CHILEAN JOURNAL
of AGRICULTURAL RESEARCH

RESEARCH ARTICLE

Diversity of endosymbiotic bacteria in the pea aphid (*Acyrthosiphon pisum*) on alfalfa in Chile

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Received: 13 May 2022; Accepted: 3 August 2022; doi: 10.4067/S0718-58392023000100063

ABSTRACT

Facultative bacterial endosymbionts are prevalent in aphid pest species and key organisms for the rapid adaptation to changing environment conditions under agricultural management. Facultative endosymbiont interactions allow beneficial phenotypes of insect hosts, providing resistance against natural enemies, fungal infections as well as enhanced resistance to thermal conditions, among others. These interactions have been highlighted as important modulators in the evolution of aphid defenses, being a key aspect for the invasive potential of aphids as pests. In this study, we have examined the bacterial community diversity associated to the pea aphid *Acyrthosiphon pisum* on alfalfa (*Medicago sativa* L.) crops in Central Chile by determining the presence and abundance of the seven most common facultative endosymbionts reported for aphid species, including: *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, *Spiroplasma* sp., *Rickettsia* sp., *Rickettsiella viridis* and *Fukatsuia symbiotica* (or pea aphid X-type symbiont' PAXS), using a PCR-based approach across species-specific primers, in 16 alfalfa fields during the Chilean spring in the Maule Region. We reported six of the seven endosymbiont species studied, observing a high frequency of infected aphids being the endosymbiont *H. defensa*, the most predominant among the aphid populations in the different alfalfa fields studied. As *H. defensa* has been extensively studied in its defensive role to parasitoids, we discuss implications for biological control of this aphid pests. We also discuss the absence of a relevant defensive endosymbiont such as *F. symbiotica* in the Chilean pea aphid populations and potential implications for the success of the pea aphid biological control program.

Key words: *Acyrthosiphon pisum*, endosymbiont bacteria, *Medicago sativa*, pea aphid, *Hamiltonellla defensa*, PAXS, multiple infections.

INTRODUCTION

Aphids (Hemiptera: Aphididae) are phytophagous insects and constitute important global crop pests, they present predominant asexual reproduction (clonal parthenogenesis) and act as vectors of phytopathogens causing major plant damage, yield losses and reducing crop duration (Simon and Peccoud, 2018; Hullé et al., 2020). In Chile, several widely distributed species that forage on crops such as alfalfa (*Medicago sativa* L.) include; the pea aphid *Acyrthosiphon pisum*, the blue alfalfa aphid *A. kondoi*, the cowpea aphid *Aphis craccivora* and the spotted alfalfa aphid *Therioaphis trifolii* (Cisternas and Torres, 2022). In particular, the pea aphid complex, *A. pisum* is considered one of the 15 aphid species with the greatest agricultural impacts, compromising a complex of specialized lineages on different leguminous plants (van Emden and Harrington, 2017; Simon and Peccoud, 2018). Aphid pest control in leguminous crops, in Chile, is mainly carried out by specialized natural enemies including different introduced aphid parasitoid wasps (Hymenoptera: Braconidae) of the genus *Praon* (*Praon volucre* and *P. gallicum*) and *Aphidius* (*Aphidius ervi*, *A. matricariacae* and *A. colemani*) (Zepeda-Paulo et al., 2013) as well as a set of native and exotic predators (mostly Coccinellids) (Grez et al., 2021). However, aphids often maintain associations with facultative endosymbiotic bacteria (i.e., not essential for host insect's survival) that confer conditional benefits to their aphid hosts, such as protection against natural enemies, which could affect the biological control of aphid pests in the field, but also, they can incur in significant costs to their aphid hosts (review in Oliver et al., 2014; Smee et al., 2021). In addition to its obligate nutritional-providing endosymbiont, *Buchnera aphidicola*, aphids can harbor several facultative endosymbionts; including Gammaproteobacteria as *Hamiltonella defensa*, *Serratia symbiotica*, *Regiella insecticola*, *Fukatsuia symbiotica* (also called PAXS for pea aphid X-type symbiont), *Rickettsiella viridis*, *Rickettsia* sp. (Alphaproteobacteria) and *Spiroplasma* sp. (Mollicutes) (Zytynska and Weisser, 2016).

Besides the defense against parasitoid wasps, facultative endosymbionts can confer additional beneficial traits on their aphid hosts, such as protection against fungal pathogens (Lukasik et al., 2013; Parker et al., 2013; Smee et al., 2021), tolerance to heat stress (Heyworth and Ferrari, 2015; Heyworth et al., 2020) or influencing host-plant use (Guo et al., 2017). For the pea aphid, the symbiont-mediated defense has been well-studied. Lab-based studies on pea aphids have evidenced that the endosymbiont *H. defensa* can confer resistance against several parasitoid wasps (Heyworth and Ferrari, 2015), as well as observing higher levels of protection for co-infected aphids with another species of endosymbiont, *F. symbiotica* (Donald et al., 2016). For the case of the protection provided by *H. defensa* in the pea aphid, this is directly associated with specific APSE phage variants (*A. pisum* secondary endosymbiont) that encodes lethal toxins for the parasitoid immatures (Oliver and Perlman, 2020). Furthermore, the infection with *F. symbiotica* efficiently protects pea aphids from the lethal fungal entomopathogen *Pandora neoaphidis*, a common aphid pathogenic fungus which causes natural epizootics in several aphid species (Lukasik et al., 2013; Heyworth and Ferrari, 2015). Therefore, facultative endosymbionts can be important modulators of the aphid defenses conferring adaptive advantages which could increment their invasive potential (Simon and Peccoud, 2018). Preceding studies on endosymbiont bacterial diversity associated to aphid species have found that facultative endosymbionts are prevalent in aphid populations, suggesting that these could be key for the rapid adaptation of aphid pests to changing environment conditions in agriculture (Leclair et al., 2021; Smith et al., 2021; Zepeda-Paulo and Lavandero, 2021).

Thereby, determining the bacterial community diversity associated with the main aphid pests will be relevant for pest management-based decisions and the biological control of this pest group in agricultural environments. A previous survey in Chile studied the incidence of five endosymbiont species in the pea aphid (Sepulveda et al., 2017), finding at least two frequent endosymbiont species in the pea aphid populations; however, this study did not include the determination of some more important endosymbiont species for the pea aphid (e.g., *F. symbiotica*). In this study, we have examined the bacterial community diversity associated to the pea aphid *A. pisum* on alfalfa crops in Central Chile by determining the presence and abundance of the seven most common facultative endosymbionts reported for aphid species (*Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, *Spiroplasma* sp., *Rickettsia* sp., *Rickettsiella viridis* and *Fukatsuia symbiotica*) using a PCR-based approach across species-specific primers, in 16 alfalfa fields during the Chilean spring in the Maule Region.

MATERIALS AND METHODS

Aphid sampling and molecular screening of bacterial endosymbionts

Live aphids were collected on 16 conventionally managed alfalfa (*Medicago sativa* L.) fields during the period of aphid colonization during the spring of one growing season (September 2013), fields were located in the Maule Region in central Chile (Table 1). Aphids were actively collected by sampling 20 tillers separately, each tiller separated by at least 20 m within each farm. DNA extraction was individually performed for each aphid specimen using the “Salting out” method described by Sunnucks and Hales (1996). The quantification and quality of the extracted DNA was examined using a spectrophotometer Epoch (Agilent BioTek, Santa Clara, California, USA) and by electrophoresis in 0.8% agarose gels. Each individual DNA extraction was normalized to a concentration of 5 ng μL^{-1} and kept at -20 °C until later PCR analysis. In order to determinate the identity and relative frequency of endosymbiont species infecting pea aphids, the molecular identification of bacterial endosymbiont infections was individually performed using whole-body DNA extracts from individual aphids, and a posteriori multiplex PCR diagnostic protocol using bacterial 16S rDNA specific primers for the seven most common facultative endosymbionts of aphids reported by Peccoud et al. (2014), which included: *Hamiltonella defensa* (primer PABS480R), *Regiella insecticola* (primer PAUS16SR), *Serratia symbiotica* (primer PASS1140R), *Spiroplasma* sp. (primer Spi1500R), *Rickettsia* sp. (primer Ric600R), *Rickettsiella viridis* (P136Ric-470R, P136F) and *Fukatsuia symbiotica* (primers PAXSF, PAXSR), together with a universal forward primer (primer16SA1). The obligate nutritional endosymbiont *Buchnera aphidicola* of aphids (bacterial endosymbiont present in all aphids) (primer Buch270R) was used as positive control of the reaction, following the PCR condition used by Peccoud et al. (2014). The different facultative endosymbionts were discriminated according to the size of the amplicons (bp) visualized in 1.5% agarose gels stained with Redgel (Biotium, Hayward, California, USA) using GeneRuler 100 bp plus a DNA ladder (Thermo Scientific, Waltham, Massachusetts, USA) as previously described by Peccoud et al. (2014).

Table 1. Number of pea aphids studied for endosymbiont diversity from 16 alfalfa fields in central Chile.

Field name	S lat	W long	Nr of aphids
f01	-35.544	-71.594	32
f02	-35.634	-71.463	16
f03	-35.597	-71.304	10
f04	-35.633	-71.427	27
f05	-35.572	-71.683	74
f06	-35.591	-71.604	12
f07	-35.632	-71.450	45
f08	-35.596	-71.561	31
f09	-35.493	-71.539	42
f10	-35.577	-71.399	57
f11	-35.571	-71.365	17
f12	-35.598	-71.300	19
f13	-35.553	-71.570	22
f14	-35.618	-71.429	41
f15	-35.629	-71.272	10
f16	-35.632	-71.413	10
Total			465

Data analysis

Differences between the proportion of infected and uninfected aphids as well as single and co-infected aphids from the whole sample were analyzed using Exact Binomial Tests (Crawley, 2013) in the R software v3.2.1 (R Foundation for Statistical Computing, Vienna, Austria). The proportion of endosymbiont infections with the most common endosymbiont in the different alfalfa fields were analyzed using a generalized linear model with a binomial distribution in the R software. Randomness of the residuals and overdispersion were checked and the significance of models was evaluated with the ANOVA function implemented in R car package (Fox et al., 2013).

RESULTS AND DISCUSSION

A total of 465 pea aphids were collected from 16 alfalfa fields and all of them were positive for the obligate nutritional endosymbiont, *Buchnera aphidicola* (positive control in the multiplex PCR diagnostic). The bacterial endosymbiont community associated with pea aphid in alfalfa was more diverse than previously reported (*Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica* and *Rickettsia* sp.) (Sepulveda et al., 2017), confirming six of the seven facultative endosymbionts here studied (*H. defensa*, *R. insecticola*, *S. symbiotica*, *Spiroplasma* sp., *Rickettsia* sp. and *Rickettsiella viridis*) in the whole pea aphid sample occurring as single (i.e., aphid harboring only one facultative symbiont species) and multiple-infections (co-infections) (Figure 1).

The relative frequency of facultative endosymbiont-infected aphids was significantly greater (57.8% of total aphids studied) than uninfected pea aphids (42.2% of total aphids) in alfalfa crops (Chi-squared = 11.172, df = 1, p value = 0.0008), being the most prevalent single infections in comparison with multiple-infections (Chi-squared = 490.65, df = 1, p value < 0.0001). A lesser prevalence of multiple-infections is likely due to additional costs on the aphid fecundity associated to multiple endosymbionts (Smee et al., 2021), being usually reported at lower frequencies in natural aphid populations (Zytynska and Weisser, 2016). The most prevalent endosymbiont in the pea aphid on alfalfa was *H. defensa*, with a 71% (average proportion in the aphid populations from different fields) of symbiont-infected aphids across all sampled fields, although a significant variation among the fields studied (ranging between 6%-100% of the total infections) was observed (factor field: Chi-squared = 158.54, df = 15, p value < 0.0001) (Figure 2). It should be noted that *H. defensa* was present in all the multiple infections observed in the sample studied (with the exception of the *Rickettsia*/*S. symbiotica* infection) (Figures 1 and 2). *Hamiltonella defensa* was followed by *R. insecticola* (14%), *Spiroplasma* sp. (6%), *S. symbiotica* (2%), *Rickettsia* sp. (5%) and *R. viridis* (2%), while the endosymbiont *F. symbiotica* was not found in the aphid samples here studied. The prevalence of infections with the endosymbiont *H. defensa* in pea aphids attacking alfalfa crops is consistent with similar studies from different regions (Smith et al., 2021; Leclair et al., 2021), which has been explained in part by the demonstrated defensive benefit conferred by this endosymbiont on pea aphids.

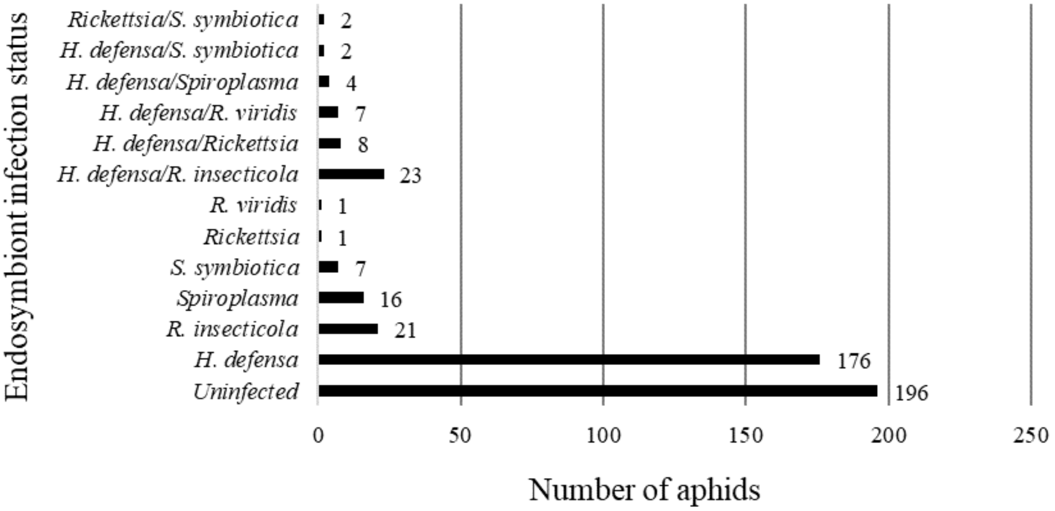

Figure 1. Endosymbiont infection statuses of pea aphids in alfalfa crops of central Chile. Numbers on bars indicate total frequency of pea aphids uninfected and infected with multiple or single facultative endosymbionts in the total aphid sample collected on alfalfa crops. Endosymbionts: *Rickettsia* sp., *Serratia symbiotica*, *Hamiltonella defensa*, *Spiroplasma* sp., *Rickettsiella viridis*, *Regiella insecticola*.

Hamiltonella-infected aphids can exhibit high levels of resistance to parasitoid egg development (> 60% resistance) of the species *Aphidius ervi* (Guay et al., 2009; Oliver et al., 2009; Smee et al., 2021), such as that the frequency of *H. defensa*-harbored aphids may depend on the intensity and fluctuations of the parasitism pressures under natural conditions (Doremus and Oliver, 2017), however multiple factors could mediate the endosymbiont prevalence in nature (Smith et al., 2021). In this respect, previous studies in the pea aphid associated to leguminous crops in Chile have shown that these aphid populations are under strong parasitism pressures in the field, observing high parasitism rates in the pea aphids on alfalfa and pea crops, being *A. ervi* the dominant parasitoid species (> 94% of the parasitoid individuals) (Zepeda-Paulo et al., 2013), which could favor the prevalence of *H. defensa*-infected aphids among the Chilean populations of pea aphid in alfalfa crops.

Furthermore, different studies have shown that the co-infection *H. defensa* + *Fukatsuia symbiotica* can enhance resistance levels provided by single infections with *Hamiltonella* (Leclair et al., 2016). Besides, aphids carrying *F. symbiotica* (as well co-infected *H. defensa* + *F. symbiotica*) can be more resistant to the fungal pathogen *Pandora neoaphidis* (Smee et al., 2021), being this co-infection recurrently observed (albeit in variable frequencies) in pea aphid populations on alfalfa crops from other regions, including Western France (native range) and North America (introduced range) (Doremus and Oliver, 2017; Rock et al., 2018; Leclair et al., 2021). Despite this, our results did not show evidence of the presence of single or co-infections with the endosymbiont *F. symbiotica* infections among the aphid populations of *A. pisum* on alfalfa crops in Central Chile. The lack of this relevant endosymbiont in Chilean populations of the pea aphid could be explained by the invasive process of aphid pest species, in which a loss of genetic variants (with respect to the native range) can occur due to founder effects and genetic drift acting during the introduction of individuals in a new area (Welles and Dlugosch, 2018).

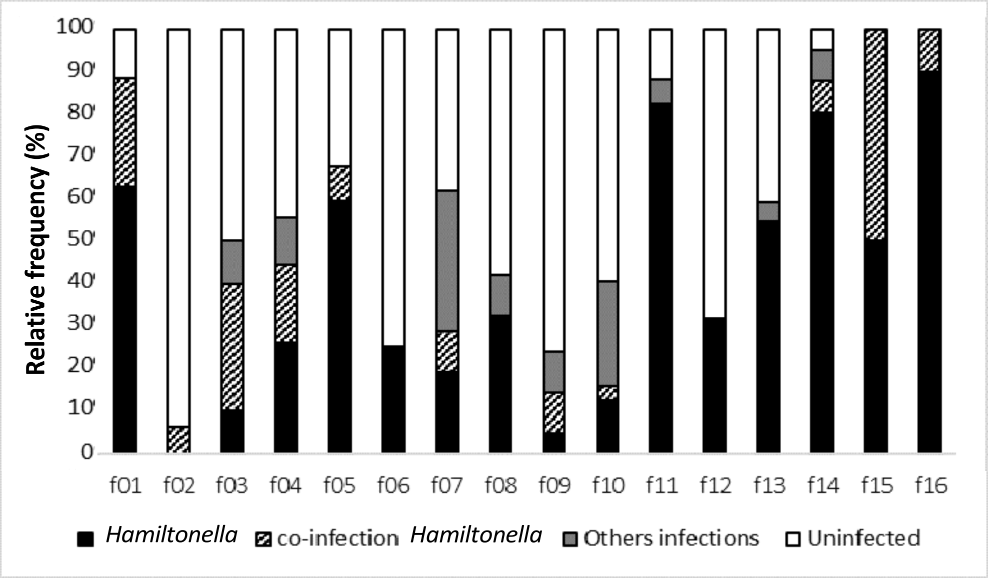

Figure 2. Relative frequency of *Hamiltonella defensa* in single and co-infected pea aphids in the 16 alfalfa fields studied.

In effect, this evolutionary scenario has been described for the Chilean populations of the pea aphid, which exhibited a decreased genotypic diversity (compared to native populations) as result of their introduction (Peccoud et al., 2008). Similarly, facultative endosymbionts could be loosed during the introduction of their hosts resulting in fewer endosymbiont taxa associated to introduced aphid populations, as observed by other studies (Desneux et al., 2018; Zepeda-Paulo et al., 2018). These conditions then, may have kept a fewer diversity of defensive endosymbionts as well as avoiding the introduction of new endosymbiont taxa in the introduced aphid populations, reducing the variability of defensive mechanisms available for these aphid populations against their natural enemies (parasitoids and pathogenic fungi), and then increasing the likelihood of successful biological control in this aphid species in Chile. Finally, from the perspective of the application of biological control programs (e.g., augmentative biological control) should be evaluated the diversity of defensive endosymbionts present in target pest populations which could allow a better selection and optimization of the biological control agents used for aphid pest control.

CONCLUSIONS

The diversity of facultative endosymbionts associated with pea aphid (*Acyrthosiphon pisum*) populations in alfalfa crops in Chile, was similar to that observed in comparable endosymbiont surveys in other regions. Our results identified six of the seven most common facultative endosymbionts carried by aphids (*Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, *Spiroplasma* sp., *Rickettsia* sp. and *Rickettsiella viridis*), observing higher rates of infections (single and multiple infections) with the recognized defensive endosymbiont *H. defensa*. However, we observed a lack of infections with *Fukatsuia symbiotica*, an important defensive endosymbiont usually reported in pea aphid populations, which could have reduced the defensive potential of this aphid pests against biological control agents allowing a better suppression of aphid pests on alfalfa crops in Chile.

Author contributions
Conceptualization: F.Z-P., B.L. Methodology: F.Z-P. Software, F.Z-P. Validation: F.Z-P. Formal analysis: F.Z-P. Investigation: F.Z-P. Resources: B.L. Data curation: B.L. Writing-original draft: F.Z-P. Writing-review & editing:

B.L. Visualization: F.Z-P. Supervision: B.L. Project administration: B.L. Funding acquisition: B.L. All co-authors reviewed the final version and approved the manuscript before submission.

Acknowledgements

This research was funded by the FONDECYT (Nr 1140632) grant "Understanding the networks of natural enemy assemblages involved in aphid control: repercussions for sustainable pest management." BL and FZ would like to thank ANID/PIA/ACT192027 for funding provided during the writing of this manuscript and the support of the Instituto de Investigación Interdisciplinario, Universidad de Talca. BL would like to thank Hans Castro for help with the fieldwork and support in the laboratory.

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