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The involvement of the microbiota in insect physiology: focus on B vitamins

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Abstract

Insects are highly successful in colonizing a wide spectrum of ecological niches and in feeding on a wide diversity of diets. This is notably linked to their capacity to get from their microbiota any essential component lacking in the diet such as vitamins and amino acids. Over one century of research based on dietary analysis, antimicrobial treatment, gnotobiotic rearing, culture-independent microbe detection progressively gathered detailed information on the implication of the microbiota in specific aspects of insect fitness. Thanks to the recent increase in sequencing capacities, whole-genome sequencing of a number of symbionts allowed to trace biosynthesis pathways and thus to validate experimental data and to assess their evolution. This field of research generated a considerable set of data in a diversity of hosts harboring specific symbionts or non-specific microbiota members. Here, we review the current knowledge on the involvement of the microbiota in insect and tick nutrition, with a particular focus on B vitamin provision. We notably question if there is any specificity of B vitamin provision by symbionts compared to the redundant yet essential contribution of non-specific microbes. We successively highlight the known aspects of microbial vitamin provision during three main life stages of invertebrates, post-embryonic development, adulthood and reproduction.

Introduction

Insects are the most diverse and broadest clade in the animal kingdom. As other animals, they are not able to produce some essential metabolites, including some amino-acids, which are crucial building blocks of the organism, thus required in large quantities, and

vitamins, which are nutrients required in small quantities as they catalyze central metabolic pathways. The insect's ability to occupy a great diversity of ecological niches largely depends on the variability of their diet and on the beneficial microorganisms that they harbor, which provide them with essential nutrients lacking in their diet (Douglas, 2017; Kucuk, 2020; Yoshii et al., 2019). Host-microbe relationships in insects influence diverse aspects of their physiology related to digestion, nutrition, defense against pathogens, behavior, immunity, detoxification, reproduction, renewal of the intestinal epithelium (Consuegra et al., 2020; Engel & Moran, 2013; Shin et al., 2011; Storelli et al., 2011). The study of nutritional contribution of microbes to insect development started in the first half of the 20th century with the identification of minimal nutritional requirements of insects in sterile conditions. Early research suggested an essential role of bacteria in provisioning amino acids and B vitamins and thus supporting the development and survival of various insects (Blewett & Fraenkel, 1944; Brecher & Wigglesworth, 1944; Dadd, 1973). More recent data were overall in line with such observations, yet also reported inconsistencies where previously reported diets may not support full development in the complete absence of bacteria (Correa et al., 2018). Methodological errors such as dietary impurities or undetected microbial contamination in germ-free individuals may have led to undermine the importance of specific microbe-derived nutrients (Dadd, 1973). Alternatively, some vitamins may have not been found as essential because they are required in minute amounts, hence present in sufficient quantity in the egg to complete development (Piper et al., 2014). The improvement of techniques in molecular biology, chemistry and high-throughput sequencing now allows to better control experimental conditions and to combine experimental work with genomic analyses in order to produce clearer data on nutritional contributions of microbiota members to their insect hosts (Douglas, 2017).

Our current knowledge on host-microbe nutritional interactions is mainly focused on host-bacteria relationships, even though there is also some evidence of nutritional symbiosis with yeasts and viruses (Biedermann & Vega, 2020; Gurung et al., 2019; Voronovsky et al., 2004), and of nutritional competition between hosts and eukaryotic parasites (Hart et al., 2015; Onchuru et al., 2018; Rio et al., 2019). Among the insect-associated bacteria, endosymbionts are intracellular bacteria which are vertically transmitted from mother to

offspring in the egg cytoplasm. Among them, *Wolbachia* is an Alphaproteobacterium colonizing 40% of the insect species; *Wigglesworthia* and *Buchnera* are two Gammaproteobacteria found in tsetse flies and aphids respectively, carried in a specific organ called the bacteriocyte; several *Spiroplasma* bacterial species, which belong to *Mollicutes*, colonize 5-10% of the insect species and have extracellular and intracellular forms (Herren et al., 2014; Michalkova et al., 2014; Nakabachi & Ishikawa, 1999; Snyder & Rio, 2015). Endosymbionts generally have a reduced genome size characterized by increased A-T content and gene loss (Husnik & McCutcheon, 2018; McCutcheon & Moran, 2012). These features may be explained by a combination of two evolution biology models. Firstly, Muller's ratchet hypothesis states that the constant evolution of organisms produces mutations, which accumulate and become costly to repair, triggering the loss of non-essential features; Secondly, the Black Queen hypothesis stresses that in the evolution of a community resources may be shared, which reduces the selection pressure on essential metabolic pathways in each member, leading to genome size reduction and enhanced interdependence of the members (Felsenstein, 1974; Morris et al., 2012). In line with these genomic features, nutritional interactions have been reported between hosts and endosymbionts, and have been classified as primary and secondary symbioses, whether the bacterium is essential or not to host physiology, respectively (Gupta & Nair, 2020; Putnam & Goodman, 2020).

Insects also harbor ectosymbionts, extracellular bacteria with a facultative association with their host, yet with closely-related genome characteristics to endosymbionts, including genome reduction, gene loss and signatures of horizontal gene transfers from/to genomes of the host or other microbes (Engel & Moran, 2013; Husnik & McCutcheon, 2018; Kucuk, 2020). The microbiota of insect also encompasses bacteria which do not have any specific association with their host, they may participate to the host nutrition but can be replaced by almost any other bacterium able to colonize the host (Coon et al., 2014). Some of these bacteria cannot strictly be defined as extracellular or intracellular. Besides the above-mentioned example of *Spiroplasma* endosymbionts, the known extracellular bacteria *Cedecea neteri* and *Serratia marcescens* have been reported to invade cells of mosquito tissues and to replicate intracellularly (Hegde et al., 2019). Here, we review the current knowledge on B vitamin provision by the microbiota to its insect

host, considering as “microbiota” any microbe hosted by the insect, whether strictly symbiont or not. In line with the literature, a large part of this review focuses on bacteria. As ticks share a similar life cycle with some insects and harbor similar bacteria as some insects, we also included tick-microbe interactions in our review. Indeed, we hypothesized that a wide focus on different types of diets and of host-microbe associations would allow us to see the big picture on conserved and specific features of host-microbe nutritional mutualism. By gathering information and cross-analyzing the role of B vitamins in various relationships, we successively highlight their importance in (i) fostering post-embryonic development, (ii) affecting adult lifespan and homeostasis and (iii) supporting reproduction.

Textbox 1. B vitamins, vitamers and known metabolic functions

B vitamins are a group of water-soluble micronutrients that can act as cofactors of diverse metabolic process in the cell. Each vitamin can be present in different forms, referred to as vitamers, the chemical structures that can complement each other to perform its biological activity (Douglas, 2017). Insects generally cannot produce B-vitamins themselves (Duron & Gottlieb, 2020). This box describes the different B vitamins and indicates which taxa can produce them.

Thiamine (B1, $C_{12}H_{17}N_4OS$) is a cofactor required for the biosynthesis of acetyl coenzyme A from pyruvate, thus linking glycolysis to tricarboxylic acid cycle (TCA). Therefore, it has essential roles in cellular bioenergetic processes leading to ATP production, as well as in the metabolism of glucose, amino acids and lipids. It is biosynthesized by bacteria, plants, fungi and archaea.

Riboflavin (B2, $C_{17}H_{20}N_4O_6$) is a precursor of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), essential cofactors of enzymes that belong to the electron transport chain, the TCA cycle and β-oxidation of fatty acids. They are biosynthesized by bacteria, plants, fungi and archaea.

Niacin, nicotinamide and nicotinic acid are the B3 vitamers ($C_6H_5NO_2$), all precursors of nicotinamide adenine dinucleotide (NAD), a coenzyme involved in cellular redox

balance reactions, the TCA cycle, the electron transport chain and the synthesis of lipids and nucleic acids. It is biosynthesized by animals, plants, fungi and bacteria.
Pantothenate, or pantothenic acid (B5, $C_9H_{17}NO_5$), is a precursor of coenzyme A (CoA). As such, it is an essential factor for the TCA cycle and fatty acid oxidation. Panthenol and pantetheine are two B5 vitamers. All three B5 vitamers are biosynthesized by bacteria, plants and fungi.
B6 ($C_8H_{11}NO_3$) exists in six main forms, namely pyridoxine, pyridoxal and pyridoxamine and their respective phosphorylated forms, pyridoxine 5'-phosphate, pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate. Pyridoxal 5'-phosphate is the main active form which plays as an important cofactor in more than 140 enzymes, transaminases, methionine catabolism as cystathione synthase and cystathionase; glycogen phosphorylase and biosynthesis of sphingolipids, among the most relevants. They are biosynthesized by archaea, bacteria, protozoan, fungi and plants.
Biotin (D-(+)-biotin, B7, $C10H_{16}N_2O_3S$) is a cofactor for several carboxylases, especially acetyl CoA carboxylase involved in fatty acid synthesis, pyruvate CoA carboxylase in gluconeogenesis, β-methyl-crotonyl CoA carboxylase in leucine degradation and propionyl CoA carboxylase in amino acid and fatty acid degradation. It is biosynthesized by plants, fungi and bacteria.
Folate (B9, $C_{19}H_{19}N_7O_6$) refers to folic acid and its related compounds, notably its main active form tetrahydrofolate (THF). It is a methyl (one carbon) donor which plays a central role in the metabolism of nucleic acids and amino acids. It is biosynthesized by bacteria, fungi and plants.
Cobalamin (B12, $C_{63}H_{88}CoN_{14}O_{14}P$) is present in several forms, methyl-, hydroxy- and adenosyl- and cyano-cobalamin, which act as coenzymes of isomerases, methyltransferases, or dehalogenases. It is notably involved in the breakdown of amino acids "fueling" the citric acid cycle and in the synthesis of methionine and of THF (thus involved in the metabolism of proteins and nucleic acids). It is biosynthesized by a few bacteria and archaea (Acevedo-Rocha et al., 2019; Douglas, 2017; Kanehisa et al., 2021; McCutcheon & Moran, 2012; Roje, 2007).

Involvement of B vitamins during development

The life cycle of an insect usually begins with an egg covered by a resistant shell which contains its own nutrient reserves to develop until structures are in place for the hatching process to begin. Thereafter, immature mobile individuals will consume large amounts of food relative to their size to sustain their nutritional status as they move from one stage to the next, growing and gaining weight. The majority of immature arthropods are completely independent in foraging their own nutrients, except in a few viviparous species such as tsetse flies. Endosymbionts are inherently acquired through direct vertical transmission into the egg. The other microbiota members are acquired from the environment and via vertical transmission, as they contaminate the egg external envelope and are ingested when the immature individuals start to feed (Bakula, 1969).

Blood-feeding immatures

Firstly, the importance of B vitamins in microbiota-host interactions was found in obligate blood feeders (Figure 1), as the blood is poor in B vitamins compared to what is generally required for insect development (Douglas, 2017). Kissing bugs *Rhodnius prolixus* notably require *Rhodococcus* bacteria for nymph development, but the addition of B vitamins in the diet can rescue nymph development in the absence of *Rhodococcus* (Baines, 1956; Ben-Yakir, 1987; Brecher & Wigglesworth, 1944). More generally, an antibiotic treatment of immature bedbugs, kissing bugs or ticks leads to development delay, arrest and/or to the death and such effects can at least partly be rescued by the addition of a mixture of B vitamins (Duron et al., 2017; Hosokawa et al., 2010; Tobias et al., 2020). These experimental observations were corroborated with genomic analyses suggesting that such symbionts can produce B vitamins. Notably, tick-associated endosymbionts of the *Francisella*, *Coxiella*, *Arsenophonus* and *Rickettsia* genera have reduced genomes compared to free-living species but keep intact B vitamin biosynthesis pathways (Duron et al., 2017; Zhong et al., 2007). A predicted ability for B vitamin production is also reported in the genomes of kissing-bug-associated extracellular symbionts *Rhodococcus* and *Dickeya* (Salcedo-Porras et al., 2020; Tobias et al., 2020). In *Cimex* and *Paracimex* bedbugs, *Wolbachia*-cured nymphs can only develop if they receive riboflavin and biotin supplements. This is quite an original case in arthropods, where colonization success of *Wolbachia* is generally linked to reproductive manipulation rather than to nutritional

symbiosis. In these species, *Wolbachia* behaves as a primary endosymbiont; it is located in a bacteriocyte and biosynthesizes riboflavin and biotin (Balvín et al., 2018; Gerth & Bleidorn, 2017; Hosokawa et al., 2010; Scholz et al., 2020).

Sap-feeding immatures

Secondly, sap-feeding insects have been found to rely to a lesser extent on microbial B vitamins for their post-embryonic development. This has specifically been evidenced in several species of hemipterans including aphids, planthoppers and leafhoppers (**Figure 1**). Many aphids species harbor *Buchnera*, a primary endosymbiont present in a bacteriocyte and able to produce riboflavin (Nakabachi & Ishikawa, 1999). Removing this symbiont via antibiotic-treatment delays the development of aphids fed on plants (Ishikawa, 1982) and impedes development of nymphs fed on a riboflavin-deprived diet (Blow et al., 2020; Moriyama et al., 2015; Nikoh et al., 2014). However, a recent study showed that, when curing two-day old larvae from *Buchnera*, aposymbiotic aphids (i.e. symbiont-cured) had a lower development success than their symbiotic controls, yet their development success was not affected by any deprivation in B vitamins (Blow et al., 2020). Intriguingly, dietary pantothenate appeared critical for aposymbiotic and symbiotic aphid development, while the genome of *Buchnera* encodes a full pantothenate biosynthesis pathway. A transcriptome analysis showed that *Buchnera* strongly expresses an antisense RNA in the *panC* and *CoaE* genes, which encode enzymes involved in pantothenate biosynthesis and pantothenate conversion to CoA, respectively. Accordingly, their proteins are undetectable, suggesting that these antisense RNAs repress the production of pantothenate and CoA in *Buchnera*. Functional implications of this repression is unclear.

Some aphid species additionally host other secondary endosymbionts such as *Spiroplasma*, *Rickettsia*, *Erwinia* and *Wolbachia*, but their colonization success related to reproductive manipulation rather than to nutritional mutualism (Romanov et al., 2020). In contrast, some planthoppers harbor mutualist *Wolbachia*; as mentioned above for bedbugs, *Wolbachia* is present in a bacteriocyte and cured immatures have a limited ability to reach adulthood unless receiving riboflavin and biotin supplements (Hosokawa et al., 2010; Kaur et al., 2021; Moriyama et al., 2015; Nikoh et al., 2014). Similarly, the

Arsenophonus endosymbiont of the date palm leafhopper *Ommatissus lybicus* is required for nymph development and its genomic sequence suggests that it can biosynthesize B vitamins (Fan et al., 2016; Karimi et al., 2019). *O. lybicus* also harbors a *Wolbachia* strain that is very similar to planthopper *Wolbachia* and predicted to biosynthesize biotin. Moreover, individuals which lack *Arsenophonus* always carry *Wolbachia* (Fan et al., 2016; Karimi et al., 2019; Qu et al., 2013; Xue et al., 2014). Hence, *Wolbachia* and *Arsenophonus* may be two alternative nutritional mutualists promoting development in *O. lybicus*. While the success of *Wolbachia* in arthropods is generally linked to reproductive manipulation rather than to nutritional symbiosis, we have cited several examples of hemipterans carrying a mutualist *Wolbachia* endosymbiont (bedbugs, plant hoppers and potentially leafhoppers). Whether a mutualistic role is more widely spread in this order of insects is unclear, but *Wolbachia* symbionts appear to be slightly more prevalent in hemipteran species (69%) than overall in terrestrial insects (50%) (Sazama et al., 2017).

Immatures feeding on microbe-containing diets

Thirdly, the importance of microbial B vitamins has been investigated in dipterans including *Drosophila* and mosquitoes (Figure 1), whose larvae develop on rotting fruit and in standing water, respectively. A standard laboratory *Drosophila* diet, based on yeast extract and corn meal, provides enough vitamins to allow full development of *Drosophila* larvae even in sterile conditions. Yet, this yeast-containing diet is already rich in microbe-derived vitamins. Hence, impoverished diets have been used to analyze the role of the microbiota in B vitamin provision. Notably, antibiotic-treated *Drosophila* larvae require dietary folate for successful development while conventionally-reared larvae do not (Blatch et al., 2010, 2015). A further requirement on microbially-sourced riboflavin and pantothenate was shown by diet manipulation in conventional and germ-free flies (Wong et al., 2014). Finally, a recent study thoroughly assessed the role of the microbiota in the provision of 50 single nutrients, including B vitamins by rearing gnotobiotic and germ-free *Drosophila* larvae on 50 chemically-defined diets, each deficient for a specific nutrient. This study showed that *Acetobacter pomorum* and *Lactobacillus plantarum* are both able to provide larvae with thiamin, riboflavin, nicotinic acid, biotin and folate, which are all essential for larval development. *A. pomorum* is additionally able to support development in the absence of pyridoxine. None of these bacterial strains can support development in

the absence of pantothenate, even though the growth of *A. pomorum* is able to grow without this vitamin (Consuegra et al., 2020). In a second study, these authors showed that both bacteria together are able to support larval development in the absence of pantothenate. *A. pomorum* provides pantothenate and biotin to *L. plantarum* which feeds *A. pomorum* with lactate, bacteria thus enhance each other's growth and provide nutrients for larval development (Consuegra et al., 2020). Considering mosquitoes, microbes are required for normal development (Coon et al., 2014), yet it is possible to produce adults with specific diets and rearing conditions, indicating that the microbiota participates to larval nutrition (Correa et al., 2018). Our laboratory has recently set up a transient bacterial colonization system to investigate the role of bacteria during *Aedes aegypti* larval development (Romoli et al., 2021). Larvae are colonized with bacteria which are auxotroph for some bacteria-specific amino acids. As long as these amino acids are present in larval food, bacteria proliferate and support larval development. Bacteria are rapidly lost in the absence of these amino acids. When decolonizing larvae at the middle of larval development, the folate pathway is strongly upregulated and development rate is reduced, suggesting that bacterial folate participates to mosquito development. Accordingly, dietary supplementation in folate partly rescued the end of development of germ-free larvae. Decolonization also leads to a defect in aminoacid storage and in lipid incorporation in tissues, yet their link with folate or any other microbial metabolite is not established. Wang et al further explored the nutritional requirements of germ-free larvae by diet manipulation. A holidic diet supplemented with commercial bovine lactalbumin supported development of germ-free larvae in the dark, while removing riboflavin, pyridoxin, thiamin or folate from this diet reduced development success; in contrast, removing pantothenate, nicotinic acid or biotin had no impact (Wang et al., 2021). It is yet unclear whether these B vitamins are also essential for mosquitoes, as their presence or absence in commercial bovine lactalbumin has not yet been documented. Focusing on riboflavin, the same study showed that a ribC-deficient *E. coli* mutant, which cannot produce riboflavin, does not support larval development. The absence of riboflavin can be complemented by FAD and/or FMN, but not by its light-degradation product, lumichrome, consistent with the fact that the holidic diet with bovine lactalbumine cannot support development with normal light:dark cycle.

Hence, B vitamin metabolism is a focal point of bacteria-insect interactions during post-embryonic development. Microbe-derived vitamins are provided either by vertically-transmitted symbionts or by unspecific microbiota species and affect several parameters, including survival of immatures, development success and development speed. They are required for the development of immatures on vitamin-poor diets such as blood, while they appear redundant with dietary vitamins for immatures feeding on other diets, notably sap. In the latter case, the microbiota plays the role of an insurance, enabling immatures to face nutritional constraints from the environment.

Involvement of B vitamins in adult physiology and survival

The adult stage is particularly studied in insects because of its numerous implications in terms of ecology, agriculture and human health: insect species account for a number of pollinators, nutrient recyclers, soil caregivers, predators and preys, seed dispersers, crop pests and pathogen vectors. In adults, the insect microbiota commonly behaves as commensal but can additionally participate to the digestion of recalcitrant diets, provision of micronutrients and production of short chain fatty acids. A normal density of microbiota induces a basal activity of antimicrobial peptides and epithelial tissue turnover, whereas an imbalance due to the presence of pathogens (viruses or bacteria) and/or induced by ageing may lead to increased induction of basal immune responses with the production of reactive oxygen species and antimicrobial peptides and to increased cell proliferation (Buchon et al., 2009; Engel & Moran, 2013; McCutcheon et al., 2019).

When specifically focusing on the role of the microbiota as a provider of B vitamins, a standard readout for fitness is adult lifespan, but other quantified parameters also include gene expression, resistance to stress, symbiont density or B vitamin levels. Four relevant examples of microbiota contribution in vitamin provision to the adult hosts are reported in this section and in Figure 2. Reproductive phenotypes are reviewed in the next section as they affect the offspring, yet are largely influenced by the parental metabolism.

Among hemipterans, *Wolbachia* is essential to adult survival in the bedbug *Cimex lectularius*. The elimination of the symbiont leads to mortality of the bedbugs, which

recovers its fitness when a B vitamin cocktail containing riboflavin and biotin is added to the blood diet (Moriyama et al., 2015). The case of phytophagous hemipterans, notably *Bemisia tabaci* whiteflies, reveals a particularly strong symbiotic integration between the host and its *Portiera* and *Hamiltonella* symbionts for pantothenate and biotin synthesis, respectively. Firstly, the genome of *B. tabaci* includes *BioA*, *BioD* and *BioB*, which encode functional enzymes of the biotin biosynthesis pathway (Ren et al., 2020). These horizontally-transferred genes have similar sequences to their *Wolbachia*, *Cardinium* and *Rickettsia* orthologs and are also present in *Hamiltonella*. An antibiotic treatment specifically targeting *Hamiltonella* leads to a reduction of biotin titers while whitefly *BioA*, *BioD* and *BioB* are induced in bacteriocytes. Silencing any of these host genes increases female mortality, unless if diet is supplemented in biotin. Secondly, the whitefly genome encodes *PanBC*, a gene with a similar sequence to two genes of the pantothenate biosynthesis pathway in *Pseudomonas*, *PanB* and *PanC* (Ren et al., 2021). Among whitefly symbionts, *Portiera* lacks these genes in the pantothenate biosynthesis pathway and *Hamiltonella* and *Rickettsia* completely lack this pathway. A rifampicin treatment of whiteflies leads to the elimination of the three main symbionts, reduces pantothenate levels and tends to increase mortality in adult females. Silencing on *PanBC* expression also reduces *Portiera* abundance, pantothenate level and female lifespan, which can partly be rescued with pantothenate supplements. Together, these studies indicate that *B. tabaci* cooperates with *Hamiltonella* to produce biotin and with *Portiera* to produce pantothenate, using genes acquired from bacteria via Horizontal Gene Transfers.

Focusing on mosquitoes, adult colonies are maintained with sugar solutions, in which multivitamin syrups are sometimes added to improve fitness. Such diet supplementation notably enhances the lifespan of *Anopheles* and *Culex* mosquitoes and this effect even remains in *Culex* until the next generation (Phasomkusolsil et al., 2017; Tan et al., 2016). Conversely, an antibiotic treatment has been found to reduce the lifespan of *Anopheles* mosquitoes, which can be recovered after reintroduction of *Serratia* and *Enterobacter*. In *Aedes*, a positive impact of colonization with *Escherichia coli* on adult lifespan has also been detected, but only if larvae have been grown in axenic conditions: colonization of adults with *E. coli* did not impact lifespan when compared with germ-free adults produced by transient colonization (Correa et al., 2018; Romoli et al., 2021), suggesting that this

positive impact of the microbiota depends on larva-to-adult carryover effects. Along these lines, adults originating from larvae carrying a conventional microbiota generally have a longer lifespan than adults coming from mono-colonized larvae (Giraud et al., 2022). Whether the positive impact of the microbiota on adult lifespan is due to vitamin provision needs to be investigated. A transcriptomic analysis detected an enrichment in the folate biosynthesis pathway in antibiotic-treated blood-fed *Anopheles* mosquitoes, suggesting that the microbiota may well have an important role in B vitamin provision to adults (Rodgers et al., 2017). Such impact of the microbiota after the blood meal may be linked to bacterial biosynthesis and/or to a participation in harvesting vitamins present in red blood cells via hemolysis and in microbes via antimicrobial activity (Gaio et al., 2011; Hyde et al., 2020).

In fruit flies, the specific activities of thiamin, riboflavin and biotin have been analyzed. Biotin-deficiency in the diet decreases lifespan in males and females, but increases resistance to stress in males (Landenberger et al., 2004). This vitamin is particularly important for mitosis of intestinal stem cells. When these cells are unable to import biotin, they do not proliferate properly, which notably increases the susceptibility of flies to bacterial infection. Flies fed with a biotin-deficient diet have a lower mitosis rate in the intestine, but *E. coli* colonization can restore it to normal levels (Neophytou & Pitsouli, 2022). Thiamine deficiency in the diet does not seem to affect adult lifespan whether in conventionally-reared or axenic individuals (Sannino et al., 2018). Riboflavin has been proposed as an anti-aging agent, as conventionally-reared flies supplied with additional riboflavin in the diet have a prolonged life expectancy in normal conditions and upon oxidative stress (Zou et al., 2017), but it is not clear whether riboflavin has to be provided by the microbiota in adults.

Besides the examples described in Figure 2, antibiotic treatment leads to a decrease in adult lifespan in several other insect species, where bacteria have been found to carry B vitamins in their genome. This has notably been found in date palm leafhoppers *Ommatissus lybicus* carrying *Wolbachia* (Fan et al., 2016; Karimi et al., 2019; Xue et al., 2014) and in hematophagous flies *Hippoboscoidea, Streblidae* and

Nycteribiidae carrying several *Arsenophonus, Sodalis* and/or *Aschnera* (Duron et al., 2014; Hosokawa et al., 2012; Šochová et al., 2017).

Provision of microbial B vitamins to the host has also been found to affect other traits than lifespan. A recent ecological study indicates that an invasion by *Solenopsis invicta* ants induces a change of diet of the endemic ants workers *Tapinoma melanocephalum* towards a protein-rich diet, which affects microbiota composition, notably increasing *Wolbachia* relative abundance (Cheng et al., 2019). This study further shows that supplementing the diet of *T. melanocephalum* in non-invaded and invaded colonies with protein-rich or sugar respectively reverses the effect on several taxa including *Wolbachia,* and leads to a loss of riboflavin and nicotinic acid in invaded ants, while some other B vitamins are no affect. This suggests that the change in diet may allow ants to behaviorally respond to invasion by favoring a microbiota producing riboflavin and nicotinic acid.

Considering vectorial transmission, the impact of microbe-derived vitamins on vector competence has been less extensively studied, yet it has been evidenced in tsetse flies (Rio et al., 2019). *Trypanosome* infections induces folate biosynthesis genes of the fly symbiont *Wigglesworthia*. In the major trypanosome vector *Glossina morsitans*, an antifolate treatment decreases infection prevalence in the gut, without affecting subsequent parasitic development towards salivary gland infection. In the inefficient vector *Glossina brevipalpis*, diet supplementation in folate leads to a strong increase in infection prevalence. Finally, folate gene expression by *Wigglesworthia* is higher in *Glossina morsitans* than in less efficient vectors and induced by parasitic infection, suggesting that folate expression may be a critical factor determining vector competence towards the parasite. In other vectors, a link with the microbiota is less clear but there is evidence that parasites require B vitamins from the host and/or the environment. Diet supplementation in folic acid or one of its precursors, para-aminobenzoate, positively affects *Brugia malayi* development in *Ae. aegypti* (Kirkness et al., 2010). Genetic studies have found that pantothenate transporter and predicted pantothenate kinases Pank1 and Pank2 are required for *Plasmodium* development in the mosquito, while they are dispensable for growth in red blood cells and differentiation to gametocytes (Hart et al., 2015, 2016).

B vitamins and reproduction

Arthropod reproduction is a highly-demanding process in terms of energetic investment, macromolecule synthesis and accumulation. This involves several processes including oogenesis, spermatogenesis, copulation and embryogenesis, ultimately leading to hatching of viable larvae. Typical readouts of reproduction are fertilization success, the proportion of egg laying females, the size of their clutch as well as egg hatching rate. In addition, the viability of larvae and the sex ratio of the resulting adult offspring may be considered as parameters of reproduction if the focus of the study is a treatment of the parents (Gnainsky et al., 2021; Mao et al., 2020; Wu et al., 2021).

Requirements on B vitamins for insect reproduction has first been studied via diet manipulation and via antivitamin treatments. Several antivitamins were notably reported to inhibit egg production by *Musca viscinis*, an effect that could partly be rescued using the corresponding vitamin (Bergmann et al., 1959). Later, Saxena and Kaul were surprised to see how little effect vitamin deficiency in the diet had on *Oryzaephilus mercator* (*Coleoptera*) fertility and discussed that this may be due to microbe-dependent production of vitamins (Saxena & Kaul, 1974). More recent studies on mosquitoes and bedbugs also pointed to a positive effect of sugar diet supplementation with B vitamins on male and female fertility. These dietary supplements specifically increased male fertilization capacity during forced-mating experiments, egg production by females, egg hatching success and/or viability of the hatched larvae (Hosokawa et al., 2010; Phasomkusolsil et al., 2017; Tan et al., 2016). This correlates with negative effects on female fecundity and/or fertility found in *Anopheles* and *Aedes* upon antibiotic-treatment, which is rescued by *Serratia* or *Enterobacter* colonization in *Anopheles* (Ezemuoka et al., 2020; Gaio et al., 2011). In *Aedes*, the microbiota promotes fecundity via hemolysis in the blood bolus, while colonization with *E. coli* HS, a non-hemolytic bacterial strain, does not affect fertility (Figure 3; Gaio et al., 2011; Romoli et al., 2021). While the link has not experimentally been established, these observations would fit with a model where B vitamins released by bacteria promote mosquito fertility. Bacteria may produce vitamins themselves and/or participate to their release via hemolysis after the blood meal. In ticks, maternally-inherited endosymbionts are essential for oviposition (lone star tick) and egg development (lone star tick and *Riphicephalus*), but the involvement of B vitamins has not

been specifically investigated so far (Figure 3; Guizzo et al., 2017; Hunter et al., 2015; L. H. Li et al., 2018; Zhong et al., 2007).

When focusing on specific vitamins, there is evidence that riboflavin and biotin particularly affect insect fecundity (Figure 3). When riboflavin is added to diet, it improves egg production in conventionally-reared fruit flies (Zou et al., 2017). While oogenesis is reduced in germ-free flies as a result of decreased ATP levels, riboflavin supplementation is sufficient to reverse the effect (Gnainsky et al., 2021). Riboflavin supplementation has also been found to restore fecundity in bedbugs cured from *Wolbachia* via antibiotic-treatment (Moriyama et al., 2015). A biotin dietary supplement also promotes fecundity in *Wolbachia*-cured bedbugs (Nikoh et al., 2014). Genomic analyses showed that the riboflavin biosynthesis pathway is often complete in *Wolbachia,* while the biotin biosynthesis pathway is complete in some *Cimex* and *Paracimex* bedbugs' species as well as in some planthoppers and missing in the other insect genera tested (Balvín et al., 2018; Ju et al., 2020; Moriyama et al., 2015). A similar situation occurs in planthoppers, where the presence of *Wolbachia* enhances egg production. Experiments based on the depletion or addition of dietary biotin and/or riboflavin indicate that this impact is driven by the provision of these vitamins by *Wolbachia*, the role of biotin being particular important (Ju et al., 2020). In whiteflies as well, removal of symbionts *Portiera* and *Hamiltonella* leads to reduced titers of pantothenate and biotin respectively and scant egg production (Ren et al., 2020, 2021). Silencing host genes of the pantothenate and biotin biosynthesis pathways (inherited from bacteria by horizontal-gene transfer, as mentioned in the previous section) recapitulates antibiotic treatments against *Portiera* and *Hamiltonella*, respectively. Again, this indicates that whiteflies and their symbionts collaborate to support the adult host requirement on B vitamins.

On the contrary, thiamine deficiency does not appear to affect egg production in *Drosophila* (Figure 3). Larvae hatched from those eggs can only fulfill development if they are conventionally reared; they are blocked during development if they are germ-free (Sannino et al., 2018). This latter observation however points back to an impact of microbial thiamine in development rather than reproduction, as mentioned above (Consuegra et al., 2020).

Tsetse flies are adenotrophic viviparous, their larvae are carried and fed with milk within the female uterus and get born in a prepupal stage. They harbor an obligatory symbiont, *Wigglesworthia*, so no cured line can be established over generations, yet it is possible to get a *Wigglesworthia*-cured progeny of “aposymbionts” after treating a female with antibiotics. These aposymbionts are reproductively sterile and do not produce folate. To study the role of folate in tsetse flies, Snyder and Rio treated conventionally-reared flies with glyphosate, which inhibits the biosynthesis of chorismate, a precursor of folate (Snyder & Rio, 2015). As this pathway is present in *Wigglesworthia* but absent in the fly genome, this treatment specifically targets folate production by the symbiont. This treatment leads to a delay in blood meal digestion, a reduction in the intrauterine area and a delay in time to pupation, which can partly be rescued by folate supplementation (Snyder & Rio, 2015). Proline is a cornerstone of tsetse fly metabolism during lactation and its biosynthesis depends on alanine-glyoxylate transaminase, a protein which requires pyridoxine as a cofactor. Experiments based on antibiotic treatments and vitamin supplementation have shown that *Wigglesworthia* pyridoxine is essential for lactation (Michalkova et al., 2014; Snyder et al., 2010).

Besides such “typical” effects of vitamins to support metabolism and egg production, microbe-derived B vitamins have also been suggested to impact other aspects of reproduction, affecting sex ratio and compatibility between mating partners. Firstly, the removal of whitefly symbionts has been found to affect fertilization process. Antibiotic treatments targeting *Hamiltonella* or *Arsenophonous* lead to an imbalance in the sex ratio of the progeny, reducing the proportion of females from 60% to 20%. This is due to a loss in fertilization leading to arrhenotokous (i.e. male-producing) parthenogenesis, which can be rescued using a cocktail of B vitamins (Wang et al., 2020). Thus, these symbionts increase the proportion of females in the progeny by avoiding a male-producing parthenogenesis, which would be detrimental for their vertical transmission. Interestingly, some other endosymbionts have been found to promote thelytokous (i.e. female-producing) parthenogenesis (albeit not reportedly via B vitamin related mechanisms). Whether by avoiding arrhenotokous parthenogenesis or by inducing thelytokous parthenogenesis, in both cases they are increasing their chance of vertical transmission (Massey & Newton, 2022). *Wolbachia* is also known to manipulate reproduction via

cytoplasmic incompatibility; colonized males are infertile unless they mate with a female colonized with the same strain of *Wolbachia*. It promotes *Wolbachia* colonization in a population as it favors colonized females, which can be fertilized by any male and will vertically transmit the endosymbiont. A recent comparison of *Wolbachia* genomes from 500 host species including nematodes and arthropods showed a correlation between the presence of a riboflavin transporter in *Wolbachia* genome and cytoplasmic incompatibility (Scholz et al., 2020), yet functional links have not yet been characterized.

Conclusion

In sum, the microbiota, including symbionts and transient microbes, strongly affects the physiology of its host throughout the reproductive cycle via the provision of B vitamins. While it is convenient to classify microbes as primary, secondary symbionts or reproductive manipulator for instance, examples treated in this review highlight that one microbe may be differently classified depending on the conditions. Host-microbe interactions can be placed on a gradient of parasitism/mutualism rather than in strict classes. For instance, while *Wolbachia* is generally a reproductive manipulator in insects and a mutualist in nematodes (Brown et al., 2018; Z. Li & Carlow, 2012), some lines of evidence point to its ability to provide B vitamins to some insects (Gerth & Bleidorn, 2017; Hosokawa et al., 2010; Ju et al., 2020; Moriyama et al., 2015; Qu et al., 2013). Such infections, favored by a combination between a pressure (here, reproductive manipulation) and a slight positive outcome of colonization on the host, are termed “Jekyll and Hyde” infections (Moriyama et al., 2015). It remains unclear whether they also happen in a wider range of insect species, where the positive fitness component may be undetected due to redundancy with nutrient provision by other members of the microbiota.

In immature stages, microbe-derived B vitamins are important for completion of the development and survival. Depending on the associations, microbes provide B vitamins that are absent in the host diet or complement diet provision, ensuring development in scarce dietary conditions. In adults, they tend to prolong the lifespan, affect vector competence and participate in reproductive success via egg production, fertilization and lactation. Interestingly, it seems that some vitamins are non-specifically cited in studies on development, lifespan or reproduction while some others appear more often in studies

on larvae or adults. For instance, folate is implicated in the development of *Drosophila*, tsetse flies, mosquitoes, bedbugs and aphids but is not reportedly so critical in adult lifespan studies. Biotin is important for lifespan and/or egg production in adult *Drosophila*, whiteflies, bedbugs and planthoppers. Riboflavin is found to be important during development as well as during adulthood in dipterans, bedbugs and aphids. These observations may be biased by the fact that most diets include unknown concentrations of the different vitamins. This issue could be addressed using chemically defined diets in germ-free and gnotobiotic hosts in order to clearly define the contribution of microbes to their host's physiology.

While a number of requirements on vitamins for specific phenotypic readouts have been reported, the underlying mechanisms have rarely been uncovered. An exception to this rule is the demonstration of the role of pyridoxine in tsetse flies as a specific cofactor of *agat* for milk production. Considering that vitamins have conserved roles in eukaryotic cells, a better understanding of such mechanisms in insects can be instrumental to better understand the role of the microbiota in animals, which may have a wide spectrum of applications

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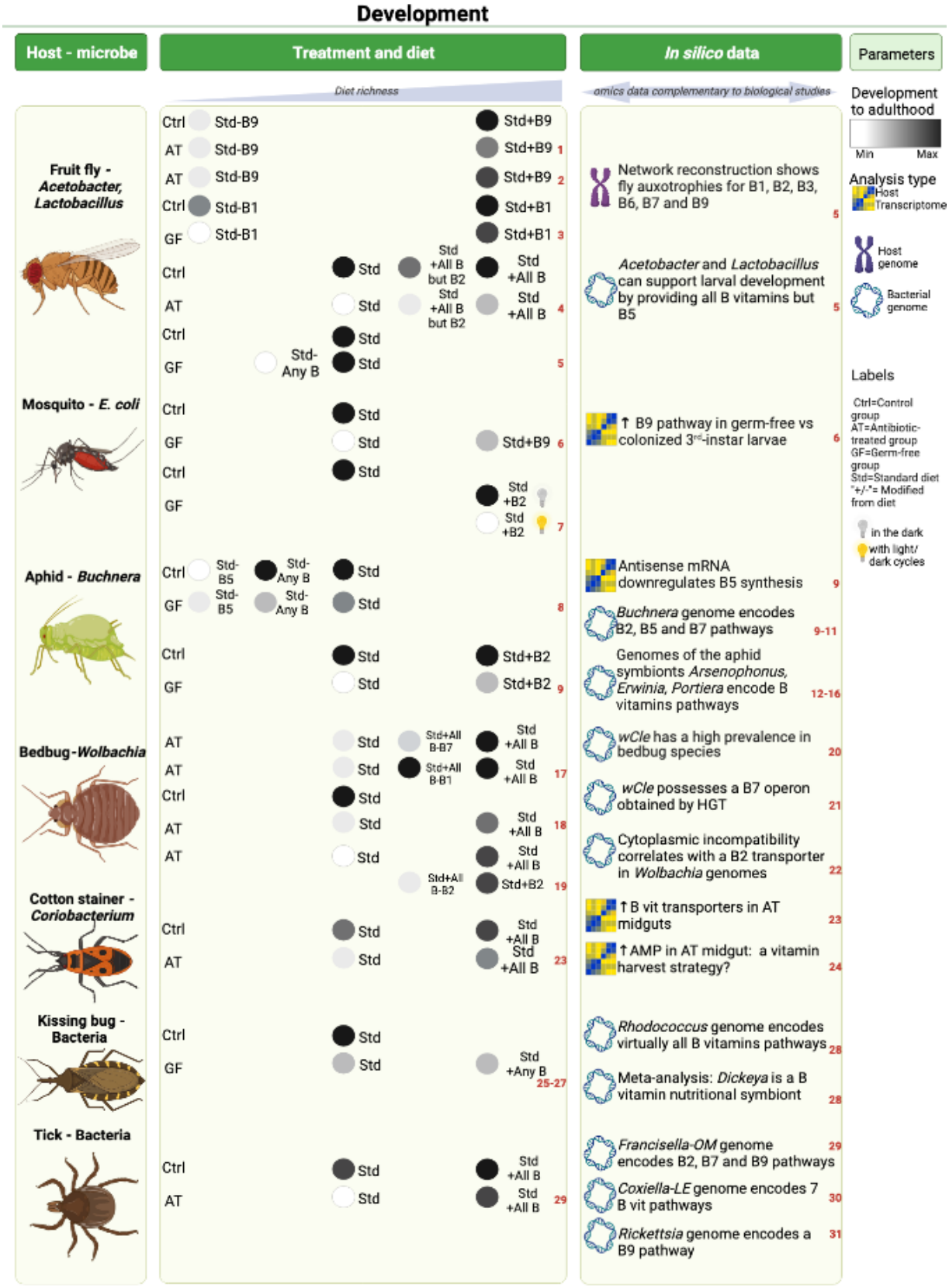
Development
Host - microbe
Treatment and diet
In silico data
Parameters
Diet richness
omics data complementary to biological studies
Fruit fly - Acetobacter, Lactobacillus
Ctrl Std-B9 Std+B9
AT Std-B9 Std+B9 1
AT Std-B9 Std+B9 2
Ctrl Std-B1 Std+B1
GF Std-B1 Std+B1 3
Ctrl Std Std +All B but B2 Std +All B
AT Std Std +All B but B2 Std +All B 4
Ctrl Std
GF Std-Any B Std 5
Network reconstruction shows fly auxotrophies for B1, B2, B3, B6, B7 and B9 5
Acetobacter and Lactobacillus can support larval development by providing all B vitamins but B5 5
Mosquito - E. coli
Ctrl Std
GF Std Std+B9 6
Ctrl Std
GF Std +B2 Std +B2 7
↑ B9 pathway in germ-free vs colonized 3rd-instar larvae 6
Aphid - Buchnera
Ctrl Std-B5 Std-Any B Std
GF Std-B5 Std-Any B Std 8
Antisense mRNA downregulates B5 synthesis 9
Buchnera genome encodes B2, B5 and B7 pathways 9-11
Ctrl Std Std+B2
GF Std Std+B2 9
Genomes of the aphid symbionts Arsenophonus, Erwinia, Portiera encode B vitamins pathways 12-16
Bedbug-Wolbachia
AT Std Std+All B-B7 Std +All B
AT Std Std+All B-B1 Std +All B 17
Ctrl Std
AT Std Std +All B 18
AT Std Std +All B
Std+All B-B2 Std+B2 19
wCle has a high prevalence in bedbug species 20
wCle possesses a B7 operon obtained by HGT 21
Cytoplasmic incompatibility correlates with a B2 transporter in Wolbachia genomes 22
Cotton stainer - Coriobacterium
Ctrl Std Std +All B
AT Std Std +All B 23
↑ B vit transporters in AT midguts 23
↑ AMP in AT midgut: a vitamin harvest strategy? 24
Kissing bug - Bacteria
Ctrl Std
GF Std Std +Any B 25-27
Rhodococcus genome encodes virtually all B vitamins pathways 28
Meta-analysis: Dickeya is a B vitamin nutritional symbiont 28
Tick - Bacteria
Ctrl Std Std +All B
AT Std Std +All B 29
Francisella-OM genome encodes B2, B7 and B9 pathways 29
Coxiella-LE genome encodes 7 B vit pathways 30
Rickettsia genome encodes a B9 pathway 31
Development to adulthood
Min Max
Analysis type
Host Transcriptome
Host genome
Bacterial genome
Labels
Ctrl=Control group
AT=Antibiotic-treated group
GF=Germ-free group
Std=Standard diet
"+/-"= Modified from diet
in the dark
with light/ dark cycles

Figure 1. Influence of B vitamins on insect development. AT – antibiotic treated; GF – Germ free; Ctrl – microbiota control (which can either mean conventionally reared or gnotobiotic, with AT and GF respectively); Std – Standard diet, neither impoverished nor enriched; a holidic diet is considered as standard when it contains all the requirements for normal physiology. Figure 1 references: 1-2 - Blatch et al., 2010, 2015; 3 - Sannino et al., 2018; 4 - Wong et al., 2014; 5 - Consuegra et al., 2020; 6 - Romoli et al., 2021; 7 - Wang et al., 2021; 8 - Blow et al., 2020; 9 - Nakabachi & Ishikawa, 1999; 10 - Nikoh et al., 2010; 11 - Price & Wilson, 2014; 12 - Weglarz et al., 2018; 13 - Manzano-Marín et al., 2020; 14 - Meseguer et al., 2017; 15 - Santos-Garcia et al., 2018; 16 - Romanov et al., 2020; 17 - Nikoh et al., 2014; 18 - Hosokawa et al., 2010; 19 - Moriyama et al., 2015; 20 - Balvín et al., 2018; 21 - Gerth & Bleidorn, 2017; 22 - Scholz et al., 2020; 23 - Salem et al., 2014; 24 - Bauer et al., 2014; 25 - Ben-Yakir, 1987; 26 - Brecher & Wigglesworth, 1944; 27 - Tobias et al., 2020; 28 - Duron et al., 2018; 29 - Guizzo et al., 2017; 30 - Smith et al., 2015; 31 - Hunter et al., 2015.

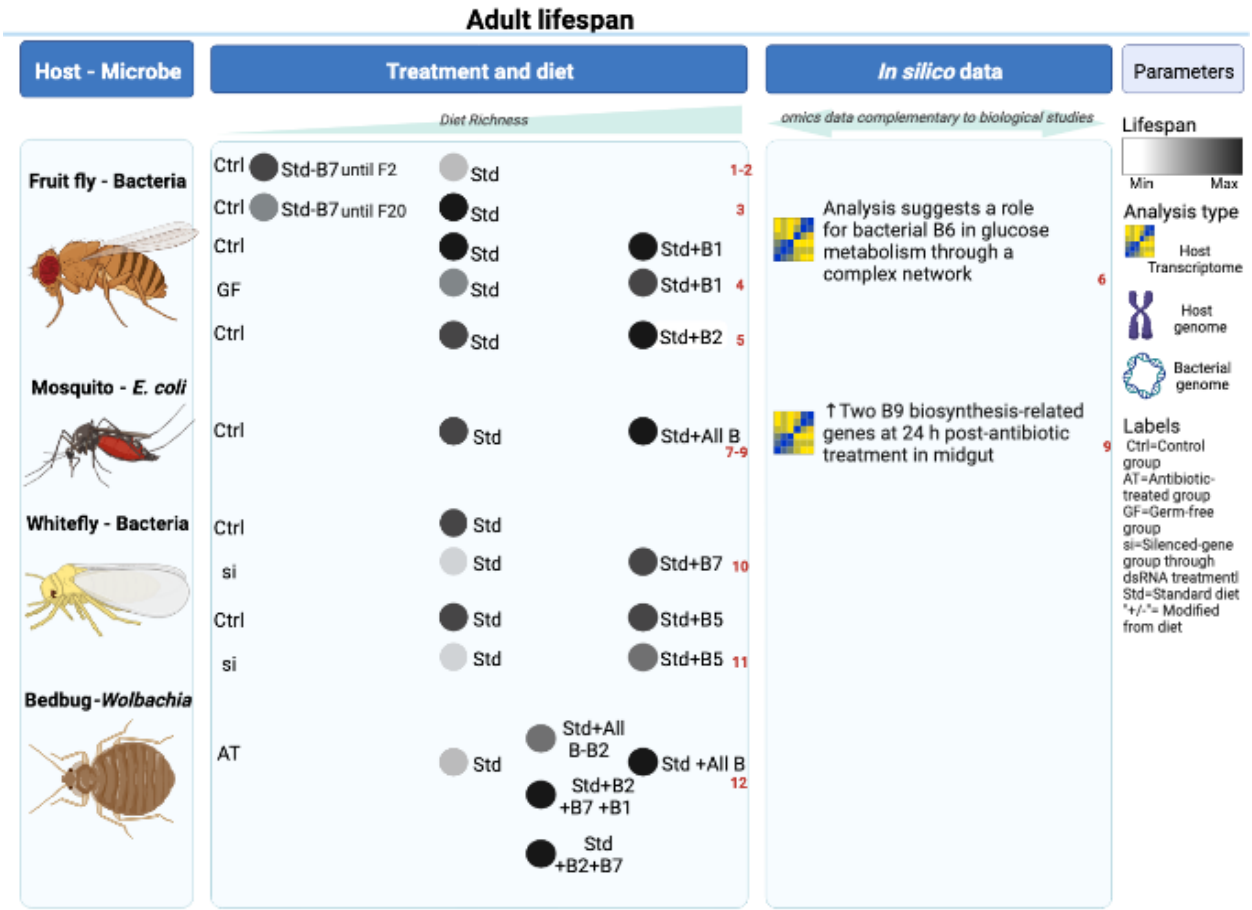

Figure 2. Influence of B vitamins on insect lifespan. AT – antibiotic treated; GF – Germ free; Ctrl – microbiota control (which can either mean conventionally reared or gnotobiotic, with AT and GF respectively); Std – Standard diet, neither impoverished nor enriched; a holidic diet is considered as standard when it contains all the requirements for normal physiology. Figure 2 references: 1- Sannino et al., 2018; 2 - Erin. Smith et al., 2007; 3 - Landenberger et al., 2004; 4 - Camporeale et al., 2006; 5 - Zou et al., 2017; 6 - Matthews et al., 2020; 7 - Tan et al., 2016; 8 - Phasomkusolsil et al., 2017; 9 - Rodgers et al., 2017; 10, 11 - Ren et al., 2020, 2021; 12 - Moriyama et al., 2015.

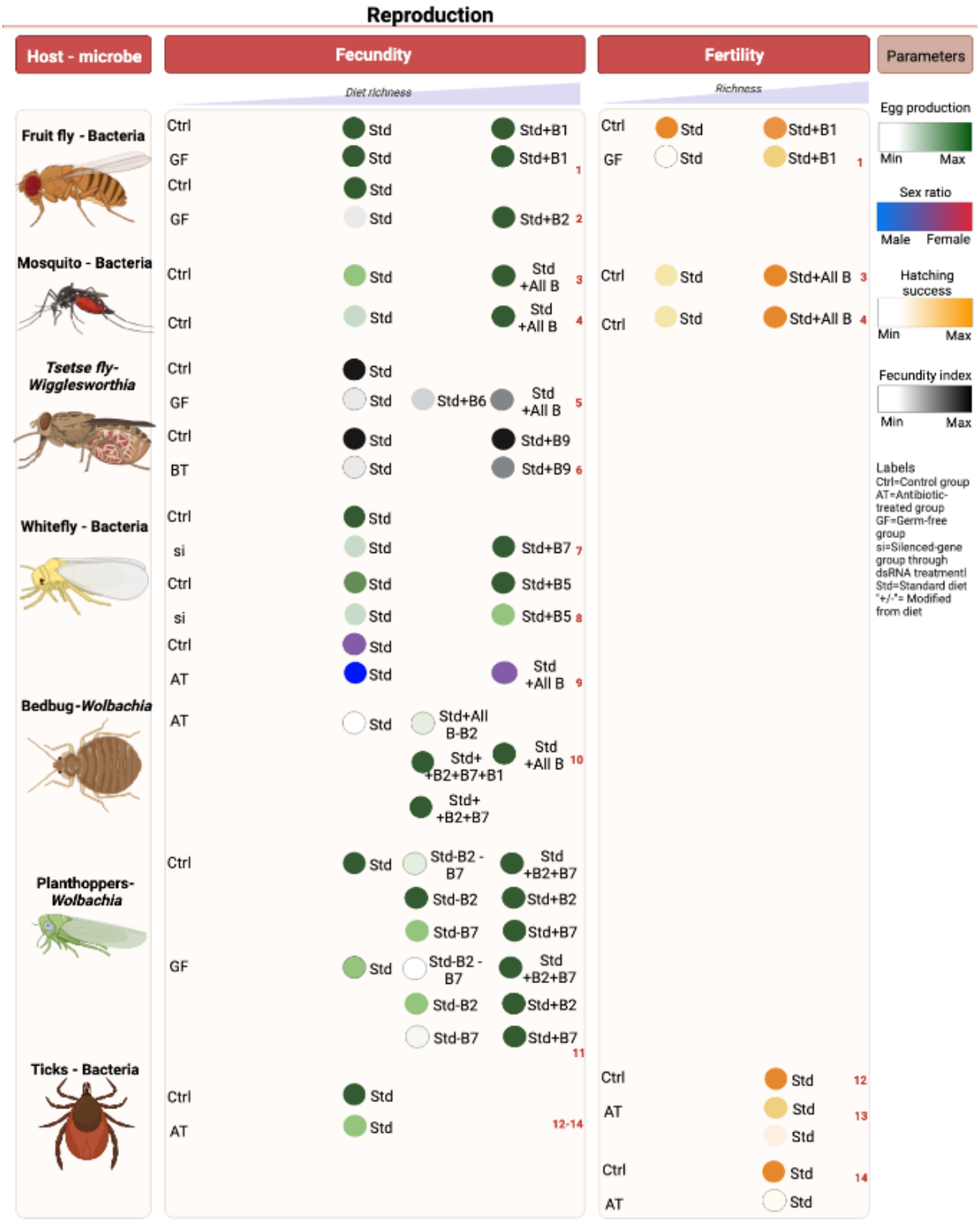

Figure 3. Influence of B vitamins on insect reproduction. AT – antibiotic treated; GF – Germ free; Ctrl – microbiota control (which can either mean conventionally reared or

gnotobiotic, with AT and GF respectively); Std – Standard diet, neither impoverished nor enriched; a holidic diet is considered as standard when it contains all the requirements for normal physiology. Figure 3 references: 1,2 - Ren et al., 2020, 2021; 3 - Wang et al., 2020; 4 - Moriyama et al., 2015; 5 - Ju et al., 2020; 6 - Phasomkusolsil et al., 2017; 7 - Tan et al., 2016; 8 - Sannino et al., 2018; 9 - Gnainsky et al., 2021; 10 - Michalkova et al., 2014; 11 - Snyder & Rio, 2015; 12 - Zhong et al., 2007; 13 - L. H. Li et al., 2018; 14 - Guizzo et al., 2017

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