Characterization and Comparison of Intestinal Bacterial Microbiomes of Euschistus Heros and Piezodorus Guildinii Collected in Brazil and the United States

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Abstract

Background: Herbaceous insects are one of the main biological threats to crops. One such group of insects, stink bugs, do not eat large amounts of tissue when feeding on soybean, but are extremely damaging to the quality of the seed yield as they feed directly on green developing seeds leading to poorly marketable harvests. In addition to causing physical damage to the seed during feeding, the insects can also transmit microbial pathogens, leading to even greater yield loss. Conducting surveys of the insect intestinal microbiome can help identify possible pathogens, as well as detail what healthy stink bug digestive systems have in common.

Methods: We used the conserved V4 515-806 region of the 16S rRNA gene to characterize the bacterial microbiome of the red-banded stink bug *Piezodorus guildinii* collected in Brazil and the United States, as well as the neotropical brown stink bug *Euschistus heros* collected in Brazil.

Results: After quality filtering of the data, 192 samples were kept for analyses: 117 samples from *P. guildinii* covering three sites in Brazil and four sites in the US, and 75 samples for *E. heros* covering 10 sites in Brazil. The most interesting observations were that the diversity and abundance of some bacterial families were different in the different ecoregions of Brazil and the United States.

Conclusions: Some families may be related to the better adaptation in some localities in provide nutrients, break down cellulose, detoxify phytochemicals, and degrade organic compounds, which makes it difficult to control these species.

Introduction

One of the largest threats to crops are insects, either directly through their feeding or indirectly via pathogen transmission [1–3]. An example of important crop-damaging insects is the stink bugs. This group of insects is classified in the order Hemiptera and feed by piercing their rostrum-encased stylets into the tissue of their plant host [4], which can cause direct damage, inject toxic substances, or transmit pathogens, reducing host tissue health [1, 5–8]. Stink bugs like to feed on legumes such as soybean (Glycine max), common bean (*Phaseolus vulgaris* L.), peas (*Pisum sativum* L.), and alfalfa (*Medicago sativa* L.), in addition to multiple alternative host plants that might be found within or near an agricultural field [9]. The attack on legume crops can be very damaging as the insects prefer feeding on the nutrient-rich developing seed. The rich food source and diversity of food options will influence the gut microbiome, which in turn can affect the health of the insects, as well as how likely they transmit pathogens to the plant host [10].

The bacterial community present in the digestive tract of most insect species varies in population size, composition, location, and function within the gut. Most of the microorganisms that have entered into the gut lumen transiently pass through or are eliminated by the host immune system, but some of them remain longer and proliferate in the healthy host gut lumen. The wide range of insect associations with intestinal microorganisms is illustrated within Hemiptera [11, 12].

The microbiota of an insect can be strictly hereditary, allowing nutrient supply to the host who lives in a restricted environment [11]. In stink bugs, the gut microbiome can be divided into three categories. One class would be the vertically transmitted core taxa, that coevolve with the host and are important for basic host functions, such as developmental programs, or essential adaptations (e.g., herbivory). Another group are members of the flexible microbial pool, which could be acquired through horizontal transmission and exchanged with the environment, but are better adapted for host colonization than environmental strains, and can facilitate host adaptation to new niches or environmental conditions. And lastly, there are the environmentally acquired microbes, which are advantageous in facilitating host adaptation to new conditions but are not adapted to the host [13].

Endosymbiont-bearing stink bugs have special crypts in the terminal region of their midgut that is filled with extracellular symbiotic bacteria. These bacteria play an important role in stink bug health, as stink bugs without the endosymbionts have been shown to have retarded growth and increased mortality and sterility [14]. Insect intestinal microbes have the ability to metabolize nutrients, produce secondary metabolites, and interact with the host immune system. Prolonged interaction with a specific member of the gut microbiota may have an impact on a diverse range of host physiologies [11, 12]. Stink bugs acquire beneficial gut symbionts deposited on eggs every generation, which resides in crypts of the intestinal tract [8, 15]. A mini-review on the genomic architecture and content of stink bug bacterial symbionts have been published by Otero-Bravo [16].

The stink bug *Riptortus pedestris* takes part in a mutualistic symbiosis with members of the *Burkholderia* genus, which are acquired from the soil during larval development. This genus is associated with insecticide resistance and was found in treated fields which showed enrichment for insecticide-degrading *Burkholderia* [17]. Similar to *R. pedestris*, the intestinal symbiotic bacteria *Citrobacter freundii* enhances the fruit flies' resistance to trichlorphon insecticide [18].

In addition to containing beneficial microbes, the stink bug intestinal track may also contain pathogens that could be passed to their host plant [19]. Conducting surveys of the insect intestinal microbiome can help identify possible pathogens, as well as detail what healthy stink bug digestive systems have in common. A study on bacteria associated with *Piezodorus guildinii* included isolating those that can or cannot be transmitted by feeding, via the sequencing of PCR products amplified from the V4 region of the 16S rRNA gene [20]. Of the 19 *P. guildinii* screened for the ability to release microbes while feeding, only 12 transmitted a culturable microbe, and seven out 12 transmitted only one that grew, with the maximum transmitted by a single insect being four. Somewhat similar results were reported in a study of bacteria transmitted by *N. viridula* [21]. The sequencing of the V4 region of the 16S rRNA gene from *P. guildinii* gut DNA [20] identified 51 putative bacteria species, including several potential plant pathogens.

The work presented here focuses on the gut microbiome of two stink bugs that are major pests of soybean, the red-banded stink bug (*P. guildinii*) collected in Brazil and the US, and the neotropical brown stink bug (*Euschistus heros*) collected in Brazil. These pests normally colonize pre-flowering soybean and peak infestations are seen at the end of grain filling. They feed by inserting their stylets into the pods,

directly reaching the seed [22–25]. According to Guedes et al. [26], one stink bug per square meter can cause losses of 125 $kg.ha^{-1}$, and losses can reach US$3.06 billion [27, 28]. Here, we hypothesize that the intestinal microbiome between species and within species diverge and this allows the best adaptation of some individuals. To elucidate this hypothesis, we characterized and compared the intestinal microbiome of these two species collected from different locations in Brazil and the United States [29], and investigated possible microbial families associated with the adaptation of these stink bugs to environments modified by agricultural management.

Material And Methods

Insect collection and dissection

Insects were collected in soybean fields in Brazil and the United States and stored in falcon tubes with 70% ethanol at -20ºC (Supplemental Table 1). Insects were dissected by removing heads and digestive tracts (including fore-, mid-, and hind guts with attached Malpighian tubes) using sterile forceps and storing them in 1.5 ml microfuge tubes at -20ºC until used for DNA extraction.

DNA extraction

For bacterial DNA extraction, a modified CTAB method was used [33] as follows. The digestive tracts samples were placed in 1.5 mL microtubes containing 350 µL 2% CTAB extraction buffer [20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB, plus 0.4% b-mercaptoethanol added just before use], 10 µL proteinase k, and 5 stainless steel 2.8 mm beads. An additional 350 µL of 2% CTAB was added per tube and the solution incubated at 65ºC for 60 min, and gently mixed by inversion every 15 min to macerate. Next, 600 µL chloroform-isoamylalcohol (24:1) was added and the tubes were gently mixed for 1 min, followed by centrifugation for 15 min at 10,000 rpm. Immediately after centrifuging, 600 µL of the supernatant of each tube was transferred to a fresh tube with 350 µL cold isopropanol (-20ºC). Samples were gently mixed by inversion and held at -80ºC for 60 min, followed by centrifugation at 14,000 rpm for 10 min. After centrifugation, it was possible to visualize the DNA at the bottom of each tube. The supernatants were removed, and the DNA pellets were washed with 1000 µL of 70% ethanol, centrifuged at 14000 rpm for 5 min. The ethanol was discarded and 500 µL 100% ethanol was added, centrifuged at 14000 rpm for 10 min, the ethanol discarded, and the tubes were set to dry for at least 3 min in a sterile cabinet with the tubes inverted over a filter paper at room temperature. The DNA pellets were suspended in 50 µL TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA pH 7.6) plus 2 µL ribonuclease (RNAse 20 mg/mL), incubated at 37ºC for 1 h, and stored at -20ºC [30].

PCR amplification and sequencing

To fully characterize the microbiome at the *Bacteria* Domain, the PCR primer pair V4_515F_New (5'-GTGYCAGCMGCCGCGGTAA) and V4_806R_New (5'-GGACTACNVGGGTWTCTAAT) was used for amplifying the V4 515–806 region of the bacterial 16S rRNA, resulting in an amplicon length of 292 bp. PCR reactions were performed on a high-throughput Fluidigm PCR platform (Biomark HD) at the Roy J.

Carver Biotechnology Center, the University of Illinois following the procedure outlined by Muturi et al. 2017. DNA samples were diluted to 2 ng/µl prior to amplification and processed with the Roche High Fidelity Fast Start Kit and 20X Access Array loading reagent according to Fluidigm protocols. To generate final PCR amplicons prepared for subsequent Illumina sequencing, two sets of primers were utilized simultaneously in one reaction. The first primer set had the Fluidigm-specific primers CS1 (5'-ACACTGACGACATGGTTCTACA) and CS2 (5'-TACGGTAGCAGAGACTTGGTCT) added to the 5' end of all the ribosomal-specific primers mentioned above. The second primer set contained the same Fluidigm-specific primers attached to the Illumina i5 primer (5'-AATGATACGGCGACCACCGAGATCT) and barcoded i7 primer (5'-CAAGCAGAAGACGGCATACGAGAT-XXXXXXXXXX). All primers were synthesized by IDT Corp. (Coralville, IA). The mastermix was aliquoted into 48 wells of a PCR plate. To each well, 1 µl DNA sample and 1 µl Fluidigm Illumina linkers with unique barcodes were added. On a separate plate, primer pairs were prepared and aliquoted. 20X primer solutions were prepared by adding 2 µl of each forward and reverse primer (50 µM), 5 µl of 20X Access Array Loading Reagent, and water to a final volume of 100 µl. The final primer concentration in the reactions was 50 nM each. Samples (4 µl each) were loaded in the sample inlets and 4 µl of primer loaded in primer inlets of a previously primed Fluidigm 48.48 Access Array IFC. The IFC was placed in The Juno microfluidic machine (Fluidigm Corp.) for the loading of all primer/sample combinations, amplification, and harvest. All samples were run on a Fragment Analyzer (Advanced Analytics, Ames, IA), and amplicon regions and expected sizes confirmed. Samples were then pooled in equal amounts according to product concentrations. The pooled products were size selected on a 2% agarose E-gel (Life Technologies) and extracted from the isolated gel slices with a Qiagen Gel Extraction kit (Qiagen) using a Qiacube robot. Cleaned, size-selected products were run on an Agilent Bioanalyzer to confirm appropriate profiles and determination of average sizes.

The PCR amplicons for the 288 samples (286 insects and 2 water negative control) were sequenced on two MiSeq flow cells of 301 cycles from each end of the fragments using a MiSeq 600-cycle sequencing kit version 3 at the Roy J. Carver Biotechnology Center, University of Illinois. Read length was 300 nt. The resulting fastq files were demultiplexed with the bcl2fastq v2.17.1.14 Conversion Software (Illumina).

Sequence data processing

For 288 sequence libraries of the bacterial 16S rRNA gene V4 region, the software Cutadapt v1.12 was used to trim specific V4 primer sequences on both 5' and 3' ends of the reads[31]. Since the primers for amplifying this region contained ambiguous positions, the option -match-read-wildcards was set to 'on'. Adapter-trimmed paired-end reads were imported into software Pear v0.9.5 [32] that merged sequences into single fragments using overlapping regions to correct sequencing errors and yield higher quality. Reverse complement sequences of merged fragments were created with an in-house script.

The software IM-TORNADO v2.0.3.2 was used to do quality filtering and taxonomy assignment for processed sequences generated by the V4 regions above [33]. The Trimmomatic program was implemented by IM-TORNADO to do quality trimming, with a hard cutoff of a PHRED score of Q3 at ends of reads (LEADING: 3 and TRAILING: 3), a 4-base average score cutoff of Q15 (SLIDING WINDOW: 4:15) and a minimum read length cutoff of 75% of the original read length. For the taxonomy assignment,

reads from the bacterial V4 region were aligned against the Ribosomal Database Project 10 (RDP10) [34]. Three BIOM files were generated separately by forward-reads, reverse-reads, and paired-reads for the region, containing the OTUs and counts corresponding to each OTU in each sample; forward-read BIOM files were used for downstream analyses. Quality filtering was performed to remove low-quality OTUs and samples using QIIME-v1.9.1 [35].

Data analysis

To compare the bacterial community structure and composition among the samples we used the Non-metric Multidimensional Scaling (NMDS) using Bray-Curtis as a distance metric, and the Phyloseq [36] package in R. To test whether the samples harbored significantly different bacterial community compositions, permutational multivariate analysis of variance (PERMANOVA) [37] was performed with the software PAST 3 [38].

Diversity measurements were calculated using the Phyloseq package in R and included alpha diversity (Shannon index) and richness (number of observed OTUs, and Chao1 richness estimator). The Kruskal Wallis non-parametric test was used to compare multiple groups and the pairwise Wilcoxon non-parametric test was used to compare samples grouped in ecoregions. Further, to determine the differential abundance of the top ten bacterial families between the ecoregions, we performed the Wilcoxon nonparametric parameter test for each bacterial family used.

Results

Sequences and samples quality control

For the 286 stink bug PCR samples of the bacterial 16S rRNA V4 sequence, the Illumina MiSeq sequencing produced approximately 7.5 million reads in one lane, and the mean number of raw reads ranges from about 7,000 to 53,000 per sample for different locations. Reads filtering procedures including merging reads and trimming low-quality bases removed large percentages of reads from most locations, with 33%-73% reads remaining, but the stringency of filtering left high-quality sequence data for subsequent analyses (Supplemental Table 2). After OTU identification, low-quality samples were filtered out for downstream analysis, leaving a total of 192 samples out of 286, with 75 samples from *E. heros* collected from 10 sites in Brazil and 117 samples from *P. guildinii* collected from three sites in Brazil and four sites in the US (Supplemental Table 2).

The sufficiency of sequencing coverage was evaluated by alpha diversity and rarefaction analyses. The depth of sequencing was sufficient as shown by *P. guildinii* rarefaction plot that began to plateau by 1000–1500 reads per sample (Supplemental Fig. 1B). Similarly, the *E. heros* rarefaction plot also began to plateau by 1000–1500 reads per sample for most locations, although the Teresina and Uberlandia locations might have benefitted from additional sequencing as those plotlines still had a slightly positive slope at 1500.

Microbiome characterization of Euschistus heros

Comparing the different collection sites of *E. heros* using the non-metric multidimensional scaling (NMDS) analysis, the bacterial communities present in the gut of these stink bugs clustered according to the following ecoregions of Brazil: tropical dry forests, tropical moist forests, and tropical savanna (Fig. 1A and B, PERMANOVA P = 0.0001). Therefore, subsequent analyses were based on these ecoregions.

Three metrics were used to estimate the alpha diversity of the microbiome among all the stink bug samples, i.e. the number of observed OTUs, Chao1, and Shannon. The species richness, based on the number of observed OTUs, is different among the ecoregions (p-value = 0.015). *E. heros* collected in tropical dry forests (p-value = 0.0059) and tropical savanna (p-value = 0.034) had significantly higher observed OTUs than those collected in tropical moist forests. This result was confirmed by the richness estimator Chao1 (p-value = 0.0004). *E. heros* collected in sites from tropical dry forests (p-value = 3.8e-05) and Tropical Savanna (p-value = 0.012) had more richness of species than *E. heros* collected in tropical moist forests. However, Shannon's diversity, an index that takes into account both abundance and evenness of the species present, did not show significant difference among ecoregions. A noticeable observation would be that the *E. heros* microbiome from tropical dry forest showed the highest richness of species (OTUs and Chao1) but the lowest Shannon diversity index, indicative of low evenness/equitability of species within this bacterial community (Fig. 1C).

The most abundant bacterial families in the intestinal microbiome of *E. heros* collected in tropical dry forests were Bradyrhizobiaceae, Enterobacteriaceae, and Sphingomonadaceae. Additionally, *E. heros* from this ecoregion contained the lowest number of bacterial families. The most abundant bacterial families identified from insects collected in the tropical moist forests region were: Acetobacteraceae, Bacillaceae, Bradyrhizobiaceae, Enterobacteriaceae, Enterococcaceae, Hyphomicrobiaceae, Methylobacteriaceae, Polyangiaceae, and Sphingomonadaceae. Among these families, the Acetobacteraceae and Bacillaceae were unique to insects sampled from the tropical moist forests. From the tropical savanna insects, we identified the top bacteria families being the Bradyrhizobiaceae, Enterobacteriaceae, Enterococcaceae, Hyphomicrobiaceae, Methylobacteriaceae, Moraxellaceae, Polyangiaceae, and Sphingomonadaceae (Fig. 1D).

Comparing the bacterial families across ecoregions, the Bradyrhizobiaceae, Enterobacteriaceae, and Sphingomonadaceae were found in the intestinal microbiome of insects from all three regions. However, striking differences in abundances could be observed for the Bradyrhizobiaceae and the Enterobacteriaceae. More specifically, stink bugs from the tropical moist forests had a significantly higher mean proportion of the Bradyrhizobiaceae than dry forests (p-value = 0.012) and savanna (p-value = 0.016) (Fig. 1E). Although Enterobacteriaceae was the most abundant family in stink bugs from all three ecoregions (Fig. 1D), it was more abundant in the tropical dry forests, followed by the tropical savanna and moist forests (Fig. 1E). The Moraxellaceae only existed abundantly in stink bugs from tropical

savanna (~ 20%) whereas in the other two ecoregions they were not detected, or present at a very low level.

Recent discoveries indicated that *E.* heros from the north and the south of Brazil were composed of two distinguished lineages. With the expansion of soybean in the Brazilian Cerrado, these two lineages could meet and produce hybrids more adapted to the climates and even to new hosts, such as cotton [39, 40]. Thus, we performed analyses to investigate the relationships between the stink bug intestinal microbiomes and the proposed lineages of *E. heros*: north (Teresina/PI, Palmeirante/TO), south (Piracicaba/SP, Anhembi/SP and Ponta Grossa/PR), and hybrids (the other localities). The NMDS analysis showed that the bacterial species present in our samples clustered significantly by stink bug lineages (PERMANOVA p-value = 0.0001) (Supplemental Fig. 2A). The species richness, based on Observed OTU and Chao1 index, is higher in samples from the north lineage than the south lineage, and the hybrids have an intermediate species richness. The species diversity, based on the Shannon index, is bigger on the north and hybrid lineages than the south lineage of *E. heros* (Supplemental Fig. 2B).

Analyzing the relative abundance of the 10 most abundant bacterial families in each lineage, we can see that the hybrid lineages had more bacterial families than the north and south lineages and the proportion of these families present in the gut of the hybrids lineages are more evenly distributed than in pure lineages (Supplemental Fig. 2C). Furthermore, the family Bacillaceae is unique in the hybrids lineages and the Acetobacteraceae family is unique in the south lineage.

Comparing the bacteria families among lineages, the family Bradyrhizobiaceae was the most abundant in *E. heros* from hybrids lineages than either of the pure lineages. The family Hyphomicrobiacea was the most abundant in *E. heros* from hybrid lineages than north lineages. The families Moraxellaceae and Sphingomonadaceas were the most abundant in hybrid lineages than south lineages and the Enterobacteriaceae family was the most abundant in both pure lineage than hybrids (Supplemental Fig. 3).

Microbiome characterization of Piezodorus guildinii

The comparison of the different collection sites of *P. guildinii* by NMDS analysis (Fig. 2B) revealed that the bacterial species present in the gut of these stink bugs also clustered (p-value of 0.0001) according to the ecoregions of Brazil and the United States: temperate broadleaf forests, temperate conifer forests, temperate grasslands, and tropical savanna (Fig. 2A). Therefore, subsequent analyses were based on these ecoregions.

The bacterial species diversity across ecoregions were significantly different based on the alpha diversity index, the number of OTUs, and Chao1 index (p-value = 0.0072 and p-value = 0.0035, respectively) (Fig. 2C). On average, *P. guildinii* collected in temperate grasslands had more bacterial species than *P. guildinii* collected in the other ecoregions. Considering the richness estimator Chao1, the values were also significantly different (p-value = 0.0035). *P. guildinii* from tropical savanna were richer in species than insects from temperate broadleaf forests (p-value = 0.0025). The Shannon's diversity showed a

significant difference among ecoregions (p-value = 0.018). *P. guildinii* from temperate conifer forests (p-value = 0.015) and tropical savanna (p-value = 0.044) had higher equitability than temperate broadleaf forests, confirming the lowest species diversity from insects of temperate broadleaf forests at Shannon index (Fig. 2C).

When focusing on the microbial abundance of *P. guildinni* at the family level, *P. guildinii* from temperate broadleaf forests had mostly gut microbiota bacteria of the families Acetobacteraceae, Aurantimonadaceae, Bradyrhizobiaceae, Enterobacteriaceae, Enterococcaceae, Moraxellaceae, Rhodocyclaceae, Sinobacteraceae, and Sphingomonadaceae. Among these families, the Sinobacteraceae family was found only in insects from temperate broadleaf forests and it existed at a low proportion (Fig. 2D). Six major families Acetobacteraceae, Aurantimonadaceae, Bradyrhizobiaceae, Enterobacteriaceae, Moraxellaceae, and Sphingomonadaceae were found in the insect guts from temperate conifer forests and one major family, Enterobacteriaceae, was found in the intestinal microbiome of insects from temperate grasslands forests. The insects collected in tropical savanna had in the intestinal microbiome the families Acetobacteraceae, Aurantimonadaceae, Bradyrhizobiaceae, Deinococcaceae, Enterobacteriaceae, Enterococcaceae, Moraxellaceae, Rhodocyclaceae, and Sphingomonadaceae. The Rhodocyclaceae family was found only in insects from this ecoregion and temperate broadleaf forests in low proportion (Fig. 2D).

Comparing the bacterial families present in the intestine of *P. guildinii* insects shared by more than one ecoregions, the Acetobacteraceae family was significantly more abundant in insect guts from temperate broadleaf forests than insect guts from tropical savanna with a p-value at 0.044. The Aurantimonadaceae was more abundant in *P. guildinii* from temperate conifer forests than temperate broadleaf forest and tropical savanna (p-value 0.015 and 0.029). The Bradyrhidobiaceae family was more abundant in *P. guildinii* from temperate broadleaf forests than tropical savanna. Lastly, the Enterobacteriaceae family, as a major component in all four ecoregions, exhibited a lower proportion in temperate conifer forests than the other three ecoregions, but the difference did not reach statistical significance either, due to large variation (Fig. 2E).

Since the species *P. guildinii* co-occurred in both Brazil and the US, we further explored the association between its intestinal microbiome and the country of origin. The NMDS analysis (Fig. 3A) showed distinct groups of bacterial species in insects from Brazil and the US with a p-value of 0.0107. According to the number of observed OTU (p-value = 0.015) and the richness estimator Chao1 (p-value = 0.0087), *P. guildinii* sampled from Brazil were richer in intestinal bacterial species than insects from the US (Fig. 3B).

The most abundant bacterial families in *P. guildinii* collected in Brazil were Acetobacteraceae, Aurantimonadaceae, Bradyrhizobiaceae, Deinococcaceae, Enterobacteriaceae, Enterococcaceae, Moraxellaceae, Rhodocyclaceae, and Sphingomonadaceae. In comparison, the most abundant bacterial families in *P. guildinii* collected in the United States were Acetobacteraceae, Aurantimonadaceae, Bradyrhizobiaceae, Enterobacteriaceae, Enterococcaceae, Moraxellaceae, Rhodocyclaceae, Sinobacteraceae, and Sphingomonadaceae (Fig. 3C). However, the Deinococcaceae family occurred only

in stink bugs collected from Brazil, and the Sinobacteraceae occurred only in insects collected from the US (Fig. 3C). The families Acetobacteraceae, Bradyrhizobiaceae, and Moraxellaceae were significantly more abundant in *P. guildinii* from the United States than Brazil, and the family Rhodocyclaceae was more abundant from Brazilian insects (Supplemental Fig. 4). Enterobacteriaceae, the most abundant bacterial family in both countries, constituted about 80% of the gut microbiome of both countries and showed no significant difference (Fig. 3C; Supplemental Fig. 4).

Analysis of E. heros **and** P. guildinii **combined**

We further combined the microbiome data of *E. heros* and *P. guildinii* and clustered samples by ecoregions. With PERMANOVA, the bacterial species present in stink bugs displayed distinct compositions by different ecoregions with a significant p-value at 0.0001(Fig. 4B). As seen in the NMDS analysis (Fig. 4B), samples from tropical dry forests clearly grouped together and were separated from samples from tropical moist forests and tropical savanna. Therefore, subsequent analyzes were based on ecoregions.

According to the number of observed OTUs, we found that stink bugs from tropical dry forests contained more abundant bacterial species than stink bugs from the tropical moist forests (p-value = 0.0059) and tropical savanna (p-value = 0.041). The same occurs when the richness estimator Chao1 is compared (p-value = 3.8e-05 and p-value = 0.0042, respectively). Lastly, Shannon's index showed that the species diversity of all three ecoregions did not differ significantly from each (Fig. 4C).

The most abundant bacterial families present in the digestive tracts of *E. heros* and *P. guildinii* in tropical dry forests were Bradyrhizobiaceae, Enterobacteriaceae, and Sphingomonadaceae, with Enterobacteriaceae comprising more than 80% of the bacterial community. In turn, the abundant families in insects from tropical moist forests were Acetobacteraceae, Bacillaceae, Bradyrhizobiaceae, Enterobacteriaceae Enterococcaceae, Hyphomicrobiaceae, Rhodocyclaceae, and Sphingomonadaceae (Fig. 4D). Insects from tropical savanna had gut bacteria of the families Acetobacteraceae, Bacillaceae, Bradyrhizobiaceae, Deinococcaceae, Enterobacteriaceae Enterococcaceae, Hyphomicrobiaceae, Moraxellaceae, Rhodocyclaceae, and Sphingomonadaceae. The families Deinococcaceae and Moraxellaceae only existed in the tropical savanna group (Fig. 4D). Comparing the bacterial families present in two or more ecoregions, the Bradyrhizobiaceae family was significantly more abundant in insects from tropical moist forests than tropical dry forests and tropical savanna. Enterobacteriaceae family was most abundant in insects from tropical dry forests, followed by insects from tropical savanna (Supplemental Fig. 5).

Comparing between the species of stink bugs *E. heros* and *P. guildinii*, the NMDS analysis showed that these pentatomids had different bacterial species in their intestines, which can be separated into two groups with a p-value equal to 0.0001 (Fig. 5A). Analyzing the diversity indexes, the number of observed OTUs showed that *E. heros* had more bacterial species than *P. guildinii*. Based on Shannon's index, the bacterial community in the gut of *E. heros* had significantly higher diversity than *P. guildinii*, which also suggests a relatively even distribution of species in the *E. heros* intestines (Fig. 5B).

The composition of bacterial communities in the two stink bugs was similar at the family level (Fig. 5C). Enterobacteriaceae was the major family and constitute more than 50% of the bacterial species in both stink bugs. The two other major bacterial families shared by the two stink bug species were Bradyrhizobiaceae and Moraxellaceae. However, differences can be observed as well. Hyphomicrobiaceae occurred only in *E. heros* and Deinococcaceae occurred only in *P. guildinii* (Supplemental Fig. 6).

The phylogenetic relationship of the OTUs from the top 10 most abundant bacterial families was identified to understand their distribution (Fig. 6). Analyzing the OTUs among *P. guildinii* from Brazil and the United States, 13 unique OTUs belonged only to Brazil and 19 unique OTUs belonged only to the United States. Most OTUs were not related among countries. The families Enterococcaceae (2 OTUs), Acetobacteraceae (1 OTU), Enterobacteriaceae (15 OTUs), Aurantimonadaceae (1 OTU), and Bradyrhizobiaceae (1 OTU) belonged to *P. guildinii* from both countries. This difference may be related to bacterial diversity among environments. On the other hand, comparing the OTU from the ten most abundant bacterial families among *E. heros* and *P. guildinii* from Brazil, 16 unique OTUs belong only to *E. heros* and 7 unique OTUs belong only to *P. guildinii.* The families Enterococcaceae (2 OTUs), Bacillaceae (2 OTUs), Acetobacteraceae (1 OTU), Rhodocyclaceae (2 OTUs), Moraxellaceae (3 OTUs), Enterobacteriaceae (12 OTUs), Sphyngomonadaceae (7 OTUs), and Bradyrhizobiaceae (3 OTUs) belonged to both stink bug species. This difference is most interesting, *E. heros* had twice the number of unique bacteria than *P. guildinii*, which may be favoring the adaptation of this species over the other since it is the most difficult to control today (Fig. 6).

Discussion

This study aimed to evaluate the insect intestinal microbiome, seeking to describe the structure and composition among different regions. Our results revealed a higher bacterial species richness in the stink bug intestinal tract from dry regions in *E. heros* and temperate grasslands in *P. guildinii*. Comparing the *P. guildinii* intestinal microbiome in both countries, we observed a higher bacterial species richness in stink bugs collected in Brazil than in the US. On the other hand, comparing both species in Brazil, *E. heros* was richer in bacterial species than *P. guildinii*.

Microbiome structure and diversity

Comparing the bacterial species richness indices (number of observed OTUs and chao1) by ecoregion, *E. heros* had higher species richness in tropical dry forests, followed by tropical savanna and tropical moist forests. *P. guildinii* had higher species richness in temperate grasslands than temperate conifer forests, tropical savanna, and temperate broadleaf forests. Comparing the two stink bug species in Brazil, individuals from tropical dry forests had the highest average species richness, followed by tropical savanna and tropical moist forests.

These differences can be explained mainly by species behavioral, environmental, and genetic factors. *E. heros* and *P. guildinii* can feed on different food sources, including native and weed plants. This feeding pattern allows individuals to frequently transfer to various areas throughout their development [9]. According to Engel and Moran [11], the microorganisms present in the insect gut can be acquired in each generation from the outside environment, such as from soil, and some authors studying the diversity and biogeography of soil bacterial communities found that soil bacterial diversity varied across the ecosystem types. For example, soils from the dry forest and dry grasslands were more diverse than soils from the humid temperate forest and tropical forests [41]. Our results showed that *E. heros* had higher species richness in tropical dry forests than other ecoregions, which might support the previous finding that the intestinal bacterial diversity could be affected by environmental factors.

Once acquired from the environment, the microorganism goes through a series of physicochemical conditions of gut compartments, such as pH, redox potential, and availability of particular substrates, that can be selective for particular species [11]. In many insects, gut bacterial communities vary among individuals within a species and appear to consist largely of bacteria not specifically adapted to living in the guts of their host species [11]. Corroborating our study that the insects from different locations and host plants have a variable gut microbiota, Priya et al. [42], found highly variable gut communities in field-collected *Helicoverpa armigera* from different locations with the influence of host plant.

The main explanation for the higher microbiome diversity in tropical dry forests could be that the major regions of soybean production in Brazil are areas from tropical savanna and tropical moist forests [43]. Thereby, the indiscriminate use of phytosanitary products and the large-scale in these ecoregions can affect the insect's gut microbiome and even soil microbiome. A previous study on the gut microbiota composition of honey bees indicated that the neonicotinoids pesticides exposition may influence dominant honey bee gut bacteria [44]. As stated earlier, stink bugs can feed on native plants. Tropical dry forests, due to the less intensive cultivation of soybeans than other ecoregions, may have greater availability of native plants to feed these insects.

Analyzing the species *E. heros* separated by northern and southern lineages, individuals of the northern lineage present higher species richness, followed by individuals of the hybrid and southern lineage. An explanation would be that individuals of the northern lineage (specialized in cotton) have genetic characteristics that when expressed allow the establishment of a greater number of bacterial species when compared to the southern lineage (specialized in soy) [39, 40]. Thus, the hybrids of these two lineages present intermediate species richness.

We found that *P. guildinii* displayed a higher intestinal bacterial diversity from Brazil than the US, which might be explained mainly by genetic and environmental factors. For genetic factors, Zucchi et al. [45] evidenced distinct *P. guildinii* lineages from Brazil and the United States using Genotype-By-Sequencing. These genetic differences in stink bug lineages might account for the differences in the intestinal microbiome. More specifically, this genetic difference may be changing the physicochemical conditions of the intestine, thus changing the microbial composition of the different lineages. For environmental

factors, the discrepancy of climatic conditions of each country could be relevant because the performance of seed-sucking heteropterans is affected by a variety of abiotic factors. For example, the high temperature could increase food ingestion, and consequently, the bacteria from the environment can be ingested on a larger scale [46].

Tropical areas feature broad offerings of resources to bacteria species due to a higher diversity of environment and microclimates. Other pentatomids such as *E. heros*, enter in reproductive diapause under a photoperiod of 12 hours or less, reducing feeding activity [47]. Collections in Brazil were made at latitudes below 25º, while in the United States they were made at latitudes above 30º. This difference means that in winter Brazil has more hours of light than the United States, possibly resulting in greater food activity of stink bugs in Brazil and thus affecting the diversity of the intestinal microbiota. The bacterial diversity found in the gut of insects in different ecoregions of the country and in both countries is possibly related to the microbial diversity of the environment in which this insect is inserted.

Microbiome composition

Bacterial abundance may be related to the stink bug environment and eating habits. Previous studies of insect gut symbionts have shown that diet change shifts relative abundances of bacteria in the insect gut rather quickly [48]. Besides that, within the insect, symbiotic microorganisms have to face the host's innate immune system [49].

A vast majority of microbes constituting the gut microbiota are referred to as commensal microbes. By definition, commensals gain benefits from the host but are neither beneficial nor harmful to the host. However, commensals can be viewed as a pool of gut-interacting microbes with potential regulatory functions in host physiology [12].

The Acetobacteraceae family was found in both species of stink bugs. However, they were the majority in insect guts collected in the tropical moist forests and temperate broadleaf and mixed forests. Members of this family are symbionts of a wide variety of insects, providing nutrition to insects on limited sugar-rich diets [50] and are commonly found in the guts of honey bee [51]. These locations have less intensive soybean cultivation than other collection sites, allowing these insects to feed on less nutrient-rich crops. Possibly these bacteria are providing nutrients necessary for the survival of these insects in this environment.

The family Bacillaceae was found only in *E. heros* individuals collected in tropical moist forests. As discussed earlier, this ecoregion has less intensive cultivation of soybean, forcing this species to remove nutrients from the cultural remains between two cycles [9]. So, the Bacillaceae family has a fundamental role in the survival of this stink bug, as many Bacillus isolates have the ability to break down cellulose, hemicellulose, and pectin. They are saprophytes that participate in the carbon, nitrogen, sulfur, and phosphorus cycles in natural habitats [52], enabling the acquisition of nutrients from these previously inaccessible cells. According to Hussender et al [53], the genus *Bacillus* is the second major genus transmitted to the plant by *P. guildinii* feeding. Species belonging to this genus is known as a promoter of

plant growth, biocontrol agent, and crop protective inhibit fungal and bacterial pathogens on seedlings. Bacteria belonging to the γ-Proteobacteria class, as Enterobacteriaceae and Moraxellaceae, supplement the nutritionally poor diet of phloem sap through the provision of essential amino acids [54].

The Moraxellaceae family was found in both stink bug species. *E. heros* had this family only in individuals collected in the tropical savanna, known as the Cerrado. However, this family was found in *P. guildinii* in all ecoregions. This family can have an important function for these insects to acquire new hosts such as cotton. According to Zucchi [40], hybrids lineages of *E. heros* are colonizing the cotton crop in Cerrado due to its improvement of the detoxification capability acquired of existing adaptations from recombination of genes from northern populations (specialized in cotton) with southern populations (specialized in soybean). It is interesting because this family was found in all sites where the use of agrochemicals is high. Moreover, some Acinetobacter species are supposedly aiding herbivores in overcoming plant defenses through detoxification of phytochemicals [55]. Furthermore, some bacteria of the Acinetobacter genus can be transmitted by the red-banded stink bug feeding and have potentially beneficial effects on insects [20].

The Enterobacteriaceae was the most abundant family in both stink bugs. Corroborating our study, Enterobacteriaceae was found in *Spodoptera frugiperda* as the most abundant family [56]. Many species can exist as free-living in diverse ecological niches, both terrestrial and aquatic environments, and some are associated with animals, plants, or insects only [57]. Besides that, the genus Erwinia of the family Enterobacteriaceae contains mostly plant pathogens. *Erwinia persicina*, one of the major bacteria species found in the guts of red-banded stink bug, is a pathogen of legume plants [20]. Enterobacteriaceae was found mostly in insect guts from the tropical dry forest and tropical savanna. Endosymbionts from this family may be performing some function assisted in adapting these insects to new hosts.

The Rhodocyclaceae family was found mostly in stink bugs from Brazilian tropical savanna. Representatives of this family have been isolated from diverse environments have considerable potential for biodegradation of organic waste material and bioremediation of polluted environments [58]. So, members of this family may be associated with insecticides degradation and consequently better adaptations of these stink bugs.

Conclusion

In this work, we assessed the microbiome of the red-banded stink bug *Piezodorus guildinii* collected in Brazil and the United States, as well as the neotropical brown stink bug *Euschistus heros* collected in Brazil. Our analysis showed that the diversity and abundance of intestinal bacteria vary among the two species, ecoregions, and countries within the same species. This variability allows individuals to perform different functions to better adapt to the environments in which they live. The capacity of endosymbionts bacteria for nutrient supply, cellulose breakdown, phytochemical detoxification, and degradation of organic compounds suggested by the analysis is directly related to adaptability, survival, and control difficulty in the field of these stink bugs. As Brazil and the United States are countries with great

environmental diversity, we cannot generalize the control method. To find a more sustainable way to control stink bugs, we need to understand the relationship and dependence on intestinal bacteria, and this relationship must be better explored in future studies.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets generated and/or analysed during the current study will still be used for another publications, the dataset will be made available in NCBI repository in the future.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Matheus Sartori Moro – Conceptualization, data analysis, original draft writing, review & editing

Xing Wu – Investigation methodology, data analysis, review & editing

Wei Wei – Data analysis, review & editing

Lucas William Mendes – Data analysis, review & editing

Clint Allen - Insect collection, review & editing

José Baldin Pinheiro – Insect collection, review & editing

Steven J. Clough – Funding, conceptualization, review & editing

Maria Imaculada Zucchi – Funding, conceptualization, insect collection, review & editing

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Figures

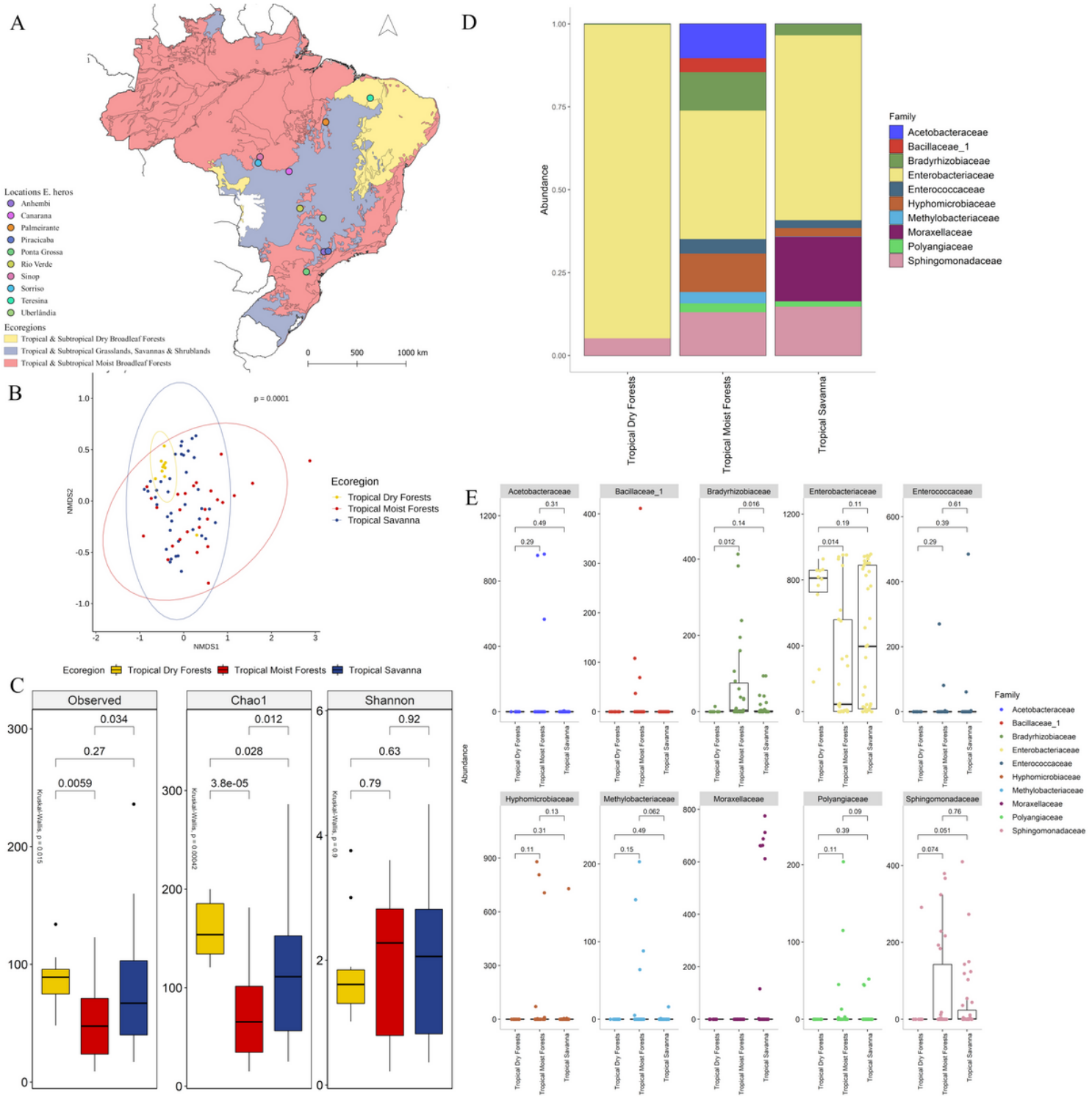

Figure 1

(A) Map of collection sites of E. heros, classified by ecoregion in Brazil. (B) NMDS of Bray-Curtis similarity matrix among 75 samples from the intestinal microbiome of E. heros, for the different ecoregions from Brazil. The ellipses are showing clustering between ecoregions. Permutational multivariate analysis of variance (PERMANOVA) is indicated in the upper right. (C) Alpha diversity levels based on Observed OTU, Chao1 index and Shannon index for the different ecoregions. The nonparametric test (Kruskal Wallis) is indicated in the upper left of each graph. Pairwise comparison (Wilcoxon) is indicated between

Ecoregions (D). Analysis of the ten most abundant bacteria families in the intestinal microbiome of E. heros collected in Brazil, classified by ecoregion. (E) Abundance analysis of each bacteria families in the intestinal microbiome of E. heros collected in Brazil, classified by ecoregion. Pairwise comparison (Wilcoxon) is indicated between ecoregions.

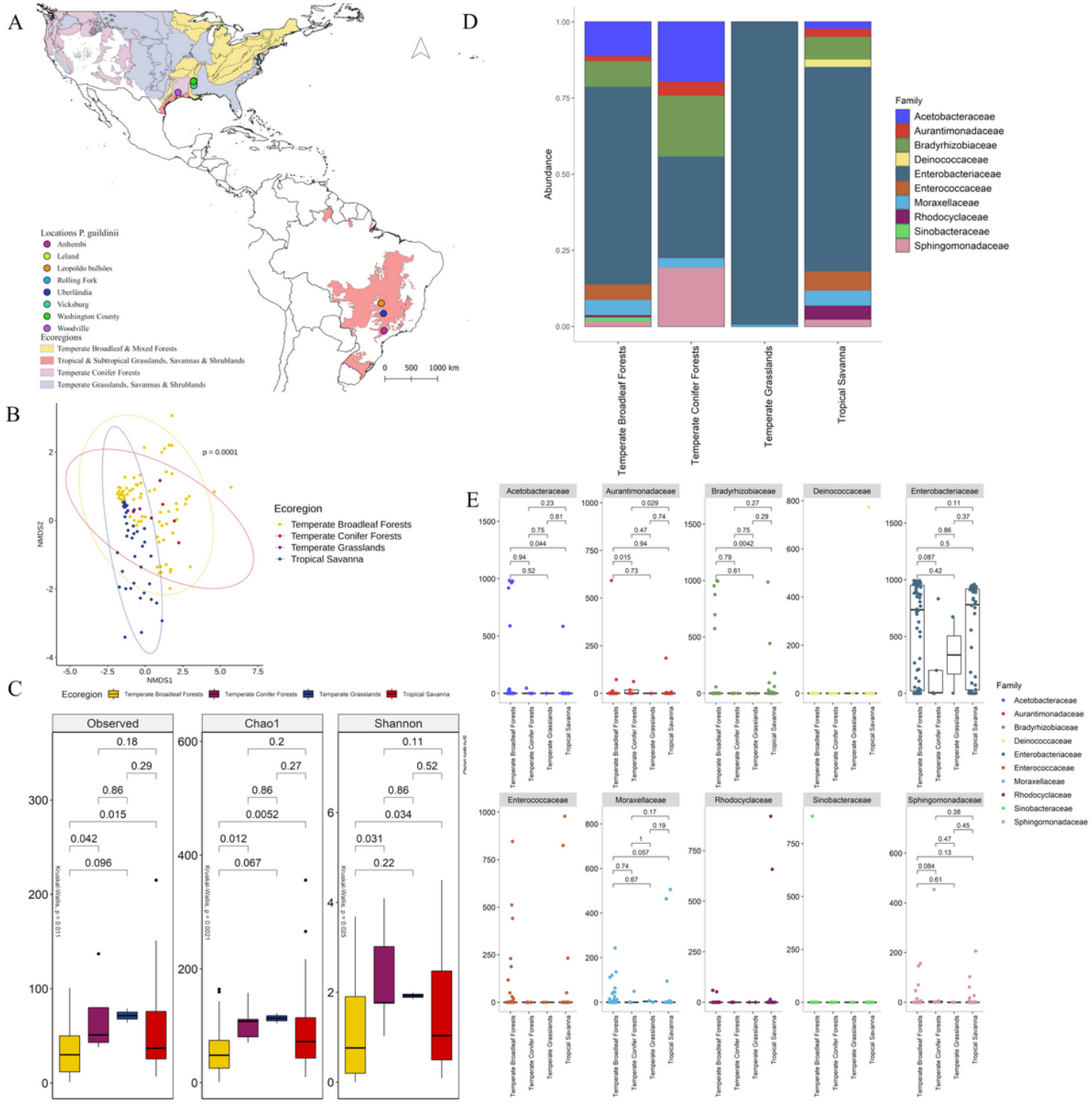

Figure 2

(A) Mapping of collection sites of P. guildinii, classified by ecoregion in Brazil and the United States. (B) NMDS of Bray-Curtis similarity matrix among 117 samples from the intestinal microbiome of P. guildinii,

for the different ecoregions from Brazil and United States. The ellipses are showing clustering between ecoregions. Permutational multivariate analysis of variance (PERMANOVA) is indicated in the upper right. (C) Alpha diversity levels based on Observed OTU, Chao1 index and Shannon index for the different ecoregion. The nonparametric test (Kruskal Wallis) is indicated in the upper left of each graph. Pairwise comparison (Wilcoxon) is indicated between Ecoregions. (D) Analysis of the ten most abundant bacteria families in the intestinal microbiome of P. guildinii collected in Brazil and the US, classified by ecoregion. (E) Abundance analysis of each bacteria families in the intestinal microbiome of P. guildinii collected in Brazil and the United States, classified by ecoregion. Pairwise comparison (Wilcoxon) is indicated between ecoregions.

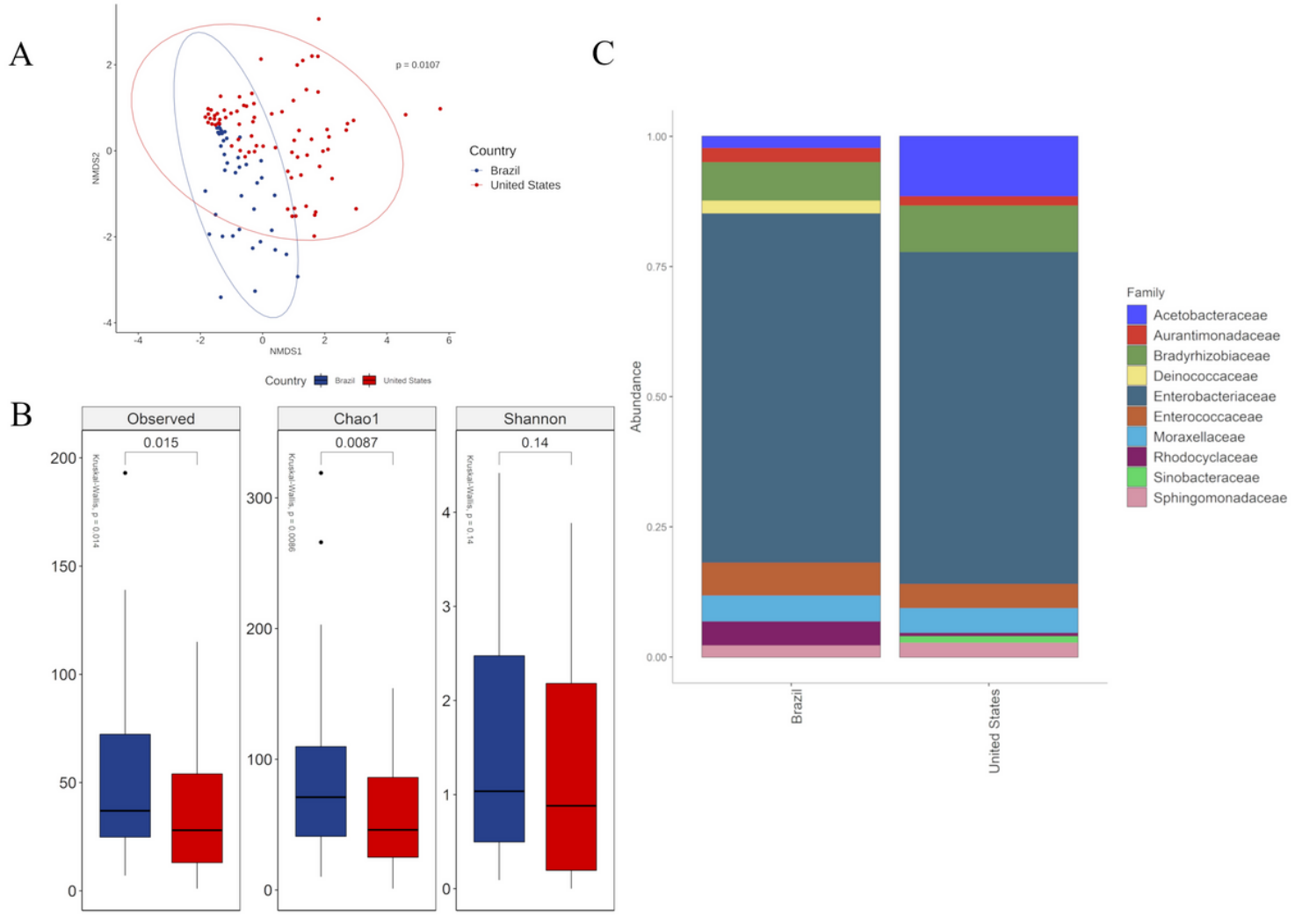

Figure 3

(A) NMDS of Bray-Curtis similarity matrix among 117 samples from the intestinal microbiome of P. guildinii for the different countries, Brazil and United States. The ellipses are showing clustering between countries. Multivariate analysis of variance (PERMANOVA) is indicated in the upper right. (B) Alpha diversity levels based on Observed OTU, Chao1 index and Shannon index for the different countries. The nonparametric test (Kruskal Wallis) is indicated in the upper left of each graph. Pairwise comparison

(Wilcoxon) is indicated between Ecoregions. (C) Analysis of the ten most abundant bacteria families in the intestinal microbiome of P. guildinii collected in Brazil and the US, classified by country.

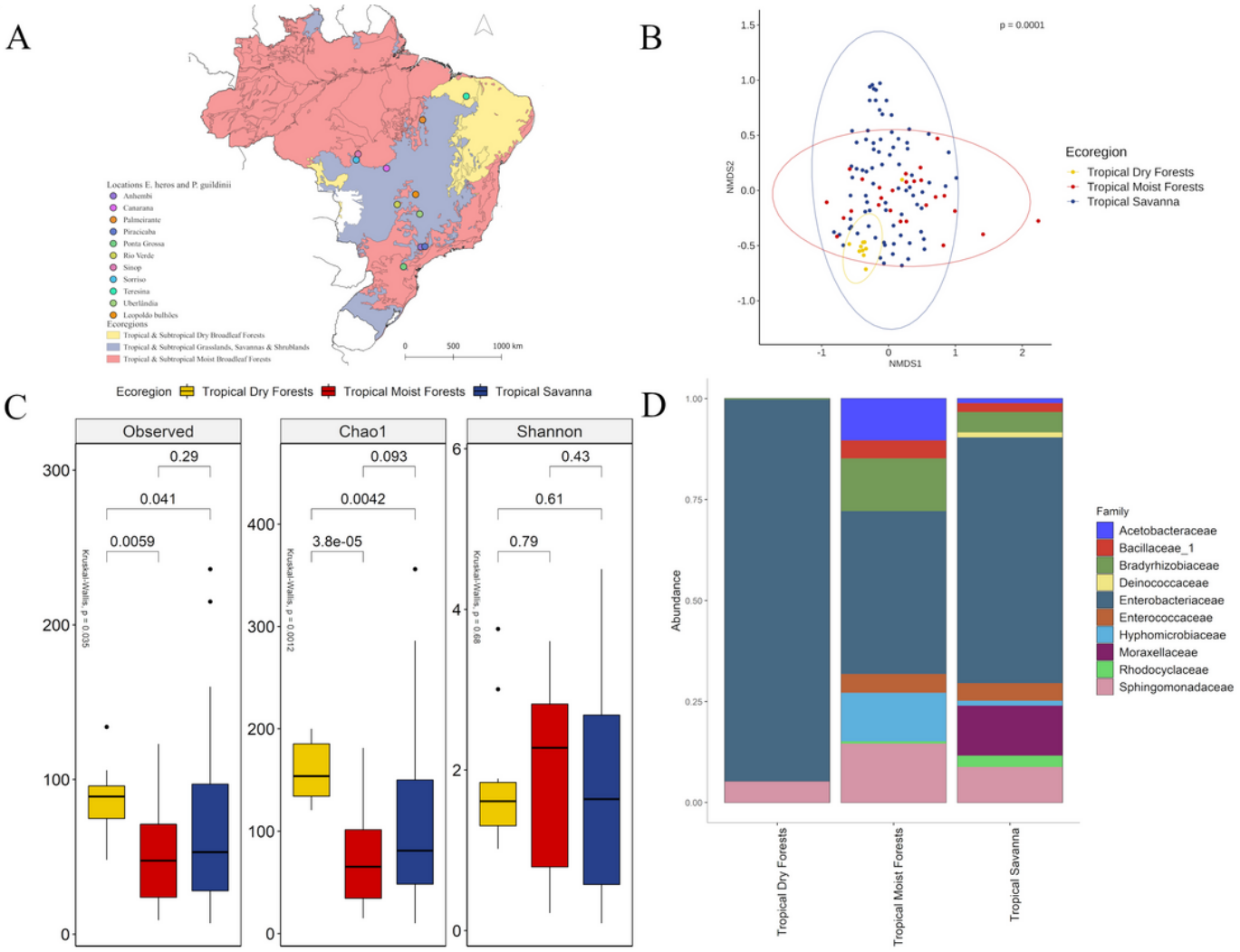

Figure 4

(A) Mapping of collection sites of E. heros and P. guildinii, classified by ecoregion in Brazil. (B) NMDS of Bray-Curtis similarity matrix among 115 samples from the intestinal microbiome of E. heros and P. guildinii, for the different ecoregion from Brazil. The ellipses are showing clustering between ecoregion. Multivariate analysis of variance (PERMANOVA) is indicated in the upper right. (C) Alpha diversity levels based on Observed OTU, Chao1 index and Shannon index for the different ecoregion. The nonparametric test (Kruskal Wallis) is indicated in the upper left of each graph. Pairwise comparison (Wilcoxon) is indicated between Ecoregions. (D) Analysis of the ten most abundant bacteria families in the intestinal microbiome of E. heros and P. guildinii collected in Brazil, classified by ecoregion.

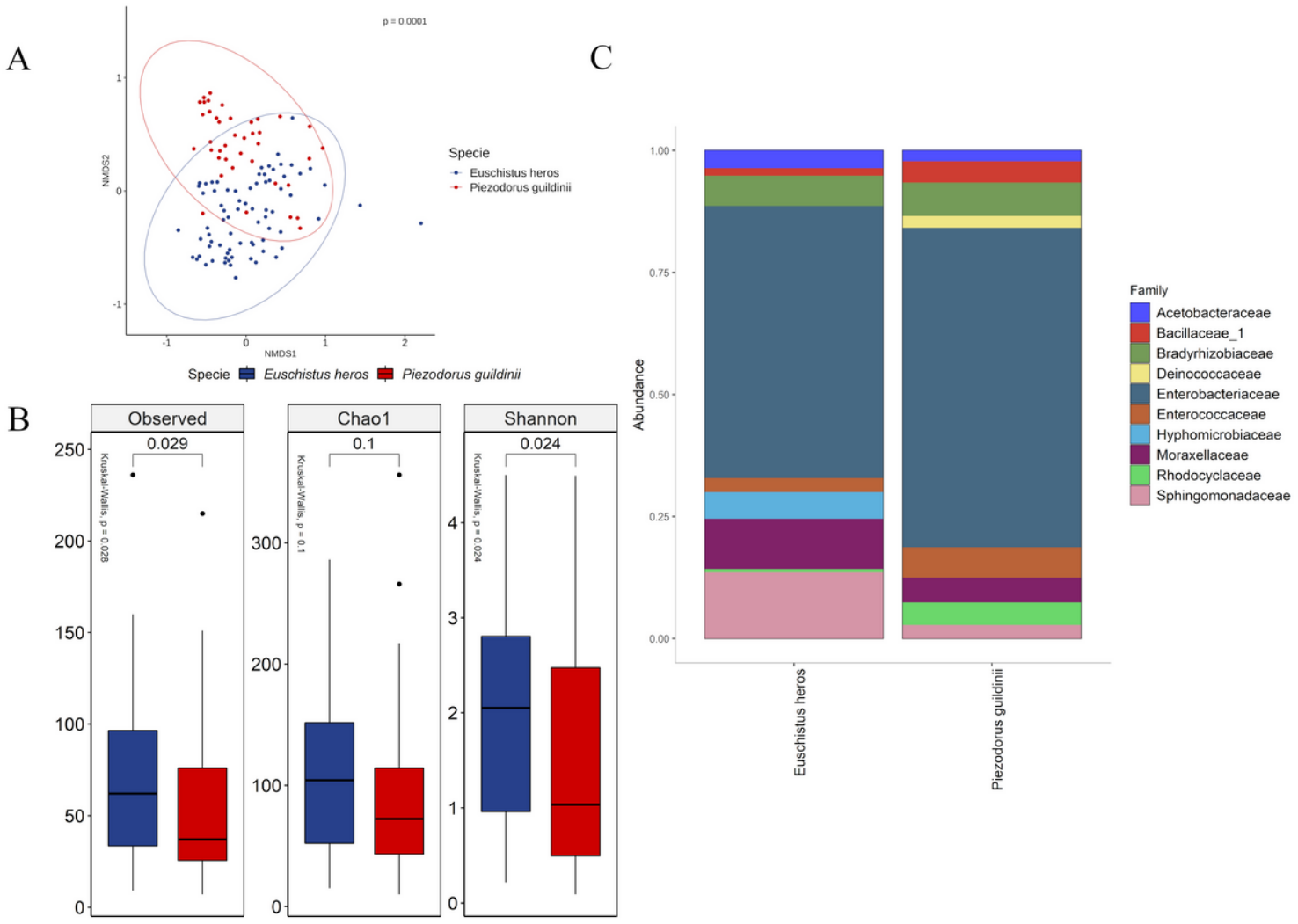

Figure 5

(A) NMDS of Bray-Curtis similarity matrix among 115 samples from the intestinal microbiome of E. heros and P. guildinii collected in Brazil. The ellipses are showing clustering between species. Multivariate analysis of variance (PERMANOVA) is indicated in the upper right. (B) Alpha diversity levels based on Observed OTU, Chao1 index and Shannon index for the different species. The nonparametric test (Kruskal Wallis) is indicated in the upper left of each graph. Pairwise comparison (Wilcoxon) is indicated between Ecoregions. (C) Analysis of the ten most abundant bacteria families in the intestinal microbiome of E. heros and P. guildinii collected in Brazil, classified by species.

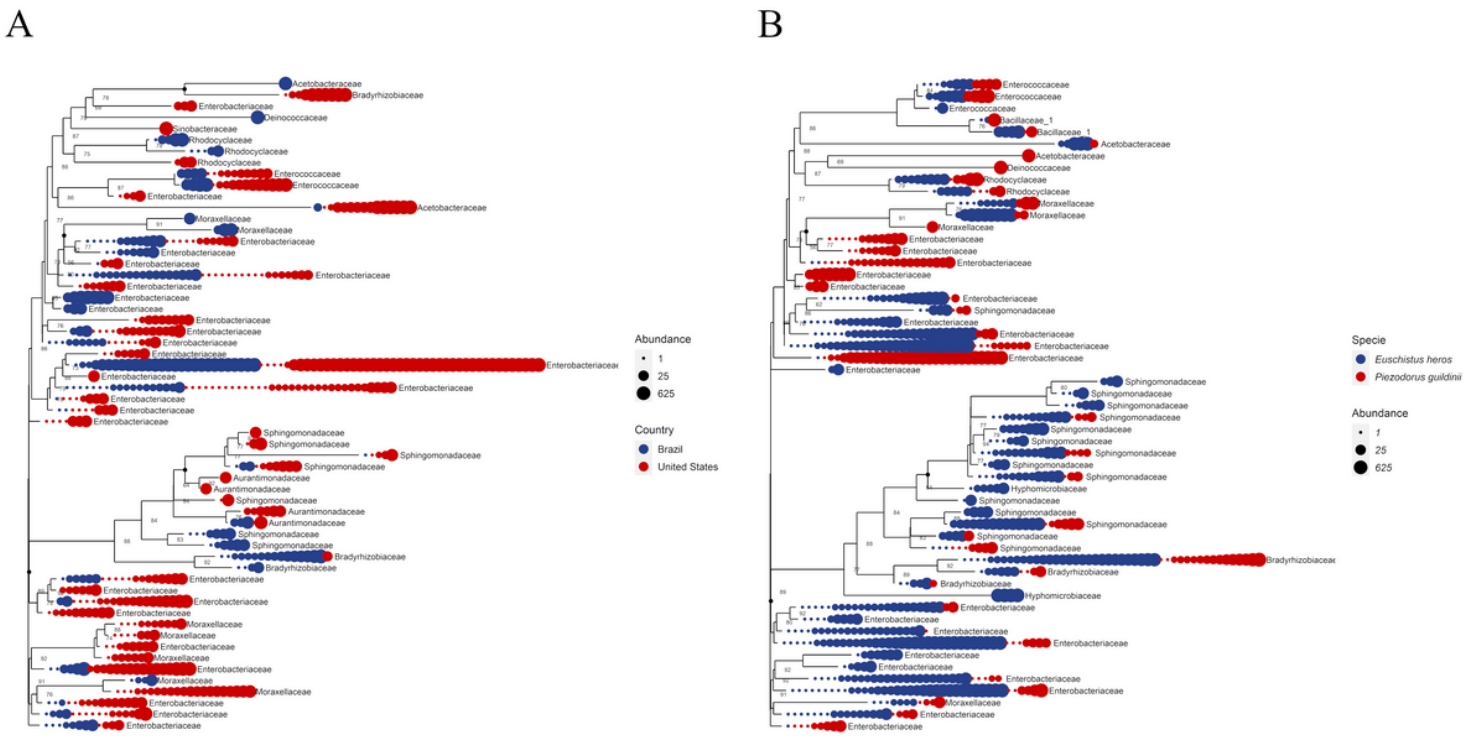

Figure 6

(A) Phylogenetic tree comparing top 10 bacteria abundance at family level among countries. (B) Phylogenetic tree comparing top 10 bacteria abundance at family level among species.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 03112020Supplementarymaterial.docx