



The nutritional dimension of facultative bacterial symbiosis in aphids: Current status and methodological considerations for future research

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ABSTRACT

Aphids are valuable models for studying the functional diversity of bacterial symbiosis in insects. In addition to their ancestral obligate nutritional symbiont *Buchnera aphidicola*, these insects can host a myriad of so-called facultative symbionts. The diversity of these heritable bacterial associates is now well known, and some of the ecologically important traits associated with them have been well documented. Some twenty years ago, it was suggested that facultative symbionts could play an important role in aphid nutrition, notably by improving feeding performance on specific host plants, thus influencing the adaptation of these insects to host plants. However, the underlying mechanisms have never been elucidated, and the nutritional role that facultative symbionts might perform in aphids remains enigmatic. In this opinion piece, I put forward a series of arguments in support of the hypothesis that facultative symbionts play a central role in aphid nutrition and emphasize methodological considerations for testing this hypothesis in future work. In particular, I hypothesize that the metabolic capacities of *B. aphidicola* alone may not always be able to counterbalance the nutritional deficiencies of phloem sap. The association with one or several facultative symbionts with extensive metabolic capabilities would then be necessary to buffer the insect from host plant-derived nutrient deficiencies, thus enabling it to gain access to certain host plants.

1. Introduction

Aphids (Hemiptera: Aphididae) are historical models in the study of symbiotic interactions that many insect taxa have evolved with heritable bacteria (Moran et al., 2008; Oliver et al., 2010). Like most insect taxa that feed on diets whose composition is incapable of fully satisfying their nutritional requirements, aphids have evolved a dependence on bacterial associates that enable them to obtain nutrients insufficiently supplied by the phloem sap: almost all species are associated with *Buchnera aphidicola*, an obligate (or primary) endosymbiont acquired tens of millions of years ago that supplies them with essential amino acids and vitamins (Douglas, 1998; Nakabachi and Ishikawa, 1999). *B. aphidicola* is essential for aphid development and reproduction and dwells intracellularly in bacteriocytes, the specialized host cells that control symbiont populations and mediate metabolic exchanges between the insect and its symbionts (Braendle et al., 2003). Constrained to strict vertical transmission and an intracellular lifestyle, *B. aphidicola* undergoes advanced genomic reduction that has resulted in a small, stabilized genome comprising only the genetic information essential for

maintaining the association (Moran et al., 2009).

In addition to *B. aphidicola*, aphids can host a plethora of more recently acquired heritable symbionts, often referred to as secondary symbionts. Secondary symbionts include so-called co-obligate symbionts that metabolically complement *B. aphidicola* in aphid species whose the ancestral symbiont has become unable to perform its nutritional function alone due to an extremely advanced state of genome reduction (Sudakaran et al., 2017). Secondary symbionts also include facultative symbionts that can infect a wide range of aphid species, but which are not fixed in aphid the species and have a scattered distribution in aphid populations (Smith et al., 2015; Sepúlveda et al., 2017). Having been acquired more recently, facultative symbionts are less genomically eroded than obligate symbionts and can still be transmitted horizontally, although their main route of transmission is vertical (Oliver et al., 2010). Unlike obligate symbionts, facultative symbionts can infect a variety of host tissues, including bacteriocytes, hemolymph, oenocytes and sheath cells (Moran et al., 2005; Koga et al., 2012; Tsuchida et al., 2014). The nature of the interaction between facultative symbionts and insects oscillates between parasitism and mutualism, with the environmental

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context having a major influence: facultative symbionts can be associated with beneficial phenotypic effects in specific environmental conditions, while in others they incur fitness costs by negatively affecting host development and reproduction (Zytyńska et al., 2021). Heritable facultative symbionts have been found to evolve with a range of sap- and blood-feeding insects (Feldhaar, 2011; Doudoumis et al., 2017; Zytyńska et al., 2021), but the ecologically important traits with which they are associated and the underlying mechanisms have mainly been examined in aphids (Oliver et al., 2010). These include resistance to heat shock, protection against parasitoids and entomopathogenic fungi, body color modification and adaptation to the host plant (Oliver et al., 2003; Tsuchida et al., 2004; Scarborough et al., 2005; Tsuchida et al., 2010a; Burke et al., 2010). Facultative symbionts are now considered important players in the evolutionary ecology of aphids, with protection against parasitoids being the associated effect that has received the most attention (Oliver and Higashi, 2019). Some twenty years ago, it was suggested that facultative symbionts might play an important role in aphid nutrition (Tsuchida et al., 2004). However, subsequent paradoxical findings, together with a lack of concrete evidence and in-depth studies, mean that the nutritional function that facultative symbionts may perform in aphids remains fairly enigmatic and is probably underappreciated today. In this opinion piece, I argue that heritable facultative symbionts of aphids may be essential nutritional partners for these insects as part of a metabolic complementation service. I discuss a number of arguments in support of this hypothesis, explain why the nutritional dimension of these bacterial associates has been rather overlooked, and suggest avenues for addressing this hypothesis in future work.

2. Facultative symbionts as nutritional partners: some evidence but many gaps

The hypothesis that facultative aphid symbionts are associated with nutritional benefits is not new, and was highlighted by Tsuchida et al. (2004). In this study, the authors found that the facultative symbiont *Regiella insecticola* significantly improved the performance of the pea aphid *Acyrtosiphon pisum* on white clover (*Trifolium pretense*), but not on vetch (*Medicago sativa*). Furthermore, experimental transfer of this symbiont strain to naturally *Regiella*-free vetch aphids (*Megoura crassicauda*) improved survival on white clover, suggesting that facultative symbionts can influence insect performance on specific host plants (T. Tsuchida, Koga, Matsumoto, et al., 2010b). These studies paved the way for research into the nutritional dimension of facultative symbionts. However, other studies performed on *A. pisum* and inspired by the Tsuchida et al. (2004) failed to find consistent plant-specific effects of facultative symbionts on aphid performance, suggesting that symbiont-incurred costs and benefits in the context of plant adaptation depend on the genotype of the facultative symbiont and that of the aphid host (Ferrari et al., 2004, 2007; Leonardo, 2004; McLean et al., 2010). Only Wagner et al. (2015) found that the facultative symbiont *Arsenophonus* confers a host plant-specific benefit in the cowpea aphid *Aphis craccivora*: the symbiont increases aphid performance on black locust (*Robinia pseudoacacia*), while causing a significant decrease in performance on alfalfa (*Medicago sativa*) (Wagner et al., 2015). A further indication of the putative role of facultative symbionts in aphid nutrition is that antibiotic-mediated elimination of *B. aphidicola* in a doubly infected pea aphid harboring a facultative strain of *Serratia symbiotica* in addition to the obligate symbiont results in the replacement of the latter by the facultative associate in the *Buchnera*-containing bacteriocytes (primary bacteriocytes) (Koga et al., 2003). This study suggests that, thanks to their metabolic capabilities, facultative symbionts can potentially compensate for the loss of the nutritional obligate symbiont. Finally, a number of field studies have pointed to the non-random distribution of facultative symbionts in insect populations, which appears to be influenced by the host plant, suggesting a nutritional role for facultative symbionts (Tsuchida et al., 2002; Leonardo and Muir, 2003;

Ferrari et al., 2004, 2012; McLean et al., 2010; Henry et al., 2013, 2015; Zytyńska et al., 2016; Guidolin and Cònsoli, 2017). For example, the symbiont *H. defensa* is present at particularly high frequencies in the pea aphid populations that feed on the plants *Medicago sativa*, *Ononis spinosa* and *Lotus pedunculatus*, while pea aphids that feed on *Lathyrus* species are rarely infected by facultative symbionts (McLean et al., 2010; Henry et al., 2013). Despite some evidence that facultative symbionts may play a nutritional role in aphids, it remains unclear how they fulfill this role. The few studies that have addressed the nutritional dimension of facultative symbiosis raise many questions. For example, what are the mechanisms underlying the ability of certain facultative symbionts to enhance aphid performance on certain plant species? What is it about the host genotype that influences the relationship between the symbiont and the host plant? Does the genotype of *B. aphidicola* influence aphids' propensity to associate with facultative symbionts? What genetic factors influence the propensity of a facultative symbiont to perform a nutritional service? The nutritional role of facultative symbionts in aphids may depend on many factors that may have been overlooked, and its study is far from conclusive.

3. Metabolic complementation of *Buchnera* by co-obligate symbionts

Answers to these questions may lie in studying the interactions that some aphid species have with co-obligate symbionts, another type of secondary symbiont. Indeed, the nutritional role of these symbionts has been well examined in different aphid species via genome-based metabolic inference approaches (Manzano-Marín and Latorre, 2014; Manzano-Marín et al., 2016, 2017, 2018; Monnin et al., 2020; Renoz et al., 2022a; Manzano-Marín et al., 2023). Co-obligate symbioses have evolved in some aphid subfamilies that include species in which the production of certain nutrients is not ensured by the ancient obligate symbiont *B. aphidicola* alone, but by the consortium it forms with a more recently acquired nutritional symbiont (Manzano-Marín et al., 2023). *B. aphidicola* genomes have evolved very differently in the various aphid lineages: in some lineages, such as the Lachninae and Chaitophorinae subfamilies, *B. aphidicola* exhibits genomes of 400–450 kb, which is significantly smaller than the genomes of *B. aphidicola* strains evolving in the Aphidinae and Eriosomatinae subfamilies, for example, which are typically over 600 kb in size (Chong et al., 2019). These “tiny-genome” *Buchnera* strains have become unable to biosynthesize certain amino acids (often tryptophan) and vitamins (often riboflavin) on their own, and the synthesis of these nutrients is supported by a complementary (“co-obligate”) symbiont that is often hosted in bacteriocytes (secondary bacteriocytes) distinct from those harboring *Buchnera* (primary bacteriocytes), and which forms a metabolic unit with the ancestral symbiont (Renoz et al., 2022a; Yorimoto et al., 2022; Manzano-Marín et al., 2023). These co-obligate symbionts, which infect 100 % of the species' individuals, are thought to originate from facultative symbionts on which the host species has become dependent to compensate for *B. aphidicola*'s metabolic deficiencies (Renoz et al., 2021). Interestingly, metabolic complementation patterns between *B. aphidicola* and the co-obligate symbiont have sometimes evolved very differently among the aphid species harboring these di-symbiotic systems (Renoz et al., 2022a; Manzano-Marín et al., 2023). Three key lessons can be drawn from the study of co-obligate symbioses in aphids to better appreciate the nutritional dimension of facultative symbiosis. The first lesson is that an additional symbiont may be required to rescue just a few of the obligate symbiont's missing genes for the biosynthesis of a nutrient deficient in the aphid diet. This reliance on an additional nutritional symbiont has major Evo-Devo implications for the insect species, since it had to evolve a second type of bacteriocyte to regulate the populations of the co-obligate symbiont and the metabolic exchanges between the different nutritional symbionts, and to do so solely to reap the benefits of a handful of genes required for nutrient biosynthesis (Manzano-Marín et al., 2017; Renoz et al., 2022a). The second lesson is the importance of

considering the evolution and genetic diversity of *B. aphidicola* when assessing the possible need for the system to evolve towards dependence on an additional nutritional symbiont. Indeed, the metabolic capacities of *B. aphidicola* strains evolving in di-symbiotic systems can differ greatly from one aphid species to another (Renoz et al., 2022a; Manzano-Marín et al., 2023). For example, *Buchnera*'s ability to synthesize tryptophan is variable in aphid species of the Lachninae subfamily, and while the *Buchnera* strain associated with the cereal aphid *Sipha maydis* can synthesize histidine, the strain associated with the Norway maple aphid *Periphyllus lyropictus*, also a Chaitophorinae aphid, is unable to do so. Finally, it is interesting to note that aphid species that have evolved a dependence on a di-symbiotic nutritional system tend to exhibit a rather specialized diet, i.e. they tend to feed on a restricted range of host plants (e.g. a restricted range of conifer species, cereal species, maple species, etc.) (Meseguer et al., 2017; Manzano-Marín et al., 2023). This suggests that diet is correlated with the insect's need to acquire additional nutritional partners, highlighting the importance of examining phloem sap composition to comprehend the aphid's actual nutritional demands (Douglas, 1993; Sandström and Pettersson, 1994; Sandström and Moran, 1999; Wilkinson and Douglas, 2003; Akman Gündüz and Douglas, 2009).

An instructive study concerning nutritional multi-symbiotic systems is that of Monnin et al. (2020). This study, which looked at the anatomical integration of co-obligate symbionts in aphids, is a valuable source of information for rethinking the nutritional dimension of facultative symbiosis in these insects. In their study, the authors claim to have identified five new cases of co-obligate symbioses consisting in the metabolic complementation of the ancestral obligate symbiont *B. aphidicola* by a co-obligate strain of *S. symbiotica*: three cases in the Chaitophorinae subfamily (*Periphyllus lyropictus*, *Periphyllus aceris* and *Periphyllus acericola*) and two cases in the Aphidinae subfamily (*Aphis urticae* and *Microlophium carnosum*). Close examination of the data confirms that all three *Periphyllus* species depend on a di-symbiotic system in which *B. aphidicola* has a highly eroded genome (around 400 kb) and is metabolically complemented by *S. symbiotica* for riboflavin biosynthesis. However, the co-obligate status of *S. symbiotica* in the species *A. urticae* and *M. carnosum* appears more questionable, as they both host *Buchnera* strains with genomes larger than 600 kb, indicating genomes that are little degraded compared to those of *Buchnera* strains evolving in Lachninae and Chaitophorinae aphids, for example, which require the metabolic assistance of an additional obligate symbiont (Manzano-Marín et al., 2023). Also, a previous study suggests that *S. symbiotica* is not systematically present in *A. urticae* and *M. carnosum*, and may not be a fully fixed symbiont in these species and therefore an obligate associate (Henry et al., 2015). That said, genome-based inference approaches conducted on *A. urticae* and *M. carnosum* aphids feeding on common nettle (*Urtica dioica*) suggest in both cases a metabolic complementarity between *Buchnera* and *S. symbiotica* (Monnin et al., 2020). In the case of *M. carnosum*, analyses show that the *ribD* gene, whose encoded enzyme catalyzes the EC 3.5.4.26 and EC 1.1.1.193 reactions, is pseudogenized in the associated *Buchnera* strain (Fig. 1). This indicates that, in this aphid species, *Buchnera* is probably deficient in riboflavin synthesis and may need to be metabolically supplemented by *S. symbiotica*. This case is remarkable as it suggests that a secondary symbiont may be necessary to rescue a biosynthetic pathway jeopardized by only a single mutation in *B. aphidicola*. It should be noted, however, that while *M. carnosum* is reputed to be a specialist species on common nettle, it has been collected on other plant species (*Sonchus oleraceus*, *Cirsium* sp, *Rubus fruticosus* and *Circaea lutetiana*) on which the aphid species was not found to be consistently infected with *S. symbiotica* (Henry et al., 2015). In light of these data, the question arises as to whether *M. carnosum* is capable of feeding on other host plants, and whether its infection status influences its adaptive abilities to feed on a range of different host plants. Another interesting observation from Monnin et al. (2020) is that, like many facultative symbionts associated with the pea aphid *A. pisum*, the

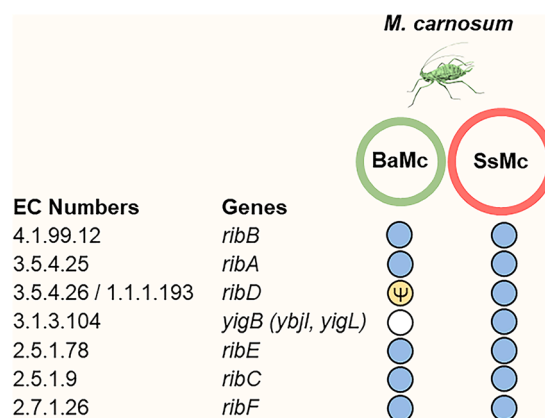


Fig. 1. B₂ vitamin (riboflavin) biosynthetic metabolic capabilities of the di-symbiotic system *B. aphidicola* (Ba)-*S. symbiotica* (Ss) in *M. carnosum* (Mc). Genomic analyses were performed using the MicroScope platform and MicroCyc as in (Renoz et al., 2021; Renoz et al., 2022) and confirmed the results of (Monnin et al., 2020). On the left are the names of the genes encoding the enzymes involved in the biosynthetic pathway and the EC numbers of the chemical reaction that the encoded enzymes catalyze. Each box is associated with the gene encoded by the genome of a symbiont. The following code is used to characterize the state of each gene: the blue color means that the gene (or an alternative gene that can perform the same enzymatic function) is present and not pseudogenized, the white color indicates that the gene is missing, and the yellow color with the psi (Ψ) symbol means that the gene is pseudogenized. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

allegedly co-obligate symbiont *S. symbiotica* of *M. carnosum* appears to be compartmentalized exclusively in sheath cells flanking the *Buchnera*-containing bacteriocytes (Monnin et al., 2020). This is an atypical localization for an aphid co-obligate symbiont, as these nutritional partners typically reside in syncytial secondary bacteriocytes (and, depending on the aphid species, occasionally in other cells and tissues) (Manzano-Marín et al., 2017, 2023; Renoz et al., 2022a, 2022b). This raises many questions, including how populations of these putative nutritional symbionts are regulated in host cells other than bacteriocytes, and the mechanisms that govern interactions between primary bacteriocytes and sheath cells in a context of nutritional cooperation.

In the case of *A. urticae*, genome-based metabolic inference analyses indicate that the *Buchnera* strain possesses all the genes for the riboflavin biosynthetic pathway and does not need to be complemented by *S. symbiotica* for nutrient supply (Monnin et al., 2020). However, the analyses suggest that the obligate symbiont may have become dependent on *S. symbiotica* for peptidoglycan biosynthesis in a similar way to what has been reported in mealybugs (Bublitz et al., 2019). This raises the possibility that in aphids, metabolic complementarity between *B. aphidicola* and secondary symbionts is not always linked to direct nutritional supply. This scenario is supported by the recent finding that in the eusocial aphid *Ceratovacuna japonica*, *Buchnera* and a co-obligate *Arsenophonus* symbiont complement each other metabolically in the riboflavin and peptidoglycan synthesis pathways (Yorimoto et al., 2022).

4. *Buchnera*'s genetic variability in amino acid and vitamin biosynthesis

The host genetic background has been suggested as a factor explaining the absence of consistent plant-specific effects of facultative symbionts on aphid performance (Ferrari et al., 2007, 2012; McLean et al., 2010). However, aphids co-evolve with bacterial associates and the genetic background should not be examined on the insect alone, but on the unit of organisms maintaining an interdependent relationship, which includes, in particular, their ancient obligate associate

B. aphidicola. It is now clearly established that, from one insect host species to another, the same species of ancestral obligate symbiont can exhibit different biosynthetic capacities (Hansen and Moran, 2014). For example, *Carsonella ruddii*, the ancestral obligate symbiont of psyllids, exhibit different amino acid capacities depending on the psyllid species with which it is associated. The same applies to *B. aphidicola*. However, apart from cases of associations where the ancestral obligate symbiont is complemented by a co-obligate symbiont (Manzano-Marín and Latorre, 2014; Manzano-Marín et al., 2016, 2017, 2018, 2023; Meseguer et al., 2017; Renoz et al., 2022), *Buchnera*'s genetic diversity and inter-strain variability in terms of metabolic capacity (for amino acid and vitamin biosynthesis in particular) are aspects that have received rather limited attention, particularly within the same aphid species (Macdonald et al., 2011; Vogel and Moran, 2011; Chung et al., 2020). No large-scale comparative genomics studies have investigated these aspects. Yet the significance of this inter-strain genetic variability has been well demonstrated in *A. pisum* with the case of the *Buchnera* gene *ibpA* which encodes a heat shock protein: a mutation in this gene can severely affect the heat tolerance of pea aphids, showing that microevolution of symbionts can have major ecological effects for insect hosts (Dunbar et al., 2007; Moran and Yun, 2015). It is likely that microevolution also affect genes related to metabolism and nutritional symbiosis in *Buchnera*. For instance, a quick comparative genomic analysis based on the genome sequences of the eight *Buchnera* strains associated with the pea aphid *A. pisum* available on NCBI shows that there may be small inter-strain differences in the ability to biosynthesize lysine (Fig. 2). The possibility of inter-strain genetic variability in *Buchnera*'s metabolic capacities has also been put forward by previous experimental studies. For instance, the interclonal variation in the amino acid requirement of the aphid *Aphis fabae* reported by Wilkinson and Douglas (2003) suggests that *Buchnera* symbionts inhabiting different aphid lines may differ in their biosynthetic capacities due to mutational inactivation of certain pathways. This hypothesis is supported by Vogel and Moran (2011), who also reports a variation in *Buchnera*'s contribution to the supply of certain amino acids in the pea aphid (Vogel and Moran, 2011). More recently, a genome-wide association study (GWAS) on 208 *A. pisum* genotypes revealed associations between *Buchnera* genome variation and pea aphid performance on a histidine-free diet, suggesting that genetic variation in *Buchnera* lines could result in different nutrient acquisition efficiencies in host aphids, thus affecting aphids' ability to feed on different host plants (Chung et al., 2020). All these findings suggest strain variation in *Buchnera* amino acid biosynthesis genes (and probably other metabolic pathways) within the same aphid species, with, in some cases, a loss of host supply capacity due to mutations. This

could be explained by a local relaxation of selection on certain genes linked to nutrient synthesis, depending on the nutrient content of the host plant's phloem sap (Sandström and Moran, 1999), but also by the host genotype and resulting metabolic capacities, which can vary slightly from one line to another within the same aphid species (Moran and Degnan, 2006). This is an important point regarding the feeding ecology of aphids, as it suggests that variation in the dietary requirements of aphid lines may affect their ability to use different plants, and hence the structure of their populations. Concentrations of nutrients such as amino acids and vitamins in phloem sap vary from one host plant species to another, and aphid performance depends on the concentration levels of these nutrients (Sandström and Pettersson, 1994). Adaptation of the host aphid and/or *Buchnera* to the phloem profile of specific host plants could therefore be instrumental in the establishment of host aphid races (Peccoud et al., 2009).

In light of this inter-strain variability in *Buchnera*, one hypothesis is that the metabolic capabilities of additional symbionts might be required in certain aphid lines to fill gaps in the biosynthetic capabilities of the obligate symbiont (and the host), and thus buffer the insect from host plant-derived nutrient deficiencies. In this context, the acquisition of one or several facultative symbionts to fill *Buchnera*'s few metabolic gaps could enable the aphid to perform well on plants that would otherwise be less suitable. However, to test this hypothesis thoroughly, a larger number of genomes from *Buchnera* strains evolving in different lines of the same aphid species need to be properly sequenced and annotated for mapping genetic variation in metabolism-related genes. To date, the genomic data required for such large-scale comparative genomics studies are sorely lacking. Yet they are essential for a comprehensive picture of the biosynthetic capacities of *Buchnera* strains evolving in different aphid lines of the same species, for amino acids and vitamins in particular, but possibly also for peptidoglycan. To complete the picture, it is essential to acquire the genomic sequences of facultative symbionts evolving alongside *Buchnera* within the same aphid line, as these data are essential for robust genome-based inference approaches aimed at highlighting possible metabolic complementarities between the obligate symbiont and facultative associates, along the lines of what has been achieved in the study of co-obligate symbiosis (Manzano-Marín et al., 2023).

To date, the only endeavor in the use of the genome-based inference approach to highlight the potential role of facultative symbionts in a metabolic complementation service comes from (Peng et al., 2023). The authors examined the community structure of heritable facultative symbionts across US populations of the pea aphid *A. pisum*. After identifying the heritable symbionts present in the sampled populations, they

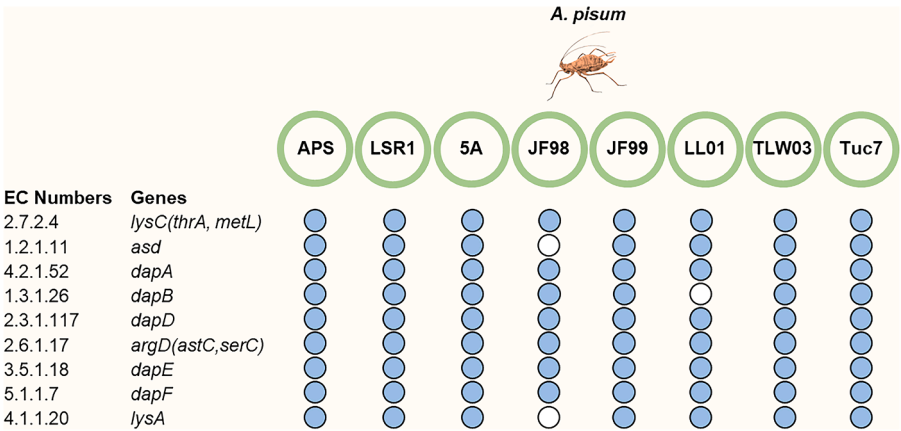


Fig. 2. Lysine biosynthetic metabolic capabilities of different *B. aphidicola* strains associated with *A. pisum*. Genomic analyses were performed using the MicroScope platform and MicroCyc as in (Renoz et al., 2021; Renoz et al., 2022). On the left are the names of the genes encoding the enzymes involved in the biosynthetic pathway and the EC numbers. The following code is used to characterize the state of each gene: the blue color means that the gene (or an alternative gene that can perform the same enzymatic function) is present and not pseudogenized, and the white color indicates that the gene is missing. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

genotyped them. Then, in order to detect possible metabolic complementarities between members of the different symbiotic communities identified, they conducted genome-based metabolic inference based not directly on the genomic sequences of the genotyped strains, but on those of the identified symbionts available in the databases and identified as being closest to the genotyped strains. Their analyses suggest a metabolic cooperation between the pea aphid *A. pisum*, *Buchnera* and the facultative symbionts *S. symbiotica* and *Rickettsiella viridis* for biotin biosynthesis (Fig. 3). This clever, low-cost approach thus suggests that facultative symbionts can fill the metabolic gaps of obligate symbionts through multiparty mutualism, in the same way as has been reported for co-obligate symbioses in certain aphid lineages. However, while this approach has the merit of taking into account the variability of symbiont strains, it has a number of drawbacks. For example, the genome-based inference approach used by the authors is highly extrapolative, as it is not based on the genomic sequences of the original partners. Furthermore, the study provides no information on the tissue tropism of the symbionts and how they are anatomically integrated into the host, although these are potentially valuable clues to support metabolic collaboration between symbionts (e.g. compartmentalization within bacteriocytes and physical proximity to *Buchnera*) (Renoz et al., 2022; Michalik et al., 2023). Finally, while the study addresses the symbiont strain level, it does not take into account another potentially pivotal parameter: variability at host level. Indeed, the analyses were conducted using one of the only *A. pisum* genomic sequences available in the databases as the host reference genome: that of *A. pisum* isolate AL4F (*Medicago sativa* biotype) (Li et al., 2019).

5. Biotypes, cryptic species and lessons from *Bemisia tabaci*

In general, when facultative symbionts are identified in aphids, they are assigned to the host species with which they have been found associated (e.g. *Acyrtosiphon pisum*, *Aphis fabae* or *Sitobion avenae*, etc.). However, this affiliation does not take into account the genetic divergence that exists within a same host species. Indeed, one aphid species may harbor complexes of biotypes (or races), i.e. complexes of subspecies that may present minute morphological variations and, above all, feed preferentially on specific host plant species (Peccoud et al., 2009, 2010, 2015; Khanal et al., 2023). Thus, many aphid species can include

diverse biotypes with varying degrees of sympatry, hybridization capacity, plant host specificity and nutritional needs. For example, (Srivastava et al., 1985) reported that amino acid requirements can differ between different *A. pisum* biotypes. Interestingly, the authors had already hypothesized that symbiotic bacteria could provide the aphids with the missing amino acids. Laboratory experiments attempting to conditionate aphid biotypes on alternative host plants have produced mixed results. For example, in the pea aphid, the performance of biotypes on alternative hosts is only marginally altered by conditioning (Via, 1991), and strong specialization of biotypes on their native host plant is still observed after a period of rearing on a shared host plant species (Peccoud et al., 2009; Ferrari et al., 2012). This suggest that the genome of the aphid biotype, but perhaps also that of the *B. aphidicola* symbiont specifically associated with it, conditions the insect's feeding performance on the host plant. This is notably supported by the evidence that different aphid lines within the same species (*A. pisum*) exhibit variations in metabolic capacity, particularly with regard to amino acid synthesis, with a correlation between molecular variations in the genomes of the host aphid and the *Buchnera* symbiont (Chung et al., 2020). Surprisingly, while a number of studies have focused on aphid biotypes and their ability to adapt to different host plant ranges, few have attempted to correlate aphid performance with the metabolic capabilities of the associated *B. aphidicola* strain. One hypothesis is that different aphid biotypes host strains of *B. aphidicola* that tend to have evolved slightly different metabolic capacities, and that this contributes to conditioning the aphids' performance on the host plants with which they deal. Depending on the composition of phloem sap, the metabolic capacities of the aphid biotype and the ability of the associated *Buchnera* strain to compensate for nutrient deficiencies, the metabolic capacities of one or more facultative symbionts may be necessary to enable the aphid to perform well on certain host plants. This hypothesis is supported by the propensity of pea aphid biotypes to carry specific symbiont combinations (Peccoud et al., 2015). For example, *S. symbiotica* is almost always present in the *Securigera varia*/*Hippocrepis comosa* biotype and the combination *Hamiltonella defensa*+*S. symbiotica* is almost always present in the *Genista sagittalis* biotype. The facultative symbiont *H. defensa* is found at high frequencies in aphid biotypes feeding on the plants *Medicago sativa*, *Ononis spinosa*, and *Lotus pedunculatus* (Ferrari et al., 2012), but is rarely present in pea aphids feeding on *Lotus corniculatus* (Henry et al., 2013). This hypothesis is further supported by the fact that a facultative symbiont is also more likely to establish a symbiotic relationship with aphid clones from a biotype that typically carries the symbiont in nature, compared with clones from a biotype that does not normally carry the symbiont (Niepoth et al., 2018). While the host genotype could explain this trend, a role played by the *B. aphidicola* genotype cannot be ruled out either. The hypothesis of metabolic complementation of the obligate symbiont *Buchnera* by facultative symbionts in certain biotypes highlights the importance of examining the performance of aphid biotypes in relation to the metabolic capacities of the *B. aphidicola* strains with which they are respectively associated, and those of possible facultative associates. To this end, more genomic sequences of *B. aphidicola* and facultative symbionts residing within the same host and associated with different biotypes of an aphid species are needed for robust genome-based metabolic inference approaches. And to complete the picture, it would also be desirable to have annotated genomic sequences of the original hosts (or at least of the different aphid host biotypes) in order to correlate the insect's metabolic capacities with those of its bacterial associates.

Studies on the whitefly *Bemisia tabaci* and its symbionts can provide some insights into the nature of interactions between aphids and their facultative symbionts. *B. tabaci* is a polyphagous insect composed of a complex of morphologically identical cryptic species, of which over thirty biotypes have been described (MacLeod et al., 2022; Brown et al., 2023). Like aphids, *B. tabaci* hosts an ancestral obligate symbiont, *Portiera aleyrodidarum*, which supplies essential amino acids to the insect host (Santos-Garcia et al., 2012). It has a tiny genome (around 355 kb)

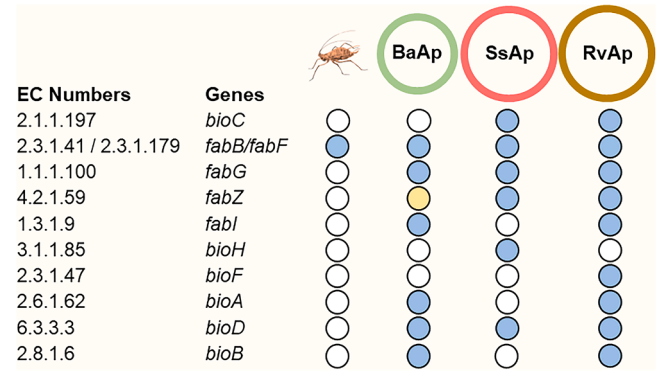


Fig. 3. Metabolic capacity of the *A. pisum*-*B. aphidicola* (BaAp)-*S. symbiotica* (SsAp)-*R. viridis* (RvAp) system for vitamin B₇ (biotin) biosynthesis, adapted from (Peng et al., 2023). On the left are the names of the genes encoding the enzymes involved in the biosynthetic pathway and the EC numbers. The following code is used to characterize the state of each gene: the blue color means that the gene (or an alternative gene that can perform the same enzymatic function) is present and not pseudogenized, the white color indicates that the gene is missing, and the yellow color indicates that the gene is encoded by a subset of strains. Interestingly, the fact that the *fabZ* gene is only encoded by a subset of strains suggests inter-strain variability in *Buchnera*'s ability to synthesize riboflavin in pea aphids. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Santos-Garcia et al., 2012; Jiang et al., 2013; Sloan and Moran, 2013) and, although it has the capacity to synthesize most essential amino acids, it lacks almost all the genes involved in vitamin and cofactor synthesis. In addition, pathways involved in the synthesis of some essential amino acids are incomplete (Rao et al., 2015). Seven secondary symbionts have been identified in the cryptic species complex of *B. tabaci*: *Hamiltonella*, *Arsenophonus*, *Cardinium*, *Rickettsia*, *Wolbachia*, *Fritschea* and *Hemipteriphilus*, with up to four present in the same insect body (Zchori-Fein et al., 2014). The existence of biotype-dependent secondary symbiont communities in sympatric populations of *B. tabaci* is not new: it has long been known that a biotype is specifically characterized by the presence of certain secondary symbionts (Chiel et al., 2007). The role of these secondary symbionts in *B. tabaci* also remains largely unknown. However, the analysis of their genomes suggests that some of them could play a nutritional role, such as *Hamiltonella* symbionts that can provide vitamins and cofactors and could also complete the missing steps of the *P. aleyrodidarum* lysine pathway (Rao et al., 2015). Moreover, these secondary symbionts often reside in the same bacteriocytes together with the obligate symbiont, suggesting a metabolic complicity that goes hand in hand with a high degree of anatomical integration. Finally, it has recently been suggested that the host plant utilization by *B. tabaci* is determined by the composition of its secondary symbiont community, probably through a metabolic contribution (Benhamou et al., 2021). The case of *B. tabaci* thus teaches us that secondary symbionts considered facultative at the species level are possibly co-obligate nutritional associates in specific genetic groups. Thus, although the nutritional role played by secondary symbionts in *B. tabaci* is far from elucidated, the case of this insect suggests that, in a similar fashion, aphids may have evolved mutualistic associations with various secondary symbionts in cryptic species complexes to compensate for the limited metabolic capacities of the host and the ancestral symbiont *Buchnera* on certain host plants. This calls for further research into the genetic diversity of aphids with regard to their metabolic capacities. An additional approach to consider is the use of the *Buchnera* genome to resolve ambiguities in aphid taxonomy (Jousselin et al., 2009).

6. Current research and perspectives

Facultative symbionts, with their enhanced metabolic capacities compared to ancient obligate symbionts, and their ability to undergo horizontal transfer, are a potential source of metabolic innovation for insects. While they can behave as opportunistic players and have negative effects on host fitness, they can also confer advantages, including nutritional benefits. Several studies have demonstrated that facultative symbionts can improve aphid performance on specific host plants. However, the mechanisms behind this nutritional advantage remain largely unexplained. The main hypothesis I argue in this opinion piece is that heritable facultative symbionts of aphids can render nutritional services by being metabolic compensators, either directly by complementing *B. aphidicola* in the biosynthetic pathways of nutrients essential for aphid development (typically amino acids and vitamins), and/or perhaps indirectly by ensuring the integrity of the obligate symbiont *Buchnera* (e.g. by contributing to the synthesis of its peptidoglycan), and that in so doing they can improve aphid performance on certain host plants. In this context, the nutritional benefits provided by these microbial partners would be conditioned by the nutritional quality of the phloem sap, the biosynthesis capacity of *B. aphidicola* and the genotype of the host insect. In particular, this may explain why *R. insecticola* can improve the performance of *A. pisum* and *M. crassicauda* on white clover and why *Arsenophonus* can improve the performance of *A. craccivora* on black locust (Tsuchida et al., 2004, 2010b; Wagner et al., 2015). This could also explain why *Arsenophonus* is so prevalent in the *A. gossypii* biotype feeding on cotton plants, whose phloem sap is poor in many amino acids: the symbiont could be compensating for the lack of amino acids in this plant that the host insect and *Buchnera* are unable to supply adequately (Tian et al., 2019). On the

other hand, when the aphid feeds on a plant for which the facultative symbiont provides no nutritional benefit, the symbiont may become an opportunistic associate that incurs fitness costs for the aphid. This could explain, for example, why *Arsenophonus* causes a decline in the performance of *A. craccivora* on alfalfa (Wagner et al., 2015). The combined effect of these double-edged effects of the facultative symbiont depending on the host plant and its phloem sap composition, the host genotype, the *Buchnera* genotype and the genotype of the facultative symbiont itself may explain why it is so difficult to reveal consistent plant-specific effects of facultative symbionts on aphid performance, and why the nutritional dimension of facultative symbiosis is so challenging to address.

It should be noted, however, that this potential role as a metabolic compensator alone cannot explain the influence of facultative symbionts on aphid performance on host plants and their non-random distribution in aphid populations. Other factors come into play. Heritable facultative symbionts are bacteria that can move rapidly horizontally within insect populations and can therefore be opportunistic passengers involved in young and tenuous interactions with the host insect, either as commensals or parasites (Gu et al., 2023). For instance, some infections may result from the direct acquisition of symbiont strains circulating in the plant or vectored by parasitoids (Gehrer and Vorburger, 2012; Pons et al., 2019; Gu et al., 2023). The distribution of facultative symbionts in insect populations reflect different histories and constraints (Guyomar et al., 2018). For example, it can be shaped by different factor including selection from natural enemies (some symbionts provide their host with varying degrees of protection against parasitoids) and from thermal conditions (which can influence the efficiency of vertical transmission) (Chang et al., 2022; Wu et al., 2022). In addition, aphids often host several facultative symbionts: by leading to community-wide effects, microbe-microbe interactions in the context of symbiont co-infections may also be instrumental in shaping infection patterns (McLean et al., 2018; Weldon et al., 2020). Last but not least, there is evidence that heritable facultative symbionts of aphids can play nutritional functions other than metabolic complementation. They can enhance the insect's lipid metabolism or interfere with host plant biology to facilitate aphid colonization and adaptation. For instance, the facultative symbiont *S. symbiotica* can boost the fatty acid metabolism of the pea aphid, thereby promoting its development and enhancing its fitness (Zhou et al., 2021). The same symbiont species can interfere with plant defenses: symbiont modulation of specific gene expression in the salivary glands of the pea aphid induces repression of reactive oxygen species (ROS) production and salicylic acid (SA) and jasmonic acid (JA) pathways. As a result, the infected aphids feed longer on *M. truncatula* than the uninfected ones (Wang et al., 2020). Similarly, Li et al. (2019) reported that a clone of the wheat aphid *Sitobion miscanthi* infected with *H. defensa* grew faster and had higher fecundity on wheat than an uninfected clone (Li et al., 2019). The authors showed that these results were correlated with lower accumulation of SA and JA and repression of downstream genes in the infected clone, indicating that facultative symbionts can mediate anti-plant defense responses. These studies on different aphid species suggest that plant modulation by facultative symbionts, resulting in improved aphid feeding, could be a generalizable effect to other aphid-symbionts consortia. However, studies addressing these aspects remain limited, and further research will need to test the different nutritional services that facultative symbionts can potentially render to aphids, including the hijacking of plant signaling to benefit the insect, the digestion of certain nutrients and the detoxification of certain metabolites (Giron et al., 2017; Sharma et al., 2021; Shih et al., 2023). Fig. 4 summarizes the currently known beneficial effects of heritable facultative symbionts on aphid nutrition.

In the paragraphs that follow, I propose a non-exhaustive inventory of methodological considerations for testing the hypothesis of the nutritional role of facultative symbionts in the context of metabolic complementation.

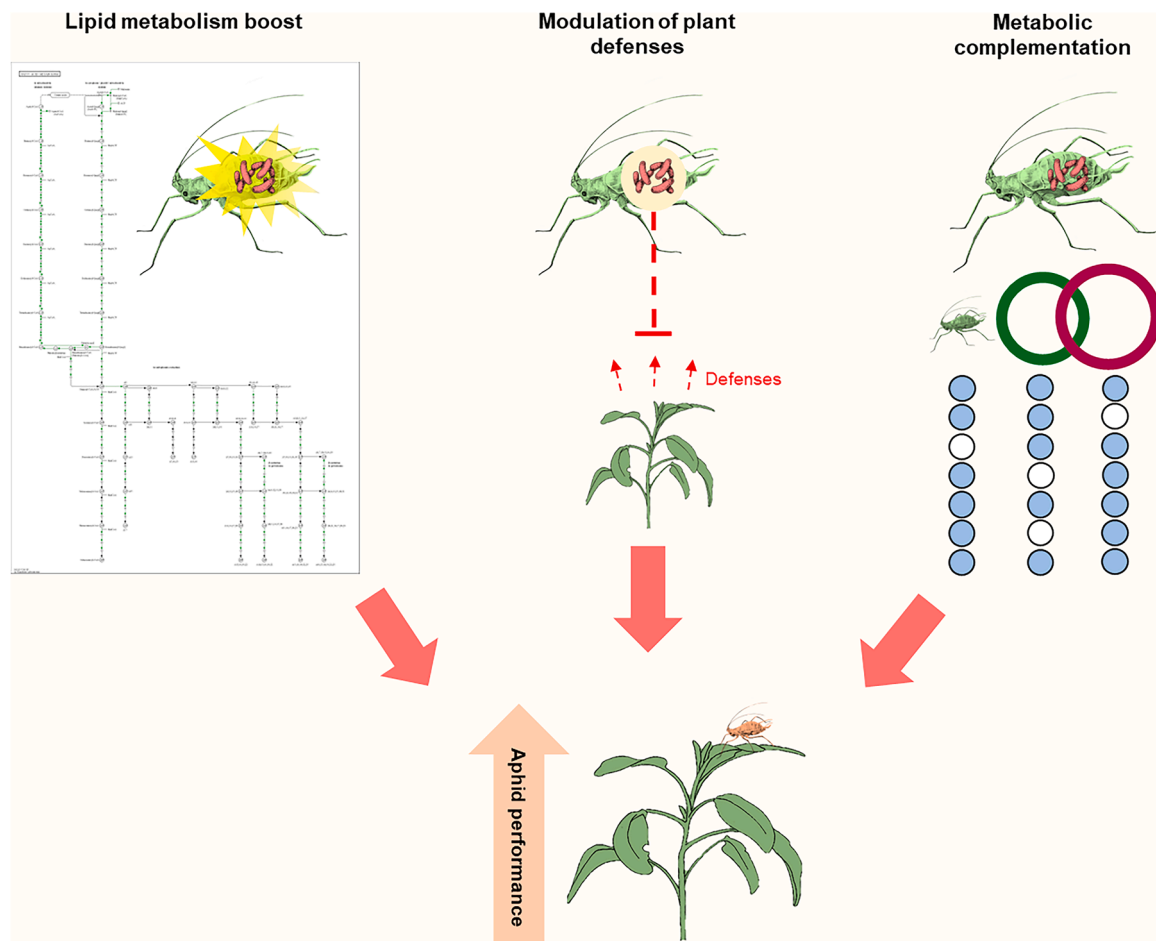


Fig. 4. Currently known nutritional services that facultative heritable symbionts can render to aphids. Facultative symbionts can 1) enhance host fatty acid metabolism, 2) help quell host plant defenses, and 3) could complement the metabolic deficiencies of the insect and its ancestral obligate symbiont *B. aphidicola*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

6.1. A better characterization of the intraspecific genetic diversity of aphids

It is pivotal to determine the genotypic variations of hosts in terms of metabolic capacity. This implies a better identification of cryptic aphid species and biotypes, accompanied by in-depth analysis of their respective metabolic capacities. Indeed, it cannot be ruled out that, depending on the nutritional composition of the phloem sap of the plant on which they evolve, a relaxation has taken place in certain genes involved in biosynthetic pathways, leading to different biosynthesis capacities in different biotypes of the same aphid species. To test this hypothesis, it is essential to sequence and annotate a larger number of aphid genomes (e.g. different biotypes of the same species). This is also important because genome-based inference approaches that are often performed to identify metabolic complementarities between symbiotic bacteria rarely take into account the host genome, which can lead to biased analyses and erroneous assertions regarding the actual nutritional requirements of the aphids studied and the nutritional role of their heritable bacterial symbionts. The pea aphid complex is a valuable model to conduct this research, but other species known to be polyphagous and to host a wide diversity of facultative symbionts, for example those of the genus *Aphis*, have great potential to address these aspects (Henry et al., 2015; Tian et al., 2019). In this context, taking into account *Buchnera*'s genomic sequence is also important, as it can help resolve ambiguities in aphid taxonomy and better delineate aphid biotypes (Jousselin et al., 2009). Finally, another advantage of obtaining the genomic sequence of aphid hosts is to examine the extent to which

horizontally transferred genes (HGTs) are involved in the host's metabolic capabilities. Indeed, some HGTs are involved in the synthesis of important metabolites in the insect-symbiosis system (Moran and Bennett, 2014). For instance, HGTs of bacterial origin in the mealybug genome cooperate with the *Moranella* symbiont in the synthesis of peptidoglycan (Bublitz et al., 2019). Similarly, biotin genes of bacterial origin transferred horizontally in the whitefly *B. tabaci* contributes to biotin synthesis (Ren et al., 2020). Studies on mealybugs, psyllids and whiteflies suggest that HGTs can complement missing genes involved in the synthesis of several essential amino acids in symbionts (Husnik et al., 2013; Sloan et al., 2014; Luan et al., 2015). In light of what has been demonstrated in various sap-feeding insects, it is timely to determine the extent to which bacterial HGTs are also involved in the biosynthesis of important metabolites in aphids.

6.2. A more in-depth analysis of the intraspecific genetic diversity of *B. aphidicola* and of facultative symbionts evolving in the same original host

It is essential to understand how microevolution and polymorphism affect genes related to the metabolism of the obligate symbiont, and to determine whether and how additional symbionts can compensate for the metabolic limitations of the former. To this end, more genomes of *B. aphidicola* and facultative symbionts residing in the same original host, need to be sequenced and annotated, so that robust genome-based metabolic inferences can be drawn. Ideally, these analyses should be mapped to the genetics of the host biotype and phloem sap composition

of the host plant. In particular, this could help explain how *Buchnera*'s metabolic capacity to buffer the insect from host-plant nutrient deficiencies influences the distribution of aphid biotypes on host plant species, as well as the non-random distribution of facultative symbionts in the different biotypes. Studies that have examined the prevalence of infection by facultative symbionts across aphid species and biotypes provide a valuable resource for identifying biotypes of interest (Ferrari et al., 2012; Henry et al., 2015; Peccoud et al., 2015). For example, interesting candidate biotypes to examine in *A. pisum* could be *Genista sagittalis* and *Genista tinctoria*, as they include specimens that are almost all systematically infected with the *Hamiltonella*+*Serratia* combination (Peccoud et al., 2015). The *Securigera varia*/*Hippocrepis comosa* biotype is also of interest, as *S. symbiotica* infections are very widespread. Conversely, the *Onobrychis viciifolia* biotype is interesting because it is essentially infected by *Buchnera* alone and only very sporadically infected by facultative symbionts. Acquiring the genomic sequence of facultative symbionts is also essential for examining the origin of their metabolic capacities and the nutritional services they can provide. Indeed, the study of co-obligate symbiosis in aphids has taught us that nutritional genes essential for the establishment of endosymbiosis can be acquired horizontally and then passed on to other secondary symbionts, suggesting that cohabitation of secondary symbionts within the same host and the resulting HGTs can be important drivers of the evolution of nutritional symbiosis in aphids (Manzano-Marín et al., 2020).

6.3. In-depth knowledge of the composition of the phloem sap of the host plant

This is essential to determine the actual nutritional deficiencies faced by aphids, which may require metabolic support from bacterial associates. This aspect is particularly relevant to explore in the case of so-called polyphagous aphid species, in order to understand the adaptive mechanisms that enable them to cope with a phloem sap composition that can vary greatly from one host plant species to another, but also within the same plant depending on its development (Sandström and Pettersson, 1994; Karley et al., 2002; Xu et al., 2020). For example, it would be worthwhile to map the phloem sap composition of the plants on which the different biotypes of the pea aphid *A. pisum* feed, in order to examine the correlation between plant nutritional inputs, the metabolic capacities of each partner in the insect system (assessed on the basis of their genomic sequences) and aphid performance. Deciphering the chemical composition of phloem sap is challenging because it is not readily collected, but this can be done using different approaches, including incisions made in the bark of the stem (Hall and Baker, 1972), EDTA-facilitated exudation (King and Zeevaert, 1974) and aphid stylectomy (Lohaus, 2022). Aphid stylectomy is probably the most fitting approach, as the liquid obtained by this method is considered the most authentic source of phloem sap.

6.4. In-depth characterization of the tissue tropism of facultative symbionts

The biological significance of the anatomical compartmentalization of facultative symbionts in secondary bacteriocytes and sheath cells is an important issue. Do these host cells play a role solely in the sequestration and control of bacteria potentially pathogenic to the insect host (Ferrarini et al., 2022)? Or do they also serve as a hub for metabolic exchanges between the facultative symbionts, *B. aphidicola*, and the host, as in the case of an obligatory symbiosis (Smith and Moran, 2020)? Can facultative symbionts dwelling more freely in the hemolymph and oenocytes provide nutritional services? There are still no answers to these questions, and the developmental biology of facultative symbiosis is still a largely unexplored field. As the physical proximity of symbionts and their degree of anatomical integration into the host are possible indications of metabolic functions and cooperation between nutritional symbionts (Renoz et al., 2022; A et al., 2023; Michalik et al., 2023),

these aspects should be examined to complement genome-based metabolic inferences using, for example, fluorescent in situ hybridization (FISH) approaches. In addition, aphids can often host more than one facultative symbiont, sometimes up to four (Renoz et al., 2020; Zhang et al., 2021). It is important to determine how such a large number of bacterial associates are spatially arranged relative to each other within the aphid host in order to correlate tissue tropism with potential metabolic interactions.

6.5. The use of experimental approaches

Correlating the composition of phloem sap of the host plant with the metabolic capacities of the insect and its symbionts deduced from their genomes is a prerequisite for deciphering the nutritional needs of the symbiotic system and the respective contribution of each partner. However, experimental approaches are required to confirm in silico predictions. Here, I propose four experimental approaches that can be performed using the pea aphid *A. pisum* as a model.

- 1) Once the phloem sap composition of each biotype's host plants has been mapped and the metabolic capacities of the different members of the symbiotic system have been projected, the performance of each biotype on its original host plant and on alternative host plants should be assayed. This would make it possible to test the metabolic compensation capacities of a facultative symbiont (or even a consortium of facultative symbionts) on host plants of different nutritional quality, and to test whether a facultative symbiont that improves aphid performance on a host plant can become a cumbersome, even parasitic associate, when the host plant is able to compensate for the nutritional deficiencies of a specific biotype. Indeed, it cannot be ruled out that if certain facultative symbionts provide access to certain host plants, the opposite situation could arise. This second hypothesis could, for example, be tested by feeding the different biotypes on the host plant with the highest nutritional quality (e.g. *Vicia faba* suggested to be a "universal host plant" for pea aphids) and assessing the impact of facultative symbionts on the fitness of the different biotypes on this common food source. These analyses could be combined with an assessment of the titer of facultative symbionts in the biotypes (e.g. by qPCR-absolute quantification approach or flow cytometry) and an analysis of the differential expression of targeted metabolism-related genes in each partner (Simonet et al., 2016; Nichols et al., 2021; Parker et al., 2021) to determine the influence of the host plant on symbiont populations and on the coordination of the different partners in the symbiotic system.
- 2) A second approach would be to eliminate the facultative symbionts from their native biotypes to determine how this impacts the biotype's performance on its specific host range versus on alternative host plants. This approach would test the hypothesis that eliminating the facultative symbiont in its original biotype, if it does indeed fulfill a metabolic role, will lead to a reduction in the aphid's performance on its specific host plant(s), but could potentially improve its performance on alternative host plants. In the same vein, replacing the biotypes' original facultative symbionts with those present in other biotypes would make it possible to test the hypothesis that alternative facultative symbionts can improve the performance of biotypes on host plants to which they do not originally have access, or at least on which their performance is poor.
- 3) Another experimental approach to test the potential metabolic compensatory role of facultative symbionts would be to examine the performance of different aphid biotypes on artificial diets of different nutritional quality, the composition of which would be established on the basis of the results of genome-based metabolic inference approaches (deduction of the system's nutritional needs). Such an approach has already been used to understand the impact of the phloem sap composition on the obligate symbiosis involving

Buchnera in particular (Zhang et al., 2016; Colella et al., 2018; Ribeiro Lopes et al., 2022). The use of artificial diets has the advantage of ensuring full control over the composition of the aphid diet (e.g. the amount of amino acids and vitamins) and improving the standardization procedure. However, artificial diets have the disadvantage of not fully reflecting the quality of phloem sap, and of hindering the development of aphids, which cannot maintain themselves on these substrates for several generations (Sandström, 1994).

- 4) Finally, omics approaches for differential gene expression analysis could highlight the aphid and symbiont genes involved in host-symbiont interactions in the context of a possible metabolic consortium with facultative symbionts and decipher how the different partners coordinate. Approaches of this type (e.g. RNA-Seq) have notably shed light on the sophisticated coordination between the aphid and its obligate symbiont *Buchnera* (Smith and Moran, 2020) and to understand how intraspecific genetic variation in the pea aphid *A. pisum* affects the regulation of *B. aphidicola* (Chong and Moran, 2016). However, the use of these approaches remains limited when it comes to studying the functioning of secondary symbioses, whether co-obligate or facultative in nature.

6.6. Experimental evolution

Experimental evolution is also a relevant approach for understanding how interactions between partners in a symbiotic system are shaped, and how their genomes evolve in a context of nutritional mutualism (Hoang et al., 2016). With this in mind, it would be interesting to test the mutual influence of the different partners on the evolution of their respective metabolism-related genes, as well as the impact of the composition of the phloem sap. Does the nutritional quality of phloem sap drive relaxed selection on metabolism-related genes and metabolic pathways in aphids and the obligate symbiont *Buchnera*? Does the acquisition of a facultative symbiont break *Buchnera*'s genomic stability by relaxing selection on genes redundant with the newly acquired symbiont? These are just some of the many important questions that underline the importance of studying the microevolution of symbiotic systems to understand how the metabolic capacities of the different partners adjust to each other. Tracking the evolution of genotypes of aphids and of their obligate symbionts from the same clonal line, but feeding in parallel on host plants featured by contrasting nutritional qualities, is an approach worth considering to address these questions. Similarly, monitoring the evolution of aphid and *Buchnera* genotypes after the addition of a facultative symbiont is an appealing strategy for understanding the influence of facultative symbionts on *Buchnera*'s nutritional functions. To conduct this kind of experiment, it is also essential to have high-quality genomic sequences for all partners from the outset.

6.7. Additional methodological approaches and final considerations

Additional methodological approaches may also be envisaged. Modeling the predicted genomic data and sap content to establish interactome networks is a potential avenue to consider for conclusive functional insights. MicroRNA-mediated regulation is another area to explore, as microRNAs can contribute to metabolic cooperation between symbionts and insect hosts, notably by regulating HGTs (Sun et al., 2022). Electrical penetration graph (EPG) is an appealing approach to test the influence of facultative symbionts on aphid feeding behavior, an aspect that has surprisingly received little attention (Wilkinson and Douglas, 1995). Finally, it is important to study the nutritional dimension of facultative symbioses under conditions that correspond as closely as possible to their natural context. For example, with regard to rearing conditions, it is preferable to maintain aphid biotypes on their native host plants. Indeed, rearing aphids in the laboratory on host plants other than those of origin can lead to biased results (e.g. a different

composition of phloem sap could lead to aphid and *Buchnera* microevolution). Similarly, it is advisable to conduct experiments with aphid clonal lines that have been naturally infected by their facultative symbionts, rather than with clonal lines derived from artificial infections. Indeed, the latter approach ignores the complex evolutionary history of the system and the way in which the different partners that make it up have influenced each other in their respective metabolic capacities. Thus, a facultative symbiont that maintains a mutualistic interaction in its native host by providing a nutritional service can potentially become a parasitic associate in an alternative host if its services are not required. Furthermore, by potentially driving *Buchnera* microevolution (and potentially host microevolution), newly acquired facultative symbionts can alter the genetics of the system, which may ultimately lead to biased results in the context of experimental comparative studies.

7. Conclusions

Aphids have developed different strategies for adapting to plants. The ancestral, multimillion-year-old symbiotic interaction these insects have evolved with *Buchnera* contributes to their adaptation to the host plant. However, a series of secondary facultative symbionts are suspected of mediating plant-aphid interactions, and may also be part of the aphids' tools for adaptation and specialization to different host plants. But some twenty years after this hypothesis was first put forward, how they contribute remains an enigma. The main hypothesis argued in this opinion piece is that facultative symbionts may play a major and underestimated role in the metabolic complementation of *Buchnera* and/or the host. In particular, this article aims to stress the urgent need to look at the intraspecific genetic variation of all the partners that compose symbiotic system to robustly test this hypothesis through a range of approaches. The nutritional dimension of facultative symbiosis in aphids and other insects still conceals many enigmas that future experimental, genomic and field studies will need to elucidate to better appreciate the role of bacterial symbiosis as a major driver of insect ecological adaptation.

CRediT authorship contribution statement

François Renoz: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

François Renoz reports financial support was provided by Fund for Scientific Research. François Renoz reports a relationship with Fund for Scientific Research that includes: funding grants.

Data availability

No data was used for the research described in the article.

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