the World Health Organization in 2001: “Live microorganisms which when administered in adequate amounts confer health benefit on the host”. The majority of probiotic microorganisms belong to the genera *Lactobacillus* and *Bifidobacterium*, commonly present in the human and animal

intestinal tract. Starting from the work of Elie Metchnikoff, Minoru Shirota, a Japanese microbiologist, discovered that some bacteria of the intestinal flora contribute to defense from pathogens. The following studies led to isolate *Lactobacillus casei*, which started the worldwide marketing of fermented milk drinks (*Malago et al., 2014*). However, the European Food Safety Authority has so far rejected most claims on probiotics benefits in Europe due to insufficient research, despite the intensive advertising campaigns by food and pharmaceutical companies. This aspect and the increased incidence of metabolic and intestinal inflammatory diseases in western societies with consequent influence on the gut microbiota (*Kau et al., 2011*) confirm the need to invest research effort in this field.

## 1.2 *Drosophila melanogaster* as animal model

### 1.2.1 What *Drosophila* is, life cycle and advantages in using it in research

Given the complexity of the interactions between metazoans and microbial communities residing on their mucosal surfaces, the use of experimental animal models to evaluate and clarify those relationships could be of great help. Furthermore, there is a need for simple models. In this context, the use of the fruit fly *Drosophila melanogaster* has proven to be a powerful animal model for studying different aspects of host-bacterial symbiosis (*Erkosar et al., 2013*). This is particularly due to the low complexity of its microbiota in contrast with the richness and diversity of the microbiota composition observed in mammals (*Spor et al., 2011*). Additionally, the organ function and physiology of this animal model are similar to those of mammals. These characteristics easily allow to translate the results to mammalian models (*Erkosar et al., 2013*). The genus *Drosophila* belongs to the family Drosophilidae, order of Diptera, and contains about 1500 species, the most important of which in scientific research is *D. melanogaster*. Its use as animal model particularly in genetics and developmental biology is due to several reasons. First, fruit flies have simple nutritional requirements

and occupy little space. They produce large number of offspring to allow sufficient data to be collected. Moreover, the life cycle of *Drosophila* is short allowing quick analysis. The duration of the cycle varies with the temperature: at 25°C the life cycle may be completed in about 10 days, but at 20°C about 15 days are required. Like most insects, the life cycle of fruit flies consists of four phases (Figure 1): egg, larva, pupa and adult. The egg is about 0.5 of a millimeter long. It is composed of an outer investing membrane, called chorion. A pair of filaments extending from the surface keeps the egg from sinking into soft food on which it may be laid. Embryonic development, which follows fertilization and the formation of the zygote, takes place inside the egg membrane. Subsequently, the egg hatches into a larva. There are three larval stages, the last of which allows to reach a length of 4.5 millimeters. The primary mechanism by which the larva grows is molting: at each molt, the entire cuticle of the insect is shed and has to be rebuilt again. The larva is composed of 14 segments (3 head segments, 3 thoracic segments and 8 abdominal segments) and its body is soft and flexible. This allows it to easily dig into food. Later, the larva undergoes a series of developmental changes that led it to the adult stage. This transformation process is called *metamorphosis* and takes place during the pupal stage. Immediately before pupation the larva leaves the food and goes up the sides of the culture bottle where it finally comes to rest. Here the hardening and the darkening of the cuticle take place. When metamorphosis is completed, the

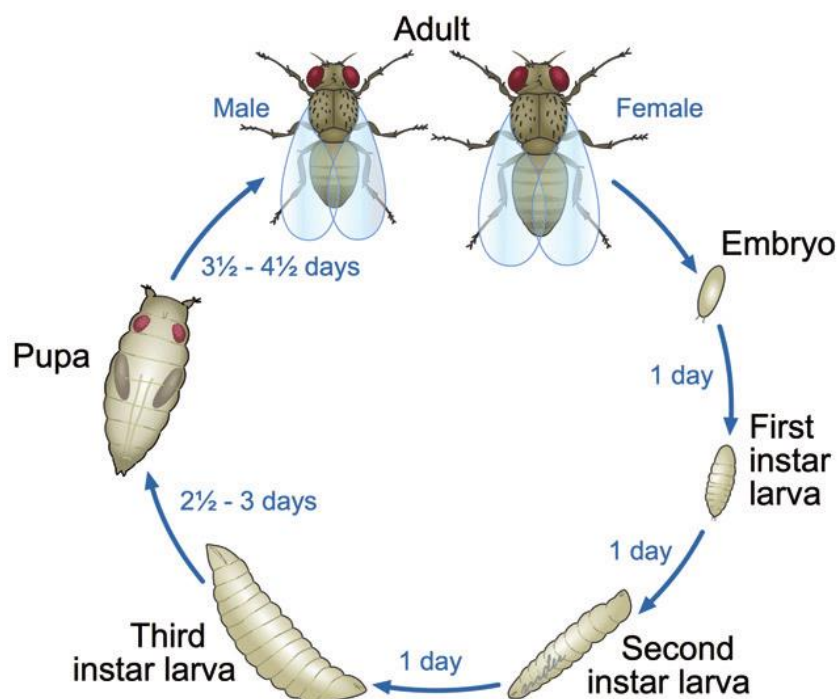


Figure 1: Life cycle of *Drosophila melanogaster* (Ong et al., 2015)

adult flies emerge from the pupal case. Finally, females begin to mate 10-12 hours after emerging from the pupa (Va et al., 2009). Another important advantage in using fruit flies in research is due to the ease of sterilizing *Drosophila* eggs in order to produce gnotobiotic flies inoculating the germ-free subjects with various bacterial strains of predefined quantity and composition. In this way, researchers can observe the phenotypic changes that occur in different aspects of host physiology and correlate them with a specific function from the microbiome. This allows to identify the genetic components of the crosstalk between *Drosophila* and its commensal bacteria. The first gnotobiotic *Drosophila* model was developed in 1969. It demonstrated that the essential mode of microbial transmission in fruit flies is through larval ingestion of contaminated chorion. This means that the dechoriation of eggs can render a fly stock germ-free (Ma et al., 2015).

### **1.2.2 *Drosophila* gut microbiota**

Numerous studies have been conducted in order to identify the composition of the gut microbiota of the fruit fly. These researches were carried out on laboratory-raised *Drosophila* strains or on flies captured in the wild and all revealed a simple microbial composition (Erkosar et al., 2013). Indeed, most of the time, only five to twenty bacterial species are found to be associated with a given fly population (Chandler et al., 2011, Wong et al., 2011). Generally speaking, the *Drosophila* microbiota is composed of two predominant phyla, Firmicutes and Proteobacteria. Two major families, Lactobacillaceae and Acetobacteraceae, and two minor families, Enterococceae and Enterobacteriaceae dominate. The four species identified as prevalent in most studies are *Lactobacillus plantarum* (Lp), *Lactobacillus brevis*, *Acetobacter pomorum* and *Enterococcus faecalis* (Erkosar and Leulier, 2014). This is why they can be considered as the core components of the *Drosophila melanogaster* microbiota, defined as a community of bacterial species that would co-exist in all individuals, although this hypothesis is debated. Anyway, all these species are aerobic or aerotolerant, making them easy to culture in laboratory. Moreover, these bacteria, with the exception of

Acetobacteraceae, are also present in mammals, including humans (*Erkosar et al., 2013*).

In several studies, it has been observed that the composition of the *Drosophila* gut microbiota can vary between wild and laboratory-grown populations. In particular, the bacterial communities in laboratory raised flies lack both richness and diversity (*Erkosar and Leulier, 2014*). In addition, it has been shown that changes in the microbiota composition among populations are largely due to dietary factors. Indeed, microbial communities of diverse *Drosophila* species feeding on the same substrate were more similar to each other than to more closely related species that were feeding on different substrates (*Broderick and Lemaitre, 2012*). In addition, growing isogenic *D. melanogaster* populations on different sterile foods led to changes in gut microbiota composition over time. This means that, like in mammals, the major factor shaping the intestinal microbiota composition in fruit flies is the diet, even if it is not the only one (*Erkosar and Leulier, 2014*). Indeed, the host physiology also contributes to determine the structure of commensal communities. During the entire *Drosophila* life cycle feeding behavior and nutritional needs vary. If at larval stage the flies have to sustain a massive growth, thus requiring a protein-rich diet, adult flies feed less regularly, mainly to maintain homeostasis, except for females that have a strong energy demand to support vitellogenesis. Therefore, the microbiota must constantly adapt to the physiological status of its host (*Erkosar et al., 2013*).

The reduced diversity of bacterial taxa reported for most insects than that observed in mammals seems to be due to two factors. First, the adaptive immune system of higher metazoans facilitates association with a greater number of microbes. Second, the frequent perturbations of the insect gut niche represent a limit to a higher diversity. In fact, the intestine of insects tends to be transient, given the frequent disturbance episodes: foregut and hindgut are shed during molting and the entire larval gut is replaced by a new adult gut during metamorphosis in fruit flies (*Broderick and Lemaitre, 2012*). This suggests that the establishment and maintenance of adult *Drosophila* microbiota depends on the constant ingestion of bacteria from the medium, confirming the close link between bacterial composition and diet previously mentioned. The strategy to maintain the commensal bacteria by continual re-association may reflect an evolutionary process: in the wild, *D. melanogaster* is saprophytic and feeds on microbe



rich-environments such as rotten fruits; therefore, little evolutionary pressure exists for the flies to invest energy to maintain a resident gut microbiota (*Erkosar and Leulier, 2014*).

As a result of what has been said, the relative simplicity of the *D. melanogaster* microbiota composition contributes to make this species a useful model to understand host-microbiota interactions.

### 1.3 *Lactobacillus plantarum*

#### 1.3.1 General characteristics of the genus *Lactobacillus*

As previously mentioned, the genus *Lactobacillus* is one of the most important bacteria in both human and *Drosophila* gut microbiota. Moreover, this genus includes a high number of GRAS (Generally Recognized As Safe) species, which are involved in fermented food production and in food preservation. Other species of human origin are exploited as probiotics. The genus *Lactobacillus* belongs to Lactic Acid Bacteria (LAB), a functional group of Gram-positive and catalase negative bacteria that produce lactic acid as the major metabolic end-product of carbohydrate fermentation (*Salvetti et al., 2012*). Table 1 shows the current classification of this genus, whose members are non-sporeforming, obligate saccharolytic rods or coccobacilli, characterized by a low GC (guanine and cytosine) content in their genome. Besides lactic acid, other products of their fermentative metabolism are acetate, ethanol, CO<sub>2</sub>, formate and succinate. Lactobacilli are generally aero-tolerant or anaerobic. They are aciduric or acidophilic and grow at temperatures ranging from 2°C to 53°C. Moreover, their nutritional requirements in terms of amino acids, peptides, vitamins, salts, fatty acids and fatty acid esters are complex. Indeed, they are present in rich environments as food, water, soil, sewage, mouth, gastrointestinal and genital tracts of humans and many animals (*Salvetti et al., 2012*).

Domain	Prokaryota
Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Lactobacillales
Family	Lactobacillaceae
Genus	<i>Lactobacillus</i>

Table 1: scientific classification of the genus *Lactobacillus*

The accepted subdivision of the genus *Lactobacillus* is the one given by Hammes and Vogel, which divides these bacteria into three groups, based on the type of metabolism (Wood and Holzapfel, 1992):

- obligate homofermentative (i.e.: *L. acidophilus*, *L. delbrueckii*, *L. helveticus*, *L. salivarius*): in this case hexoses are fermented almost exclusively to lactic acid through the Embden-Meyerhof-Parnas (EMP) pathway. These bacteria lack phosphoketolase and therefore they are unable to ferment pentoses and gluconate;
- facultative heterofermentative (i.e.: *L. plantarum*, *L. casei*, *L. sakei*, *L. curvatus*): also in this case hexoses are fermented to lactic acid by the EMP pathway. Additionally, they are able to ferment pentoses (and often gluconate);
- obligate heterofermentative (i.e.: *L. brevis*, *L. fermentum*, *L. reuteri*, *L. buchneri*): hexoses are fermented by the phosphogluconate pathway to lactate, ethanol (or acetic acid) and CO<sub>2</sub> in equimolar amounts. Pentoses also exploit this pathway and may be fermented.

The genus *Lactobacillus* comprises more than 200 species, whose genome range in size from 1.23 Mb to 4.91 Mb. Beyond a core genome composed of about seventy genes, many of which encode essential proteins involved in cell growth and replication, the genetic diversity of this genus is larger than that of a typical family, a feature that allows its members to adapt and survive in diverse environments (Sun et al., 2015).

### 1.3.2 The species *Lactobacillus plantarum*

Among the species belonging to the genus *Lactobacillus*, *L. plantarum* is one of the most important due to its versatility and flexibility. Indeed, it is frequently encountered as a natural inhabitant of human mouth, intestine and vagina as well as in a variety of ecological niches of interest to the agro-alimentary industry (i.e.: dairy products, meat, fermented vegetable products). In addition, specific strains of *L. plantarum* are used as probiotics, which may confer various health beneficial effects to the consumer. *L. plantarum* ecological flexibility is also confirmed at genetic level since this species has one of the largest genomes among lactic acid bacteria. *L. plantarum* was also the first *Lactobacillus* species to be sequenced in 2002 (Kleerebezem et al., 2003). Specifically, the strain WCFS1, that was originally isolated from human saliva, was the first to be fully sequenced. Its genome consists of a circular chromosome of 3.3 Mb (with a GC content of 44.5%), two small plasmids of 1.9 kb and 2.3 kb and a larger one of 36.1 kb. The sequence of *L. plantarum* WCFS1 revealed that this microbe focuses on carbon catabolism, encoding a large variety of protein involved in sugar uptake and utilization. Moreover, the discovery of a large collection of surface-anchored proteins indicates that *L. plantarum* has the potential to associate with many substrates for growth. These information confirm the flexible and adaptive behavior of this species (Kleerebezem et al., 2003).

Many bacteria, including species of the genus *Lactobacillus*, adapt to defined environments thanks to changes in genome content and regulation (i.e.: *L. delbrueckii* strains adapt specifically to dairy environments through a process characterized by genome decay (Smokvina et al., 2013)). Unlike these bacteria, the evolutionary history of *L. plantarum* does not appear to be related to environmental features. Indeed, strains belonging to this species maintain the acquired abilities independently of their habitat by means of a universal set of genes, that make *L. plantarum* a nomadic bacterial species. Thus, in nomadic lactobacilli genomic adaptation occurs not through environmental pressure but thanks to other selective factors (Martino et al., 2016).

### 1.3.3 Interactions with *Drosophila*

*L. plantarum* is one of the dominant commensal species present both in human and in *Drosophila* gut microbiota. Additionally, it is vertically transmitted to the progenies. Regarding its beneficial effects, it has been demonstrated that it is able to promote larval growth upon nutrient scarcity, recapitulating, on its own, the benefit of a more complex natural microbiota (Storelli *et al.*, 2011). Consequently, the association with *L. plantarum* accelerates larval development and results in a greater adult size and in an earlier emergence of the adults compared to germ-free animals.

Several evidences support the notion that *L. plantarum* does not act via a diet-derived sugar metabolism, but rather by promoting protein assimilation from the food by the larvae. In this context, TOR (target of rapamycin) kinase and the amino acids transporter *slimfast* are essential for *L. plantarum* beneficial effect on larval growth. These molecules are indeed involved in the host nutrient sensing system, which governs *Drosophila* development: activated by diet-derived amino acids, TOR kinase regulates the production of ecdysone in the prothoracic gland and the production of insulin-like peptides in the fat body, which in turn control the length of the terminal growth period and the larval growth rate respectively (Storelli *et al.*, 2011). In addition to this mechanism, *Drosophila* larval growth is also promoted through other pathways. In particular, *L. plantarum* influences juvenile growth partly through the increased expression of a set of host digestive enzymes in the intestine. As a result, the digestion of dietary proteins into dipeptides and amino acids, and their uptake increase (Combe *et al.*, 2014). In this context the activity of *pbpX2-dlt* operon, which is involved in the bacterial cell wall biogenesis, plays a key role. Specifically, it participates in D-alanine esterification of teichoic acids (TA). It has been demonstrated that the absence of *pbpX2-dlt* operon in *L. plantarum* depletes D-alanine from TA modifying the architecture of the bacterial cell. In this way, the *Drosophila* enterocytes cannot sense the bacterial signal that triggers intestinal peptidase induction and systemic growth (Matos *et al.*, 2017). Moreover, larval growth is also promoted through a pathway in which peptidoglycan is involved. Diaminopimelic acid (DAP) containing peptidoglycan, which is a fundamental cell component of many bacteria, is sensed by peptidoglycan recognition proteins (PGRPs) of the host. This molecular recognition triggers the IMD (Immune

Deficiency)/Relish signaling cascade, a pathway associated to the regulation of processes related to immune responses. As a consequence, the production of peptidase by the host improves the *Drosophila* digestive functions (Combe et al., 2014, Matos et al., 2017).

## 1.4 Experimental evolution

### 1.4.1 Definition, advantages and limits

One of the most successful approaches used to better understand evolutionary processes is experimental evolution. This is defined as the study of evolutionary changes occurring in experimental populations as a consequence of conditions (environmental, demographic, genetic, social and so forth) imposed by the experimenter. It is an alternative research framework that offers the opportunity to study the evolution mechanisms in real time, unlike traditional methods based on patterns of phylogeny, divergence between species or populations, variation within populations, genome structure and genome sequence, which all reflect past evolution (Kawecki et al., 2012). The essence of experimental evolution is conceptually quite simple: a specific population is propagated for many generations in a controlled and reproducible environment, where a biotic or abiotic variable is altered; a sample of the ancestral population and samples from different time points in the experiment are stored indefinitely (i.e.: frozen at -80°C); after population has been propagated for some time, ancestral and derived genotypes can be compared (Garland and Rose, 2009).

The strengths of this approach are replication and control. By replicating the number of populations exposed to the novel environment, it is indeed possible to evaluate the statistical significance of any differences between the experimental and control groups. Thanks to this it also provides rigorous testing of evolutionary hypotheses and theories that formerly were matters only of assumption. Additionally, this method can define the degree of environmental change, limiting it to a single element or to a combination of

elements. In this way, the experimenter can isolate and analyze the adaptive response to specific environmental factors. Because of its emphasis on control and replication, experimental evolution is more suited to laboratory rather than to natural situations (even if this approach has been successfully applied also in the field). Consequently, these experiments sacrifice the ecological realism provided by nature (*Garland and Rose, 2009*). In addition to the simplified nature of laboratory environment, there are also other limitations of experimental evolution. First, some evolutionary processes may be too slow to be seen within the span of a research. Second, the intended regimes can be altered by unexpected factors (i.e.: contamination). Third, laboratory environments often confine mobile animals to small space, changing the context of social and sexual interactions. Moreover, the population genetics of laboratory evolution may differ in important ways from evolution in nature, due for example to the small effective population size (*Kawecki et al., 2012*).

The first to set up an experimental evolution study was William Dallinger, a Liverpool minister and amateur scientist, who was curious to know if the flagellates might be able to adapt to the warming water through natural selection. The experiment lasted seven years, from 1880 to 1886. Raising the temperature of water to 150 degrees Fahrenheit over the course of months, Dallinger found that the flagellates continued to reproduce. He concluded that flagellates had indeed evolved resistance to heat, losing at the same time the ability to survive at cooler temperatures. This experiment happened more than twenty years before the term *gene* was coined. Indeed, experimental evolution did not immediately bloom into a new kind of science: not even Darwin fully appreciated how important this study was to his own theory. Because of this, no one followed up on his work for decades, until Richard Lenski started in 1988 one of the earliest and biggest experimental evolution studies, which is still ongoing after 31 years (*Weber, 2012*).

#### **1.4.2 The use of microorganisms in experimental evolution**

In last decades, a field of research has developed around the idea of using microorganisms, and not only plants or vertebrates, to study evolution in action, due to the fact that many microbes are of great importance to humans not only as pathogens

but for numerous essential ecosystem services. So, it became fundamental to understand the dynamics of microbial evolution. Furthermore, microbes offer numerous advantages in experimental evolution studies, summarized in the following list (*Elena and Lenski, 2003*):

- they are easy to propagate and enumerate;
- they reproduce quickly allowing experiments to run for many generations;
- small spaces are needed;
- they can be easily stored and later revived for direct comparisons among ancestral and evolved strains;
- asexual reproduction allows the precision of experimental replication;
- asexuality also facilitates fitness measurements;
- environmental variables can be easily manipulated;
- there are abundant molecular and genomic data for many species.

The most important experiment in this context is the one conducted by Lenski, previously mentioned, started in 1988 and still running. This long-term evolution experiment, LTEE for short, has now passed 60.000 bacterial generations. Lenski chose to use *Escherichia coli* for his experiment because it was the best understood microbe known to science and because it reproduces quickly. The initial purpose was to understand the dynamics of adaptation and divergence in twelve populations coming from the same strain, but over time analyses have expanded in order to investigate other aspects such as the evolution of mutation rate, the origin of new functions and so on. The structure of the LTEE is simple: the populations live in a simple environment where glucose represents the limiting resource; every day someone takes 1% of each population from its flask and transfers it to a flask containing fresh medium; the cells then grown until they run out of glucose and then wait until next day, when the process is repeated (*Kawecki et al., 2012, Lenski and Burnham, 2018*). According to the fact that adaptation occurs through mutations that improve the performance of an organism and its reproductive success in a specific environment, after 50.000 generations the evolved bacteria had a fitness of 1.7 relative to the ancestor (*Lenski and Burnham, 2018*).

More broadly, this study and other microbial evolution experiments confirm the following assertions. First of all, fitness gains are initially rapid when a population is introduced into a new environment. Then, microbes continue to improve, although

more slowly, even in a constant environment because beneficial mutations with ever smaller effects become increasingly accessible to selection. Second, the use of replicate populations allows the study of parallel molecular evolution. Third, genetic adaptation to one environment could be associated with fitness loss in other environments. Fourth, rapidly evolving asexual populations have more opportunities to spread, thanks to the beneficial mutation that they occasionally generate. Lastly, in very small populations genetic drift may prevail over natural selection and fitness tends to decline (*Elena and Lenski, 2003*).

#### **1.4.3 *Drosophila/L. plantarum* experimental evolution**

As previously mentioned (see *Interactions with Drosophila*, 1.3.3), specific strains of *L. plantarum* promote larval growth of *Drosophila melanogaster*. In particular, two strains are conceptually important to better understand this mechanism: *L. plantarum* WJL and *L. plantarum* NIZO2877. The first is a potent growth-promoting strain, originally isolated from the intestine of the fruit flies (*Martino et al., 2015*). The second has been isolated from processed meat in Vietnam; unlike the first, this strain moderately promotes larval growth. However, a recent study has revealed that *Lp*<sup>NIZO2877</sup> experimentally evolved in symbiosis with *Drosophila*, exhibits the same effects of *Lp*<sup>WJL</sup> after only two fly generations. This means that NIZO2877 is able to adapt in the context of symbiosis leading to a rapid improvement of its beneficial effect in terms of host growth promotion. Comparing the genome of the ancestral (mid-growth promoting) NIZO2877 strain and the NIZO2877-evolved (growth promoting) strain, the authors found a mutation on the acetate kinase A (*ackA*) gene, which was demonstrated to be responsible for the gain of function. This mutation triggers the increased production of N-acetylated amino acids, including N-acetyl-glutamine, a compound that has been shown to confer improved growth-promoting capabilities to the evolved strain. Remarkably, the mutations of the *ackA* gene appeared both in strains evolving with the host and also in strains evolving in the host nutritional environment, without their host. This means that ***L. plantarum* improves its symbiotic benefit by adapting to the host diet**. The authors thus demonstrated that the host **nutritional environment**, rather than



the host *per se*, **is the first step in the emergence and perpetuation of facultative animal-microbe symbiosis**. Interestingly, also WJL strain has been found to bear two nucleotide substitutions in *ackA* gene, compared to the ancestral NIZO2877 strain, which might concur with its beneficial effect (*Martino et al., 2018*).

If the role of the diet is now clear, the influence of the host in shaping the evolution of its gut microbes remains to be elucidated. We hypothesize that the evolution of host/microbe mutualism is temporally shaped first by the bacterial adaptation to the host nutritional environment and then to the host. To address this hypothesis, in the present work, two parallel experimental microbiota evolution studies have been set up at the same time starting from the ancestor strain NIZO2877 +/- *Drosophila*. In this way, we will be able to assess if and how *Drosophila* has any effect on the tempo of evolution of its gut microbes, thus **revealing role of the animal host** in the evolution and perpetuation of host/microbiota symbiosis.

## 2. AIM

It has been demonstrated that the host diet represents the predominant driving force in the emergence and perpetuation of facultative animal-microbe symbioses. This means that the nutritional environment, rather than the host itself, is the first selective factor that acts on microbiota evolution, determining its beneficial effect.

It is now necessary to clarify the role of the host in shaping the evolution of its gut microbes. We hypothesize that the evolution of host/microbe mutualism is temporally shaped first by the bacterial adaptation to the host nutritional environment and then to the host, which could play a role on the evolutionary rate.

To address this hypothesis, in the present work, two parallel experimental microbiota evolution studies have been set up at the same time starting from the ancestor strain NIZO2877 with and without its animal host, *Drosophila melanogaster*. In this way, we will be able to assess if and how *Drosophila* has any effect on the tempo of evolution of its gut microbes, thus revealing the role of the animal host in the evolution and perpetuation of host/microbiota symbiosis.

### 3. MATERIALS AND METHODS

#### 3.1 The *Drosophila melanogaster* food

##### 3.1.1 Media composition

The first step for setting up an experimental evolution protocol with fruit flies as animal model is the preparation of their food, which is poured into proper tubes where the insects can develop.

In this context, the recipe for the production of the food differs depending on the type of use of the flies. Thus, it is useful to consider three different recipes corresponding to as many *Drosophila* stocks:

- diet A. It is specific for flies with their own bacterial flora, called CR (conventionally reared):

COMPONENT	QUANTITY (per liter)
Deionized water	1 l
Agar	7.14 g
Cornmeal	80 g
<b>Yeast</b>	<b>50 g</b>
Moldex	5.2 g
Propionic acid	4 ml

Table 2: food of CR flies

- diet B. It is specific for germ-free flies, called GF. These flies derive from eggs laid from the CR stock, which have been sterilized (see *Embryo bleaching protocol*, 3.2), and they are kept on medium supplemented with antibiotic in order to maintain the stock in sterile conditions. GF flies will be used to perform the mono-association with *L. plantarum*:

COMPONENT	QUANTITY (per liter)
Deionized water	1 l
Agar	7.14 g
Cornmeal	80 g
<b>Yeast</b>	<b>50 g</b>
Moldex	5.2 g
Propionic acid	4 ml
<b>Antibiotic</b>	<b>4 ml</b>

Table 3: food of GF flies

- diet C. It is specific for flies associated with *L. plantarum*. These flies will be used in experimental evolution:

COMPONENT	QUANTITY (per liter)
Deionized water	1 l
Agar	7.14 g
Cornmeal	80 g
<b>Yeast</b>	<b>8 g</b>
Moldex	5.2 g
Propionic acid	4 ml

Table 4: food of flies associated with *L. plantarum*

While the cornmeal acts as source of carbohydrates, the inactivated dried yeast is the protein source. Moldex (methyl 4-hydroxybenzoate sodium salt) and propionic acid are used to prevent fungal and microbial contamination, respectively. Regarding the antibiotic, a mixture of four molecules is needed in diet B to maintain sterility in the tubes containing GF flies:

- 50 µg/mL Ampicilline;
- 50 µg/mL Tetracycline;
- 50 µg/mL Kanamycine;
- 15 µg/mL Erythromycine (or 50 µg/mL Chloramphenicol).

This last consideration summarizes the only difference between the first two diets. On the other hand, diet A and B differ from C because the latter has a lower concentration of yeast. This detail is particularly important and has the role of putting flies in a condition of nutrient deprivation. Indeed, as specified in the Introduction, *L. plantarum* confers benefit to the host upon nutrient scarcity and not in a nutrient rich environment.

### **3.1.2 Food preparation procedure**

The first thing to do when cooking food for flies is to weigh the ingredients according to the number of *Drosophila* tubes needed, adding 50 ml of water to the quantity needed for the tubes as these will evaporate during the cooking process. Water is heated in a becher with the help of a laboratory stove, and, as soon as it boils, agar must be added slowly. It is important to let it boil for ten minutes, taking care that it is always kept mixed thanks to a magnet. After this time, the becher must be removed from the stove. At this point, the yeast and the cornmeal can be added to the mixture keeping it constantly mixed, for example using a graduate pipette, in order to avoid the formation of lumps. Subsequently, when temperature drops below 65°C, Moldex and propionic acid are added. In the case of diet B, the antibiotic is also added after thawing it from -20°C freezer where it is stored. In this case it is essential to check that the food temperature has dropped below 50°C to prevent the degradation of the antibiotic. Later it is necessary to pour the food in the *Drosophila* tubes or cages where flies can lay the eggs and grow. About 10 ml of food is usually added per tube. This last operation must be performed under the hood to avoid contamination deriving from the environment, which could affect the development of the host. After letting the food to solidify, the tubes are closed using cotton flugs.

It is good practice to prepare the food as close as possible to its use, to avoid the influence of environmental factors (i.e.: contamination, condensation, etc.). However, if the tubes are not used immediately, they can be stored in fridge at 4°C. Then, at the time of their use they are placed in an incubator at 25°C (*Panasonic MIR-154 Cooled Incubator*), the temperature at which flies develop.

### 3.2 Embryo bleaching protocol

In order to investigate the symbiosis between *Drosophila* and *Lactobacillus plantarum*, the use of gnotobiotic fruit flies, that is flies associated with the bacterial strain or community of interest, is essential. To obtain them it is first necessary to produce germ-free flies.

The initial step to develop sterile embryos is the preparation of egg-laying cages, in which 400 ml/l of fruit juice (apple, orange, grape, etc.) and 30 g/l of Agar are contained. Moreover, the addition of yeast paste, prepared mixing yeast powder with enough water to make it look like a paste, in the center of the caps helps the flies lay more eggs. Then, four to ten day-old flies must be transferred from their tubes to the cages, taking into account that approximately four medium-sized tubes per cage are required to have enough flies. It is advisable to carry out the latter operation in the afternoon of the day 0 in order to avoid having too many larvae (which cannot be sterilized) the next day. Subsequently, flies are incubated at 25°C overnight.

The first job to do in the morning of the following day (day 1) is to remove from cages the larvae that have already hatched with the help of the microscope. Then, working under a safety hood to maintain sterility, three trays containing 2.7% bleach, 70% ethanol and sterile water are prepared. At the same time, eggs must be removed from the medium and placed in a sieve, by gently scraping them with a spatula after adding sterile water to the caps. Next, the immersion of the sieve in every tray for two minutes is required, taking care to drain it at each step and respecting the following order: bleach, ethanol and sterile water. Using a spatula previously sterilized with 70% ethanol, the clean embryos are collected and ready to be placed in new tubes containing normal medium (50 g yeast) supplemented with antibiotics (diet B). Finally, eggs are incubated at 25°C with a twelve-hour light cycle.

At the time of emergence of the first adults (around day 10), a sterility test on the flies must be performed to assess whether the described procedure was carried out correctly. For this purpose, a piece of food is taken from the tubes using a sterile loop, and put it into 2 ml microtubes containing 1 mm beads. After adding 1 ml of phosphate buffered saline (PBS), the food is homogenized using *Tissue Lyser II (Qiagen)*, setting a

frequency of 30 Hz for one minute. Then, 150  $\mu$ l of the contents of the tubes are plated on Luria Bertani (LB) Agar medium. Finally, the plates are incubated for 48 hours at a temperature of 37°C. The complete absence of bacteria on the plates indicates that the germ-free stock has been successfully obtained. A new GF stock must be set up every 4-5 months.

### 3.3 Experimental evolution design

In this work, two parallel experimental evolution protocols have been developed with the aim of studying the evolution of the *Drosophila*/microbiota relationship, in the presence of the host (*niche*) and in its absence (*medium*), as shown in Figure 2. In this way, it is possible to understand whether the evolution of *L. plantarum* is due to the host or to its diet.

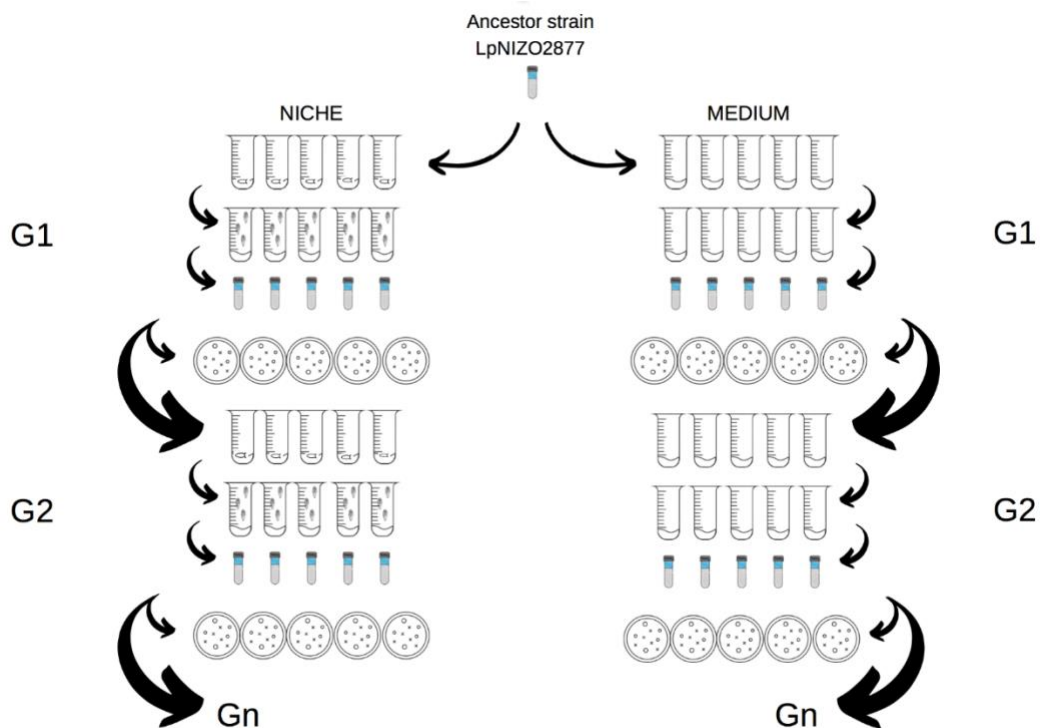


Figure 2: schematic representation of the experimental evolution study

Ancestral culture. The preparation of an experimental evolution study using microorganisms begins with the culture of the strain of interest in a specific medium. In this case, the ancestor strain *Lp*<sup>NIZO2877</sup> is cultivated in 10 ml of MRS broth on day 0 of the study. The abbreviation MRS stands for De Man, Rogosa and Sharpe and indicates a selective culture medium, developed in 1960, specific for *Lactobacillus* spp., which can be solid or liquid. This inoculum must be incubated at 37°C overnight. On day 0 it is also necessary to cook 10 tubes and 2 cages (diameter=55 mm) of food containing 8 g/l of yeast (diet C). Additionally, germ-free flies are inserted in the two cages so that they lay the eggs. This last step must be performed in the afternoon of the day 0, in order to avoid the presence of too many larvae the following day, respecting in this way the life cycle of *Drosophila*. Then, the cages are incubated at 25°C overnight.

On the day after (day 1), the mono-association is performed. It represents therefore the first day of the first generation (G1) of the experiment.

Preparation of Niche and Medium tubes. The initial step is to cut five small pieces of medium containing 40 eggs from the cages prepared on day 0 (where fruit flies laid their eggs during the night) with the help of scalpel and microscope. The five parts of medium must be placed in five of the ten tubes previously prepared. These are the *niche* tubes, while the other five are called *medium*. Unlike the niche tubes, the medium tubes are characterized by the absence of flies: thus, they will contain only food and *L. plantarum*. This difference permits to investigate how the microbiota evolve with and without its host, thus assessing the influence of the animal partner on the evolution of its gut microbes.

Purification of the ancestral culture and inoculum. Regarding the inoculum, it is necessary to take 1 ml of the overnight bacterial culture (*Lp*<sup>NIZO2877</sup>) and put it in a sterile Eppendorf. After it has been centrifuged at 4000 rpm for 10 minutes, the supernatant must be removed in order to re-suspend the pellet in 1 ml of PBS. Since the bacterial concentration is about 10<sup>9</sup> CFU/ml, serial dilutions are needed to bring it to 10<sup>6</sup> CFU/ml. Repeating these steps at least three times is required to get enough inoculum for the ten tubes. Subsequently, 150 µl of the dilution (containing then about 10<sup>5</sup> total CFUs) are added to each tube. All the last operations must be done under the hood to avoid any contamination. Finally, the tubes are placed at 25°C.



When pupae begin to develop, it is fundamental to count them every day in the niche tubes order to evaluate their developmental time. As soon as there are at least 15 pupae in all niche tubes, a small amount of food is transferred, using a sterile loop, from each of the ten tubes into as many new microtubes where beads were previously introduced. After adding 1 ml of MRS broth, the content of the microtubes is dissolved with the help of the *Tissue Lyser II* (30 Hz for 1 minute). Assuming that the bacterial load in the microtubes is about  $10^7$  CFU/ml, four serial dilutions are needed to be able to plate a concentration of  $10^2$  total CFUs. So, 100  $\mu$ l of the last dilution ( $10^3$  CFU/ml) deriving from each microtube are plated on MRS agar (10 plates in total). The plates are then incubated at 37°C for 48 hours, after which colonies are counted. Finally, 200  $\mu$ l of sterile glycerol (80%) are added in each microtube in order to store the bacteria at -80°C. The preparation of the subsequent generations (G2, G3, etc.) reflects the described procedure and starts from the frozen microtubes obtained in the previous generation. Depending on their bacterial concentration, an adequate number of dilutions is performed in order to add  $10^5$  CFUs to the new medium and niche tubes for each of the following generations. The starting inoculum consists of  $10^5$  CFUs in such a way that *L. plantarum* can reach the exponential phase, which is central for the bacterial evolution, inside the tubes. From a practical point of view, the key step in this experimental evolution study is the transfer of the food during each generation, which represents the evolutionary bottleneck. Indeed, no further inoculation of the ancestor strain *Lp*<sup>NIZO2877</sup> must be performed because evolved bacteria are propagated along with the food.

### 3.4 Analysis of *Drosophila* developmental time

At the end of each *Drosophila* generation over the course of experimental evolution, the NIZO2877-evolved strains are tested in order to investigate their effect on the host developmental time, as a measure of bacterial phenotypic evolution.

First, the homogenized media (both in niche and medium setup) are plated on MRS agar, after proper dilution and the plates are incubated for 48 hours at 37°C. There are

therefore 10 total plates, five obtained starting from the niche tubes and five from the medium ones.

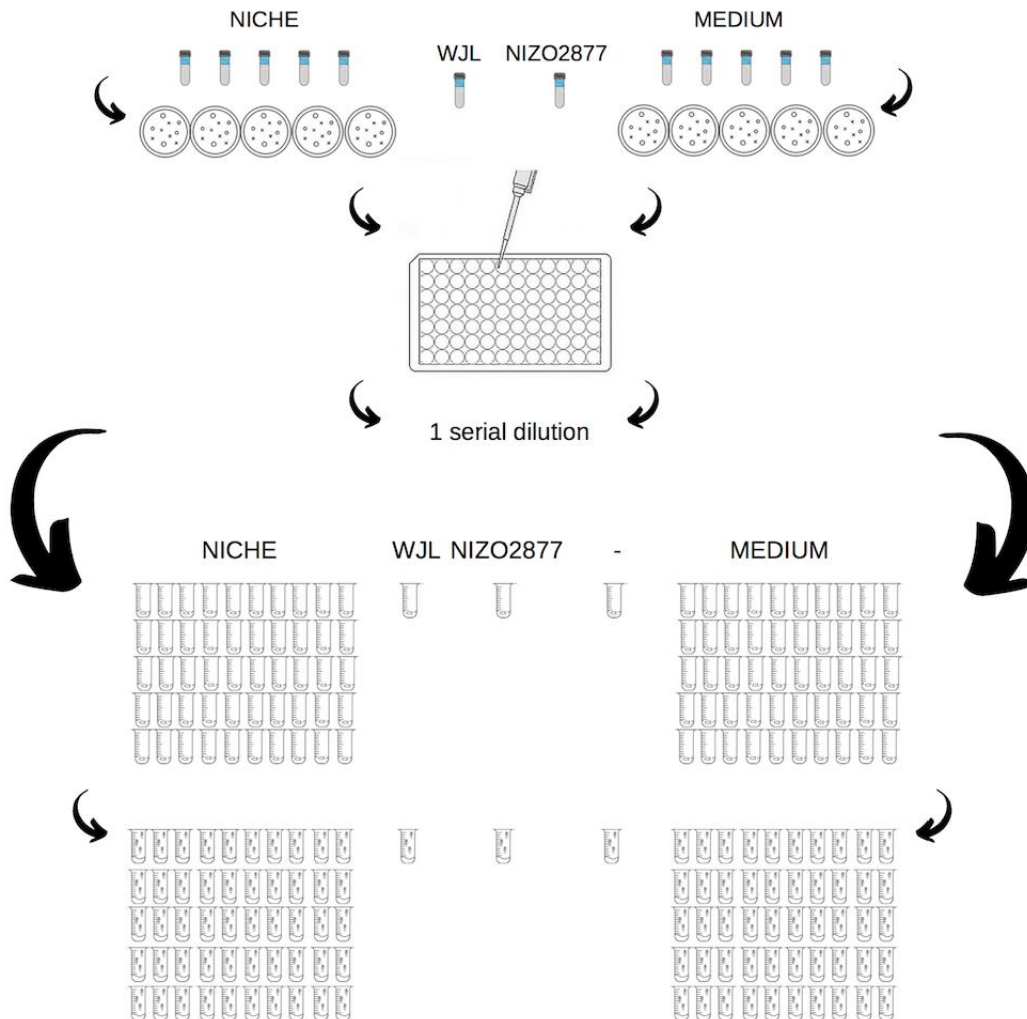


Figure 3: schematic representation of the analysis of *Drosophila* developmental time

**Preparation of bacterial cultures.** At day 0 of the experiment, 10 colonies are randomly selected from each plate and re-suspended in 1.8 ml of MRS broth, using a 96-well plate and 7 microtubes (including the negative control and WJL – growth promoting *L. plantarum* strain - and NIZO2877 – ancestor - positive controls). Moreover, 103 tubes (considering 100 strains from the ten plates + NIZO2877, WJL and a negative GF control) and an adequate number of cages with 8g/l of yeast must be prepared.

**Preparation of egg-laying cages.** In the afternoon, germ-free flies are placed to lay eggs inside the cages.

**Mono-association.** On the next day (day 1), a piece of food containing 20 eggs is transferred from cages to each of the 102 tubes. Regarding the inoculum, the following

steps are needed. First, 1 ml of the overnight culture must be transferred from each well of the plate into 102 sterile Eppendorf. Then, after they have been centrifuged at 4000 rpm for 10 minutes, the supernatant must be removed in order to re-suspend the pellet in 1 ml of PBS. Now, bacterial load is about  $10^9$  CFU/ml. So, a serial dilution in 900 ml of PBS is required in order to reach a concentration of  $10^8$  CFU/ml. Finally, 150  $\mu$ l of the dilution are added in each tube, thus containing  $10^7$  total CFUs. Also in this case, the tubes are incubated at 25°C.

Pupae emergence was scored every day until all pupae have emerged.

### 3.5 Bacterial DNA extraction

For bacterial DNA extraction, the *microLYSIS-Plus* protocol developed by *Microzone* is applied. First, the strains are plated on MRS agar, which are then incubated for 48 hours at 37°C. Subsequently, the colonies are dissolved in PCR tubes where 20  $\mu$ l of *microLYSIS-Plus*, a complex solution which releases the DNA from the cells, have been previously added. Finally, the tubes must be inserted in a thermal cycler, setting the following cycle:

- step 1: 65°C for 15 minutes;
- step 2: 96°C for 2 minutes;
- step 3: 65°C for 4 minutes;
- step 4: 96°C for 1 minutes;
- step 5: 65°C for 1 minutes;
- step 6: 96°C for 30 seconds;
- step 7: 20°C hold.

After lysis, the *microLYSIS-Plus*/DNA mixture can be used directly in PCR. Alternatively, it can be stored at -20°C for future use.

### 3.6 PCR amplification and sequencing

PCR amplification and electrophoresis. Starting from the bacterial DNA previously extracted (see 3.5 Bacterial DNA extraction), the *ackA* gene must be amplified using the following primers:

- *ackA\_F1*: 5'-TGAACAAATCCTGAAAGCGT-3' (forward);
- *ackA\_R1*: 5'-ACCATGATCAAAAGCCGTGA-3' (reverse);
- *ackA\_F2*: 5'-GGAAACCATTGGATTCGCTTAA-3' (forward);
- *ackA\_R2*: 5'-GGGGACAAGAGCTGACTTAG-3' (reverse)

with the following combinations:

1. *ackA\_F1/ackA\_R1*
2. *ackA\_F2/ackA\_R2*

Table 5 shows the PCR mixture components, which in total contains 20  $\mu$ l per sample.

COMPONENT	QUANTITY (per sample)
10x PCR buffer	2 $\mu$ l
25 mM dNTPs	0.2 $\mu$ l
Taq DNA polymerase	0.2 $\mu$ l
H <sub>2</sub> O	14.6 $\mu$ l
10 $\mu$ M <i>ackA_F1/ ackA_R1</i>	0.5 $\mu$ l
10 $\mu$ M <i>ackA_R1/ ackA_R2</i>	0.5 $\mu$ l
1:10-diluted DNA	2 $\mu$ l

Table 5: PCR mixture required for the *ackA* gene amplification

After preparing the mixture, samples are placed in a thermal cycler with the following conditions:

- 95°C for 4 minutes;
- 35 cycles of 95°C for 15 s, 57°C for 30 s and 72°C for 60 s;
- 72°C for 7 minutes.

Then, the products are separated by electrophoresis in 1.8% agarose gel, obtained by dissolving the agarose powder in TAE (Tris-Acetate-EDTA) buffer. During the preparation of the gel, the addition of *SYBR Safe (Invitrogen)* is needed to allow the subsequent

visualization of the DNA to UV rays. Next, 5  $\mu$ l of the amplified DNA and 5  $\mu$ l of *Green GoTaq Flexi* loading buffer (*Promega*) are added to each well of the gel. Subsequently, after the run of the DNA through the gel, the fragments are revealed on the transilluminator (*Gel Doc XR<sup>TM</sup>*, *Biorad*).

*Purification and sequencing.* The purification procedure consists of adding 1  $\mu$ l of *ExoSAP-IT<sup>TM</sup>* reagent to 5  $\mu$ l of each sample. Subsequently, samples must be incubated first at 37°C for 15 minutes to degrade remaining primers and nucleotides and then at 80°C for 15 minutes in order to inactivate *ExoSAP-IT<sup>TM</sup>* reagent. The Sanger method is then used for the sequencing of the *ackA* gene, which is performed at *Macrogen Europe* using the primer listed above.

### 3.7 Data analysis

Bacterial growth data in Niche and Medium samples were analyzed using *Prism 8* (*GraphPad*) software (*GraphPad* software, [www.graphpad.com](http://www.graphpad.com)), which analyzes the data (expressed in CFU/ml) through the *unpaired t-test* (significance  $p < 0.05$ ; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ). It compares the means of two unmatched groups assuming that the values follow a Gaussian distribution, focusing on each point of the curve. Moreover, data concerning the bacterial growth are also analyzed through the *ANCOVA Non Parametric* test (significance  $p < 0.05$ ; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ), which compares the function of the two curves (niche e medium), unlike the unpaired t-test.

Data regarding the developmental time of *Drosophila melanogaster* have been analyzed through a dedicated script previously developed on *Rstudio* software (*RStudio* Team, [www.rstudio.com](http://www.rstudio.com)) that calculates the day when fifty percent of the pupae emerged. It takes as input a table with the number of pupae emerged every day for each condition and calculates with a local linear regression the day when fifty percent of the pupae emerged (D50). These data have been also converted in z-scores. In addition, the unpaired t-test has been used to compare the DeltaD50 (difference between the D50 relative to the ancestral and the evolved strain) obtained from niche and medium tubes.

## 4. RESULTS

### 4.1 Phenotypic evolution of *Lp*<sup>NIZO2877</sup>

In order to analyze the phenotypic evolution of *L. plantarum* NIZO2877, two parameters were considered: bacterial growth and host developmental time. These data were collected at the end of each generation of the experimental evolution.

#### 4.1.1 *L. plantarum* growth

Bacterial growth was monitored during the experimental evolution study, both in presence (niche) and in absence (medium) of the host. This allows to verify whether the presence of the host affects the evolution of bacterial growth and adaptation to the environment. A significant difference has been detected between the two setups, showing that **bacterial growth was overall higher when the host was not present** (Medium setup, Figure 4A - Ancova). However, when bacterial growth was analyzed separately at the end of each generation, we detected a **significant difference between the two setups** (with bacterial growth higher in the Medium setup) **only at the end of Generation 7** (Figure 4A – unpaired t test, Table 6).

Fly Gen.	1	2	3	4	5	MEAN	DEV.ST	1	2	3	4	5	MEAN	DEV.ST
	NICHE	NICHE	NICHE	NICHE	NICHE			MEDIUM	MEDIUM	MEDIUM	MEDIUM	MEDIUM		
<b>G1</b>	1.10x10 <sup>7</sup>	2.70x10 <sup>7</sup>	2.20x10 <sup>7</sup>	2.90x10 <sup>7</sup>	5.70x10 <sup>7</sup>	2.92x10 <sup>7</sup>	1.70x10 <sup>7</sup>	3.20x10 <sup>5</sup>	7.40x10 <sup>6</sup>	4.60x10 <sup>6</sup>	2.90x10 <sup>5</sup>	2.80x10 <sup>6</sup>	3.08x10 <sup>6</sup>	3.02x10 <sup>6</sup>
<b>G2</b>	1.10x10 <sup>6</sup>	2.70x10 <sup>6</sup>	1.37x10 <sup>7</sup>	1.79x10 <sup>7</sup>	1.00x10 <sup>7</sup>	9.08x10 <sup>6</sup>	7.15x10 <sup>6</sup>	4.70x10 <sup>6</sup>	1.35x10 <sup>7</sup>	1.03x10 <sup>7</sup>	8.00x10 <sup>6</sup>	1.64x10 <sup>7</sup>	1.06x10 <sup>7</sup>	4.57x10 <sup>6</sup>
<b>G3</b>	1.89x10 <sup>7</sup>	2.88x10 <sup>7</sup>	2.29x10 <sup>7</sup>	4.55x10 <sup>7</sup>	1.65x10 <sup>7</sup>	2.65x10 <sup>7</sup>	1.16x10 <sup>7</sup>	1.65x10 <sup>7</sup>	2.05x10 <sup>8</sup>	1.45x10 <sup>8</sup>	1.53x10 <sup>7</sup>	1.64x10 <sup>7</sup>	7.96x10 <sup>7</sup>	8.96x10 <sup>7</sup>
<b>G4</b>	1.00x10 <sup>6</sup>	3.10x10 <sup>6</sup>	1.10x10 <sup>7</sup>	5.80x10 <sup>6</sup>	3.18x10 <sup>7</sup>	1.05x10 <sup>7</sup>	1.25x10 <sup>7</sup>	2.07x10 <sup>7</sup>	2.64x10 <sup>7</sup>	7.14x10 <sup>7</sup>	3.73x10 <sup>7</sup>	6.45x10 <sup>7</sup>	4.41x10 <sup>7</sup>	2.27x10 <sup>7</sup>
<b>G5</b>	2.67x10 <sup>7</sup>	1.24x10 <sup>7</sup>	4.45x10 <sup>7</sup>	1.85x10 <sup>7</sup>	3.47x10 <sup>7</sup>	2.74x10 <sup>7</sup>	1.28x10 <sup>7</sup>	2.28x10 <sup>7</sup>	6.30x10 <sup>7</sup>	5.74x10 <sup>7</sup>	3.29x10 <sup>7</sup>	3.45x10 <sup>7</sup>	4.21x10 <sup>7</sup>	1.72x10 <sup>7</sup>
<b>G6</b>	2.10x10 <sup>6</sup>	4.40x10 <sup>6</sup>	5.40x10 <sup>6</sup>	3.20x10 <sup>6</sup>	3.40x10 <sup>6</sup>	3.70x10 <sup>6</sup>	1.25x10 <sup>6</sup>	1.47x10 <sup>7</sup>	6.10x10 <sup>6</sup>	1.82x10 <sup>7</sup>	1.02x10 <sup>7</sup>	5.90x10 <sup>6</sup>	1.10x10 <sup>7</sup>	5.39x10 <sup>6</sup>
<b>G7</b>	1.00x10 <sup>6</sup>	1.70x10 <sup>6</sup>	5.00x10 <sup>5</sup>	3.10x10 <sup>6</sup>	2.70x10 <sup>6</sup>	1.80x10 <sup>6</sup>	1.10x10 <sup>6</sup>	5.40x10 <sup>6</sup>	4.90x10 <sup>6</sup>	7.90x10 <sup>6</sup>	6.20x10 <sup>6</sup>	7.70x10 <sup>6</sup>	6.42x10 <sup>6</sup>	1.34x10 <sup>6</sup>
<b>G8</b>	1.20x10 <sup>6</sup>	1.60x10 <sup>6</sup>	1.20x10 <sup>6</sup>	3.70x10 <sup>6</sup>	2.10x10 <sup>6</sup>	1.96x10 <sup>6</sup>	1.04x10 <sup>6</sup>	1.70x10 <sup>6</sup>	1.05x10 <sup>7</sup>	4.20x10 <sup>6</sup>	1.50x10 <sup>6</sup>	3.50x10 <sup>6</sup>	4.28x10 <sup>6</sup>	3.66x10 <sup>6</sup>
<b>G9</b>	4.70x10 <sup>5</sup>	3.90x10 <sup>5</sup>	9.60x10 <sup>5</sup>	6.80x10 <sup>5</sup>	2.70x10 <sup>5</sup>	5.45x10 <sup>5</sup>	2.72x10 <sup>5</sup>	1.00x10 <sup>6</sup>	3.50x10 <sup>6</sup>	8.30x10 <sup>5</sup>	5.40x10 <sup>5</sup>	6.50x10 <sup>5</sup>	1.30x10 <sup>6</sup>	1.24x10 <sup>6</sup>
<b>G10</b>	1.70x10 <sup>6</sup>	1.50x10 <sup>6</sup>	6.00x10 <sup>5</sup>	1.30x10 <sup>6</sup>	1.20x10 <sup>6</sup>	1.26x10 <sup>6</sup>	4.16x10 <sup>5</sup>	1.00x10 <sup>5</sup>	2.51x10 <sup>7</sup>	7.08x10 <sup>7</sup>	9.90x10 <sup>6</sup>	2.00x10 <sup>3</sup>	2.12x10 <sup>7</sup>	2.96x10 <sup>7</sup>

Table 6: number of colonies (CFU/ml) obtained from Niche and Medium setup during the course of the experimental evolution when there were at least 15 pupae for each tube

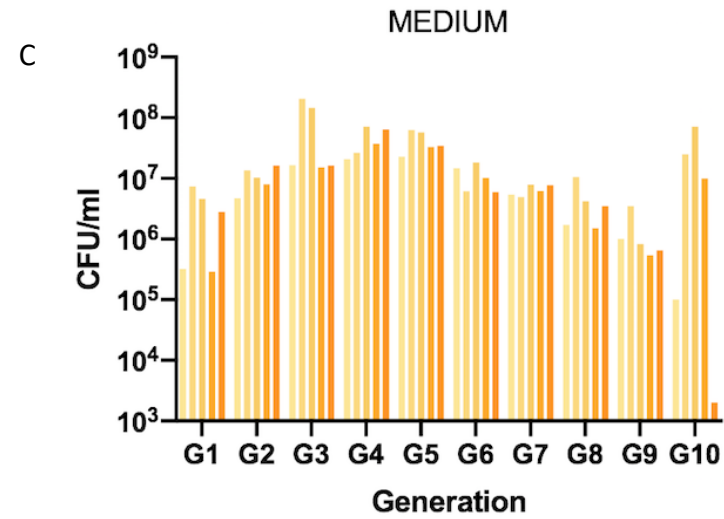
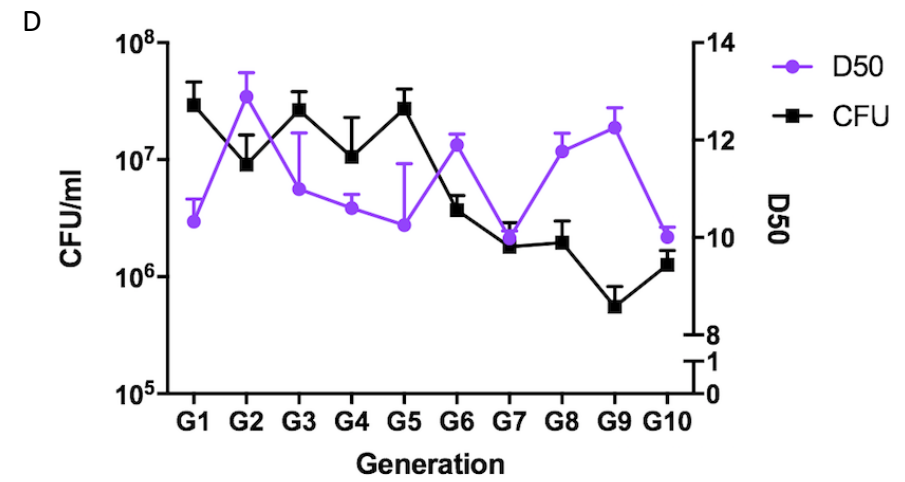
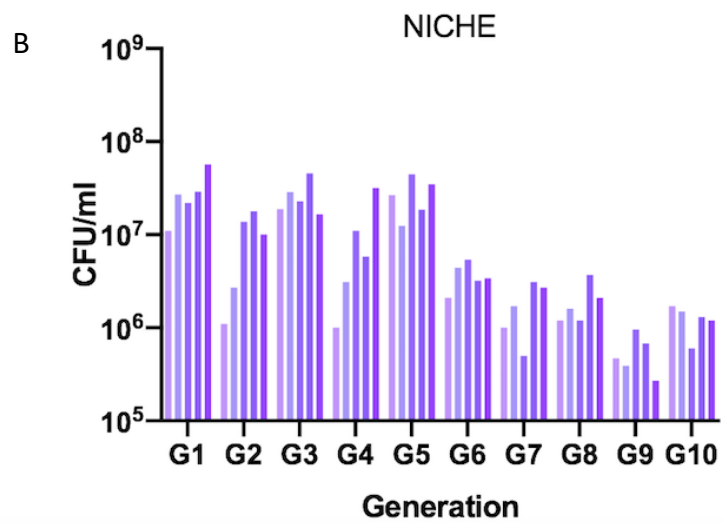
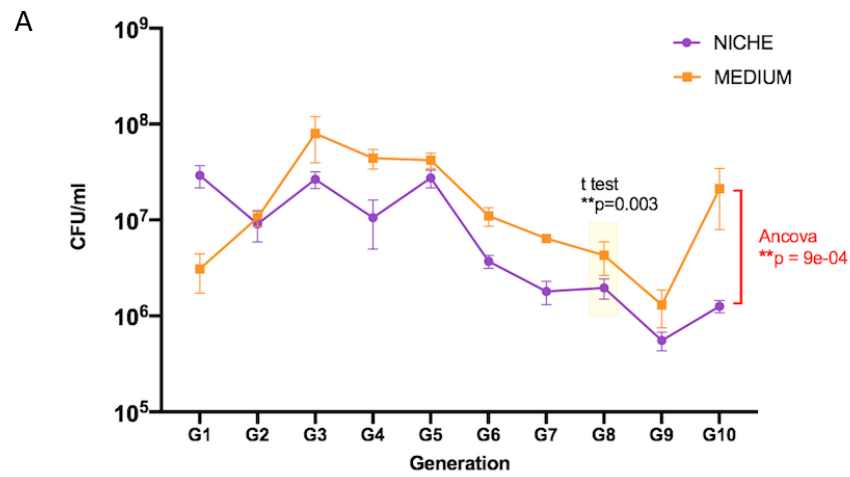


Figure 4: (A) The curves represent *L. plantarum* growth in the Niche and Medium setup. Each point represents the mean with the standard error of the mean (SEM) for each generation; (B) Bacterial CFU of each replicate of the experimental evolution study for the Niche (B) and Medium (C) setup.; (D) Comparison between D50 (Figure 4A) and bacterial growth in the Niche setup



#### 4.1.2 *L. plantarum* growth promotion

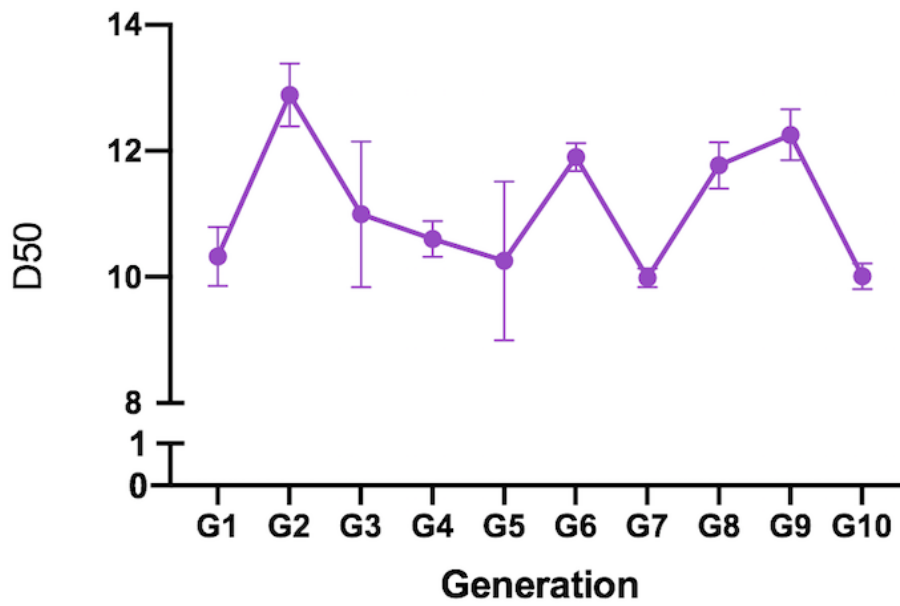
In order to test the evolution of *L. plantarum* growth-promotion, we analyzed the developmental time of *D. melanogaster* larvae associated with *Lp*<sup>NIZ02877</sup>-evolved strains (Niche setup) during each fly generation of the experimental evolution study. To this end, we used the D50 parameter, that is the day when half of the *D. melanogaster* larvae turn into pupae (Table 7).

<b>Fly Generation</b>	<b>1 NICHE</b>	<b>2 NICHE</b>	<b>3 NICHE</b>	<b>4 NICHE</b>	<b>5 NICHE</b>	<b>MEAN</b>	<b>DEV.ST</b>
<b>G1</b>	10.46	10.62	9.57	10.76	10.21	10.32	0.47
<b>G2</b>	12.57	12.45	12.57	13.50	13.36	12.89	0.50
<b>G3</b>	9.83	9.67	11.58	11.73	12.15	10.99	1.15
<b>G4</b>	10.52	10.53	10.24	10.72	11.00	10.60	0.28
<b>G5</b>	9.58	9.38	12.19	9.25	10.90	10.26	1.26
<b>G6</b>	11.81	11.63	11.82	12.10	12.15	11.90	0.22
<b>G7</b>	9.85	10.17	9.82	10.09	10.00	9.99	0.15
<b>G8</b>	12.23	12.03	11.30	11.57	11.72	11.77	0.37
<b>G9</b>	11.58	12.25	12.61	12.34	12.50	12.26	0.40
<b>G10</b>	9.96	10.24	9.70	10.03	10.11	10.01	0.20

Table 7: D50 obtained from the niche tubes during the experimental evolution study

Figure 5 shows the developmental time of the flies throughout the entire experimental evolution study.

A



B

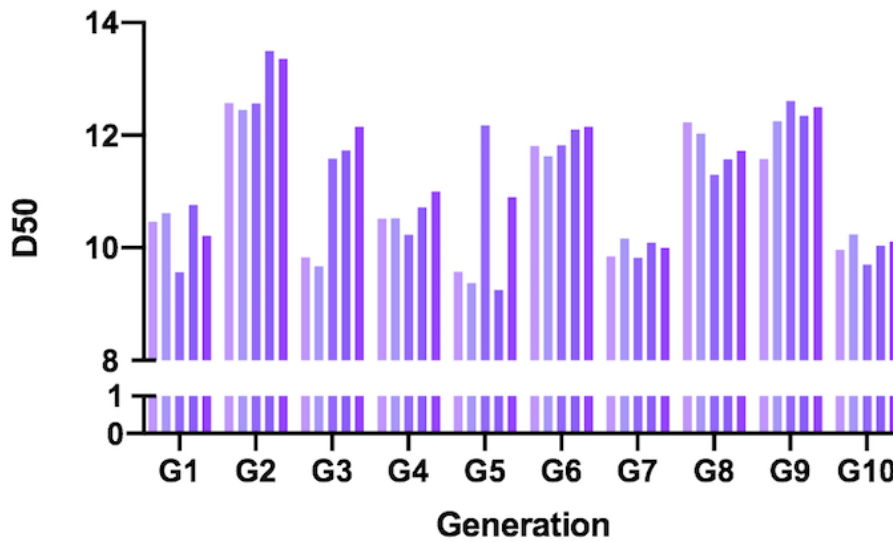


Figure 5: D50 values over the experimental evolution study. Figure 5A shows the trend of the D50 over time (mean with standard deviation -SD-), Figure 5B reports the data of the five replicates separately.

#### 4.1.3 Comparison of the developmental time of Niche- and Medium- evolved strains

With the aim of investigating whether the presence of the host affected the phenotypic evolution of *Lp*, and specifically of its growth promoting effect, single strains isolated at the end of each *Drosophila* generation (From Generation 2 to Generation 6) from both setup (Medium and Niche) have been tested. Specifically, this allows to compare strains that have been isolated at the same time, thus being at the same evolutionary stage, that only differed in the presence of their host

Figure 6 illustrates the distribution of the  $Lp^{NIZO2877}$ -evolved strains obtained during the developmental time analysis compared to their ancestor ( $Lp^{NIZO2877}$ ), a growth-promoting *Lp* strain ( $Lp^{WJL}$ ) and to germ-free conditions (GF). Two panels are present regarding generation 4. This is due to the fact that the eggs laid by the flies during the first experiment (panel C) were not sufficient for all the samples. As a consequence, the experiment was set up in two stages.

In order to understand whether Medium- or Niche-evolved strains showed a faster improvement in the growth promoting capabilities, the difference between the D50 of the ancestral *Lp* strain (mid-growth promoting) and the D50 of each of the evolved strains analyzed ( $\Delta D50$ ) has been calculated and compared between the two setups for each Generation (Figure 7, Table 8). A statistical difference between the *Lp* strains evolved in the Medium and Niche setup is visible in generations 2 and 6. Specifically, in Generation 2, the strains isolated from the Medium setup exhibited significantly better growth-promoting abilities (\*\*  $p=0.0013$ ). From G3 to G5, no significant difference has been detected, while, in G6, the strains isolated from the Niche resulted to be significantly better than the Medium-isolated strains in terms of growth-promoting abilities (\*\*  $p=0.0034$ ).

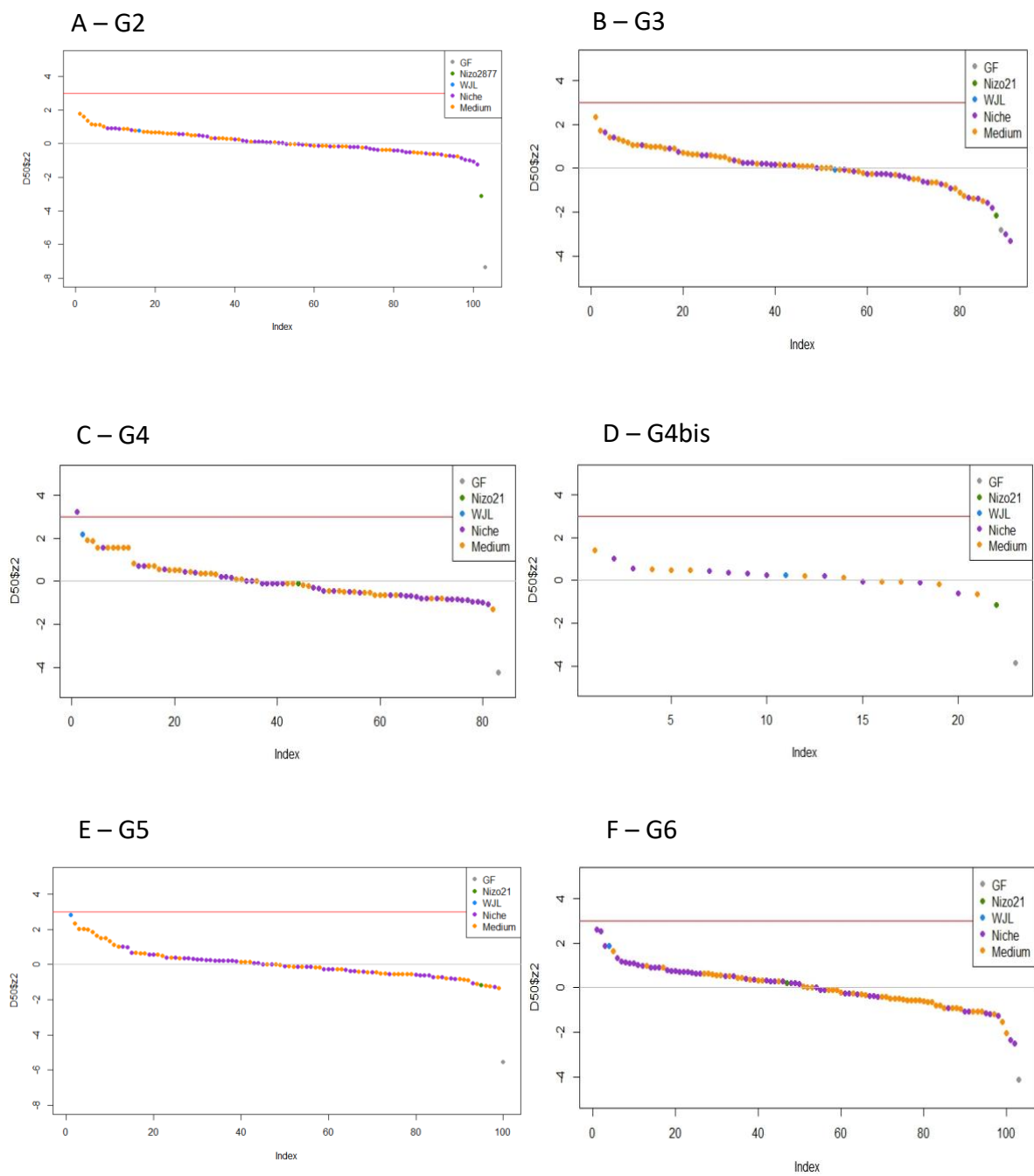


Figure 6: distribution of Niche- and Medium-derived samples on the basis of their Z-score. In each graph germ-free (GF), WJL and ancestor NIZO2877 (NIZO21) are reported as controls. Every graph refers to a specific generation (G).

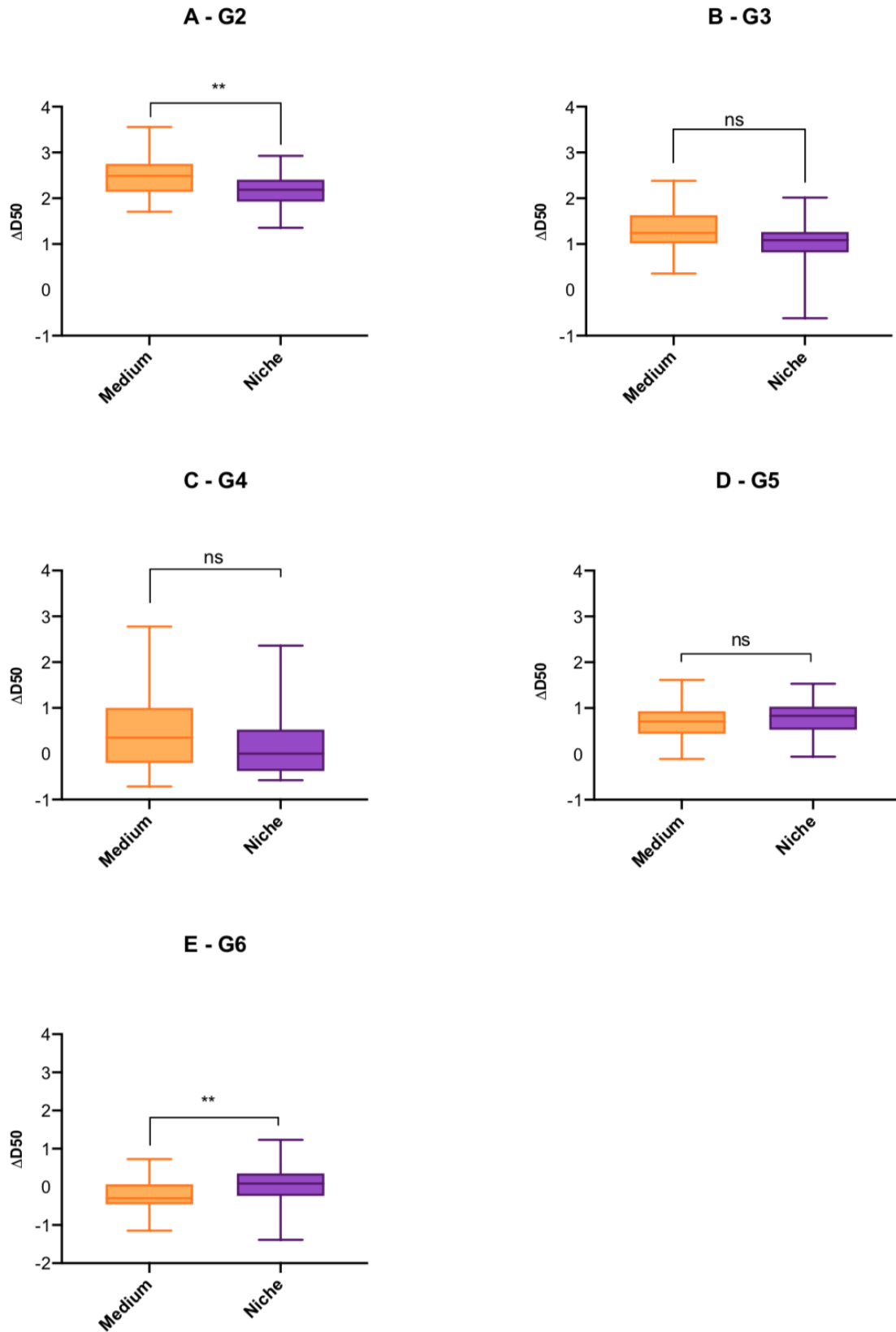


Figure 7: comparison of niche- and medium- derived samples on the basis of their  $\Delta D50$ , using the unpaired t-test.

	MEAN NICHE	DEV. ST NICHE	MEAN MEDIUM	DEV.ST MEDIUM
<b>G2</b>	9.31	0.30	9.02	0.47
<b>G3</b>	9.64	0.54	9.38	0.43
<b>G4</b>	9.11	0.48	8.86	0.55
<b>G5</b>	8.61	0.36	8.76	0.43
<b>G6</b>	8.81	0.52	9.08	0.36

Table 8: mean values and standard deviations of D50 (day) obtained from the analysis of developmental time

With the aim of understanding whether specific replicates of evolution repeatedly showed the *Lp* strains with the highest growth-promoting capabilities, the ranking of the first ten samples that present the greater Z-score for each generation is reported (Table 9). Sample 4M is the only one that is present in each generation. Regarding the samples derived from the Niche setup, 1N is the most repeated, being in all generations except for the fifth.

	1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>	4 <sup>th</sup>	5 <sup>th</sup>	6 <sup>th</sup>	7 <sup>th</sup>	8 <sup>th</sup>	9 <sup>th</sup>	10 <sup>th</sup>
<b>G2</b>	2M	1M	1M	4M	1M	1M	5M	1N	4N	1N
<b>G3</b>	1M	3M	4N	2M	1N	1M	3M	2M	4M	2N
<b>G4</b>	1N	WJL	3M	4M	4M	3N	1M	1M	2M	4M
<b>G5</b>	WJL	5M	3M	5M	3M	5M	5M	5M	4M	3M
<b>G6</b>	2N	1N	2N	WJL	4M	3N	2N	2N	3N	3N

Table 9: distribution of the first ten samples considering their Z-score (M=medium, N=niche; 1, 2, 3, 4, 5 represents the replicate from which the strain was obtained)

## 4.2 Genotypic evolution of *Lp*<sup>NIZO2877</sup>

To investigate whether the *Lp*<sup>NIZO2877</sup>-evolved strains show any mutations in the *ackA* gene that was previously found responsible for the improvement of *L. plantarum* growth-promoting capabilities, amplification and sequencing of this gene were conducted. To this end, a total of twenty-nine evolved strains selected among the best performing strains (Table 9 - 14 Niche samples, 15 Medium samples) were analyzed (Table 10).

Figure 8A shows the percentages of the strains that exhibited mutations in the *ackA* gene. 24 out of 29 strains, corresponding to 83%, showed mutations. Thirteen (54%) of these derived from the Niche setup, while the other eleven (46%) from the Medium setup (Figure 8B). Every mutation consisted of a single nucleotide polymorphism (SNP) with the exception of sample E07, in which there is an insertion of 12 bp.

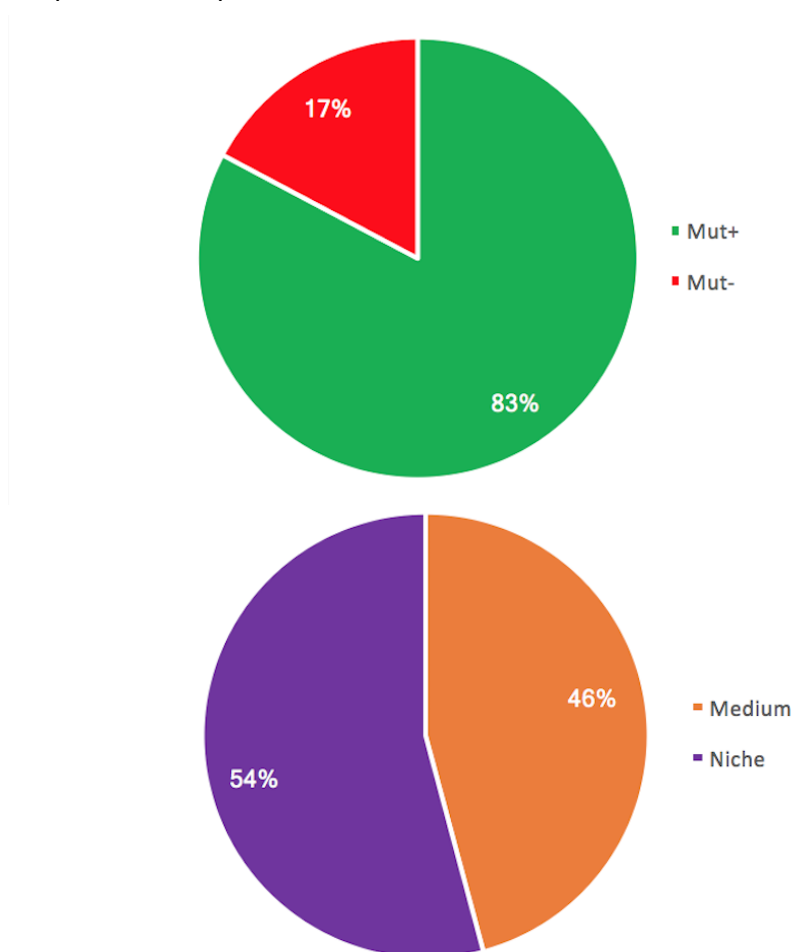


Figure 8: percentages of mutated and not mutated strains (8A) in the *ackA* gene; percentages of medium- and niche-derived strains that show mutations (8B)

Strain	Generation	Setup	Mutation	Type
D05	2	Niche	+	SNP
A03	2	Niche	+	SNP
F07	2	Medium	+	SNP
F03	2	Medium	+	SNP
G08	2	Medium	+	SNP
A09	3	Niche	+	SNP
D03	3	Niche	-	
B03	3	Niche	+	SNP
G09	3	Medium	+	SNP
H02	3	Medium	+	SNP
F06	3	Medium	+	SNP
C03	4	Niche	+	SNP
C04	4	Niche	+	SNP
A01	4	Niche	+	SNP
A11	4	Medium	+	SNP
H08	4	Medium	+	SNP
A12	4	Medium	-	
E07	5	Niche	+	12 bp insertion
A04	5	Niche	+	SNP
E05	5	Niche	+	SNP
5M8	5	Medium	-	
H09	5	Medium	-	
5M7	5	Medium	+	SNP
A06	6	Niche	+	SNP
B06	6	Niche	+	SNP
B04	6	Niche	+	SNP
E11	6	Medium	+	SNP
H06	6	Medium	+	SNP
B12	6	Medium	-	

Table 10: results obtained from *ackA* gene sequencing of twenty-nine high-performing strains in terms of fly growth promotion, derived both from Medium and Niche setups (SNP=single nucleotide polymorphism; bp=base pair)



## 5. DISCUSSION

### 5.1 Phenotypic evolution of *Lp*<sup>NIZO2877</sup>

In order to clarify the role of the host in shaping the evolution of its gut microbes, we first evaluated the phenotypic evolution of *Lp*<sup>NIZO2877</sup> in terms of bacterial growth and host-growth promotion.

#### 5.1.1 Does *L. plantarum* evolve to improve its host's development?

First, we analyzed whether the evolution of *L. plantarum* affected the developmental time of its host during the course of the ten *Drosophila melanogaster* generations (G). Observing the values of D50, no significant differences were observed from G1 to G10. Having 5 replicates of experimental evolution was crucial to understand whether our results could be replicated independently. As shown in Fig. 5B, we indeed found no significant differences in D50 among the five samples within each generation. The only exceptions are represented by G3 and G5, where standard deviations are slightly higher than the others (1.15 and 1.26, respectively): in fact, samples 3, 4 and 5 have a greater D50 than 1 and 2 in G3; samples 3 and 5 have a greater D50 than 1, 2 and 4 in G5. Considering the phenotypic evolution of *Lp* in terms of its ability to promote *Drosophila* development, an initial decrease in D50 from G2 to G5 is visible, showing a continuous improvement of the beneficial effect of the bacterium on fly growth. However, this aspect has not been confirmed in the following generations (from G6 to G9) where the D50 value increases again, suggesting a reversion of the *Lp* growth-promoting capabilities to the ancestral state. This phenomenon is particularly noticeable in G8 and G9. We hypothesize that this result is related to the decrease of bacterial concentration that occurs specifically during these generations (Figure 4A). Indeed, the increase in the

*Drosophila* developmental time could be explained by the fact that fewer bacteria were isolated during these generations (Figure 4D), since it is known that host growth is directly proportional to the concentration of its microbiota. Specifically, it can be observed that, for both medium and niche samples, there is a clear drop in CFU/ml from G6 to G9, exactly when D50 increased. In fact, the starting inoculum used in generations 8, 9 e 10 was not  $10^5$  CFU/ml, but  $\sim 10^4$  CFU/ml.

Nevertheless, it is interesting to note that in G10 there is again an important improvement (reduction) of D50 compared to the previous generations ( $D50_{G9}=12.26$ ;  $D50_{G10}=10.01$ ), although the bacterial CFU remained low (the same as in G7 and G8). This suggests that the bacterium might have adapted to our experimental settings and, even if present in lower concentrations, might start to be more beneficial to its host (decrease in D50). However, this should be confirmed with further studies on subsequent fly generations.

### 5.1.2 Does *L. plantarum* grow better with or without its host?

The experimental evolution study also allowed to monitor bacterial growth in presence and in absence of its animal host during evolution. From Figure 4A, it is possible to observe that, starting from G3, the bacterial count tends to be higher in medium samples compared to the niche ones. This difference is statistically confirmed only in G7 (unpaired t-test \*\*  $p=0.003$ ). A possible explanation is that the standard deviations (SD) are quite high in some samples (i.e.,  $Mean_{MEDIUM\_G10}= 2.12 \times 10^7$  CFU/ml,  $SD_{MEDIUM\_G10}= 2.96 \times 10^7$  CFU/ml;  $Mean_{MEDIUM\_G3}= 7.96 \times 10^7$  CFU/ml,  $SD_{MEDIUM\_G3}= 8.96 \times 10^7$  CFU/ml), which then affects the calculation of the statistical significance. However, when we consider the overall bacterial growth over time (bacterial CFUs in medium and niche from G1 to G10 – Figure 2A), the difference between the two growth curves is significant (ANCOVA test, \*\*  $p=0.0009$ ). Specifically, we found that *Lp* CFUs are generally lower when the host is present (Niche setup), showing that bacterial concentration is higher in the host diet alone. This result is particularly interesting since it has been demonstrated that *Lp* proliferation is favored when the host is present, as *Drosophila* secretes metabolites that help *Lp* duplication (Storelli et al., 2018). We postulate that

the higher bacterial concentration found in the absence of the host in our study can be explained by the fact that the nutritional environment used in this study is very harsh both for the host and for the bacteria. Therefore, the medium setup (absence of the host) exerts a higher selective pressure (compared to the Niche setup) on the bacteria, which are then forced to adapt faster, hence, to grow more.

## 5.2 Does the diet play the key role in the phenotypic evolution of *L. plantarum*?

Considering the evolution of *L. plantarum* in promoting fly development, we found that, already starting from G2, most of the evolved *Lp* strains seem to improve host growth better than the ancestral strain NIZO2877 (greater Z-score, Figure 6) regardless of their evolutionary setup (Medium or Niche). This aspect is particularly evident in G2, G3 and G5. In particular, it can be seen that in the first generations (G2, G3 and G4) the highest Z-scores correspond mainly to medium samples, that is in strains evolved in the absence of their host. On the contrary, the situation is reversed in G6 where niche-derived strains seem to be more growth-promoting.

This has been also confirmed statistically (Figure 7, unpaired t-test). In fact, while in G2 the strains isolated from medium tubes have a greater  $\Delta D50$  (\*\*  $p=0.0013$ ), the situation is inverted in G6 (\*\*  $p=0.0034$ ). In generations 3, 4 and 5 the difference is not statistically confirmed even if the  $\Delta D50$  of the medium derived strains constantly decreases if compared with niche derived strains.

All together, these data indicate that, although medium-derived samples seem to be more growth-promoting in the first stages of the experimental evolution, niche-derived strains resulted to be better after 6 generations. We can then postulate that **the host diet alone exerts a high selection pressure on microbiota evolution at the early stages of bacterial adaptation** (as reported in paragraph 5.1.2, Figure 4A, Table 9), while the host seems to be important for microbiota adaptation in a later phase (Figure 7, Table

9). However, this hypothesis must be verified with further studies on subsequent generations.

### 5.3 Genotypic evolution of *Lp*<sup>NIZO2877</sup>

Since mutations in the *ackA* gene have been shown to be responsible for the improvement of *Lp* growth-promoting capabilities, the sequencing of this gene was conducted on twenty-nine high performing strain.

Most of the selected strains (83%) show mutations in the *ackA* gene. This data further confirms the importance of this gene on the evolution of *L. plantarum* in these environmental settings. Specifically, it can be observed that mutations appear already starting from the second generation of the experimental evolution study, as happened in previous works (Martino *et al.*, 2018). When we analyzed whether the animal host affected the mutation accumulation rate of *Lp*, no differences have been detected as both medium- (46%) and niche- (54%) derived strains were affected by *ackA* mutations in a similar percentage (Figure 5B). This suggests that the presence of the host seems not to affect the evolution of the *ackA* gene more than diet does, at least during early stages of microbiota evolution and adaptation.

However, further studies are now needed to investigate these results. Specifically, it is necessary to evaluate (1) if the identified *ackA* mutations are synonymous or non-synonymous, in order to establish whether the structure of the protein, and therefore its function, change. (2) The sequencing of the evolved strains that did not improve their symbiotic benefit is also necessary to verify whether the gene mutates regardless of the evolution of benefit. This will shed light into the link between the evolution of acetate kinase and *Lp* growth promoting effect. (3) Finally, the whole genome sequencing of the evolved strains is required in order to identify other genes that could be involved in the evolution of the bacterium and to verify whether they are regulated by the host's presence.

## 6. CONCLUSION

It has been demonstrated that diet represents the predominant driving force in the emergence and perpetuation of facultative animal-microbe symbiosis (*Martino et al., 2018*). However, the role of the animal host in the evolution of its own microbiota remain elusive.

To this end, in the present work, two parallel experimental microbiota evolution studies have been set up at the same time starting from the ancestor strain *L. plantarum* NIZO2877 with and without *Drosophila*.

All together, our data show that, at early stages, the microbiota first needs to adapt to the host diet to improve its symbiotic benefit, as no significant differences in microbiota evolution in presence and absence of its host were detected both phenotypically and genomically. This further confirms the primary role of the nutritional environment in the evolution of animal-microbe symbiosis. It is important to specify that the nutritional regime used in this study is particularly restrictive, due to the strong protein deficiency, which might hinder the effect of the host, thus explaining why a potential role of the animal host was not detected during the early phase of microbiota evolution. However, our results also show that, later in evolution, the animal host seems to concur to improve the symbiotic benefit of its microbiota, as strains evolved in the presence of the fly results to be more growth-promoting than strains evolved in the absence of the host. Further studies analyzing the following generations and also using a less restrictive diet are needed. In this way, the role of the host on microbial evolution will be finally disclosed.

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