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Antimicrobial resistance and genetic relationship among enterococci from siblings and non-siblings *Heliconius erato phyllis* caterpillars

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Background: Studies evaluating bacterial in insects could provide information about host-microorganism-environment interactions. The gut community is recognized to have a profound effect on various physiological functions of the insects. *Enterococcus* is part of the gut community in humans andanimals, as well as in the guts of insects. The presence and antimicrobialresistance profileof enterococci are well studied in different animals; however, data in *Heliconius erato phyllis* (Lepidoptera; Nymphalidae), do not yet exist. Therefore, the aims of this study were to evaluate species distribution, antimicrobial resistance profile, virulence genes and genetic relationship amongenterococci isolated from fecal samples of siblings and non-siblings *H. erato phyllis* caterpillars collected from different places in South Brazil.

Methods: Three *H. erato phyllis* females were captured (two from a forest fragment and one from an urban area), and kept individually in open-air insectaries. Eggs were collected and caterpillars (siblings and non-siblings) were fed daily with *Passiflora suberosa* leaves. Fecal samples (n=12) were collected from fifth instar caterpillars, inoculated in selective medium and fifteen colonies were randomly selected from each sample. Enterococci were identified by PCR and MALDI-TOF, submitted to *antimicrobial susceptibility tests by disk* diffusion method, and screened for resistance and virulence genes by PCR. The genetic relationships among the strains were determined using pulsed-field gel electrophoresis (PFGE).

Results: A total of 178 enterococci were identified as: *Enterococcus casseliflavus* (74.15%; n=132),*E. mundtii* (21.34%; n=38), *E. faecalis* (1.12%; n=2) and *Enterococcus* sp. (3.37%; n=6). High rates of resistance to rifampicin (56%) and erythromycin (31%) were observed. One hundred and twenty (67.41%) out of the 178 isolates showed resistance to at least one compound and 6 (3.37%) were multidrug-resistant.None of the erythromycin-resistant strains was positive to *erm*(B) and *msr*C genes. The virulence genes *esp*, *ace* and *gelE* were observed in 35%, 7% and 1% of the strains, respectively. PFGE separated the enterococci into 22 patterns, being four patterns composed by strains from sibling caterpillars.

Conclusion: *Enterococcus casseliflavus* was the dominant species in fecal samples of fifth instar caterpillars. Resistant enterococci strains could be related to environmental pollution or linked to

environmental resistome. The PFGE analysis showed related genetic relationship among some strains, suggesting that the enterococci isolated from fecal samples of fifth instar sibling caterpillars might have come from common sources, by diet (herbivory) and/or via vertical transmission (through egg surface). Further studies will be conducted to better understand the role of *Enterococcus* on the microbial gastrointestinal tract community of these insects, and the mechanisms involved in acquisition and maintenance of these bacteria.

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Abstract

Background: Studies evaluating bacterial in insects could provide information about host-microorganism-environment interactions. The gut community is recognized to have a profound effect on various physiological functions of the insects. *Enterococcus* is part of the gut community in humans and animals, as well as in the guts of insects. The presence and antimicrobial resistance profile of enterococci are well studied in different animals; however, data in *Heliconius erato phyllis* (Lepidoptera; Nymphalidae), do not yet exist. Therefore, the aims of this study were to evaluate species distribution, antimicrobial resistance profile, virulence genes and genetic relationship among enterococci isolated from fecal samples of siblings and non-siblings *H. erato phyllis* caterpillars collected from different places in South Brazil.

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samples (n=12) were collected from fifth instar caterpillars, inoculated in selective medium and fifteen colonies were randomly selected from each sample. Enterococci were identified by PCR and MALDI-TOF, submitted to antimicrobial susceptibility tests by disk diffusion method, and screened for resistance and virulence genes by PCR. The genetic relationships among the strains were determined using pulsed-field gel electrophoresis (PFGE).

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Conclusion: *Enterococcus casseliflavus* was the dominant species in fecal samples of fifth instar caterpillars. Resistant enterococci strains could be related to environmental pollution or linked to environmental resistome. The PFGE analysis showed related genetic relationship among some strains, suggesting that the enterococci isolated from fecal samples of fifth instar sibling caterpillars might have come from common sources, by diet (herbivory) and/or via vertical transmission (through egg surface). Further studies will be conducted to better understand the role of *Enterococcus* on the microbial gastrointestinal tract community of these insects, and the mechanisms involved in acquisition and maintenance of these bacteria.

Introduction

Heliconius (Lepidoptera; Nymphalidae) comprises a widespread genus of butterflies distributed throughout tropical and subtropical regions, from South America up to the southern United States (*Brown, 1981; Merril et al., 2015*). Adults feed on pollen as well as nectar (*Gilbert, 1972; Merril et al., 2015)*, and this food supply provides a better use of resources and a reduction of competition since the same flower can provide nutrients for both *Heliconius* and other butterflies that exclusively use nectar as food. This diet is rich in amino acids that allow adult females to oviposit on a daily basis during their lives (*Gilbert, 1972)*. *Heliconius erato phyllis* is a subspecies commonly found between northeastern Brazil and northern Argentina in both forests and urban environments. Adult females are monandric and lay their eggs individually in the apical meristem of a host plant, since first instar caterpillars may cannibalize other eggs (*De Nardin et al., 2016*). Several species of passionflower vines are used as a host plant for oviposition and feeding of the caterpillars, including *Passiflora suberosa*, *P. misera* and *P. capsularis*, commonly found in Southern Brazil, where this study was completed. These plants have cyanogenic glycosides, which are assimilated by the caterpillars to make them unpalatable to potential predators; however, adults of *H. erato phyllis* are also able to synthesize cyanogenic glucosides and transfer them to their eggs (*Hay-Roe & Nation, 2007*).

It has been recognized for a long time that microorganisms play key roles on various physiological functions of the host. Gut microbial community provide an especially diverse range of benefits in insect nutrition, e.g. by providing xenobiotic metabolism, synthesis of vitamins, establishment of metabolic pathways, contributing to defense against invading pathogens and immune system modulation (*Douglas, 2015; Shao et al., 2017*). The presence of microorganisms in the GI tract of insects can be explained by environmental bacteria ingested with food and/or acquired by maternal transfer (*Engel & Moran, 2013*). A growing number of studies have addressed the importance of the microbiota in the gastrointestinal (GI) tract of insects (*Engel & Moran, 2013; Chen et al., 2016; Douglas, 2018*). Studies investigating the gut microbiota in different species of order Lepidoptera identified *Enterococcus* as one of the most frequently genus in different stages of the life cycle of these animals (*Brinkmann, Matens & Tebbe, 2008; Chen et al., 2016; Ruokolainen et al., 2016; Snyman et al., 2016; van Shooten et al., 2018; Allonsius et al., 2019*).

Enterococcus genus is often found as part of the GI tract in humans and animals, as well as in the guts of insects (*Shao et al., 2017*). *Hammer et al. (2014)* reported that *Enterococcus* spp. was the most abundant genus in immature and adult in a subspecies of *H. erato* from Panamá. Furthermore, it has also been reported in insects of other orders, such as Coleoptera (*Kim et al., 2017*), Hymenoptera (*Audisio et al., 2011*), and Diptera (*Ghosh et al., 2014*). A characteristic of this genus is their intrinsic resistance to several antimicrobial agents and a great ability to transfer and acquire resistant genes (*Hollenbeck & Rice, 2012*). The anthropogenic activities such as animal husbandry, agronomic practices and wastewater treatment play an important role in the emergence and spread of antibiotic resistant enterococci and/or antibiotic resistance genes in the environment - especially in soil, water, wastewater, and food (*Gothwal & Shashidhar, 2014; Singer et al., 2016*). Notwithstanding antibiotic resistance is a great concern, few studies have addressed the importance of insects carrying resistant enterococci (*Allen et al., 2009; Lowe & Romney, 2011*).

As previously mentioned, *Enterococcus* genus is often found as part of the GI tract in Lepidoptera and it may play a fundamental role in the development of these insects. The identification of enterococcal strains and their resistance profile in insects, is an important point that must be addressed about host-microorganism-environment interactions. To our knowledge, until today, there are no studies evaluating enterococci in *H. erato phyllis*. The aim of our research was to analyze species distribution, antimicrobial resistance profile, virulence genes and genetic relationship among enterococci isolated from fecal samples of siblings and non-siblings *H. erato phyllis* caterpillars collected from different places in South Brazil.

Materials & Methods

Sample collection

The fecal samples used in the present study were collected from fifth instar caterpillars. The caterpillars were sourced from three different populations of *Heliconius erato phyllis* butterflies and consisted of sibling from the same female. The *H. erato phyllis* females (HE)

were captured with entomological nets in Rio Grande do Sul, South Brazil. The first female (HEAB2) was collected in a forest fragment located in Águas Belas Agronomical Station (30° 02' 18.1" S; 51° 01' 23.0" W), the second female (HES2) in a forest fragment located in São Francisco de Paula (29° 26' 34.1" S; 50° 36' 48.8" W) and the third female (HEV2) from a population in an intense urban area in Viamão (30° 09' 40.5" S; 50° 55' 01.5" W).

Butterflies were kept individually in open-air insectaries with dimensions of 2.3 m x 3 m x 3 m (width, length, height) approximately. Insectaries had many plants for simulation of natural conditions, including *P. suberosa*, used by females for oviposition. The butterflies were fed daily with a mixture containing water, honey and pollen.

A total of 12 eggs were collected (five from HEAB2, five from HEV2 and two from HES2) with the assistance of a paintbrush. The eggs were transported to the laboratory, and caterpillars were grown individually in cylindrical plastic pots. Immatures were fed exclusively with *P. suberosa* leaves (Fig. 1). Fecal samples were collected from each caterpillar individually after 48 h of molting to the fifth instar, with the aid of a disposable plastic spoon, stored in 1.5 mL microtubes and maintained at -80 °C until processing. The oviposition dates are shown in Table S1.

This study was carried out in accordance with the recommendations of Chico Mendes Institute for Biodiversity Conservation (ICMBio). The protocol was approved by Information Authorization System in Biodiversity (SISBIO) number 33404-1. This study has the Council for the Management of Genetic Patrimony - CGEN - under the Ministry of Environment number A720680.

Isolation and identification of Enterococci

Isolation and identification of enterococci were performed as previously described in *Santestevan et al. (2015)*, with modifications. One milligram of fecal sample was transferred to 10 mL of saline 0.85% and incubated at 37 ºC for 24 h. One mL was inoculated in 9 mL of Azide Dextrose Broth selective medium (Himedia, Mumbai, India) and incubated for 24 h at 37 ºC. Aliquots (1 mL) were placed in 9 mL of saline 0.85%, and initial samples were further diluted 10-fold. From dilution 10^{-5} and 10^{-6}, 100 µL was inoculated in brain heart infusion (BHI) agar plates (Himedia, Mumbai, India) supplemented with 6.5% NaCl, incubated for 48 h at 37 ºC.

Fifteen colonies were randomly selected from each fecal sample. Phenotypic criteria (size/volume, shape, color, gram staining, catalase production and bile aesculin reaction) were used to separate the enterococci group and the non-enterococcal strains. Selected pure colonies were stored at -20 ºC in a 10% (w/v) solution of skim milk (Difco, Sparks, MD, USA) and 10% (v/v) glycerol (Neon Comercial Ltda, São Paulo, SP, BR).

Genomic DNA was extracted by physicochemical method as previously described by *Depardieu, Perichon & Courvalin (2004)*. Genus-specific PCR assays targeting the *tuf* gene were performed (*Ke et al., 1999*) (Table 1) and *E. faecalis* ATCC 29212 was used as positive control.

Characterization of enterococci species

Isolates were screened with the species-specific PCR assay for *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus casseliflavus* and *Enterococcus mundtii* (*Cheng et al., 1997; Jackson, Fedorka-Cray & Barrett, 2004; Sedgley et al., 2005*). The primers and annealing temperature used are listed in Table 1. Strains that were not identified by PCR reactions were submitted to matrix-assisted laser desorption and ionization time-of-flight technique (MALDI-TOF) applied to *Enterococcus* sp., according to *Sauget et al. (2017)*.

Isolates classified as *Enterococcus* sp. were identified by sequence analysis. The PCR product of *16S rRNA* gene, using the 8F and R1522 primers (*Coenye et al., 1999*) (Table 1), was purified with Illustra™ GFX™ PCR DNA and gel band purification kit (GE Healthcare, Buckinghamshire, UK). Sequencing was performed with the ABI PRISM® BigDye® Primer Cycle Sequencing Ready Reaction Kit in an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems®), according to the manufacturer's protocol. The sequences obtained were compared to nucleotide sequences of reference enterococci strains deposited in GenBank.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities were determined by Kirby-Bauer disk diffusion method recommended by the Clinical and Laboratory Standards Institute (*CLSI, 2016*). Eleven antibiotics used in clinical and veterinary medicine were tested: ampicillin 10 µg (AMP), vancomycin 30 µg (VAN), erythromycin 15 µg (ERY), tetracycline 30 µg (TET), ciprofloxacin 5 µg (CIP), norfloxacin 10 µg (NOR), nitrofurantoin 300 µg (NIT), rifampicin 5 µg (RIF), chloramphenicol 30 µg (CHL), gentamicin 120 µg (GEN) and streptomycin 300 µg (STR).

Intermediate and resistant strains were considered in a single category and classified as resistant. Strains showing resistance to three or more unrelated antibiotics were considered as multidrug-resistant (MDR) (*Schwarz et al., 2010*).

Detection of resistance and virulence genes

Erythromycin-resistant strains were tested by PCR for the presence of resistance encoding genes more commonly associated to clinical and environmental enterococci: *erm*(B), which encodes a ribosomal methylase that mediates MLSB resistance; and *msr*C, which encodes for a macrolide and streptogtamin B efflux pump. The presence of virulence associated genes *gelE* (gelatinase enzyme), *cylA* (activator of cytolysin), *ace* (accessory colonization factor), *esp* (associated to biofilm formation) and *agg* (aggregation substance) was determined by PCR in all enterococcal isolates. The amplifications were performed as described in *Prichula et al. (2016)*.

The sequences of the primers and annealing temperature are described in Table 1.

***Molecular typing of Enterococci by Pulsed-field gel electrophoresis* (PFGE)**

Enterococci strains isolated from siblings and non-sibling caterpillars were selected for PFGE analysis according to the following criteria: maternal origin (females HEAB2, HEV2 or HES2), hatched larvae, enterococcal species and antimicrobial profile. Chromosomal DNA

extraction and electrophoresis conditions were prepared according to *Murray et al. (1990)* and *Saeedi et al. (2002)*. The restriction enzyme used was *Sma*I (Invitrogen®). The electrophoresis was carried out using a clamped homogeneous electric field (CHEF-DRII device; Bio-Rad Laboratories, Richmond, Calif.), with ramped pulse times recommended by *Saeedi et al. (2002)* at 11 °C. The gels were stained with ethidium bromide (0.5 µg/mL for 20 min). The PFGE patterns were interpreted using the program GelCompar II v. 11 6.6, and the percentage of similarity was estimated using the Dice coefficient. A dendrogram was generated to examine the relatedness of PFGE patterns for selected isolates, and cutoff level of 80% applied to this dendrogram (*Tenover et al., 1995*).

Statistical Analysis

Simpson's index of diversity (D) was calculated to assess the differentiation of enterococci species among the caterpillars from different maternal origins (*Hunter & Gaston, 1988*).

Results

Enterococci species present in fecal samples of H. erato phyllis caterpillars

A total of 178 strains were isolated from fecal samples from fifth instar caterpillars (Table 2). *Enterococcus casseliflavus* was the most common species identified (74.15%; n = 132), followed by *E. mundtii* (21.34%; n = 38) and *E. faecalis* (1.12%; n = 2). Six strains (3.37%) could not be identified at the species level.

Differences in the composition of enterococci were detected among the three groups of caterpillars, as shown in Table 2. The Simpson's indexes of diversity showed differences between the three populations, with higher diversity of enterococci species from fecal samples of caterpillars from HES2 (1 – D = 0.68), followed by HEV2 (1 – D = 0.49) and HEAB2 (1 - D = 0.27).

Antimicrobial susceptibility

One hundred and twenty (67.41%) enterococci were resistant to at least one evaluated antimicrobial agent. The frequency of antibiotic susceptible strains is shown in Table 3. Rifampicin resistance phenotype was the most commonly observed (56%; n=100), followed by erythromycin (31%; n=55). Eight strains (4%) were resistant to norfloxacin and five (3%) to ciprofloxacin. All investigated strains were susceptible to ampicillin, vancomycin, tetracycline, nitrofurantoin, chloramphenicol, gentamicin and streptomycin.

Among the 120 resistant strains, single (SR), double (DR), and MDR were observed in 67% (n=80), 28% (n=34), and 5% (n=6) of strains, respectively.

Resistance and virulence determinates

None of the 55 erythromycin-resistant strains was positive to *erm*(B) and *msr*C genes. The presence of virulence genes was evaluated in all strains, and *esp* gene was detected in

35.39% (n=63), *ace* in 6.74% (n=12) and *gel*E in 1.12% (n=2). None strain were positive for *cly*A and *agg* genes.

Genetic relationship among enterococci isolated from siblings and non-siblings caterpillars into fifth instar

Of the 178 strains isolated, 86 (*E. casseliflavus*, n=58; *E. mundtii*, n=23; *E. faecalis*, n=2*; Enterococcus* sp., n=3) were chosen for PFGE (Table S1). From sibling caterpillars 6, 7, 10, 11 and 14 of HEAB2 female were picked, *E. casseliflavus* (n=32), *E. mundtii* (n=8) and *E. faecalis* (n=2). All strains were isolated from caterpillars hatched closely in time and fed with the same batch of *P. suberosa* leaves. From the offspring of HEV2 female (sibling caterpillars 9, 18, 26, 27 and 29) were selected *E. casseliflavus* (n=19), *E. mundtii* (n=9) and *Enterococcus* sp. (n=3). These isolates were recovered from siblings that hatched at different periods of time and were fed with different batches of leaves. And from the offspring of HES2 female (sibling caterpillars 3 and 17) were chosen *E. casseliflavus* (n=7) and *E. mundtii* (n=6). Those strains were isolated from sibling caterpillars.

The hierarchical relationship among enterococci selected from siblings and non-siblings caterpillars, showed 22 patterns (15 patterns and 7 single strain -singleton) (Fig. 2). Four patterns generated by PFGE indicated genetic relationship by strains isolated from siblings caterpillars (P7, P8, P9 and P13), and 11 were composed by strains isolated from the same caterpillars (P1, P2, P3, P4, P5, P6, P10, P11, P12, P14 and P15). No genetic relationship was observed by strains isolated from non-siblings.

The band pattern among the 32 *E. casseliflavus* isolates from sibling caterpillars (6, 7, 10 11 and 14) from the HEAB2 female, showed six PFGE patterns (P5, P7, P8, P9, P11 and P12) and three singletons. There were three PFGE patterns (P7, P8, and P9), which included 18 of the 32 strains that were isolated from sibling caterpillars 6, 7, 10 and/or 11, with low levels of genetic variability. P5 and P11 each contained two isolates and P12 with eight isolates showed genetic variations, and the remaining three *E. casseliflavus* isolates were singletons and represented unique PFGE patterns. All *E. mundtii* from caterpillar 14 were closely genetic related and were clustered in one patterns (P10), as well as the two *E. faecalis* (P3) isolates from caterpillar 6. These results demonstrate strains may be originated from the single linage each.

Seven different PFGE patterns (P4, P6, P13, P15, and three singletons) from sibling caterpillars 9, 18, 26, 27, and 29 from the HEV2 female were obtained. The analysis of the fragment profiles of the *E. mundtii* (n=9) and *Enterococcus* sp. (n=1) strains isolated from caterpillars 18, 26, 27 and 29 demonstrate elevated genetic relationship between them (P13). The 19 *E. casseliflavus* strains showed four PFGE patterns: P4, P6 and P15, each contained six, three and nine isolates, respectively, and one singleton. These distinct patterns are suggestive of genetic events in these strains.

The seven *E. casseliflavus* strains from caterpillars 3 and 17 (offspring of HES2 female) showed two distinct patterns (P1 and P2) with high levels of genetic similarities between them (100%). P1 contained four isolates and P2 encompassed three isolates. Of the six *E. mundtii*

isolated from caterpillar 3, five showed 100% of similarity and were clustered in the P14 pattern - suggesting that these strains may be progeny from a single lineage - and one strain had distinct and unrelated PFGE by criteria of *Tenover et al.(1999)*.

In addition, most of the patterns were shared by isolates with the same antimicrobial profile.

Discussion

An increasing number of studies have aimed to investigate the microbial communities in GI tract of insects. In the order Lepidoptera, high abundance of *Enterococcus* genus has been found, both in immatures and adults, raising questions about the role of these bacteria in invertebrates and their importance in maintaining the health of individuals (*Hammer, McMillan & Fierer, 2014; Holt et al., 2015; Chen et al., 2016; Ruokolainen et al., 2016; Snyman et al., 2016; Shao et al., 2017; van Schooten et al., 2018; Allonsius et al., 2019*). To our knowledge, only the studies of *Hammer, McMillan & Fierer (2014)* and, more recently, *van Schooten et al. (2018)*, address the microbial communities in *Heliconius* GI tract and reported that the genus *Enterococcus* is dominant in samples of different *Heliconius* species.

The genus *Enterococcus* is associated with the environment and a wide range of organisms, among them plants (*Müller et al., 2001; Byappanahalli et al., 2012; Sánchez Valenzuela et al., 2012*). In the present study, *E. casseliflavus* was more frequently identified in feces of *H. erato phyllis* caterpillars. The diet constitutes an additional source of organisms in the GI tract of insects, and the presence of enterococci in the GI tract of caterpillars can play an important role with regards to protection against other pathogens, since this genus are able to produce lactic acid (causing a decrease of pH), and enterocins (peptides with antimicrobial activity). Since *E. casseliflavus* is are frequently related has part of microbial communities in plants (*Byappanahalli et al., 2012; Micallef et al., 2013; Ong et al., 2014*), the highest abundance of them in fecal samples of *H. erato phyllis* caterpillars could be explained by the herbivory of juveniles, unleashing the predominance of these species. As noted by *Chen et al. (2016)*, enterococci may act beneficially for caterpillars. However, further studies are needed to understand the role of these bacteria as components of the GI tract of caterpillars in *H. erato phyllis* subspecies.

Resistant enterococcal strains isolated from fecal samples of caterpillars are a matter of concern, since these larvae had no history of antibiotic use. Studies that highlight the resistance profile of enterococci isolated from insects in natural environments are scarce. *Channaiah et al. (2010)* describe enterococci isolated from stored-product insects resistant to tetracycline, streptomycin, erythromycin, kanamycin, ciprofloxacin, ampicillin, and chloramphenicol, and suggest that these animals can be potential vectors in disseminating of antibiotic-resistant strains. *Ahmad et al. (2011)* report multidrug resistant enterococci isolated from house flies and cockroaches in a confined swine production environment, and also point those insects maybe potential vectors and/or reservoirs of resistant enterococci. Despite the occurrence and spread of resistant strains has intensified due to the use of antimicrobials, the isolation of resistant

enterococci in the present study could be related both to anthropogenic activities (contamination of the environment) and/or linked to resistance that occurs naturally in the environment (environmental resistome) (*Martínez, 2008; Allen et al., 2010*).

The most widespread mechanism of resistance to macrolides in enterococci is mediated by *erm* and *msr*C genes (*Aarestrup et al., 2000; Santestevan et al., 2015; Prichula et al., 2016*). Nevertheless, in the present study, none of these genes was detected. It is possible that these strains harbored other erythromycin-resistance genes, such as *erm*D, E, F, and other efflux pump like *msr*A gene. A low percentage of virulence genes was detected in enterococci of *H. erato phyllis* caterpillars. Although, these genes are related to the pathogenicity of clinical enterococci strains, the presence of these genes in strains in fecal samples of the caterpillars, may be associated with the maintenance of the cells of the GI tract, and consequently with microorganism and host interactions.

The *E. casseliflavus*, *E. faecalis*, *E. mundtii*, and *Enterococcus* sp. isolated from fecal samples of siblings and non-siblings caterpillars demonstrated unrelated or related patterns based on maternal origin, on the analysis of PFGE fingerprint. The unrelated patterns found in P1, P2, P3, P4, P5, P6, P10, P11, P12, P14, and P15 demonstrated genetic diversity among these strains. The genetic variations in these strains maybe have been associated with genetic event, such as mobile elements or mutation, a common characteristic of enterococci (*Lebreton et al., 2014*). The related patterns were observed among strains isolated from siblings caterpillars (P7, P8, P9 and P13) may be associated with a common sources, by diet (herbivory) and/or via vertical transmission (through egg surface). Since the fecal samples used in the present study were collected from fifth instar caterpillars, the last stage before the pupa, the result present here may be suggestive of a vertical transmission of enterococci that are being replaced from diet. Plants are a food source for bacteria present in the GI tract of insects; these bacteria contribute to improving diets poor in nutrients, as well as a supporting role in development and maturation of the immune system to protect the host against pathogenic microorganisms (*Dillon & Dillon, 2004; Engel & Moran, 2013*). Therefore, it is likely that the herbivory of *H. erato phyllis* immature hatchlings represents an abundant supply of enterococci throughout the larval stage. Considering that the passionflower leaves were the only source of food to the immature, these leaves might was the source of *Enterococcus* sp. in the GI tract of the immatures.

As well as to the diet, the vertical transmission can also be a source of bacteria from a female to her offspring. Some studies have described the vertical transmission mechanism of bacteria in different species of Lepidoptera (*Brinkmann, Martens & Tebbe, 2008; Chen et al., 2016; Teh et al., 2016; Shao et al., 2017*). The caterpillar hatches eating the corion and forming its way out of the egg. *Brinkmann, Martens & Tebbe (2008)* reported that the *Enterococcus* spp. present in the gut of the larvae of *Manduca sexta* (Lepidoptera; Sphingidae) was acquired via ingestion of their eggshell, demonstrating the maternal transmission of microorganisms. Another target of the Lepidoptera studies regarding the microbiota is the butterfly considered agricultural pest *Spodoptera littoralis* (Lepidoptera; Noctuidae), and studies have describe the presence of enterococci in immature and adults of these insects: *Chen et al. (2016)* observed that most of the

bacteria found in the egg mass of *S. littoralis* were also found in caterpillars, and suggest that microorganisms present in the mass of eggs represent both a source of maternal origin as well as environmental bacteria. *Teh et al. (2016)* showed the route of transmission of *E. mundtii* in *S. littoralis* when administered *in vivo*; the authors reported the presence of *E. mundtii* in all stages of life of this insect, as well as established the presence of this bacteria in oocytes and the muscle tissue of the first-instar larvae of the second-generation offspring, highlighting again the vertical transmission of enterococci in this species of butterfly. The enterococci isolated in the present study may be linked to herbivory; however, despite our analysis do not include adult females and the bacterial communities of passionflower leaves, we do not discard that enterococci could also be transmitted from female to the offspring through the surface of eggs, as previously mentioned for other species of Lepidoptera.

Conclusions

In conclusion, *E. casseliflavus* was the dominate strains isolated in fecal samples of fifth instar *H. erato phyllis* caterpillars. Resistant strains present in samples of these insects could be related to environmental resistome and/or to anthropogenic activity; also the presence of MDR enterococci could also be an indication of environmental contamination with antibiotics. The results obtained by PFGE analysis suggest that the enterococci isolated from fecal samples of sibling caterpillars might have come from common sources, by diet (herbivory) and/or via vertical transmission (through egg surface). Further studies will be conducted to better understand the role of *Enterococcus* on the microbial GI tract community of these insects, and the mechanisms involved in acquisition and maintenance of these bacteria in *H. erato phyllis* butterflies. In addition, the data obtained can be used in future analyses of the microbiota present in adult females of *H. erato phyllis* compared to the microbiota of the offspring, to confirm the occurrence of vertical transmission of *Enterococcus* sp. in this model organism.

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Figure 1

Illustration of *Heliconius erato phyllis* butterflies and caterpillars used in the study.

Female HEAB2 and offspring (6, 7, 10, 11, and 14); female HEV2 and offspring (9, 18, 26, 27, and 29); female HES2 and offspring (3 and 17).

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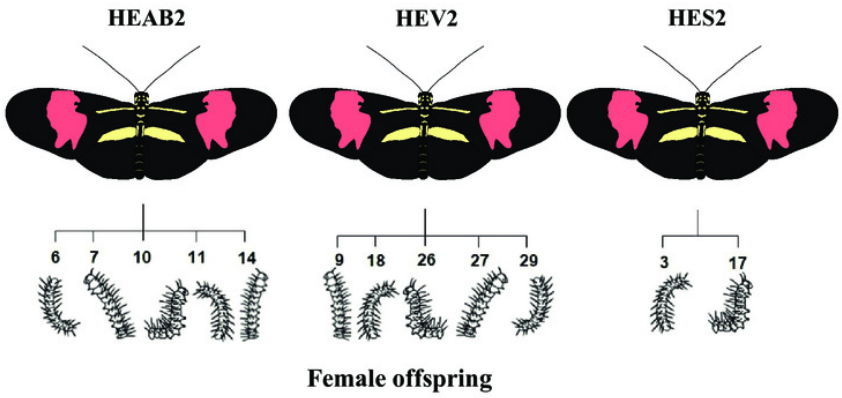

Figure 1: Illustration of *Heliconius erato phyllis* butterflies and caterpillars used in the study. Female HEAB2 and offspring (6, 7, 10, 11, and 14); female HEV2 and offspring (9, 18, 26, 27, and 29); female HES2 and offspring (3 and 17).

Figure 2

Dendrogram of enterococci isolated from fecal samples of *Heliconius erato phyllis* caterpillars.

HEAB2, *H. erato phyllis* from Águas Belas; HEV2, *H erato phyllis* from Viamão; HES2, *H. erato phyllis* from São Francisco de Paula. Antibiotics: RIF, rifampicin; ERY, erythromycin; NOR, norfloxacin; CIP, ciprofloxacin; S, susceptible; R, resistant.

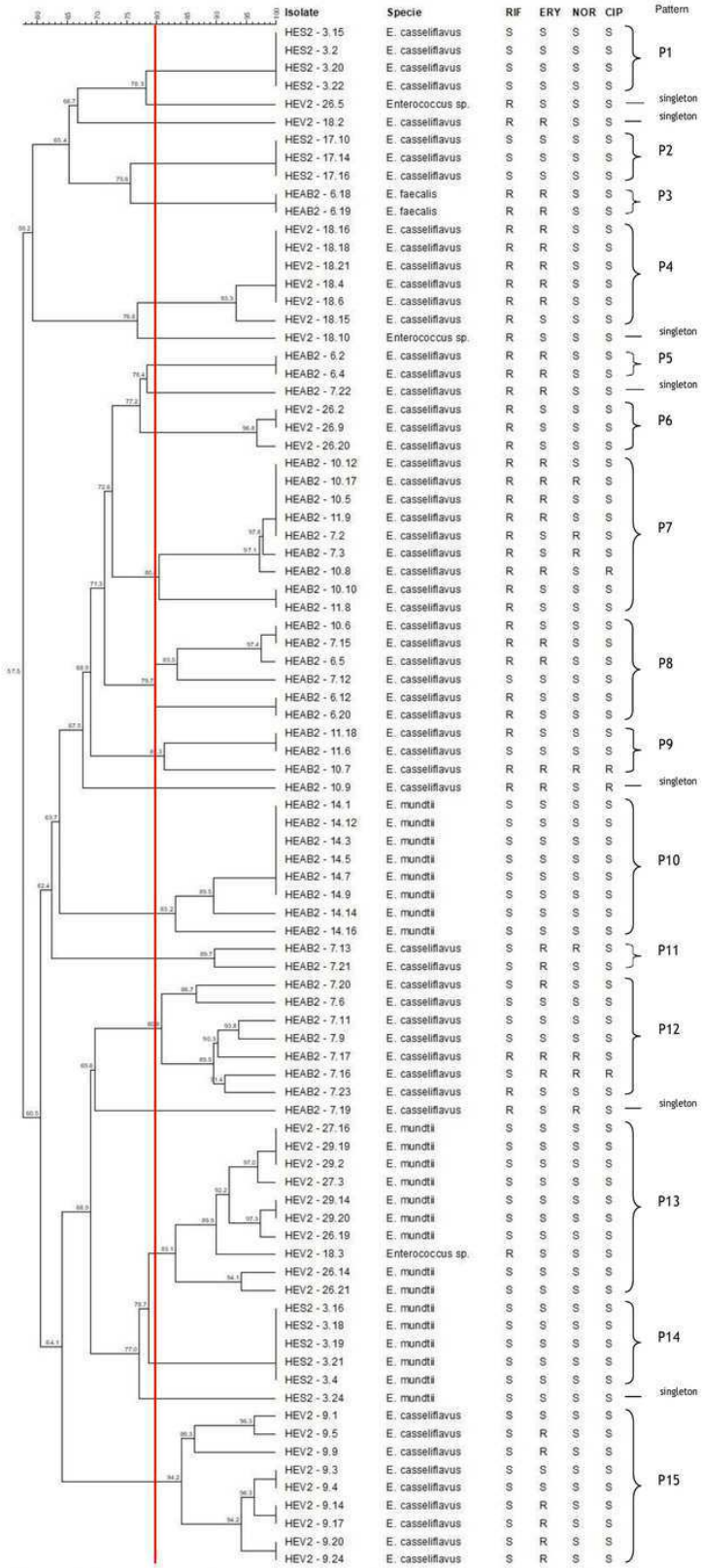

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Table 1(on next page)

Primers used in the PCRs carried out in this study

Table 1:
Primers used in the PCRs carried out in this study.

Primer	Nucleotide sequence (5'-3')	AT[1] (°C)	Amplicon (bp)	Reference
Genus				
tuf-F	TACTGACAAACCATTCATGATG	54	112	*Ke et al. (1999)*
tuf-R	AACTTCGTCACCAACGCGAAC			
E. faecalis				
E16s-F	CCGAGTGCTTGCACTCAATTGG	66	138	*Sedgley et al. (2005)*
E16s-R	CTCTTATGCCATGCGGCATAAAC			
E. faecium				
EM1A-F	TTGAGGGACACCAGATTGACG	62	658	*Cheng et al. (1997)*
EM1B-R	TATGACAGCGACTCCGATTCC			
E. casseliflavus				
CA1	TCCTGAATTAGGTGAAAAAAC	59	288	*Jackson, Fedorka-Cray & Barrett (2004)*
CA2	GCTAGTTTACCGTCTTTAACG			
E. mundtii				
MU1-F	CAGACATGGATGCTATTCCATCT	60	94	*Jackson, Fedorka-Cray & Barrett (2004)*
MU2-R	GCCATGATTTTCCAGAAGAATG			
16s rRNA				
8F	AGAGTTTGATCCTGGCTCAG	60	1514	*Coenye et al. (1999)*
1522R	AAGGAGGTGATCCAGCCGCA			
Erythromycin				
erm(B)_F	GAAAAGGTACTCAACCAAATA	52	639	*Sutcliffe, Tait-Kamradt & Wondrack (1996)*
erm(B)_R	AGTAACGGTACTTAAATTGTTTAC			
msrC_3	AAGGAATCCTTCTCTCTCCG	52	343	*Werner, Hildebrandt & Witte (2001)*
msrC_4	GTAAACAAAATCGTTCCCG			
Gelatinase				
gelE_TE9	ACCCCGTATCATTGGTTT	50	419	*Eaton & Gasson (2001)*
gelE_TE10	ACGCATTGCTTTTCCATC			
Cytolysin				
cylA_TE17	TGGATGATAGTGATAGGAAGT	59	517	*Eaton & Gasson (2001)*
cylA_TE18	TCTACAGTAAATCTTTCGTCA			

Adhesion				
ace1_F	AAAGTAGAATTAGATCCACAC	59	320	*Mannu et al. (2003)*
ace2_R	TCTATCACATTCGGTTGCG			
Biofilm				
ESP46	TTACCAAGATGGTTCTGTAGGCAC	60	1198	*Shankar et al. (1999)* *Schmidt (2009)*
ESP49	CTTTTTCTTTCCAAGTATACTTAG			
Aggregation				
agg_TE3	AAGAAAAAGAAGTAGACCAAC	60	1553	*Eaton & Gasson (2001)*
agg_TE4	AAACGGCAAGACAAGTAAATA			

3[1]AT, annealing temperature.

Table 2(on next page)

Distribution of *Enterococcus* sp. isolated from fecal samples of *Heliconius erato phyllis* caterpillars.

Table 2:

Distribution of *Enterococcus* sp. isolated from fecal samples of *Heliconius erato phyllis* caterpillars.

Species	Number (%) of enterococci isolated of caterpillars from[1]:			
	HEAB2	**HEV2**	**HES2**	**Total (%)**
E. faecalis	2 (2.04)	0	0	**2 (1.12)**
E. casseliflavus	83 (84.69)	42 (65.62)	7 (43.75)	**132 (74.15)**
E. mundtii	13 (13.26)	19 (29.68)	6 (37.50)	**38 (21.34)**
Enterococcus sp.	0	3 (4.68)	3 (18.75)	**6 (3.37)**
Total	**98 (100)**	**64 (100)**	**16 (100)**	**178 (100)**

[1]HEAB2, female from Águas Belas; HEV2, female from Viamão; HES2, female from São Francisco de Paula.

Table 3(on next page)

Antibiotic resistance profiles in enterococci isolated from fecal samples of *Heliconius erato phyllis* caterpillars

Table 3:

Antibiotic resistance profiles in enterococci isolated from fecal samples of *Heliconius erato phyllis* caterpillars.

Female[1]	Species (n)	Number (%) of resistant strains[2]				Profiles[3]		
		ERY	CIP	NOR	RIF	SR	DR	MDR
HEAB2	*E. faecalis* (2)	2 (100)	0	0	2 (100)	0	2 (100)	0
	E. casseliflavus (83)	25 (30)	4 (5)	8 (10)	70 (84)	48 (58)	21 (25)	5 (6)
	E. mundtii (13)	0	0	0	0	0	0	0
HEV2	*E. casseliflavus* (42)	27 (64)	0	0	23 (55)	28 (67)	11 (26)	0
	E. mundtii (19)	0	0	0	0	0	0	0
	Enterococcus sp. (3)	0	0	0	3 (100)	3 (100)	0	0
HES2	*E. casseliflavus* (7)	0	0	0	0	0	0	0
	E. mundtii (6)	0	0	0	0	0	0	0
	Enterococcus sp. (3)	1 (33)	1 (33)	0	2 (67)	1 (33)	0	1 (33)
Total (178)		**55 (31)**	**5 (3)**	**8 (4)**	**100 (56)**	**80 (45)**	**34 (19)**	**6 (3)**

[1]HEAB2, female from Águas Belas; HEV2, female from Viamão; HES2, female from São Francisco de Paula. [2]Antibiotics: ERY, erythromycin; CIP, ciprofloxacin; NOR, norfloxacin; RIF, rifampicin. [3]Profiles: SR, single resistant; DR, double resistant; MDR, multidrug resistant.