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Javier Serrato-Salas, Mathilde Gendrin

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

2 Authors: Javier Serrato-Salas¹ and Mathilde Gendrin^{1,2}

3 Affiliations: ¹ Microbiota of Insect Vectors Group, Institut Pasteur de la Guyane, Cayenne,
4 French Guiana; ² Department of Parasites and Insect Vectors, Institut Pasteur, Université
5 de Paris, Paris, France

6 **Abstract**

7
8 Insects are highly successful in colonizing a wide spectrum of ecological niches and in
9 feeding on a wide diversity of diets. This is notably linked to their capacity to get from their
10 microbiota any essential component lacking in the diet such as vitamins and amino acids.
11 Over one century of research based on dietary analysis, antimicrobial treatment,
12 gnotobiotic rearing, culture-independent microbe detection progressively gathered
13 detailed information on the implication of the microbiota in specific aspects of insect
14 fitness. Thanks to the recent increase in sequencing capacities, whole-genome
15 sequencing of a number of symbionts allowed to trace biosynthesis pathways and thus
16 to validate experimental data and to assess their evolution. This field of research
17 generated a considerable set of data in a diversity of hosts harboring specific symbionts
18 or non-specific microbiota members. Here, we review the current knowledge on the
19 involvement of the microbiota in insect and tick nutrition, with a particular focus on B
20 vitamin provision. We notably question if there is any specificity of B vitamin provision by
21 symbionts compared to the redundant yet essential contribution of non-specific microbes.
22 We successively highlight the known aspects of microbial vitamin provision during three
23 main life stages of invertebrates, post-embryonic development, adulthood and
24 reproduction.

25 **Introduction**

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

29 vitamins, which are nutrients required in small quantities as they catalyze central
30 metabolic pathways. The insect's ability to occupy a great diversity of ecological niches
31 largely depends on the variability of their diet and on the beneficial microorganisms that
32 they harbor, which provide them with essential nutrients lacking in their diet (Douglas,
33 2017; Kucuk, 2020; Yoshii et al., 2019). Host-microbe relationships in insects influence
34 diverse aspects of their physiology related to digestion, nutrition, defense against
35 pathogens, behavior, immunity, detoxification, reproduction, renewal of the intestinal
36 epithelium (Consuegra et al., 2020; Engel & Moran, 2013; Shin et al., 2011; Storelli et al.,
37 2011). The study of nutritional contribution of microbes to insect development started in
38 the first half of the 20th century with the identification of minimal nutritional requirements
39 of insects in sterile conditions. Early research suggested an essential role of bacteria in
40 provisioning amino acids and B vitamins and thus supporting the development and
41 survival of various insects (Blewett & Fraenkel, 1944; Brecher & Wigglesworth, 1944;
42 Dadd, 1973). More recent data were overall in line with such observations, yet also
43 reported inconsistencies where previously reported diets may not support full
44 development in the complete absence of bacteria (Correa et al., 2018). Methodological
45 errors such as dietary impurities or undetected microbial contamination in germ-free
46 individuals may have led to undermine the importance of specific microbe-derived
47 nutrients (Dadd, 1973). Alternatively, some vitamins may have not been found as
48 essential because they are required in minute amounts, hence present in sufficient
49 quantity in the egg to complete development (Piper et al., 2014). The improvement of
50 techniques in molecular biology, chemistry and high-throughput sequencing now allows
51 to better control experimental conditions and to combine experimental work with genomic
52 analyses in order to produce clearer data on nutritional contributions of microbiota
53 members to their insect hosts (Douglas, 2017).

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

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

79 Insects also harbor ectosymbionts, extracellular bacteria with a facultative association
80 with their host, yet with closely-related genome characteristics to endosymbionts,
81 including genome reduction, gene loss and signatures of horizontal gene transfers from/to
82 genomes of the host or other microbes (Engel & Moran, 2013; Husnik & McCutcheon,
83 2018; Kucuk, 2020). The microbiota of insect also encompasses bacteria which do not
84 have any specific association with their host, they may participate to the host nutrition but
85 can be replaced by almost any other bacterium able to colonize the host (Coon et al.,
86 2014). Some of these bacteria cannot strictly be defined as extracellular or intracellular.
87 Besides the above-mentioned example of *Spiroplasma* endosymbionts, the known
88 extracellular bacteria *Cedecea neteri* and *Serratia marcescens* have been reported to
89 invade cells of mosquito tissues and to replicate intracellularly (Hegde et al., 2019). Here,
90 we review the current knowledge on B vitamin provision by the microbiota to its insect

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

101

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, $C_{10}H_{16}N_2O_3S$) 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).

102 **Involvement of B vitamins during development**

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

113 *Blood-feeding immatures*

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

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

136 *Sap-feeding immatures*

137 Secondly, sap-feeding insects have been found to rely to a lesser extent on microbial B
138 vitamins for their post-embryonic development. This has specifically been evidenced in
139 several species of hemipterans including aphids, planthoppers and leafhoppers (**Figure**
140 **1**). Many aphids species harbor *Buchnera*, a primary endosymbiont present in a
141 bacteriocyte and able to produce riboflavin (Nakabachi & Ishikawa, 1999). Removing this
142 symbiont via antibiotic-treatment delays the development of aphids fed on plants
143 (Ishikawa, 1982) and impedes development of nymphs fed on a riboflavin-deprived diet
144 (Blow et al., 2020; Moriyama et al., 2015; Nikoh et al., 2014). However, a recent study
145 showed that, when curing two-day old larvae from *Buchnera*, aposymbiotic aphids (i.e.
146 symbiont-cured) had a lower development success than their symbiotic controls, yet their
147 development success was not affected by any deprivation in B vitamins (Blow et al.,
148 2020). Intriguingly, dietary pantothenate appeared critical for aposymbiotic and symbiotic
149 aphid development, while the genome of *Buchnera* encodes a full pantothenate
150 biosynthesis pathway. A transcriptome analysis showed that *Buchnera* strongly
151 expresses an antisense RNA in the *panC* and *CoaE* genes, which encode enzymes
152 involved in pantothenate biosynthesis and pantothenate conversion to CoA, respectively.
153 Accordingly, their proteins are undetectable, suggesting that these antisense RNAs
154 repress the production of pantothenate and CoA in *Buchnera*. Functional implications of
155 this repression is unclear.

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

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

176 *Immatures feeding on microbe-containing diets*

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

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

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

233 **Involvement of B vitamins in adult physiology and survival**

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

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

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

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

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

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

296 In fruit flies, the specific activities of thiamin, riboflavin and biotin have been analyzed.
297 Biotin-deficiency in the diet decreases lifespan in males and females, but increases
298 resistance to stress in males (Landenberger et al., 2004). This vitamin is particularly
299 important for mitosis of intestinal stem cells. When these cells are unable to import biotin,
300 they do not proliferate properly, which notably increases the susceptibility of flies to
301 bacterial infection. Flies fed with a biotin-deficient diet have a lower mitosis rate in the
302 intestine, but *E. coli* colonization can restore it to normal levels (Neophytou & Pitsouli,
303 2022). Thiamine deficiency in the diet does not seem to affect adult lifespan whether in
304 conventionally-reared or axenic individuals (Sannino et al., 2018). Riboflavin has been
305 proposed as an anti-aging agent, as conventionally-reared flies supplied with additional
306 riboflavin in the diet have a prolonged life expectancy in normal conditions and upon
307 oxidative stress (Zou et al., 2017), but it is not clear whether riboflavin has to be provided
308 by the microbiota in adults.

309 Besides the examples described in Figure 2, antibiotic treatment leads to a decrease
310 in adult lifespan in several other insect species, where bacteria have been found to
311 carry B vitamins in their genome. This has notably been found in date palm
312 leafhoppers *Ommatissus lybicus* carrying *Wolbachia* (Fan et al., 2016; Karimi et al.,
313 2019; Xue et al., 2014) and in hematophagous flies *Hippoboscoidea*, *Streblidae* and

314 *Nycteribiidae* carrying several *Arsenophonus*, *Sodalis* and/or *Aschnera* (Duron et al.,
315 2014; Hosokawa et al., 2012; Šochová et al., 2017).

316 Provision of microbial B vitamins to the host has also been found to affect other traits than
317 lifespan. A recent ecological study indicates that an invasion by *Solenopsis invicta* ants
318 induces a change of diet of the endemic ants workers *Tapinoma melanocephalum*
319 towards a protein-rich diet, which affects microbiota composition, notably increasing
320 *Wolbachia* relative abundance (Cheng et al., 2019). This study further shows that
321 supplementing the diet of *T. melanocephalum* in non-invaded and invaded colonies with
322 protein-rich or sugar respectively reverses the effect on several taxa including *Wolbachia*,
323 and leads to a loss of riboflavin and nicotinic acid in invaded ants, while some other B
324 vitamins are not affected. This suggests that the change in diet may allow ants to behaviorally
325 respond to invasion by favoring a microbiota producing riboflavin and nicotinic acid.

326 Considering vectorial transmission, the impact of microbe-derived vitamins on vector
327 competence has been less extensively studied, yet it has been evidenced in tsetse flies
328 (Rio et al., 2019). *Trypanosome* infections induce folate biosynthesis genes of the fly
329 symbiont *Wigglesworthia*. In the major trypanosome vector *Glossina morsitans*, an
330 antifolate treatment decreases infection prevalence in the gut, without affecting
331 subsequent parasitic development towards salivary gland infection. In the inefficient
332 vector *Glossina brevipalpis*, diet supplementation in folate leads to a strong increase in
333 infection prevalence. Finally, folate gene expression by *Wigglesworthia* is higher in
334 *Glossina morsitans* than in less efficient vectors and induced by parasitic infection,
335 suggesting that folate expression may be a critical factor determining vector competence
336 towards the parasite. In other vectors, a link with the microbiota is less clear but there is
337 evidence that parasites require B vitamins from the host and/or the environment. Diet
338 supplementation in folic acid or one of its precursors, para-aminobenzoate, positively
339 affects *Brugia malayi* development in *Ae. aegypti* (Kirkness et al., 2010). Genetic studies
340 have found that pantothenate transporter and predicted pantothenate kinases Pank1 and
341 Pank2 are required for *Plasmodium* development in the mosquito, while they are
342 dispensable for growth in red blood cells and differentiation to gametocytes (Hart et al.,
343 2015, 2016).

344 **B vitamins and reproduction**

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

353 Requirements on B vitamins for insect reproduction has first been studied via diet
354 manipulation and via antivitamin treatments. Several antivitamins were notably reported
355 to inhibit egg production by *Musca viscinis*, an effect that could partly be rescued using
356 the corresponding vitamin (Bergmann et al., 1959). Later, Saxena and Kaul were
357 surprised to see how little effect vitamin deficiency in the diet had on *Oryzaephilus*
358 *mercator* (*Coleoptera*) fertility and discussed that this may be due to microbe-dependent
359 production of vitamins (Saxena & Kaul, 1974). More recent studies on mosquitoes and
360 bedbugs also pointed to a positive effect of sugar diet supplementation with B vitamins
361 on male and female fertility. These dietary supplements specifically increased male
362 fertilization capacity during forced-mating experiments, egg production by females, egg
363 hatching success and/or viability of the hatched larvae (Hosokawa et al., 2010;
364 Phasomkusolsil et al., 2017; Tan et al., 2016). This correlates with negative effects on
365 female fecundity and/or fertility found in *Anopheles* and *Aedes* upon antibiotic-treatment,
366 which is rescued by *Serratia* or *Enterobacter* colonization in *Anopheles* (Ezemuoka et al.,
367 2020; Gaio et al., 2011). In *Aedes*, the microbiota promotes fecundity via hemolysis in the
368 blood bolus, while colonization with *E. coli* HS, a non-hemolytic bacterial strain, does not
369 affect fertility (Figure 3; Gaio et al., 2011; Romoli et al., 2021). While the link has not
370 experimentally been established, these observations would fit with a model where B
371 vitamins released by bacteria promote mosquito fertility. Bacteria may produce vitamins
372 themselves and/or participate to their release via hemolysis after the blood meal. In ticks,
373 maternally-inherited endosymbionts are essential for oviposition (lone star tick) and egg
374 development (lone star tick and *Rhipicephalus*), but the involvement of B vitamins has not

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

377 When focusing on specific vitamins, there is evidence that riboflavin and biotin particularly
378 affect insect fecundity (Figure 3). When riboflavin is added to diet, it improves egg
379 production in conventionally-reared fruit flies (Zou et al., 2017). While oogenesis is
380 reduced in germ-free flies as a result of decreased ATP levels, riboflavin supplementation
381 is sufficient to reverse the effect (Gnainsky et al., 2021). Riboflavin supplementation has
382 also been found to restore fecundity in bedbugs cured from *Wolbachia* via antibiotic-
383 treatment (Moriyama et al., 2015). A biotin dietary supplement also promotes fecundity in
384 *Wolbachia*-cured bedbugs (Nikoh et al., 2014). Genomic analyses showed that the
385 riboflavin biosynthesis pathway is often complete in *Wolbachia*, while the biotin
386 biosynthesis pathway is complete in some *Cimex* and *Paracimex* bedbugs' species as
387 well as in some planthoppers and missing in the other insect genera tested (Balvín et al.,
388 2018; Ju et al., 2020; Moriyama et al., 2015). A similar situation occurs in planthoppers,
389 where the presence of *Wolbachia* enhances egg production. Experiments based on the
390 depletion or addition of dietary biotin and/or riboflavin indicate that this impact is driven
391 by the provision of these vitamins by *Wolbachia*, the role of biotin being particular
392 important (Ju et al., 2020). In whiteflies as well, removal of symbionts *Portiera* and
393 *Hamiltonella* leads to reduced titers of pantothenate and biotin respectively and scant egg
394 production (Ren et al., 2020, 2021). Silencing host genes of the pantothenate and biotin
395 biosynthesis pathways (inherited from bacteria by horizontal-gene transfer, as mentioned
396 in the previous section) recapitulates antibiotic treatments against *Portiera* and
397 *Hamiltonella*, respectively. Again, this indicates that whiteflies and their symbionts
398 collaborate to support the adult host requirement on B vitamins.

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

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

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

436 cytoplasmic incompatibility; colonized males are infertile unless they mate with a female
437 colonized with the same strain of *Wolbachia*. It promotes *Wolbachia* colonization in a
438 population as it favors colonized females, which can be fertilized by any male and will
439 vertically transmit the endosymbiont. A recent comparison of *Wolbachia* genomes from
440 500 host species including nematodes and arthropods showed a correlation between the
441 presence of a riboflavin transporter in *Wolbachia* genome and cytoplasmic incompatibility
442 (Scholz et al., 2020), yet functional links have not yet been characterized.

443 **Conclusion**

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

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

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

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

482

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488

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.

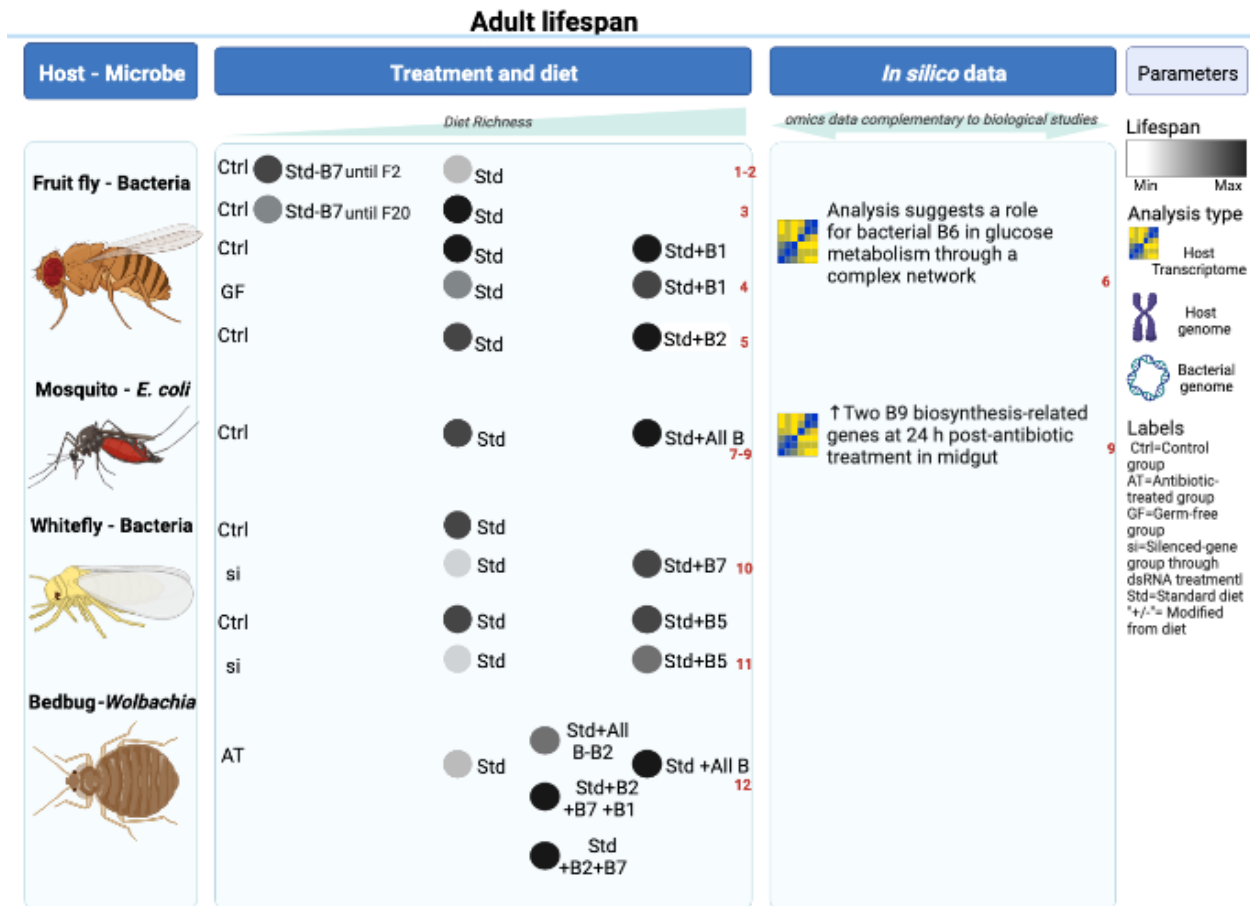


Figure 2. Influence of B vitamins on insect lifespan. AT – antibiotic treated; GF – Germ free; Ctrl – microbiota control (which can either mean conventionally reared or gnotobiotic, with AT and GF respectively); Std – Standard diet, neither impoverished nor enriched; a holidic diet is considered as standard 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.

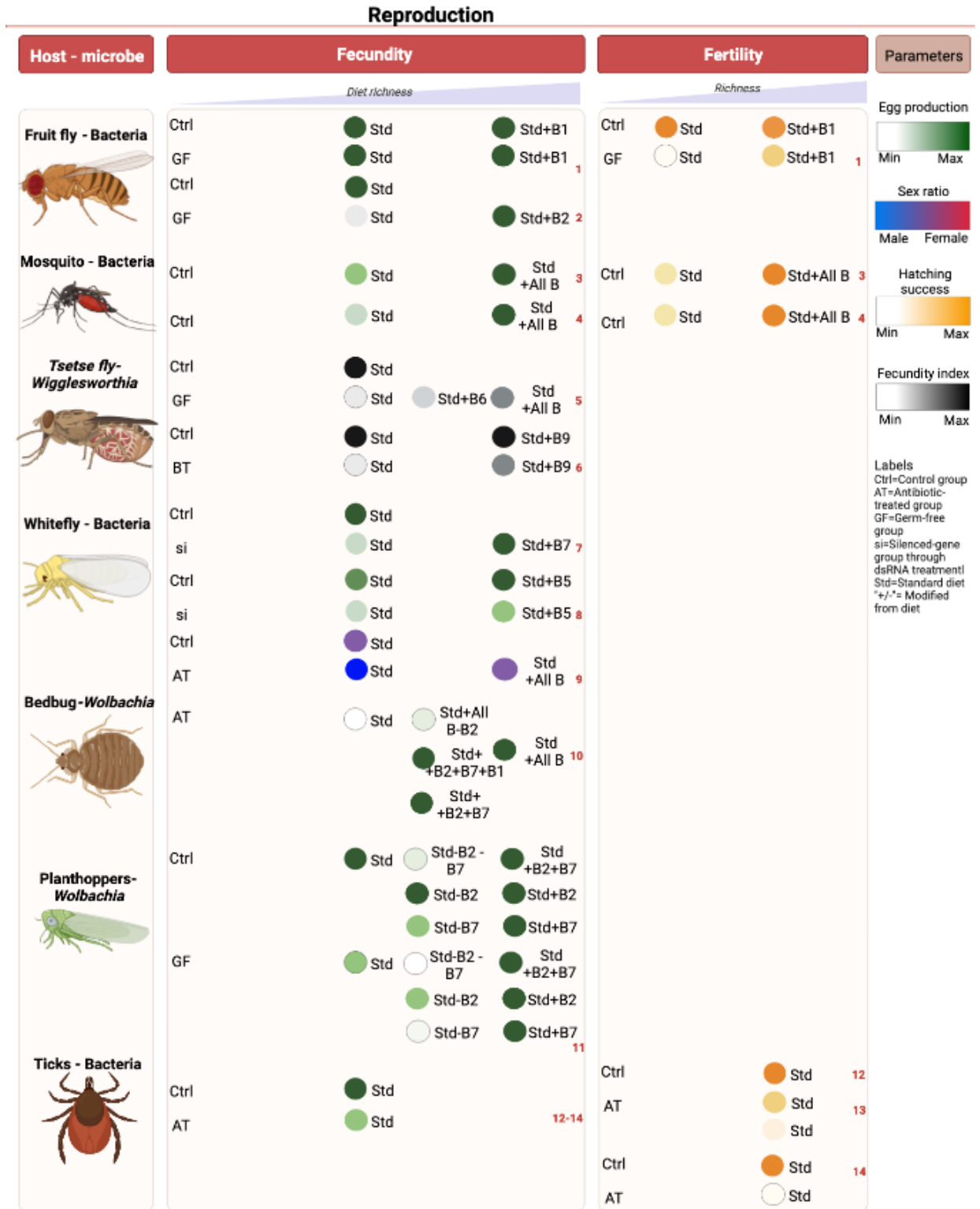


Figure 3. Influence of B vitamins on insect reproduction. AT – antibiotic treated; GF – Germ free; Ctrl – microbiota control (which can either mean conventionally reared or

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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