**Universidade de São Paulo
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**Simbiontes associados ao intestino médio e aparelho reprodutivo de
Euschistus heros (F.) (Hemiptera: Pentatomidae)**

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Tese apresentada para obtenção do título de Doutora em
Ciências. Área de concentração: Entomologia

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(F.) (Hemiptera: Pentatomidae)**

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RESUMO

Simbiontes associados ao intestino médio e aparelho reprodutivo de *Euschistus heros* (F.) (Hemiptera: Pentatomidae)

Bactérias simbiontes foram fundamentais no processo evolutivo de eucariotos. Em insetos, essa associação permitiu a exploração de novos nichos ecológicos pela íntima associação estabelecida com bactérias que fornecem nutrientes essenciais a hospedeiros cuja dieta apresenta limitação em sua composição nutritiva, assim como proteção a condições de estresse e fatores bióticos e abióticos. Um grande número de associações facultativas, aquelas em que o simbiote não é essencial para a sobrevivência do hospedeiro, mas pode impactar na aptidão biológica, tem sido descobertas. Pentatomídeos estão comumente associados a bactérias extracelulares facultativas presentes nas invaginações da região final do intestino médio, sendo alvos de investigação para a determinação da diversidade de simbiontes e, principalmente, sua função na interação com o inseto hospedeiro. Porém, na ausência destes simbiontes, o desenvolvimento e a aptidão reprodutiva do hospedeiro podem ser prejudicados. Adicionalmente, bactérias livres associadas a insetos podem se associar a outros tecidos, como as estruturas reprodutivas, e causar efeitos nocivos ao hospedeiro. A intensidade dos efeitos induzidos por essa microbiota depende de vários fatores, como a sua diversidade, a idade e o tecido do hospedeiro. Nesta tese utilizamos como modelo de estudo o percevejo *Euschistus heros* (F.) (Hemiptera: Pentatomidae), uma importante praga de diversas culturas agrícolas, para investigar i) a diversidade de simbiontes cultiváveis associada às estruturas reprodutivas (ovários, espermateca e vesícula seminal) e digestivas (criptas da região final do intestino médio); ii) a diversidade de simbiontes associada a embriões e ventrículos do intestino médio de ninfas de último ínstar e adultos; o efeito de tratamentos para a eliminação de bactérias associadas iii) à vesícula seminal e iv) região V4 do intestino médio na aptidão reprodutiva de *E. heros*. A região V4 do intestino médio, ovário, espermateca e vesícula seminal apresentou *Proteobacteria* (*Enterobacteriaceae*) e *Firmicutes* (*Enterococcaceae* e *Staphylococcaceae*) como simbiontes cultiváveis, sendo a região V4 do intestino médio a mais diversa entre as estruturas avaliadas. Entre os filotipos isolados, foram encontrados filotipos comuns a todas as estruturas analisadas, mas também aqueles associados exclusivamente ao trato reprodutivo e aparelho digestivo. A análise de metagenômica indicou o embrião de *E. heros* como sendo o mais diverso, seguido pelas regiões V1 e V2 do intestino médio de ninfas e adultos. A região V4 foi a menos diversa, sendo habitada praticamente por uma *Enterobacteriaceae*. A espermateca e a vesícula seminal são naturalmente infectadas por simbiontes e a eliminação dessas bactérias após tratamento com antibióticos não resultou em alterações na capacidade reprodutiva de fêmeas. O tratamento de adultos com antibióticos e a esterilização superficial dos ovos não interferiu no desenvolvimento e reprodução de *E. heros*, ocorrendo, porém, redução da fertilidade e na mortalidade de ninfas quando os parentais foram alimentados com antibióticos.

Palavras-chave: Microbiota; Simbiose; Caracterização molecular; Interação inseto-simbionte

ABSTRACT

Symbionts associated with the midgut and reproductive tract of *Euschistus heros* (F.) (Hemiptera: Pentatomidae)

Bacterial symbionts are fundamental in the evolutionary process of eukaryotes. In insects this association allowed the exploitation of new ecological niches, because the bacteria provide essential nutrients for those whose diet is nutritionally limited. Bacterial symbionts also provide protection against biotic and abiotic factors, allowing insects to exploit a range of food types. In recent years, a large number of facultative associations have been discovered. In such associations, symbionts are not essential for host survival, but may have a significant impact on insect host fitness. Pentatomids are commonly associated with facultative extracellular bacteria present in the invaginations of the posterior region of the midgut and have been targeted in studies for the determination of the diversity and function of these symbionts. However, associations with symbionts can impose adaptive costs to the host; therefore, in the absence of these symbionts, the development and reproduction of the host may be impaired. In addition, facultative symbionts can be potentially pathogenic bacteria, and can infect the host's reproductive system. The intensity of the effects induced by this microbiota depends of several factors, such as its diversity, age and host tissue infected. Transmission of the microbiota usually occurs vertically by specific mechanisms shortly after egg hatching, such as the contamination of the surface of the eggs by a gelatinous mass during deposition and by the deposition of bacterial capsules next to the eggs. Symbionts can be acquired from the environment at each generation, depending on the host species. In here, we used *Euschistus heros* (F.) (Hemiptera: Pentatomidae), an important agricultural pest of several crops, as a model to investigate: i) the diversity of culturable symbionts of *E. heros* (ovaries, spermatheca and seminal vesicle) and digestive tract (crypts of the posterior region of the midgut); ii) the diversity of non-culturable symbionts present along the midgut, in embryos, last instar and adults; the effects of symbionts associated with the iii) seminal vesicle and iv) the V4 region of the midgut on the reproductive fitness of *E. heros*. Our data show that the diversity of culturable symbionts associated with the V4 region of the midgut, ovary, spermatheca and seminal vesicle is represented by *Proteobacteria* (*Enterobacteriaceae*) and *Firmicutes* (*Enterococcaceae* and *Staphylococcaceae*). The V4 region of the midgut was the most diverse among the evaluated structures. In addition, unique culturable phylotypes were found in the reproductive and digestive tract. Metagenomic analysis indicated the embryo of *E. heros* carried the most diverse microbiota, followed by the V1 and V2 region of the gut. The V4 region was mostly inhabited by an *Enterobacteriaceae*. Symbionts associated with the spermatheca and the seminal vesicle naturally infect *E. heros* and do not affect the reproductive capacity of females. The sterilization of the egg surface did not interfere directly with the development and reproduction of *E. heros*, but the progenie of couples treated with antibiotics presented reduced fertility and nymphal mortality.

Keywords: Microbiota; Symbiosis; Molecular characterization; Insect-symbiont interaction

INTRODUÇÃO

Bactérias simbiontes, endocitobiontes ou endossimbiontes de vida livre, contribuíram de forma significativa na adaptação de insetos às mais diversas fontes de alimentos e habitats, visto que os simbiontes estão envolvidos no fornecimento de nutrientes essenciais aos hospedeiros (vitaminas e aminoácidos), os quais não estão disponíveis em concentrações adequadas ao inseto na fonte de alimento explorada (Klepzig *et al.* 2009; Moran & Dunbar 2006).

Os endossimbiontes podem ser divididos em primários ou obrigatórios e secundários ou facultativos. Os simbiontes obrigatórios normalmente se localizam em células especializadas isoladas, denominadas bacteriócitos, ou agrupadas, formando um órgão específico, o bacterioma, localizado na hemocele do inseto. Esses simbiontes são transmitidos via transovariana e estão envolvidos em associações de mutualismo altamente especializadas, necessárias a ambos os parceiros (Moran & Dunbar 2006). Os facultativos, por sua vez, são tipicamente de vida livre, presentes na hemolinfa, no lúmen intestinal ou associados à diferentes tipos celulares, sendo de ocorrência irregular nas populações do hospedeiro (Afkhami & Rudgers 2008; Fukatsu *et al.* 2000; Sudakaran *et al.* 2017). Porém, muitos simbiontes secundários acabam influenciando aspectos essenciais da aptidão do hospedeiro, como a tolerância a altas temperaturas (Montllor *et al.* 2002), a preferência pela planta hospedeira (Leonardo & Muiro 2003; Tsuchida *et al.* 2004), o catabolismo de pesticidas (Cheng *et al.* 2017; Kikuchi *et al.* 2012; Pan *et al.* 2013) e o aumento da resistência de hospedeiros a parasitoides (Oliver *et al.* 2003; Oliver *et al.* 2005) e fungos (Scarborough *et al.* 2005). Outros simbiontes facultativos, como *Wolbachia*, *Spiroplasma* e *Cardinium*, são conhecidos por manipular a reprodução do hospedeiro (Anbutsu & Fukatsu 2003; Werren *et al.* 2008; Zchori-Fein & Perlman 2004). Embora a maioria dos endocitobiontes facultativos sejam transmitidos verticalmente, não há congruência na filogenia de endocitobiontes e seus hospedeiros, sugerindo múltiplas origens e frequentes transmissões horizontais (Hosokawa *et al.* 2016a; Hosokawa *et al.* 2016b; Kikuchi 2009; Moran & Dunbar 2006). Ainda, os endossimbiontes secundários transmitidos via materna podem sofrer redução em sua densidade ao longo de gerações sucessivas do hospedeiro (Afkhami & Rudgers 2008; Fukatsu *et al.* 2000).

Informações sobre os mecanismos de transmissão e aquisição de endossimbiontes de vida livre, como os associados à hemolinfa e ao lúmen intestinal, são escassas. Porém, há relatos da transmissão de simbiontes associados ao trato digestivo via alimentação da

superfície coriônica contaminada (Boucias *et al.* 2012; Fukatsu & Hosokawa 2002; Kaiwa *et al.* 2014), coprofagia materna (Beard *et al.* 2002) e trofolaxia proctodeal (Currie *et al.* 2006; Hongoh 2010; Koch & Schmid-Hempel 2011; Marsh *et al.* 2014; Powell *et al.* 2014). A aquisição também pode ocorrer diretamente do ambiente, a cada nova geração (Caspi-Fluger *et al.* 2012; Kikuchi *et al.* 2007).

Os percevejos, representantes da Ordem Hemiptera, consistem de mais de 40.000 espécies no mundo. São agrupados em sete infraordens, sendo relatados simbioses em duas delas, Cimicomorpha e Pentatomomorpha. Os percevejos dessas infraordens demonstram adaptações a diversos habitats e estratégias de vida, como a dos predadores, hematófagos e fitófagos. Além disso, apresentam o intestino médio dividido em quatro segmentos, sendo o último deles (V4) caracterizado por apresentar invaginações (cecos ou criptas), sendo considerado um importante local de interação com bactérias (Kikuchi *et al.* 2008; Schaefer & Panizzi 2000; Weirauch & Schuh 2011).

Embora a maior parte das funções biológicas da microbiota intestinal permaneça obscura em percevejos, foi demonstrada que a eliminação de simbioses das criptas resultou em crescimento retardado e maior mortalidade ninfal em algumas espécies de hospedeiros (Fukatsu & Hosokawa 2002; Hosokawa *et al.* 2016a; Hosokawa *et al.* 2006; Kikuchi *et al.* 2007; Kikuchi *et al.* 2009; Prado *et al.* 2006; Tada *et al.* 2011).

Diante deste contexto, evidencia-se a importância de se conhecer a diversidade, localização e possível função dos simbioses em insetos, visando entender as implicações dessas interações na biologia e fisiologia do hospedeiro. Assim, esse projeto teve por finalidade abordar a relação de simbiose facultativa em *Euschistus heros* (F.) (Hemiptera: Pentatomidae), importante praga agrícola de diversas culturas (soja, algodão, girassol, milho), e, assim, fornecer bases para o aprimoramento e/ou desenvolvimento de técnicas alternativas de controle de pragas.

CONCLUSÃO

A região V4 do intestino médio, ovário, espermateca e vesícula seminal apresentou *Proteobacteria* (*Enterobacteriaceae*) e *Firmicutes* (*Enterococcaceae* e *Staphylococcaceae*) como simbiontes cultiváveis, sendo a região V4 do intestino médio a mais diversa entre as estruturas avaliadas. Entre os filotipos isolados, foram encontrados filotipos comuns a todas as estruturas analisadas, mas também aqueles associados exclusivamente ao trato reprodutivo e aparelho digestivo. A análise de metagenômica indicou o embrião de *E. heros* como sendo o mais diverso, seguido pelas regiões V1 e V2 do intestino médio de ninfas e adultos. A região V4 foi a menos diversa, sendo habitada praticamente por uma *Enterobacteriaceae*. A espermateca e a vesícula seminal são naturalmente infectadas por simbiontes e a eliminação dessas bactérias após tratamento com antibióticos não resultou em alterações na capacidade reprodutiva de fêmeas. O tratamento de adultos com antibióticos e a esterilização superficial dos ovos não interferiu no desenvolvimento e reprodução de *E. heros*, ocorrendo, porém, redução da fertilidade e na mortalidade de ninfas quando os parentais foram alimentados com antibióticos.

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