Commensal bacteria differentially shape the nutritional requirements of
Drosophila during juvenile growth

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**Abstract**

The interplay between nutritional and microbial environments is one of the decisive environmental inputs that determine juvenile growth trajectory. Nutritional deficiencies contribute to developmental delays, and an immature gut microbiota is a hallmark of pathologies related to childhood undernutrition. However, how commensal bacteria modulate the impact of nutrition on juvenile growth remains elusive. Here, using gnotobiotic *Drosophila melanogaster* larvae independently associated with two major commensal strains, *Acetobacter pomorum*$_{WJL}$ (Ap$_{WJL}$) and *Lactobacillus plantarum*$_{NC8}$ (Lp$_{NC8}$), we performed a large-scale, systematic nutritional screen based on larval growth in 40 different and precisely controlled nutritional environments. We combined these results with *in silico* metabolic network reconstruction to define the biosynthetic capacities of Drosophila germ-free larvae and the two commensals. We first establish that Ap$_{WJL}$ or Lp$_{NC8}$ differentially fulfills the nutritional requirements of the ex-GF larva and parsed such difference down to individual amino acids, vitamins, other micronutrients and trace metals. We found that Drosophila commensal bacteria not only fortify the host’s diet with essential nutrients but in specific instances, they functionally compensate for host auxotrophies even if the bacteria fail to synthesize the missing nutrient. Our systematic work reveals that beyond the molecular dialogue engaged between the host and its commensal partners, Drosophila engage into an integrated nutritional network with its facultative commensals centered around the sparing and utilization of nutrients. We thus uncover a novel facet of the facultative nutritional mutualism engaged between Drosophila and its commensal bacteria, which allows the juvenile host to better cope with changes in nutrients availability during the critical phase of post-natal growth; hence ensuring optimal host fitness.
Introduction

Nutrition is arguably the major environmental factor that determines to what extent an organism can realize its genetically encoded growth potential [1]. The attributes of nutrition are defined by the quantity [2], quality [3] and bioavailability [4] of different nutrients in the diet. Nutrients are classified as non-essential or essential [3] based on the organism’s biosynthetic capacities. Diets deficient in essential nutrients cause important growth and maturation delays or even growth arrest, or “stunting” characterized by low height-for-age score [5]. In addition, some nutrients are conditionally-essential (or semi-essential). These nutrients can be synthesized by the organism, but insufficiently so under certain metabolically demanding conditions such as juvenile growth. Therefore, these semi-essential nutrients must also be retrieved from diets. Deficient consumption of conditionally-essential nutrients can also be detrimental for growth [3].

The intricate relationship between nutrition and growth is transformed by the microbial environment. In a classic human twin study, Smith et al unequivocally demonstrated that the gut microbiota composition of the juvenile subject suffering from stunting is significantly different from that of the healthy twin. When the faecal microbiota from the discordant twins were transplanted into genetically identical germ-free (GF) mice, the recipients of the microbiota from the stunted human twin performed poorly in terms of growth gain and weight recovery compared to the recipients of the microbiota of the healthy twin [6]. Furthermore, functional analyses of gut microbiota from children experiencing strong acute malnutrition showed significant under-representation in pathways of amino acid biosynthesis and uptake; carbohydrate utilization and B-vitamin metabolism [7]. Diets supplemented with nutrients favoring the growth of bacteria enriched in these underrepresented pathways increase plasma
biomarkers and levels of mediators of growth, bone formation, neurodevelopment, and immune function in children with moderate acute malnutrition [7]. These studies clearly show that the microbial environment strongly impacts how the organism responds to changes in its nutritional environment, and vice versa.

Diverse animal models are employed to decipher the physiological, ecological, genetic and molecular mechanisms underpinning host/microbiota/diet interactions. Among them, Drosophila melanogaster (Dm) is frequently chosen to study the impact of the nutritional environment on growth and development thanks to its short growth period as well as easy and cost-effective rearing conditions. During the juvenile phase of the Drosophila life cycle, the larva feeds constantly and increase its body mass ~200 times until entry into metamorphosis [8]. However, the pace and duration of larval growth can be altered by the nutritional context and the microbial environment [9–11]. Like other animals, Drosophila live in constant association with commensal microbes, including bacteria and yeast [12]. The impact of the microbial environment can be systematically assessed by generating gnotobiotic flies associated with a defined set of commensals [13–15]. Lab-reared flies typically carry bacterial strains from only four to eight species. The microbiota from wild flies are more complex. Nevertheless, they are usually dominated by members from Acetobacteraceae and Lactobacilli [16–22]. Most bacterial strains from these dominant species are easy to culture in the lab, and some have even been genetically engineered for functional studies of host-microbes interactions [23–25]. These fly commensals are facultative symbionts that are constantly horizontally acquired [26–28]. Even though recent experimental evidence show that wild bacterial isolates can persistently colonize the adult crop [22,29], the larval gut commensals are in fact transient; they constantly shuttle between the larval gut and the food substrate to establish a mutualistic cycle with the host [30,31]. Thus,
The Drosophila larval growth is an attractive model to study the strong interplay between the Drosophila commensals and the nutritional environment.

We and others have previously shown that GF larvae raised in nutritional challenges show important developmental delays, and a single gut commensal strain can accelerate Drosophila development during nutritional challenge [20,25]. Specifically, *Acetobacter pomorum*\(^{WJL}\) (Ap\(^{WJL}\)) modulates developmental rate and final body size through the insulin/insulin-like growth factor signaling (IIS) pathway, and the intact acetate production machinery is critical [25]. *Lactobacillus plantarum*\(^{WJL}\) or *L. plantarum*\(^{NC8}\) (Lp\(^{NC8}\)) promote host juvenile growth and maturation partly through enhanced expression of intestinal peptidases upon sensing of bacterial cell wall components by *Drosophila* enterocytes [20,23,32]. Interestingly, the growth-promoting effect of these bacteria was only observed under nutrient scarcity, suggesting that besides the molecular dialogue engaged between the commensal bacteria and their host, bacteria-mediated growth promotion on low diets also includes some nutritional compensation, as already reported for bacteria-derived thiamine provision to the host [33]. However, how the presence of such bacteria specifically alters the host’s nutritional environment and satisfies the host’s nutritional requirements remains unexplored. To do so, we systematically assessed the microbial contribution to larval growth in 40 different and strictly controlled nutritional contexts, and we conducted this large-scale screen based on chemically defined holidic diets (HDs).

Holidic diets comprise a mixture of pure chemical ingredients that satisfy the different physiological requirements of the Drosophila host [34,35]. By altering the concentration of each or a combination of ingredient, one can exactly tailor the experiments by generating specific nutrient deficiencies or excess [36]. The first development of HD supporting the growth of Drosophila can be traced back to the 40s
and have been used to assess the direct impact of the nutritional environment on axenic larvae in the 1950s [38,39]. In this study, we adopted a recently developed FLYAA HD where the amino acids concentrations are calculated so that they match the amino acid ratios found in the translated exome of the fly [40]. The FLYAA HD is optimal for both fecundity and lifespan of adults, and was recently used to investigate the links between nutrition and lifespan [40–43], fecundity [40–42,44], food choice behavior [45], nutrient sensing [46], growth and maturation [33,40–42,47–49]. Exome-based HD can efficiently support larval growth albeit not to the optimal growth and maturation rate obtained with classic oligidic laboratory fly food [34]. Using the chemically defined HD, we aimed to deconstruct in a systematic manner the microbial contribution to the host’s nutritional requirements down to individual nutrients.

To do so, we first needed to establish the biosynthetic capacities of GF larvae and two major commensals of Drosophila: ApWJL and LpNC8 on HD. We further complemented the in vivo study with in silico metabolic network reconstruction based on the genome sequences of D. melanogaster, ApWJL and LpNC8. In recent years, metabolic approaches based on genome-driven network reconstructions have been applied to predict the potential metabolic dependencies and metabolic exchanges between hosts and associated microbes [50–55]. The mutualistic association between the pea aphid Acyrthosiphon pisum and its obligate intracellular symbiont Buchnera aphidicola was the first symbiotic association for which genomic information were available on both partners, and is a case study for a comprehensive survey of integrated host-symbiont metabolic interactions. In this model, decades of nutritional experiments using HDs and aposymbiotic aphids were reinterpreted in the light of newly available genomic data, thus changing the traditional paradigm that proposed a clear separation between the pathways of the host and its symbionts and revealing a
particularly integrated metabolic network between the insect and its obligate
endosymbionts [56,57]. Indeed, the metabolic coevolution between the pea aphid and
*Buchnera aphidicola* led to the emergence of shared metabolic pathways, such as that
for the biosynthesis of branched-chain [58] and aromatic amino acids [59,60], in which
the enzymes are encoded by both the host and the bacterial genomes. Such highly
integrated metabolic pathways have been subsequently discovered in other obligate
mutualistic associations and can involve an eukaryotic host and all its bacterial
symbionts in the case of multi-partner symbioses [61]. The availability of the genomes
of *D. melanogaster* and of its two major commensal bacterial species provides the
unique opportunity to extend these studies from obligate mutualistic bacterial
endosymbioses to facultative host-commensal bacteria mutualisms. Here we report
that association of GF larvae with Ap*WJL* or Lp*NC8* modifies the nutritional requirements
of ex-GF larvae in a specific manner each. We show that Ap*WJL* and Lp*NC8* not only
modify the nutritional environment of their host by fortifying diets with essential
nutrients but also functionally compensate for host auxotrophies despite not
synthetizing the missing nutrient.
Results and Discussion

Metabolic network reconstruction of the host (D. melanogaster) and its two commensals, Ap\textsuperscript{WJL} and Lp\textsuperscript{NC8}, was automatically generated using the CycADS pipeline [62]. The resulting BioCyc metabolism databases are available at http://artsymbiocyc.cycadsys.org for visualization and analysis purposes. We generated the enriched functional annotations of all the predicted proteins from the complete genomes of D. melanogaster (Dm, RefSeq GCF_000001215.4 release 6), A. pomorum strain DM001 (Ap\textsuperscript{WJL}, accession NCBI-Taxonomy 945681 version 1.0.1) and L. plantarum subsp. plantarum NC8 (Lp\textsuperscript{NC8}, NCBI Bioproject PRJNA67175). From the genomic analyses, we inferred all pathways allowing production of the organic compounds that are present in the exome-based FLYAA holidic diet (HD) developed by Piper et al [40]: fly’s essential and non-essential amino acids (EAA\textsuperscript{Fly} (n=10) and NEAA\textsuperscript{Fly} (n=10)), B-vitamins (n=7), cholesterol (n=1), and Nucleic Acids and Lipid precursors (NAL, n=4).

\textit{Drosophila melanogaster} biosynthetic capabilities inferred from \textit{in silico} metabolic network reconstruction

Although a BioCyc metabolic reconstruction of D. melanogaster is already publicly available (https://biocyc.org/FLY), we have reconstructed an improved and more comprehensive BioCyc database using CycADS [62]. This metabolic reconstruction comprises 22,189 protein-encoding genes including 5,061 enzymes and 156 transporters associated with 1,610 compounds assembled in a network of 331 pathways (\textit{versus} the 227 pathways found in BioCyc). Like other metazoans, Dm possesses the gene repertoire to produce all the NEAA\textsuperscript{Fly}, but is unable to produce the
EAAfly (Fig 1A and Table S1). Dm can also produce myo-inositol, inosine and uridine, but is unable to synthesize vitamins from simple precursors (Fig 1B and Table S2).

**ApWJL biosynthetic capabilities inferred from in silico metabolic network reconstruction**

According to our metabolic reconstruction, ApWJL database comprises 4,268 protein-encoding genes including 1,326 enzymes and 46 transporters associated with 1,306 compounds assembled in a network of 313 pathways. The reconstructed metabolic network reveals that ApWJL is a complete autotroph for all amino acids, (Fig 1A and Table S1). ApWJL also possesses the genetic potential to produce the DNA bases inosine and uridine and five of the seven vitamins present in the HD: biotin, folate, pantothenate, riboflavin and thiamine. The biosynthesis of nicotinate from Asp seems impossible since L-aspartate oxidase (1.4.3.16), the enzyme necessary to catalyze the first step of the pathway is absent (Fig 1B and Table S2), and further TBLASTN searches failed to retrieve any 1.4.3.16 activity based on ApWJL genome. In the case of pyridoxine, ApWJL cannot catalyze the first two reactions of its biosynthetic pathway (Table S2). A direct phosphorylation of Thr would also be needed to complete the pathway (using the 3.1.3.3 activity, Table S2). However, no evidence for such an activity in Acetobacter sp. was found in genomic data or in the literature. Pyridoxine is reported as non-essential for acetic acid bacteria [63]. Finally, ApWJL cannot produce choline and myo-inositol. In summary, ApWJL genome analysis predicts that it is able to synthesize all amino acids and five B-vitamins (biotin, folate, pantothenate, riboflavin and thiamine). ApWJL cannot synthesize choline, myo-inositol and nicotinate.
Lp<sup>NC8</sup> biosynthetic capabilities inferred from *in silico* metabolic network reconstruction

Metabolic reconstruction from Lp<sup>NC8</sup> genome generated a database that includes 2,868 protein-encoding genes, consisting of 973 enzymes and 74 transporters associated with 1,154 compounds, all assembled in a network of 246 pathways. Interestingly, although the genome of Lp<sup>NC8</sup> is about 30% smaller than *A. plantarum* WJL's genome, it encodes ~40% of supplementary transporters. This reflects the importance of metabolic exchanges for survival and adaptation of *L. plantarum* strains to a broad spectrum of environments [64].

From a genomic perspective (Fig 1A and Table S1), Lp<sup>NC8</sup> is able to produce most amino acids from glucose or inner precursors. Exceptions are Phe, sulfur-containing (Cys, Met) and branched-chain amino acids (BCAA, Ile, Leu, Val). Indeed, synthesis of Phe seems to be precluded in Lp<sup>NC8</sup> since the prephenate dehydratase activity (4.2.1.51) is not encoded by its genome (Table S1). Moreover, Lp<sup>NC8</sup> has lost the capability to reduce sulfate in sulfite, a necessary step for the integration of sulfur in Cys biosynthesis [65]. Biosynthesis of Met seems to be feasible in Lp<sup>NC8</sup>, but is probably limited by sulfite production rate and consequently by Cys availability. As previously reported, Lp<sup>NC8</sup> is unable to produce the three BCAA, as four enzymatic activities of this pathway are lacking (1.1.1.86, 4.2.1.9, 2.3.3.13 and 4.2.1.33) [66]. However, the last transamination step common to each specific BCAA pathway is conserved, suggesting rather than importing the amino acids themselves, Lp<sup>NC8</sup> may import 2-oxo-carboxylic acids, the direct precursors of Ile, Leu and Val to fulfill the BCAA requirements. Furthermore, Lp<sup>NC8</sup> genome encodes a complete Arg biosynthesis pathway, yet Arg is known to be limiting [67] or essential to certain *L. plantarum* strains [66,68]. Arg auxotrophies are currently little understood; they are
usually attributed to regulatory effects or to the presence of certain deleterious mutations in the biosynthetic pathway [68]. We further estimated the Arg biosynthetic capability of Lp\textsuperscript{NC8} by analyzing the sequence of functional gene cluster argCJBD\textsuperscript{F} and searched for possible mutations using an autotrophic strain of \textit{L. plantarum} (\textit{L. plantarum} CCM 1904, [69]) as a reference. No stop codon, frame-shifts nor deletions were identified in the genes of the operon. This suggests that Lp\textsuperscript{NC8} can be an Arg autotroph, but does not exclude the possibility that Arg may become limiting in specific conditions. Lp\textsuperscript{NC8} has the minimal gene requirements to produce Ala and Asp (Table S1), but the biosynthesis of Ala and Asp invokes secondary metabolic routes. Therefore, when possible, uptake of Ala and Asp from the culture medium should be favored instead of \textit{de-novo} biosynthesis. Similarly, biosynthesis of Thr is directly linked to Asp and Cys and is probably very limited in Lp\textsuperscript{NC8}. Accordingly, all the Thr interconnecting pathways are lost, thus limiting its production or uptake from the diet for protein synthesis.

Regarding vitamin biosynthesis, Lp\textsuperscript{NC8} is able to produce folate, riboflavin and thiamine, as well as all DNA bases including uridine and inosine (Fig 1B and Table S2). Lp\textsuperscript{NC8} cannot produce thiamine through the canonical pyrimidine pathway because it lacks the 4.1.99.17 enzyme, but it seems possible through the pyrimidine salvage pathway (2.1.7.49). Unlike Ap\textsuperscript{WJL}, pyridoxine biosynthesis seems feasible in Lp\textsuperscript{NC8} using an alternative route involving Thr phosphorylation. Conversely, biosynthesis of nicotinate seems to be impossible. Indeed, the two first steps of its biosynthetic pathway (1.4.3.16 and 2.5.1.72) are not encoded by Lp\textsuperscript{NC8} genome. Similarly, Lp\textsuperscript{NC8} has lost the capability to produce pantothenate as two enzymatic activities of the pathway (2.1.2.11 and 6.3.2.1) are not present in the genome. Finally, like Ap\textsuperscript{WJL}, Lp\textsuperscript{NC8} likely does not produce choline and myo-inositol.
In summary, our metabolic reconstruction suggests that Lp^{NC8} can synthesize all amino acids except for Phe, Cys, Ile, Leu and Val. Synthesis of Arg, Thr, Ala and Asp can easily become limiting in the contexts of high nutritional demand. Thus, Lp^{NC8} is expected to extract these amino acids from the diet, or its growth would be altered when they are absent from the diet. Met synthesis seems to be possible, but is dependent on Cys abundance. Finally, Lp^{NC8} can produce DNA bases, folate, riboflavin, thiamine and perhaps pyridoxine, but not biotin, nicotinate, pantothenate, choline and myo-inositol.

Collectively, our *in silico* metabolic network reconstruction shows that *D. melanogaster* and its commensals have differential biosynthetic capacities. Indeed, some of the complete biosynthetic pathways are only present in one organism while others are present in two or all the three partners. However, for incomplete biosynthetic pathways we did not detect potentially shared pathways between a commensal and its host as observed between obligate mutualistic partners (Fig 1-2).

**Experimental validation of commensal bacteria auxotrophies using holidic diets**

In order to test experimentally the metabolic potential of Dm and its commensals, predicted by our in silico analysis (see above), we adopted the exome based FLYAA holidic diet (HD) [40]. We systematically removed a single component at a time to generate 39 different fly nutritional substrates (henceforth named HDΔX, X being the nutrient omitted), plus one complete HD medium. This medium can also be prepared in a liquid version by omitting agar and cholesterol from the recipe. Liquid HD can then be used to assess bacterial growth in 96-well plates, increasing the experimental throughput.
We first assessed Ap\textsuperscript{WJL} and Lp\textsuperscript{NC8} growth in each of the 40 different liquid HD for 72 hours, using maximal optical densities (\(\text{OD}_{\text{Max}}\)) as a readout (Fig 3A and Table S3). In the complete HD, both Ap\textsuperscript{WJL} and Lp\textsuperscript{NC8} grow well (Fig 3A, first line). On the deficient media, Ap\textsuperscript{WJL} can grow in HD\textasciitilde Sucrose, presumably using acetate from the acetate buffer as a carbon source. Also, its growth is not altered in the absence of any of EAA\textsuperscript{Fly}, Vitamins or NAL. However, while Ap\textsuperscript{WJL} growth is not impacted by the lack of most NEAA\textsuperscript{Fly}, it grows poorly in HD\textasciitilde Ala, HD\textasciitilde Cys and HD\textasciitilde Glu. In addition, Ap\textsuperscript{WJL} fails to grow in HD\textasciitilde Cu, HD\textasciitilde Fe and HD\textasciitilde Mg (Fig 3A, first column and Table S3). The broad growth capacity of Ap\textsuperscript{WJL} in HDs correlates well with the wide range of environmental niches the genus Acetobacter can colonize. Acetobacter species are found in sugar-rich niches such as flowers and fruits, but also in poorer niches such as soil and water, where they need to synthesize all nutrients required for their own growth [70]. These findings are corroborated by our genome-based \textit{in silico} predictions (Fig 1). Furthermore, the \textit{in silico} reconstruction predicted that Ap\textsuperscript{WJL} would not be able to synthesize choline, myo-inositol and pyridoxine, but we observed that Ap\textsuperscript{WJL} grows in their absence. Choline is an important precursor of phosphatidylcholine (PC), which is a major component of Acetobacter membranes and plays an important role to confer acetic acid tolerance. Despite its importance, PC is not essential for Acetobacter growth. Indeed, mutants precluding PC synthesis show a shift towards increased membrane content of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) and do not show any growth defect [71]. Similarly, Ap\textsuperscript{WJL} likely does not need myo-inositol for its growth since inositol compounds are absent from the membrane of most bacteria [72]. Regarding nicotinate and pyridoxine, the biosynthesis pathways of these two nutrients are only partially understood and the presence of alternative pathways and enzymatic activities may be possible in Ap\textsuperscript{WJL} (see above). It is thus possible that
ApWJL is capable of synthesizing nicotinate and pyridoxine through elusive biochemical pathways permitting growth in their absence. Further investigation will be required to identify such biochemical pathway(s). Interestingly, ApWJL growth was only precluded in the absence of some metal ions: Cu, Fe, and Mg. Metal ions are important co-factors required for enzymatic activities [73]. Specifically, in acetic acid bacteria, Cu is an important co-factor of the energy-producing cytochromes of the respiratory chain [74] making it essential for ApWJL growth.

We detected far more nutritional auxotrophies for LpNC8 on HDs (Fig 3A, second column and Table S3). LpNC8 fails to grow in HDΔSucrose, since sucrose is the only suitable carbon source for Lactobacilli in the liquid HD. Also, LpNC8 growth is precluded in the absence of 9 amino acids, including 6 EAAFly (Arg, Ile, Leu, Phe, Thr, Val) and 3 NEAAFly (Ala, Asp, Cys). It also grows poorly in media lacking the EAAFly Lys, Met and Trp and the NEAAFly Asn and Glu. Moreover, LpNC8 does not grow in HDΔBiotin and HDΔPantothenate. However, it grows in absence of nicotinate, which is predicted to be essential according to our in silico analysis (see above). Finally, LpNC8 growth is not affected by the lack of any NAL. In the absence of certain metal ions such as Ca, Cu, Mg, Zn, OD_{max} is increased. On the contrary, OD_{max} is significantly reduced in HDΔMn. These relatively elevated nutritional requirements of LpNC8 are expected as L. plantarum is a species adapted to nutrient-rich environments [75]. Hence, many L. plantarum strains have lost the capacity to synthetize various nutrients that can easily be found in their natural habitats [64,75]. The inability of L. plantarum to synthetize important nutrients such as BCAA (Ile, Leu and Val) or the B-vitamin pantothenate was previously identified by both genome analyses [66] and growth studies in chemically defined minimal media [67,76,77]. Moreover, it is known that L. plantarum needs Mn to resist to oxidative stress [78], which explains its poor growth in HDΔMn. Our
experimental data only partially correlate with the results of our genomic analysis. *In silico* predicted auxotrophies to Ile, Leu, Val, Phe, Cys, pantothenate and biotin were confirmed *in vivo*. The identified Arg auxotrophy was not surprising because, as mentioned above, Arg is often described as essential to *L. plantarum* in high metabolic demanding conditions even though all genes necessary for Arg biosynthesis are present. However, auxotrophies of LpNC8 to Thr, Ala, Asp, were not expected (Fig 3A, denoted by “*”), even though these amino acids were predicted to be limiting (see above). As mentioned previously, bacterial growth in liquid HD was assessed in 96-well plates using a microplate reader (see methods). Every cycle includes an agitation step to homogenize the solution to improve OD reading accuracy. This agitation step may oxygenate the media and thus negatively affects LpNC8 growth in scarcity conditions since *L. plantarum* strains are aerotolerant but optimal growth is achieved under microaerophilic or anaerobic conditions [79]. To retest these unexpected auxotrophies, we assessed LpNC8 growth in liquid HDΔThr, HDΔAla, HDΔAsp in 15-mL closed falcon tubes without aeration. After 72 hours of incubation we determined CFU counts in each media (Fig 3B). As predicted by our genomic analyses, LpNC8 was able to grow in each of the three deficient media in static conditions to the same extent as in the complete HD (Fig 3B). Therefore, LpNC8 auxotrophies observed for Thr, Ala and Asp in 96-well plates are likely an artifact due to excessive oxygenation. This could also explain the poor growth of LpNC8 in the absence of the EAA*F*ly Lys, Met and Trp and the NEAA*F*ly Asn and Glu. The ability of LpNC8 to grow in HDΔCholine, HDΔMyo-inositol, HDΔNicotinate and HDΔPyridoxine do not correlate with *in silico* data. As for ApWJL (see above), LpNC8 growth probably does not require choline or myo-inositol. A previous study quantified choline and inositol compounds in *L. plantarum* cell extracts and found them to be extremely low and therefore most likely due to contaminations.
from the medium rather than components of *L. plantarum* biomass [80]. Moreover, pyridoxine is a precursor of pyridoxal-5-phosphate, a cofactor necessary for amino acid converting reactions. Teusink et al. [66] showed that *L. plantarum*<sup>WCSF1</sup> requires exogenous sources of pyridoxine only in a minimal medium lacking amino acids. Since the HD<sub>Δ</sub>Pyridoxine contains all amino acids, it is likely that pyridoxine is not essential for Lp<sup>NC8</sup> growth in these conditions. Finally, the capacity of Lp<sup>NC8</sup> to grow in HD<sub>Δ</sub>Nicotinate could be related with the presence of an alternative pathway of nicotinate biosynthesis. Indeed, this possibility has been previously reported in the genera *Lactobacillus* [64] which would explain the capacity to grow in absence of exogenous nicotinate.

Altogether, the complete HD is a suitable nutritional environment that allows the two prevalent Drosophila commensals, Ap<sup>WJL</sup> and Lp<sup>NC8</sup> to grow. Growth capacities in deficient media vary from one bacterium to another and are dictated by their individual genetic repertoires.

**Germ free larvae exhibit 22 auxotrophies while developing on holidic diets**

We next sought to establish the nutritional requirements of GF larvae by assessing larval developmental timing (DT) in complete HD and in each of the 39 deficient HD (see methods). DT is expressed as D<sub>50</sub>, which represents the day when 50% of the larvae population has entered metamorphosis in a specific nutritional condition. In agreement with previous studies [38,39], GF larvae fail to develop in all HD<sub>Δ</sub>EAA<sup>Fly</sup>, all HD<sub>Δ</sub>Vitamins, HD<sub>Δ</sub>Choline HD<sub>Δ</sub>Cholesterol, HD<sub>Δ</sub>Zn and HD<sub>Δ</sub>Mg (Fig 4A, first column). Over 60 years ago, Sang et al reported that Zn was dispensable for GF larval development in a classic study [38]. We suspect that the casein in the medium used in Sang et al inadvertently provided trace amount of Zn, which could
account for the discrepancy between our observation and that of Sang et al. Also in accordance with previous studies [38,39,49], GF larvae were able to reach pupariation in HDΔNEAA^{Fly} (ΔAla, ΔCys, ΔGln, ΔGlu, ΔGly, ΔPro), HDΔUridine, HDΔMyo-inositol, and HDΔMn at the same rate as on a complete HD (Fig 4A). The absence of sucrose, Tyr, inosine, Ca, Cu and Fe did not prevent pupae emergence, but increased their developmental timing significantly (Fig 4A first column, Table S4). Surprisingly, GF larvae were able to reach pupariation, albeit late, in HDΔSucrose. Indeed, all the HDs developed to date include carbohydrates (either sucrose or fructose) as a carbon source [34]. Larval development in the absence of carbohydrates suggests that GF larvae may use other components of the HD as carbon source. In summary, GF yw larvae show 22 auxotrophies while developing on sterile holidic diets.

Our observations correlate well with our in silico predictions (Fig 1) with one exception: GF larvae did not reach pupariation in HDΔAsn. This result was surprising because Asn is described as a NEAA in Drosophila and other animals [81]. To test whether Asn auxotrophy was specific to the yw fly line used in our assays, we assessed larval DT in two other Dm reference lines, DGRP-RAL-25210 [82] and w^{1118}. Unlike yw, both w^{1118} and RAL-25210 larvae were able to develop in GF condition in HDΔAsn albeit with a severe developmental delay (Fig 4B). Therefore, a complete Asn auxotrophy is specific to our yw strain, which explains why we did not detect this auxotrophy in our in silico analyses that were based on the genome sequence of the Drosophila Reference Genome strain (Bloomington stock #2057). We therefore sequenced the coding region of the enzyme AsnS that converts Asp to Asn in yw flies, and did not detect any non-synonymous mutation (Fig S1). Further studies may thus be required to determine the origin of the Asn auxotrophy in our yw line on HD.
However, these results indicate that Asn is not an EAA per se but remains a limiting NEAA, an observation that also applies to Tyr.

The molecular dialogue engaged between commensals and their host is at play on complete holidic diet

We then investigated if and how the association with commensals affects the nutritional requirements of GF larvae during juvenile growth and maturation. To this end, we mono-associated GF embryos with Ap<sup>WJL</sup> or Lp<sup>NC8</sup> and measured D<sub>50</sub> and egg-to-pupa survival in complete and deficient HDs (Fig 4A second and third columns, respectively and Table S4, S5). On a complete HD mono-association with either Ap<sup>WJL</sup> or Lp<sup>NC8</sup> accelerated larval DT with a mean D<sub>50</sub> of 8.4 and 7.7 days, respectively, whereas GF mean D<sub>50</sub> 10.1 days (Fig 3A, first line).

These growth-promoting effects upon mono-association with either Ap<sup>WJL</sup> or Lp<sup>NC8</sup> have been previously reported on complex diets and insights on the underlying molecular mechanisms were provided [20,25]. Shin <i>et al</i>. showed that when the associated larvae grow on a low casamino-acid semi-oligidic diet, the pyrroloquinoline quinone–dependent alcohol dehydrogenase (PQQ-ADH) activity of Ap<sup>WJL</sup> modulates the developmental rate and body size through insulin/insulin-like growth factor signaling (IIS). PQQ-ADH disruption in the Ap<sup>WJL::Tn<pqq</sup> mutant severely reduces acetic acid production, which has been proposed to alter the regulation of developmental and metabolic homeostasis upon mono-association [25]. Lp<sup>NC8</sup> promotes host juvenile growth and maturation on a low yeast-based oligidic diet, partly through enhanced expression of intestinal peptidases upon sensing of bacterial cell walls components by Drosophila enterocytes [20,23]. Deletion of the <i>dlt</i> operon, which encodes the molecular machinery involved in the D-alanylation of teichoic acids, leads...
to bacterial cell wall alteration with a complete loss of D-alanylation of teichoic acids
and consequently cell walls purified from the LpNC8Δdllop mutant trigger a reduced
expression of peptidases in enterocytes [23]. Therefore, we first probed the importance
of these molecular mechanisms on commensals mediated larval growth promotion on
complete HD. To this end, we tested in our HD setting the loss of function mutants
ApWJL::Tnppq [25] and LpNC8Δdllop [23]. In complete HD, only the LpNC8Δdllop mutant
failed to support larval growth, reminiscent of the previous observation on the low-
yeast oligidic diets (Fig S2). Surprisingly in complete HD, the ApWJL::Tnppq mutant
actually triggered an enhanced growth promotion as compared to its WT reference
strain. Shin et al. reported that ApWJL::Tnppq associated larvae experienced growth
delay, which can be rescued by acetic acid provision [25]. Therefore, the acetic acid-
based buffer in HD may explain why ApWJL::Tnppq no longer behaves as a loss of
function mutant in this setting; however, how it actually surpasses the WT strain on HD
remains elusive. Collectively, these results establish that sensing bacterial cell walls
containing D-alanylated teichoic acids is also an important feature of the intrinsic
growth promoting ability of LpNC8 in a complete chemically defined HD. Thus, the
previously reported molecular sensing mechanism that mediates the growth-promoting
effect of LpNC8 during chronic undernutrition is also at play in synthetic diets.

Association with ApWJL fulfills 19 of the 22 auxotrophies of GF larvae

Association with ApWJL sustained larval development in the absence of 19 out of 22
GF essential nutrients (Fig 4A second column). ApWJL associated larvae reached
pupariation in the absence of each EAA^fly (though their development was slower than
on complete HD), Asn, vitamins, choline and Zn. Association with ApWJL also rescued
the developmental delay observed in GF larvae in HDΔTyr, HDΔinosine, HDΔCu and HDΔFe. The only nutritional requirements of GF larvae that were not fulfilled by ApWJL were cholesterol, pantothenate and Mg.

Association with LpNC8 fulfills 12 of the 22 auxotrophies of GF larvae

Compared to ApWJL, mono-association with LpNC8 compensated for a reduced number of the GF larvae nutritional deficiencies (12 out of 22; Fig 4A third column). LpNC8 associated larvae reached pupariation in the absence of some EAAFly (HDΔHis, HDΔLys, HDΔMet, HDΔPhe, HDΔThr), Asn, certain vitamins (HDΔBiotin, HDΔFolate, HDΔNicotinate, HDΔRiboflavine, HDΔThiamine), and Zn. Moreover, LpNC8 rescued the developmental delay observed in GF on HDΔTyr, HDΔinosine, HDΔCu and HDΔFe.

Bacteria need to be metabolically active in order to fulfill larval nutritional requirements

Bacteria were grown in rich medium before association with larvae. Therefore, they may have accumulated nutrients that can be used by the larvae to fulfill their nutritional requirements. To test for the nutritional input brought by the initial bacterial inoculum, we associated GF larvae with 10X heat-killed (HK) bacteria and measured D50 in complete and deficient HDs (Fig 4A fourth and fifth columns). In most cases, the D50 of larvae in HK and GF conditions were similar, and they both show striking delay compared to the mono-associated larvae. Therefore, bacteria need to be metabolically active to fulfill the larval nutritional requirements on HDs. However, we found some exceptions. In HDΔAsn, HDΔBiotin, HDΔFolate, HDΔCu and HDΔFe, addition of HK bacteria allowed the larvae to develop, though not as fast as in association with living
bacteria. These results suggest that larvae only require a very small amount of these nutrients, which can be sufficiently derived from the inert bacterial inoculum.

The ability of bacteria to compensate nutritional deficiencies does not always correlate with the ability of bacteria to synthesize the nutrient

Next, based on the *in silico* predictions and the experimentally revealed auxotrophies, we correlated the ability of each commensal bacteria to synthesize a nutrient to their ability to fulfill the larval requirements in this nutrient. We identified 4 distinct scenarios:

Case 1: the bacteria synthesize a nutrient and fulfill the related larval auxotrophy

In most of the tested conditions, when the bacteria can synthesize a nutrient, they can also fulfill the related nutritional requirements of the GF larvae. For Ap<sup>WJL</sup> this includes all EAA<sup>Fly</sup>, Asn and most vitamins (except pantothenate). For Lp<sup>NC8</sup>, the correlation between the nutritional complementation of ex-GF larva and the ability of Lp<sup>NC8</sup> to synthesize the missing nutrient is more limited and only applies to the requirements of His, Lys, Met, Thr, Asn and most vitamins (except pyridoxine). Nonetheless, these results suggest that bacteria can actively supply the nutrients lacking in the HD to the larvae. This phenomenon is reminiscent of previous observations using conventional and gnotobiotic hosts, in which microbial sparing of riboflavin or thiamine by differential commensals have been proposed [33,83]. Exceptions to this case seem to be Ap<sup>WJL</sup> on HD<sup>Δ</sup>Pantothenate and Lp<sup>NC8</sup> on HD<sup>Δ</sup>Trp. Specifically, Ap<sup>WJL</sup> can produce pantothenate and grows in HD<sup>Δ</sup>Pantothenate and similarly Lp<sup>NC8</sup> can produce Trp and grows in HD<sup>Δ</sup>Trp. However, neither supported larval development on the respective depleted HD. It is therefore probable that Ap<sup>WJL</sup> and Lp<sup>NC8</sup> produce enough pantothenate and Trp, respectively, to sustain their own growth in the depleted HD but
not sufficiently or in a manner inaccessible to the larvae and thus fail to fulfill larval requirements for these nutrients.

Case 2: the bacteria do not synthesize a nutrient and they cannot fulfill larval requirements

Expectedly, we observed that when bacteria do not synthesize a nutrient, they do not fulfill ex-GF larvae requirements for this nutrient. For instance, Lp\textsuperscript{NC8} cannot produce the BCAA, nor grow in their absence, and thus it cannot fulfill larval requirements for these amino acids. In some depleted diets such as HD\textDelta Cholesterol, HD\textDelta Choline and HD\textDelta Pyridoxine, bacteria were able to grow (Fig 3A) even though they cannot synthetize these nutrients (See above and Table S2), and they failed to fulfill the larvae requirements of these specific nutrients. This is observed for Ap\textsuperscript{WJL} and Lp\textsuperscript{NC8} on HD\textDelta Cholesterol. The likely explanation is that cholesterol is an animal sterol but is dispensable for bacterial growth [72,84]. Similarly, on HD\textDelta Choline and HD\textDelta Pyridoxine, Lp\textsuperscript{NC8} is also unable to fulfill larval requirements (Fig 3A) as it cannot synthetize these compounds.

Case 3: the bacteria do not synthesize a nutrient but they can fulfill larval requirements

In most cases, we observe growth rescue by bacteria provision of the missing nutrients, but there are interesting exceptions. Ap\textsuperscript{WJL} is unable to synthesize de-novo choline and pyridoxine (See above and Table S2). Surprisingly, it fulfills larval auxotrophies on HD\textDelta Choline and HD\textDelta Pyridoxine. This could be achieved either by a functional compensation mechanism or via alternative biosynthetic pathways of these components that are undetected by our metabolic reconstruction. In the case of choline, the former seems to be the most plausible explanation, as Ap\textsuperscript{WJL} may synthesize other compounds that Drosophila can use to replace choline. As stated before, Acetobacter mutants precluding phosphatidylcholine (PC) synthesis shift their
membrane composition towards increased content of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) [71]. PE and PG have been reported to be part of the phospholipidic repertoire of Drosophila membranes [85], in which PE represents approximately 50% of their lipid composition [86]. We posit that ex-GF larvae growing on HDΔCholine may capitalize on ethanolamine or glycerol produced by ApWJL to compensate for the lack of choline in their diet. In the case of pyridoxine, ApWJL may fulfill larval requirements by either a functional compensation or through pyridoxine biosynthesis by non-canonical pathways (see above). We reach the same conclusion regarding nicotinate. Both ApWJL and LpNC8 grow well on HDΔNicotinate and also can fulfill the larval requirements in this vitamin even though our metabolic reconstruction predicts that they cannot synthesize it (see above).

Moreover, LpNC8 cannot grow in the absence of Phe (Fig 2A). The genomic analyses point to the possible loss of the gene coding for the enzyme prephenate dehydratase (4.2.1.51), the penultimate step on Phe biosynthesis. Yet LpNC8 can fulfill larval requirements for Phe (Fig 4A). We wondered if the Phe auxothrophy we observed in 96-well plates (Fig 3A) was due to the oxygenation generated by the agitation through OD readings, as for Thr, Ala and Asp (Fig 3B). To test this, we set cultures of LpNC8 in HDΔPhe in static 15-mL closed falcon tubes and assessed bacterial growth after 3 days of culture. In contrast to agitation, LpNC8 grows in HDΔPhe ~1x10^2 times in static conditions (Fig 4C), whereas in the complete media (Fig 3B), LpNC8 grows ~2x10^4 times. Therefore, these results indicate that the rescue of larvae developmental timing by LpNC8 in HDΔPhe is still mediated by bacterial nutrient supply. However, the poor growth of LpNC8 in HDΔPhe suggests the existence of an alternative pathway for Phe biosynthesis in absence of the prephenate dehydratase (Fig 1A). As suggested by Hadadi et al. [87], Phe might be produced from L-arogenate using a
derivative catalysis through the 2.5.1.47 activity which is encoded in Lp\textsuperscript{NC8} by the \textit{cysD} gene (nc8_2167).

A second such interesting case is larval developmental rescue by Lp\textsuperscript{NC8} in HD\textDelta{}Cys. Lp\textsuperscript{NC8} is auxotroph for Cys (Fig 3A), even in static conditions (Fig 4C). Lp\textsuperscript{NC8}– associated larvae develop faster than GF larvae, though GF larvae are not auxotroph to Cys (Fig4A). This beneficial effect of Lp\textsuperscript{NC8} on ex-GF larvae development in HD\textDelta{}Cys is similar to what is observed on a complete HD (Fig 4A, first row) and therefore probably reflects the basal nutrient-independent growth-promoting effect of Lp\textsuperscript{NC8}, that we previously reported and which relies on the molecular dialog between Lp\textsuperscript{NC8} and its host (Fig S2) [23]. Taken together, our results indicate that Lp\textsuperscript{NC8} is able to grow in HD\textDelta{}Cys only in the presence of Dm larvae. To test this hypothesis, we assessed Lp\textsuperscript{NC8} growth in solid HD\textDelta{}Cys in the absence (Medium Load) and the presence (Niche Load) of Dm larvae (Fig 4D). Without larvae (ML), Lp\textsuperscript{NC8} grew one log above the inoculum level in solid HD\textDelta{}Cys (Fig 4D, Medium load). This minimal growth in solid HD\textDelta{}Cys could be due to the Cys reserves from Lp\textsuperscript{NC8} growth in rich media prior to inoculation, or from contaminants in the agar and cholesterol added to prepare the solid HD. Interestingly, in the presence of larvae in the HD\textDelta{}Cys (NL), Lp\textsuperscript{NC8} CFU counts dramatically increased over time, reaching \textasciitilde{}10\textsuperscript{8} CFU/tube at day 6 (net growth: \textasciitilde{}3x10\textsuperscript{4} times inoculum. Fig 4D). These results suggest that in HD\textDelta{}Cys larvae support Lp\textsuperscript{NC8} growth, probably by supplying Cys or a precursor/derivative, which in turn promotes larval development and maturation. This observation extends the recent demonstration that Dm and \textit{L. plantarum} engage in a mutualistic symbiosis, whereby Dm benefits the growth of \textit{L. plantarum} in their shared nutritional environment [30]. Here we discover that Cys is a Dm symbiotic factors also referred to as “bacteria maintenance factors” [30].
Case 4: mineral and metal traces

We observed that both Ap\textsuperscript{WJL} and Lp\textsuperscript{NC8} could compensate for Cu, Fe and Zn deficiencies (Fig 4A, second and third column). Requirements in Cu and Fe were also fulfilled by HK bacteria (Fig 4A, fourth and fifth column) although larvae associated with HK bacteria in these conditions developed much slower than larvae associated with living bacteria. This suggests that the inert bacterial inoculum contains traces of Cu and Fe accumulated during the overnight growth in rich medium prior to the inactivation and inoculation. These accumulated quantities allowed the larvae to develop when Cu and Fe were not supplied in the HD. Surprisingly, Zn requirements were fulfilled by living bacteria only (Fig 4A). We hypothesize that bacteria may concentrate contaminating traces of these elements in the HD and make them more available to the larva. Alternatively, this could be an interesting case of functional complementation that requires further investigation. Indeed, Zn is an important enzymatic cofactor in the biosynthesis of several metabolites by the larva [88]. In the absence of Zn, these compounds would not be produced by the GF larva, instead they could be produced by the bacteria and supplied to the ex-GF larvae similarly to the nutritional complementation we observed above (examples in Case 3). Interestingly, a link between Zn response and the microbiota of Drosophila have been described in previous studies. Expression of the Zn transporter \textit{zip-3} is higher in GF Drosophila adults midguts than in their conventionally raised (CR) counterparts [27]. Moreover, the genes encoding Metallothioneins B and C (\textit{MtnB} and \textit{MtnC}) are more expressed in flies harboring a microbiota than in GF flies [89]. Metallothioneins are intracellular proteins that can store Zn. Their expression, as well as expression of Zn transporters such as \textit{zip-3}, is regulated by intracellular levels of Zn [90]. Altogether, these results suggest that commensal bacteria may play an important role in the uptake of metals.
(especially Zn) by Drosophila larvae. This idea is reminiscent of recent reports in *Caenorhabditis elegans*, whereby a commensal bacteria promotes worm development upon Fe scarcity by secreting a scavenging siderophore [91].

**Commensal bacteria spare amino acids essential to larval development**

Despite the interesting exceptions detailed above, our data establish that in general, bacterial commensals complement the nutritional requirements of their host by synthesizing and supplying essential nutrient. Bacteria can actively excrete amino acids in their environment when they are produced in excess as intracellular by-products of metabolic reactions [92]. Moreover, the bacterial cell wall is rich in D-amino acids, and it undergoes an important turnover [93,94]. In certain bacterial species, D-amino acids accumulate in the supernatant during growth and act as a signal to undergo stationary phase [95]. Thus, D-amino acids may also contribute to larval nutrition. Indeed, it has been previously shown that D-amino acids (D-Arg, D-His, D-Lys, D-Met, D-Phe, and D-Val) can support growth of GF larvae probably through the action of amino acid racemases [47]. We thus hypothesized that Ap<sup>WJL</sup> and Lp<sup>NC8</sup> could provide amino acids to their host by releasing them in the HD. To directly tested this hypothesis, we cultured Ap<sup>WJL</sup> and Lp<sup>NC8</sup> in liquid HD lacking each EAA<sup>Fly</sup> and quantified the production of the corresponding missing EAA<sup>Fly</sup>. We focused on EAA<sup>Fly</sup> whose deficiency could be compensated by the commensals in our DT experiments (Fig. 4A). In these assays, Ap<sup>WJL</sup> was cultured under agitation and Lp<sup>NC8</sup> cultures were grown in both agitated and static conditions (See Methods). After three days, we quantified the amino acids concentration from bacterial supernatants using high pressure liquid chromatography (HPLC). We quantified amino acid production by Ap<sup>WJL</sup> under agitation while growing in HD<sub>Δ</sub>Arg, HD<sub>Δ</sub>His, HD<sub>Δ</sub>Ile, HD<sub>Δ</sub>Leu, HD<sub>Δ</sub>Lys,
HD\(\Delta\)Met, HD\(\Delta\)Phe, HD\(\Delta\)Thr, and HD\(\Delta\)Val and observed accumulation all missing AAs expect for Lys and Met (Fig 5A). For Lp\(^{NC8}\), we analyzed the supernatants of HDs that support Lp\(^{NC8}\) growth under agitation (Fig 3A): HD\(\Delta\)His, HD\(\Delta\)Lys and HD\(\Delta\)Met. We also analyzed supernatants from static conditions, HD\(\Delta\)His, HD\(\Delta\)Lys, HD\(\Delta\)Met, HD\(\Delta\)Phe and HD\(\Delta\)Thr. Surprisingly, from all tested conditions we only detected His accumulation in the supernatant of Lp\(^{NC8}\) grown on HD\(\Delta\)His under agitation (Fig 5B). We did not detect Lys and Met in Ap\(^{WJL}\) culture supernatant or Lp\(^{NC8}\) culture under agitation supernatant nor His, Lys, Met, Phe or Thr in Lp\(^{NC8}\) static culture supernatants. However, Ap\(^{WJL}\) or Lp\(^{NC8}\) can both fulfill larval requirements in a HD lacking these nutrients (Fig 4A). We only analyzed supernatants after 72h of growth, it is therefore possible that we missed the peak of accumulation of the targeted AA, which may have taken place at another time point during the growth phase. Also, Ap\(^{WJL}\) and Lp\(^{NC8}\) may only secrete precursors or catabolites of these AA that we did not target in our analysis. Such AA derivatives may also be used by the larvae to compensate for the lack of the cognate amino acids in the diets. However, we detected Arg, His, Ile, Leu, Phe, Thr and Val production by Ap\(^{WJL}\) and His by Lp\(^{NC8}\), a production which correlates with the respective ability of Ap\(^{WJL}\) and Lp\(^{NC8}\) to compensate for the lack of these AAs in the respective depleted HD. Of note, the concentration of newly synthetized amino acids accumulating in the supernatant is low compared to their concentration in a complete HD (20-150 µM in the former vs 1-5 mM in the latter). However, the bacterial supply of amino acids to the larvae is probably a continuous process, which can be stimulated upon uptake and transit through the larval intestine. Thus, amino acids are directly supplied to the larvae and will fulfill its nutritional requirements without the need to accumulate in the surrounding media. Altogether, our results show that Ap\(^{WJL}\) and Lp\(^{NC8}\) are able to synthetize and excrete some EAA\(^{FLy}\) in their supernatants. These
results confirm our hypothesis that Dm commensal bacteria Ap\textsuperscript{WJL} and Lp\textsuperscript{NC8} produce
these EAA\textsuperscript{Fly} while growing on HD\Delta EAA\textsuperscript{Fly}. When associated to Dm larvae, Ap\textsuperscript{WJL} and
Lp\textsuperscript{NC8} will therefore supply these amino acids to the larvae, allowing larval development
on these deficient media as observed upon mono-associations (Fig 4A).
Conclusion

In this study, we have unraveled the interactions between the microbial and the nutritional environment of Drosophila, as well as the functional importance of these interactions for Drosophila juvenile growth. We systematically characterized, both in silico and in vivo, the biosynthetic capacities of growing GF larvae and two representative commensal strains of bacteria (Ap\textsuperscript{WJL} and Lp\textsuperscript{NC8}). We show that both commensals, each in its unique manner, alleviate the nutritional constraints in the environment to accelerate host growth and maturation in diets depleted of essential nutrients (Fig 6). The capacity of a bacterium to fulfill the larval requirements in a specific nutrient correlated with its metabolic activity and in most cases its ability to produce the missing nutrient. In contrast to obligate symbioses, our results highlight the clear separation between the metabolic pathways of the host and its commensals and reveal a particularly integrated nutritional network between the insect and its facultative commensals around the sparing and utilization of nutrients.

Importantly, we further demonstrate that active nutrient provision by metabolite biosynthesis not only evoke the canonical pathways, but also non-canonical pathways such as the ones described here, for example nicotinate by both Ap\textsuperscript{WJL} and Lp\textsuperscript{NC8}, pyridoxine by Ap\textsuperscript{WJL}, and Phe by Lp\textsuperscript{NC8}. Interestingly, we also detected two cases where nutrient compensation is not explained by a direct nutrient supply: (1) the compensation of choline deficiency by Ap\textsuperscript{WJL} and (2) the compensation of Zn deficiency by both Ap\textsuperscript{WJL} and Lp\textsuperscript{NC8}. We propose the existence of functional compensation mechanisms whereby Ap\textsuperscript{WJL} would complement choline deficiency by synthesizing and providing precursors and/or functional analogues of choline, such as ethanolamine or glycerol and both commensals can compensate Zn deficiency by uptaking, concentrating and sparing contaminant traces of Zn to the host.
Previous works have shown that the molecular dialogue between the host and its commensal partners invokes the sensing of commensal bacteria cell wall and the induction of transcriptomic rewiring of the digestive potential in the Drosophila enterocytes. Here we show that in addition, the metabolic activities of live Drosophila’s commensal bacteria correct host auxotrophies. These results reveal a novel facet of the facultative nutritional mutualism engaged between Drosophila and its commensal bacteria, which supports the host’s nutritional versatility and allows the juvenile host to better cope with changes in nutrients availability during the critical phase of post-natal growth; hence ensuring optimal host fitness. Our work lays the foundational blueprint for further mechanistic studies to investigate if and how commensals regulate the synthesis and release of essential nutrients for the host and if the host influences this regulatory process. Dissecting how commensals functionally compensate for nutrients that they cannot produce also constitute an attractive perspective for future investigations.

In some cases, the in silico prediction of bacterial biosynthetic capabilities were incongruent with our in vivo assessment of bacterial auxotrophies (Table S6). Such seeming discrepancy served as an entry point for us to discover novel phenomena and interactions that would have been missed had we only adopted a single approach. One such interesting example is Asn auxotrophy unique to the Dm yw line in GF conditions. Another one is the larval provision of Cys (or its derivatives) to LpNC8 to maintain a mutualistic nutritional exchange between host and commensal. Previously, combination of in silico and in vivo approaches has been successfully used for bacteria [66] but not applied to complex symbiotic systems such as host-commensals nutritional interactions. Indeed, reports characterize these interactions in silico [96] but they were not confirmed in vivo. Our work closes this gap and emphasizes the importance of
using parallel systematic *in silico* and *in vivo* approaches for the understanding of the intricate relationships between the microbial and the nutritional environments and their impact on animal juvenile growth.
In silico reconstruction of the biosynthetic potential of *D. melanogaster* and its commensals

We used the Cyc Annotation Database System (CycADS) [62], an automated annotation management system, to integrate protein annotations from the complete genomes of *Drosophila melanogaster* (RefSeq GCF_000001215.4 release 6), *A. pomorum* strain DM001 (accession NCBI-Taxonomy 945681 version 1.0.1) and *L. plantarum* subsp. *plantarum* NC8 (NCBI Bioproject PRJNA67175). CycADS collects protein annotation results from different annotation methods including KAAS [97], PRIAM [98], Blast2GO [99,100], and InterProScan [101] in order to obtain EC numbers and GO annotations. All annotation information was then processed in the CycADS SQL database and automatically extracted to generate appropriate input files to build the three BioCyc databases using the Pathway Tools software v22.5 [102]. The BioCyc databases and their associated metabolic networks are available in the EcoCyc database [103]. From the genomic analyses, we inferred the biosynthetic capabilities of the three organisms and manually inspected all pathways allowing production of the organic compounds that are present in the exome-based FLYAA holidic diet (HD) developed by Piper et al [40]: fly’s essential and non-essential amino acids (EAA\textsuperscript{Fly} (n=10) and NEAA\textsuperscript{Fly} (n=10)), B-vitamins (n=7), cholesterol (n=1), and Nucleic Acids and Lipid precursors (NAL, n=4). For each gap found in biosynthetic pathways or non-conventional enzymatic catalysis, TBLASTN [104] searches were performed in the three genomes to look for unpredicted protein activities. Alternative pathways were searched in the literature or using the BioCyc “Metabolic Route Search” tool [105].
Drosophila stocks were reared as described previously [32]. Briefly, flies were kept at 25°C with 12/12-hour dark/light cycles on a yeast/cornmeal medium containing 50 g/L of inactivated yeast, 80 g/L of cornmeal, 7.4 g/L of agar, 4 mL/L of propionic acid and 5.2 g/L of nipagin. Germ-free stocks were established as described previously [89] and maintained in yeast/cornmeal medium supplemented with an antibiotic cocktail composed of kanamycin (50 µg/mL), ampicillin (50 µg/mL), tetracycline (10 µg/mL) and erythromycin (5 µg/mL). Axenicity was tested by plating fly media on nutrient agar plates. Drosophila yw flies were used as the reference strain in this work.

Other Drosophila lines used include DGRP-RAL-25210 [82] and w1118 [106].

Experiments were performed on Holidic Diet (HD) without preservatives. Complete HD, with a total of 8 g/L of amino acids, was prepared as described by Piper at al. using the fly’s exome matched amino acid ratios (FLYAA) [40]. Briefly, sucrose, agar, amino acids with low solubility (Ile, Leu and Tyr) as well as stock solutions of metal ions and cholesterol were combined in an autoclavable bottle with milli-Q water up to the desired volume, minus the volume of solutions to be added after autoclaving. After autoclaving at 120°C for 15 min, the solution was allowed to cool down at room temperature to ~60°C. Acetic acid buffer and stock solutions for the essential and non-essential amino acids, vitamins, nucleic acids and lipids were added. Single nutrient deficient HD was prepared following the same recipe excluding the nutrient of interest (named HDΔX, X being the nutrient omitted). Tubes used to pour the HD were sterilized under UV for 20 min. HD was stored at 4°C until use, for no longer than one week.

Bacterial strains and growth conditions
A. pomorum\textsuperscript{WJL} (Ap\textsuperscript{WJL}) [25], L. plantarum\textsuperscript{NC8} (Lp\textsuperscript{NC8}) [107], Ap\textsuperscript{WJL::Tnppq} [25] and Lp\textsuperscript{NC8}\textsuperscript{Δdlt}op [23] were used in this study. A. pomorum strains were cultured in 10 mL of Mannitol Broth (Bacto peptone 3 g/L, yeast extract 5 g/L, D-mannitol 25 g/L) in 50 mL flask at 30°C under 180 rpm during 24h. L. plantarum strains were cultured in 10 mL of MRS broth (Carl Roth, Germany) in 15 mL culture tubes at 37°C, without agitation, overnight. Liquid or solid cultures of Ap\textsuperscript{WJL::Tnppq} were supplemented with kanamycin (Sigma-Aldrich, Germany) at a final concentration of 50 µg/mL. CFU counts were performed for all strains on MRS agar (Carl Roth, Germany) plated using the Easyspiral automatic plater (Intersciences, Saint Nom, France). The MRS agar plates were then incubated for 24-48h at 30°C for Ap\textsuperscript{WJL} or 37°C for Lp\textsuperscript{NC8}. CFU counts were done using the automatic colony counter Scan1200 (Intersciences, Saint Nom, France) and its counting software.

**Bacterial growth in liquid HD**

To assess bacterial growth in the fly nutritional environment we developed a liquid HD comprising all HD components except agar and cholesterol. Liquid HD was prepared as described for HD. Single nutrient deficient liquid HD was prepared following the same recipe excluding the nutrient of interest. After growth in rich media, PBS washed Ap\textsuperscript{WJL} or Lp\textsuperscript{NC8} were inoculated at a final concentration of ~ 10\textsuperscript{6} CFU/mL in 200 µL of either complete liquid HD or nutrient deficient liquid HD. Cultures were incubated in 96-well microtiter plates (Nunc\textsuperscript{™} Edge 2.0. Thermo Fisher Scientific) at 30°C for 72h. Growth was monitored using an SPECTROstar\textsuperscript{Nano} (BMG Labtech GmbH, Ortenberg, Germany) by measuring the optical density at 600 nm (OD\textsubscript{600}) every 30 minutes. Heatmap in Fig 3A represents the maximal OD detected during the 72h of growth (average of three replicates). The whole experiment was repeated at least
twice. Fig 3A was created using the imagesc function on MATLAB (version 2016b. MathWorks, Natick, Massachusetts). LpNC8 growth in static conditions was performed in 10 mL of liquid HD in 15-mL falcon tubes inoculated at a final concentration of \( \sim 10^4 \) CFU/mL. Cultures were incubated at 30°C for 72h. After incubation, cultures were diluted in PBS and plated on MRS agar as described above. Growth is expressed as

\[
\frac{\text{CFU}(t = 72h) - \text{CFU}(t = 0)}{\text{CFU}(t = 0)}.
\]

**Bacterial growth in solid HD**

Bacterial CFUs in HDΔCys were assessed in presence (Niche Load: NL) or absence (Medium Load: ML) of Dm larvae. Microtubes containing 400 \( \mu \)L of HD and 0.75–1 mm glass microbeads were inoculated with \( \sim 10^4 \) CFUs of LpNC8. For NL, 5 first-instar larvae, collected from eggs laid on HDΔCys, were added. The tubes were incubated at 30°C for 0, 3 or 6 days. After incubation, 600\( \mu \)L of PBS were added directly into the microtubes. Samples were homogenized with the Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Lysates dilutions (in PBS) were plated on MRS and CFU counts were assessed as described above. Growth is expressed as

\[
\frac{\text{CFU}(t = 72h) - \text{CFU}(t = 0)}{\text{CFU}(t = 0)}.
\]

**Developmental timing determination**

Axenic adults were placed in sterile breeding cages overnight to lay eggs on sterile HD. The HD used to collect embryos always matched the experimental condition. Fresh axenic embryos were collected the next morning and seeded by pools of 40 in tubes containing the HD to test. For the mono-associated conditions a total of
~10^7 CFUs of Ap^{WJL} or ~10^8 CFUs of Lp^{NC8}, washed on PBS, were inoculated on the substrate and the eggs. Inoculation of Ap^{WJL} was limited to ~10^7 CFUs because higher inoculums decreased egg-to-pupa survival. For heat killed (HK) conditions, washed cells of Ap^{WJL} or Lp^{NC8} were incubated 3h at 65°C. Once at room temperature, embryos were inoculated with ~10^8 CFUs and ~10^9 CFUs, respectively. In the germ-free conditions, bacterial suspensions were replaced with sterile PBS. Tubes were incubated at 25°C with 12/12-hour dark/light cycles. The emergence of pupae was scored every day until all pupae had emerged. The experiment was stopped when no pupae emerged after 30 days. Each gnotobiotic or nutritional condition was inoculated in five replicates. Means, standard error of the mean and statistical tests (Dunn’s test of multiple comparisons) are detailed in Table S4. Since larvae are cannibalistic and can find missing nutrients by eating their siblings [108,109] therefore we excluded replicates with low egg-to-pupa survival (<25%, i.e n<10). Moreover, we considered that larvae failed to develop in one condition if the mean egg-to-pupa survival of the five replicates was inferior to 25% (for details in egg-to-pupa survival, see Table S5). D_{50} was determined using D50App (http://paulinejoncour.shinyapps.io/D50App) as described previously [23]. The whole experiment was repeated at least twice. D_{50} heatmap represent the average of the five replicates of each gnotobiotic and nutritional condition. Fig 4A was done using the imagesc function on MATLAB (version 2016b. MathWorks, Natick, Massachusetts).

**DNA extraction and AsnS locus analyses**

Genomic DNA from 2 adults yw flies was extracted as previously described [110]. Briefly, flies were grinded in microtubes containing 0.75–1 mm glass microbeads and 500 µL of lysis buffer (Tris-HCl 10 mM, EDTA 1 mM, NaCl 1 mM at pH 8.2) using the
Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Then, we added Proteinase K (PureLink Genomic DNA extraction kit, Invotrogen) at a final concentration of 200 µg/mL and incubated the samples at 56°C under 700 rpm agitation for 1h. The samples were centrifuged at 10000 g for 2 min and we collected the supernatant. AsnS coding sequence was amplified by PCR (Q5 Pol High Fidelity M0491S, New England BioLabs) using the primers AsnS_F (CGGGCCGCTTCTGTTAAAAA) and AsnS_R (TGGATTCTCAGACTTGCCA) with a Veriti Thermal Cycler (Applied BioSystems, USA). PCR products were purified using the NucleoSpin Gel and PCR Cleanup kit (Macherey-Nagel, Germany) following manufacturer’s instructions. Sequencing was done by Sanger sequencing (Genewiz, Germany) using the following primers: AsnS_F, AsnS_R, AsnS1 (AGGATTATGGAAAGGATCTTCTGCA), AsnS2 (CTCCGTCGGATTTGCATCA), AsnS3 (TAATGCCAAGGGGTCTCGG) and AsnS4 (GTGCGGCAGCTGCATTATC). The whole coding sequence was then assembled and analyzed using Geneious (version 10.1.3. Biomatters Ltd. New Zealand) by mapping on the reference D. melanogaster genome (RefSeq GCF_000001215.4 release 6).

Amino acid quantification by HPLC

After growth in rich media, PBS washed Ap\textsuperscript{WJL} or Lp\textsuperscript{NC8} were inoculated in triplicates at a final concentration of ~10\textsuperscript{6} CFU/mL into 10 mL of each liquid HD\textsuperscript{ΔEA}\textsuperscript{Fly} shown to support their growth (Fig 1A, B) and in which they fulfill larval requirements. For Ap\textsuperscript{WJL} this includes liquid HD\textsuperscript{ΔArg}, HD\textsuperscript{ΔHis}, HD\textsuperscript{ΔIle}, HD\textsuperscript{ΔLeu}, HD\textsuperscript{ΔLys}, HD\textsuperscript{ΔMet}, HD\textsuperscript{ΔPhe}, HD\textsuperscript{ΔThr}, HD\textsuperscript{ΔVal} in agitated conditions. For Lp\textsuperscript{NC8} this includes liquid HD\textsuperscript{ΔHis}, HD\textsuperscript{ΔLys} and HD\textsuperscript{ΔMet} in agitated conditions and liquid HD\textsuperscript{ΔHis}, HD\textsuperscript{ΔLys},
HDΔMet, HDΔPhe and HDΔThr in static conditions. For agitated conditions cultures were done in 50 mL flasks and incubated at 30°C under 180 rpm. Static conditions were performed in 15-mL falcon tubes at 30°C. Samples were taken at time 0h and 72h. Samples were centrifuged (5000 rpm, 5 min). Supernatants were collected and stored at -20°C until use.

Amino acid quantification was performed by HPLC from the supernatants obtained at 0h and 72h. Samples were crushed in 320 μl of ultra-pure water with a known quantity of norvaline used as the internal standard. Each sample was submitted to a classical protein hydrolysis in sealed glass tubes with Teflon-lined screw caps (6N HCl, 115°C, during 22h). After air vacuum removal, tubes were purged with nitrogen. All samples were stored at -20°C, and then mixed with 50 μL of ultra-pure water for amino acids analyses. Amino acid analysis was performed by HPLC (Agilent 1100; Agilent Technologies, Massy, France) with a guard cartridge and a reverse phase C18 column (Zorbax Eclipse-AAA 3.5 μm, 150 × 4.6 mm, Agilent Technologies). Prior to injection, the sample was buffered with borate at pH 10.2, and primary or secondary amino acids were derivatized with ortho-phthalaldehyde (OPA) or 9-fluorenylmethyl chloroformate (FMOC), respectively. The derivatization process, at room temperature, was automated using the Agilent 1313A autosampler. Separation was carried out at 40°C, with a flow rate of 2 mL/min, using 40 mM NaH2PO4 (eluent A, pH 7.8, adjusted with NaOH) as the polar phase and an acetonitrile/methanol/water mixture (45/45/10, v/v/v) as the non-polar phase (eluent B). A gradient was applied during chromatography, starting with 20% of B and increasing to 80% at the end. Detection was performed by a fluorescence detector set at 340 and 450 nm of excitation and emission wavelengths, respectively (266/305 nm for proline). These conditions do not allow for the detection and quantification of cysteine and tryptophan, so only 18 amino
acids were quantified. For this quantification, norvaline was used as the internal
standard and the response factor of each amino acid was determined using a 250
pmol/μl standard mix of amino acids. The software used was the ChemStation for LC
3D Systems (Agilent Technologies).

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Figure legends

Fig 1. In silico metabolic network reconstruction of Dm, ApWJL and LpNC8
(A) Amino acids biosynthetic pathways. (B) Vitamins and cofactors biosynthetic
pathways. Left panels, Drosophila melanogaster. Central panels, Acetobacter
pomorumWJL. Right panels, Lactobacillus plantarumNC8. Color code: blue,
biosynthesized amino acid or vitamin; brown, limited amino acid or vitamin
biosynthesis (biosynthesis of the metabolite may be possible, but it is limited and/or
requires secondary metabolic pathways); black, non-biosynthesized amino acid or
vitamin; grey, pathway intermediary metabolite. Red cross: non-functional pathway
(lack of key enzyme(s)). Orange nods, major metabolic pathways. Abbreviations: α-cglu: α-keto-glutarate. AceCoA: Acetyl-CoA. Ant: Antranilate. Aro: Arogenate. Cho: chorismate. Cit: Citrate. Cysta: Cystathionine. Dihyn-P3: 7,8-Dihydroneopterin-3'-P3. Dm-ribi: 6,7-Dimethyl-8-ribityllumazine. Ery-4P: Erythrose-4P. Fum: Fumarate. Glc: Glucose. Gly-3P: Glycerate-3P. Homocs: Homocysteine. Homoser: Homoserine. Ind: Indole. Orn: Ornithine. Oxa: Oxaloacetate. P-ra-imi: 1-(5'-Phospho-ribosyl)-5-aminoimidazole. Phoser: Phosphoserine. Pre: Prephenate. Pyn-P: Pyridoxine phosphate. Pyr: Pyruvate. Rib-5P: Ribose-5P.

**Fig 2.** Dm, Ap<sup>WJL</sup> and Lp<sup>NC8</sup> have differential biosynthetic capacities of nutrients contained in the holidic diet

Venn diagram represents the number of HD nutrients that can be synthesized by each organism. The list of corresponding metabolites is provided. Green dotted circles: biosynthesis of this metabolite by Lp<sup>NC8</sup> may be possible, but it is limited and/or requires secondary metabolic pathways.

**Fig 3.** Ap<sup>WJL</sup> and Lp<sup>NC8</sup> auxotrophies detected in liquid fly Holidic Diet (HD)

(A) Heat map representing the mean maximal optical density (OD<sub>Max</sub>) reached by Ap<sup>WJL</sup> or Lp<sup>NC8</sup> after 72h of culture. Each line shows growth in a different version of liquid HD: complete HD (first line) or HD lacking nutrient X (ΔX, lines below). EAA<sup>Fly</sup>: Fly’s Essential Amino Acids, NEAA<sup>Fly</sup>: Fly’s Non-Essential Amino Acids, NAL: Nucleic Acids and Lipids. Cultures were made in 96-wells plate under agitation. Asterisks (*) pinpoint contradictions with in silico analysis that are explained in panel B. (B) Net growth of Lp<sup>NC8</sup> in four versions of liquid HD: complete HD, HDΔThr, HDΔAla and
HDΔAsp, in static conditions. Plot shows means with standard error based on three replicates by assay. Each dot represents an independent replicate.

Fig 4. Ap\textsuperscript{WJL} and Lp\textsuperscript{NC8} can differentially fulfill their host’s nutritional requirements

(A) Heat map representing the mean $D_{50}$ (i.e. day when 50% of the larvae population has entered metamorphosis) of GF larvae (first column) and larvae associated with Ap\textsuperscript{WJL}, Lp\textsuperscript{NC8}, Ap\textsuperscript{WJL\textsubscript{HK}} and Lp\textsuperscript{NC8\textsubscript{HK}} (columns 2, 3, 4, 5 respectively). Each line shows $D_{50}$ in a different version of HD: complete HD (first line) or HD lacking nutrient X (ΔX, lines below). EAA\textsubscript{Fly}: Fly’s Essential Amino Acids, NEAA\textsubscript{Fly}: Fly’s Non-Essential Amino Acids, NAL: Nucleic Acids and Lipids. White means larvae did not reach pupariation in these conditions. Means, standard errors of the mean and statistical tests (Dunn’s test of multiple comparisons) are detailed in Table S4. (B) $D_{50}$ of yw, DGRP-\textit{RAL}-25210 and w\textsuperscript{1118} larvae on HDΔAsn. Boxplots show minimum, maximum and median. Each dot shows an independent replicate. GF yw larvae did not reach pupariation. For the other two lines, we performed Kruskal-Wallis test followed by post-hoc Dunn’s tests to compare each gnotobiotic condition to GF. ns: non-significant, **: p-value<0.005, ***: p-value<0.0005, ****: p-value<0.0001. (C) Growth of Lp\textsuperscript{NC8} in liquid HDΔPhe and liquid HDΔCys, in static conditions, 3 days after inoculation. Plot shows mean with standard error. Each dot shows an independent replicate. (D) Growth of Lp\textsuperscript{NC8} in solid HDΔCys, in absence and in presence of larvae (Medium Load and Niche Load, respectively), 3 days and 6 days after inoculation. Plot shows mean with standard error based on five replicates by assay. Each dot represents an independent replicate. We performed two-ways ANOVA followed by post-hoc Sidak’s test. ns: non-significant, **: p-value<0.005.
Fig 5. ApWJL and LpNC8 can accumulate EAA\textsubscript{Fly} during growth

(A) HPLC measured concentration of Arg, His, Ile, Leu, Phe, Thr and Val in the supernatant of an ApWJL culture in HD\Delta Arg, HD\Delta His, HD\Delta Ile, HD\Delta Leu, HD\Delta Phe, HD\Delta Thr, HD\Delta Val, respectively, 72h after inoculation. Plot shows mean with standard error. Each dot shows an independent replicate. (B) HPLC measured concentration of His in the supernatant of a LpNC8 culture in HD\Delta His, 72h after inoculation. Plot shows mean with standard error. Each dot shows an independent replicate.

Fig 6. Commensal bacterial differentially shape the nutritional requirements of their juvenile host

For each gnotobiotic condition, essential nutrients are represented in black and non-essential nutrients in color. Color code: blue, this nutrient can be synthesized by the bacteria; red: this nutrient cannot be synthesized by the bacteria, suggesting a mechanism of functional compensation. In purple: this nutrient may be synthesized by the bacteria through still unknown metabolic pathways.

Supporting Information

Table S1: Inference from genomic analysis of the biosynthetic capabilities for amino acids production in D. melanogaster, A. pomorum\textsuperscript{WJL} and L. plantarum\textsuperscript{NC8}. f / i: targeted amino acid biosynthesis is feasible / impossible in a depleted medium.

Table S2: Inference from genomic analysis of the biosynthetic capabilities for vitamins production in D. melanogaster, A. pomorum\textsuperscript{WJL} and L. plantarum\textsuperscript{NC8}. f / i: targeted vitamin biosynthesis is feasible / impossible in a depleted medium.
Table S3: OD_{Max} of Ap^{WJL} and Lp^{NC8} grown in 39 HDs. Mean and Standard Error to
the Mean (SEM) of OD_{Max} reached by Ap^{WJL} or Lp^{NC8} grown in complete liquid HD (first
line) or liquid HD lacking nutrient X (ΔX, lines below) during 72h of growth.

Table S4: D_{50} of larvae in 40 HDs and 5 gnotobiotic conditions. Mean and Standard
Error of the Mean (SEM) of D_{50} (i.e. day when 50% of the larvae population has entered
metamorphosis) of GF larvae or larvae associated with Ap^{WJL}, Lp^{NC8}, Ap^{WJL-HK} and
Lp^{NC8-HK}. n: number of independent replicates for each condition. For each gnotobiotic
condition, we performed Kruskal-Wallis test followed by post-hoc Dunn’s test to
compare each nutritional environment to complete HD.

Table S5: Egg-to-pupa survival in 40 HDs and 5 gnotobiotic conditions. Mean and
Standard Error of Mean (SEM) of egg-to-pupa survival of GF larvae or larvae
associated with Ap^{WJL}, Lp^{NC8}, Ap^{WJL-HK} and Lp^{NC8-HK}. n: number of independent
replicates for each condition. For each gnotobiotic condition, we performed Kruskal-
Wallis test followed by post-hoc Dunn’s test to compare each nutritional environment
to complete HD.

Table S6: Comparison of in silico predictions with in vivo auxotrophies and bacterial
complementation of larval nutritional deficiencies. Can partner A synthesize nutrient
X?: prediction from in silico metabolic reconstruction (from Fig 1, Table S1 and Table
S2). Can partner A grow in the absence of nutrient X?: auxotrophy observed in vivo
(from Fig 3A-B). Can bacterial partner A promote larval growth on HD ΔX?: in vivo
complementation of ex-GF larvae requirements (from Fig 4A), y: yes (green), n: no
Hypothesis to explain contradiction: why the different approaches do not always lead to the same conclusion. NA: Non-applicable.

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Figure 2

Dm

Myo-inositol

Ala
Asn
Asp
Inosine
Gln
Uridine
Glu
Gly
Pro
Ser
Tyr
Pyrodoxine

Myo-inositol

Arg
Folate
Riboflavine
Thiamine
His
Lys
Met
Thr
Trp

Lp^{NC8}

Ap^{WJL}

11

1

9

6

3

1

0

Cholesterol
Nicotinate
Choline
Cys
Ile
Leu
Biotin
Phe
Pantothenate
Val

1

1

1

1

2

0

3
### Figure 3

#### A

| Nutritional environment | Ap<sup>WJL</sup> | Lp<sup>NC8</sup> |
|-------------------------|------------------|------------------|
| **EAA<sup>Fly</sup>**  |                  |                  |
| ΔSucrose                |                  |                  |
| ΔArg                    |                  |                  |
| ΔHis                    |                  |                  |
| ΔIle                    |                  |                  |
| ΔLeu                    |                  |                  |
| ΔLys                    |                  |                  |
| ΔMet                    |                  |                  |
| ΔPhe                    |                  |                  |
| ΔThr                    |                  |                  |
| ΔTrp                    |                  |                  |
| ΔVal                    |                  |                  |
| ΔAla                    |                  |                  |
| ΔAsn                    |                  |                  |
| ΔAsp                    |                  |                  |
| ΔCys                    |                  |                  |
| ΔGln                    |                  |                  |
| ΔGlu                    |                  |                  |
| ΔGly                    |                  |                  |
| ΔPro                    |                  |                  |
| ΔSer                    |                  |                  |
| ΔTyr                    |                  |                  |
| ΔBiotin                 |                  |                  |
| ΔFolate                 |                  |                  |
| ΔNicotinate             |                  |                  |
| ΔPantothenate           |                  |                  |
| ΔPyridoxine             |                  |                  |
| ΔRiboflavin             |                  |                  |
| ΔThiamine               |                  |                  |
| ΔCholine                |                  |                  |
| ΔMyo-inositol           |                  |                  |
| ΔInosine                |                  |                  |
| ΔUridine                |                  |                  |
| **NAL precursors**      |                  |                  |
| ΔCaCl<sub>2</sub>       |                  |                  |
| ΔCuSO<sub>4</sub>       |                  |                  |
| ΔFeSO<sub>4</sub>       |                  |                  |
| ΔMgSO<sub>4</sub>       |                  |                  |
| ΔMnCl<sub>2</sub>       |                  |                  |
| ΔZnSO<sub>4</sub>       |                  |                  |
| **Traces**              |                  |                  |

#### B

**Lp<sup>NC8</sup> growth in static conditions**

**Growth X-times inoculum (Log)**

- Complete HD
- HDΔThr
- HDΔAla
- HDΔAsp

---

*Note: The figure shows a heatmap and a bar graph representing the growth of two strains, Ap<sup>WJL</sup> and Lp<sup>NC8</sup>, under different nutritional environments. The heatmap highlights the growth of each strain under various conditions, while the bar graph compares the growth of Lp<sup>NC8</sup> under different treatments in a log scale.*
Figure 4

A Microbial environment

Complete HD

\[ \Delta \text{Sucrose} \]

\[ \Delta \text{Arg} \]

\[ \Delta \text{His} \]

\[ \Delta \text{Ile} \]

\[ \Delta \text{Leu} \]

\[ \Delta \text{Lys} \]

\[ \Delta \text{Met} \]

\[ \Delta \text{Phe} \]

\[ \Delta \text{Thr} \]

\[ \Delta \text{Trp} \]

\[ \Delta \text{Val} \]

\[ \Delta \text{Asn} \]

\[ \Delta \text{Asp} \]

\[ \Delta \text{Cys} \]

\[ \Delta \text{Gln} \]

\[ \Delta \text{Glu} \]

\[ \Delta \text{Gly} \]

\[ \Delta \text{Pro} \]

\[ \Delta \text{Ser} \]

\[ \Delta \text{Tyr} \]

\[ \Delta \text{Biotin} \]

\[ \Delta \text{Folate} \]

\[ \Delta \text{Nicotinate} \]

\[ \Delta \text{Pantothenate} \]

\[ \Delta \text{Pyridoxine} \]

\[ \Delta \text{Riboflavin} \]

\[ \Delta \text{Thiamine} \]

\[ \Delta \text{Cholesterol} \]

\[ \Delta \text{Choline} \]

\[ \Delta \text{Myo-inositol} \]

\[ \Delta \text{Inosine} \]

\[ \Delta \text{Uridine} \]

\[ \Delta \text{CaCl}_2 \]

\[ \Delta \text{CuSO}_4 \]

\[ \Delta \text{FeSO}_4 \]

\[ \Delta \text{MgSO}_4 \]

\[ \Delta \text{MnCl}_2 \]

\[ \Delta \text{ZnSO}_4 \]

B Asn essentiality is genotype-dependent

\[ yw \]

\[ \text{DGRP-RAL-25210} \]

\[ w^{118} \]

C Lp^{NC8} growth in static conditions

D Lp^{NC8} growth in solid HDΔCys

Growth X-times inoculum (Log)

\( \times 10^0 \)

\( \times 10^1 \)

\( \times 10^2 \)

\( \times 10^3 \)

\( \times 10^4 \)

\( \times 10^5 \)

Time (Days)

\( 3 \)

\( 6 \)

\( 3 \)

\( 6 \)

No pupae
Figure 5

(A) Amino acid concentration (µM) for HDΔArg, HDΔHis, HDΔIle, HDΔLeu, HDΔPhe, HDΔThr, and HDΔVal.

(B) Amino acid concentration (µM) for HDΔHis with additional data for ApWJL and LpNC8.
Figure 6

GF larva: 22 essential nutrients

Mineral and metal traces

Vitamins

Arg
His
Ile
Leu
Lys
Met
Phe
Thr
Trp
Val
Asn

NAL precursors

Cholesterol
Choline

Ap\textsuperscript{WUL}-monoassociated larva: 3 essential nutrients

Mineral and metal traces

Vitamins

Arg
His
Ile
Leu
Lys
Met
Phe
Thr
Trp
Val
Asn

NAL precursors

Cholesterol
Choline

Biotin
Folate
Nicotinate
Pantothenate
Pyridoxine
Riboflavine
Thiamine

Lp\textsuperscript{NCS}-monoassociated larva: 11 essential nutrients

Mineral and metal traces

Vitamins

Arg
His
Ile
Leu
Lys
Met
Phe
Thr
Trp
Val
Asn

NAL precursors

Cholesterol
Choline

Biotin
Folate
Nicotinate
Pantothenate
Pyridoxine
Riboflavine
Thiamine

Legend:

\textbf{aa}= Dm essential nutrient.

\textbf{aa}= Dm non-essential nutrient, can be produced by the bacteria.

\textbf{aa}= Dm non-essential nutrient. Compensation by the bacteria without synthesis= Functional compensation.

\textbf{aa}= Dm non-essential nutrient. Possible synthesis by the bacteria through undetected pathways.