# Structure and culture of the gut microbiome of the Mormon cricket *Anabrus simplex* (Orthoptera: Tettigoniidae)

Chad C. Smith
Department of Integrative Biology
University of Texas at Austin
1 University Station C0990
Austin, TX 78724
chad.smith123@gmail.com

Robert B. Srygley
USDA-Agricultural Research Service
Northern Plains Area Research Laboratory
1500 N. Central Avenue
Sidney MT 59270

Frank Healy
Department of Biology
Trinity University
1 Trinity Pl.
San Antonio, TX 78212

Karthikeyan Swaminath

Department of Integrative Biology
University of Texas at Austin
1 University Station C0990
Austin, TX 78724

Header: Structure of the Mormon cricket gut microbiome

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## ABSTRACT

The gut microbiome of insects plays an important role in their ecology and evolution, participating in nutrient acquisition, immunity, and behavior. Microbial community structure within the gut is thought to be heavily influenced by differences among gut regions in morphology and physiology, which determine the niches available for microbes to colonize. We present a high-resolution analysis of the structure of the gut microbiome in the Mormon cricket *Anabrus simplex*, an insect known for its periodic outbreaks in the Western United States and nutrition-dependent mating system. We found the Mormon cricket microbiome was dominated by eleven bacterial phylotypes from the Lactobacillaceae, Enterobacteriaceae, and Streptococcaeae. While most of these were represented in all gut regions, there were marked differences in their relative abundance, with lactic-acid bacteria (Lactobacillaceae) more common in the foregut and midgut and enteric (Enterobacteriaceae) bacteria more common in the hindgut. Differences in community structure were driven by variation in the relative prevalence of three groups: a *Lactobacillus* phylotype in the foregut, *Pediococcus* lactic-acid bacteria in the midgut, and *Pantoea agglomerans*, an enteric bacterium, in the hindgut. These taxa have been shown to have beneficial effects on their hosts in insects or other animals by improving nutrition, increasing resistance to pathogens, and modulating social behavior. Phylogenetic analysis of 16s rRNA sequences from cultured isolates indicated low levels of divergence from sequences derived from plants and other insects, suggesting that these bacteria are likely to be frequently exchanged between Mormon crickets and the environment. Our study provides the foundation for future work on an economically important insect and emerging model for the study of how social interaction influence host-microbe symbiosis.

Insects are the most speciose and abundant taxa in the animal kingdom, playing a key ecological role in many of the world's ecosystems. Symbioses between insects and their microbial associates has undoubtedly contributed to their success, providing the capability to degrade recalcitrant food items, supplementing nutrient-deficient diets, protecting them from their natural enemies, and modulating the expression of social behavior (Moran *et al.*, 2008; Engel and Moran, 2013; Douglas, 2015). Among the niches available to occupy within the host, the gut houses the largest and most diverse microbiome in insects (Engel and Moran, 2013; Douglas, 2015) and other animals (Ley *et al.*, 2008; Cho and Blaser, 2012). Gut morphology and physiology vary markedly along the alimentary tract in insects, resulting in an environmental gradient that influences, and is influenced by, the microbial communities that populate it (Dillon and Dillon, 2004; Engel and Moran, 2013; Brune and Dietrich, 2015).

The insect gut consists of three regions that are analogous to that in mammals, the foregut, the midgut and the hindgut, each of which contributes to a different aspect of gut function (Douglas, 2013). The foregut serves as the entry point for food, where it is stored in the crop before passing through the proventriculus, a valve that can also be modified to mechanically grind or and filter of food (Woodring and Lorenz, 2007; Douglas, 2013) and even microbes (Lanan *et al*., 2016). Digestion and absorption of nutrients begins at the midgut, which, in some species, contains specialized crypts that house microbes that aid in insect nutrition (Kikuchi *et al.*, 2005; Bistolas *et al.*, 2014). Host immune factors also have been shown to play an important role in regulating of commensal microbes in the midgut (Ryu *et al.*, 2010; Buchon *et al.*, 2013), some of which protect the host from pathogens (Forsgren *et al.*, 2010). Following the midgut is the hindgut, which is comprised of the ileum, colon, and rectum. Malphigian tubules permeate the anterior hindgut, depositing nitrogenous waste and other solutes from the hemocoel that can

provide nutrients for dense populations of microbes (Bignell, 1984). In some species, bristle-like structures in the ileum (Woodring and Lorenz, 2007) and rectal papillae (Hunt and Charnley, 1981) provide attachment sites for microbes, some of which fix nitrogen (Tai *et al.*, 2016), degrade recalcitrant plant polymers (Kaufman and Klug, 1991; Engel and Moran, 2013; Brune and Dietrich, 2015), and prevent infection (Dillon and Charnley, 2002).

The Mormon cricket *Anabrus simplex* (Orthoptera: Tettigoniidae) is an economically important shield-backed katydid distributed throughout the Western United States. Mormon crickets can form dense aggregations that number millions of individuals spread over 10 kilometers long and several kilometers wide, feeding on forbes, grasses, and agricultural crops as they march *en masse* in migratory bands across the landscape (Wakeland, 1959; MacVean, 1987). Mormon crickets are also emerging as a model for the study of how social interactions and diet influence the microbiome (Smith *et al.*, 2016). Differences in population density are linked to reproductive behavior, as in high density populations, protein-limited females compete for access to males to gain access a proteinaceous “nuptial gift” males produce for females during copulation (Gwynne, 1984). While consumption of male nuptial gifts by females does not influence the composition of the microbiome, sexually inactive females experience a dramatic decline in *Pediococcus* lactic-acid gut bacteria compared to sexually active females (Smith *et al.*, 2016). The mechanism underlying the change in lactic-acid bacteria is not known, however lactic-acid bacteria are common associates of the alimentary tract in animals regarded for their beneficial effects on immune function and nutrition in animals, including insects (Forsgren *et al.*, 2010; Storelli *et al.*, 2011; Erkosar *et al.*, 2015).

We characterize the structure of the gut microbiome of Mormon crickets and infer their evolutionary relationships using a combination of culture-dependent and culture-independent

approaches. Our aim is to determine whether gut microbial communities are differentiated along the alimentary tract and assess their potential to influence host function based on where they are found and their known associations with other insects. We also establish methods for isolating Mormon cricket gut microbiota in culture to permit future experimental manipulations of the gut microbiome and build genomic resources to infer their evolution and function.

## METHODS

*Spatial structure of the gut microbiome*

Mormon crickets were obtained from field (n=5) and laboratory-raised (n=8) collections. Wild females were caught in EK Mountain (43°47’58”N, 106°50’31”W, 1752 m) near Kaycee, Wyoming in the summer of 2014, immediately preserved in 100% ethanol, and stored at -80°C until dissection. Laboratory-raised Mormon crickets were derived from eggs collected from individuals caught in EK Mountain and fed a mixture of wheat bran, wheat germ, sunflower, mixed bird seeds, tropical fish flakes, fresh Romaine lettuce (added daily), and water *ad libitum*.

Mormon crickets were dissected using flame-sterilized tools after rinsing in 1% bleach for 3 minutes followed by two rinses in autoclaved distilled water to remove bacteria on exoskeleton, DNA from the foregut (crop and proventriculus), midgut (ventriculus), ileum, and rectum (Fig. 1) of laboratory-raised crickets was extracted with MoBio Powersoil© as in Smith et al. (2016). Foregut (crop and proventriculus), midgut (ventriculus), and hindgut tissue (ileum and rectum combined) from field-collected animals were bead-beat in a sterile 2ml screw cap tube with 750 µl of 0.1mm silica-zircon beads (Biospec Products), two 3.2mm stainless steel beads (Biospec Products) and 1 ml of sterile CTAB buffer (0.1M Tris, 1.4M NaCl, 20mM EDTA, 2%

PVP, 2 µl beta-mercaptanol, 20µl of 20mg/ml Proteinase-K). Tubes were bead beat at maximum speed for 2 minutes (Mini Beadbeater-96, Biospec Products) and incubated overnight at 55°C. RNAse (400µg, Qiagen) was added to the lysate and incubated at 37°C for 30 minutes. A phenol-chloroform extraction with isopropanol precipitation was performed to isolate the DNA. DNA extraction methods can influence the representation of bacterial taxa in 16s rRNA metagenomic studies (Yuan *et al.*, 2012), however our aim here is not to make inferences about differences between field and laboratory-raised animals. Instead we include the source of the animal (field or laboratory) and its interaction with tissue type in all statistical analyses to assess how the microbiome differs among gut regions exclusive of variation due to source/DNA extraction method (see Statistics).

*Sequencing and Bioinformatics*

Library preparation was done by the Genome Sequencing and Analysis Facility at the University of Texas at Austin using the NEBNext kit for Illumina. The variable V4 region of 16s rRNA gene was amplified with universal primers (Hyb515F: 5'-GTGYCAGCMGCCGCGGTA -3', Hyb806R: 5'-GGACTACHVGGGTWTCTAAT-3') and sequenced on the Illumina Miseq V3 platform. DADA2 1.1.5 (Callahan *et al.*, 2016, 2) was used to process the raw sequencing data, truncating reads when Illumina quality scores were less than two, removing sequences with a maximum expected error of one, and removing sequences flagged as chimeric. Clustering was then performed with DADA2 (Callahan *et al.*, 2016), specifying joint inference of sample composition and sequence error rates (selfConsist=T). Taxonomy was then assigned with the Greengenes 13.8 database at 97% identity. OTUs that were classified as unassigned, mitochondria, or chloroplast, and those that comprised and average of less than 1% of the reads

recovered within a given Mormon cricket, were removed for analysis using phyloseq 1.16.2 (McMurdie and Holmes, 2013).

*Bacterial Abundance*

The density of bacteria from laboratory-raised Mormon crickets was measured using qPCR following Powell et. al (2014). Universal 16S rRNA gene primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 355R (5'-CTGCTGCCTCCCGTAGGAGT-3') were used to amplify all copies of the 16S rRNA gene in tissue samples from laboratory (n=8) and field caught individuals (n=8) on an Applied Biosystems ViiA7 (Life Technologies). Triplicate 20 ul reactions were used with 10 ul of 2x PowerSYBR master mix (Applied Biosystems), 0.4 ul of each 10 mM primer and 5 ng of template DNA. PCR amplification was performed at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 1 minute at 60°C. Quantification of copy number was based on standard curves from amplification of the cloned target sequence in a pGEM-T (Promega, Madison, WI, USA) vector (Powell *et al.*, 2014).

*Culturing*

Five female Mormon crickets were surface sterilized in 1% bleach for three minutes, rinsed twice in sterile water and dissected using flame-sterilized tools. Gut tissue was homogenized for 10 seconds with a bead beater using autoclaved 3.2mm stainless steel beads in sterile PBS. Homogenates were plated onto trypsin soy agar, brain heart infusion agar, nutrient agar, or Man–Rogosa–Sharpe agar (BD), cultured in anaerobic or Campy (low $O_2$) Gaspak pouches (Becton, Dickinson and Company, Franklin Lakes, NJ) at 37°C for 24-48 hours, and individual colonies passaged three times to obtain pure isolates.

DNA was extracted by boiling cells for 15 minutes in lysis buffer (100mM NaCl and 0.5% sarcosyl), adding an equal volume of 20% chelex, and boiling for 15 additional minutes.

16s rRNA amplicons were amplified with 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers using Apex PCR master mix (Genesee Scientific, San Diego, CA) with 35 cycles (95°C for 20 s, 52°C for 1 min 30 s and 72°C for 40 s). PCR products were cleaned up with Sera-mag beads (GE Healthcare Life Sciences, Pittsburgh, PA) or ethanol precipitation and sequenced at the University of Texas at Austin on an Applied Biosystems 3730XL DNA analyzer.

We compiled 16s rRNA sequences greater than 1.2kb long reported as sourced from insect guts from NCBI Genbank, and used BLAST to find the closest matches to our Mormon cricket isolates. *Pediococcus* and *Lactobacillus* sequences were aligned with pyNAST as implemented in Qiime 1.9 (Caporaso *et al.*, 2010) using a curated alignment for *Lactobacillus* (McFrederick *et al.*, 2013) as the reference template. The alignment was then manually edited with Mesquite (Maddison and Maddison, 2016) and filtered to remove characters with less than 80% coverage across sequences using Qiime 1.9 (Caporaso *et al.*, 2010). Sequences from the Enterobacteriaceae were aligned with online implementation of the SILVA release 113 (Pruesse *et al.*, 2012; Quast *et al.*, 2013), manually checked in Mesquite (Maddison and Maddison, 2016), and filtered as above, with the additional removal of the top 10% most entropic (hypervariable) base positions. The phylogenies were constructed using maximum likelihood with a GTR + Gamma model for nucleotide evolution in RaxML 8.2.4 (Stamatakis, 2014), with 1000 bootstraps to assess branch support. Archaeopteryx 0.9916 (Han and Zmasek, 2009) was used to visualize the tree and produce the figures.

*Phenotypic assays*

Fresh overnight cultures of all isolates were used for microscopic analysis. Lactobacillaceae isolates were cultured in Man–Rogosa–Sharpe medium and Enterobacteriaceae were cultured in

nutrient broth or LB medium. Biochemical tests were done following Bridson (1998). Motility was determined using SIM medium and microscopic examination of culture wet mounts. Man–Rogosa–Sharpe or nutrient broth containing 1 g/L potassium nitrate was used for nitrate reduction tests. Fermentation tests were done anaerobically in Man–Rogosa–Sharpe and nutrient broth media with the addition of indicated sugars to 1% w/v final concentration.

*Statistics*

Analyses were performed in R 3.3.1 (R Core Development Team, 2013). OTU tables rarified at 1300 reads using phyloseq (McMurdie and Holmes, 2013), resulting in the exclusion of two hindgut sample from a field-caught female with a low number of reads from analysis. Alpha diversity was compared among tissue types and between origin of subject (field vs. lab) with a linear mixed model with the lme4 package (Bates *et al.*, 2013), entering the individual ID as a random effect to account for within-subject correlations in diversity. Three metrics were calculated: species richness, the Chao1 species richness estimator, and the Shannon-Weiner diversity index. Post-hoc comparisons among gut regions was performed using a Tukey test as implemented in the multcomp package (Hothorn *et al.*, 2008).

Beta diversity among gut tissue types and between animal source (field vs. lab) were assessed with a distance-based redundancy analysis (db-RDA) as implemented in vegan 2.3 (Oksanen *et al.*, 2015), specifying Bray-Curtis distances. Prior to analysis, the relative abundance of OTUs were calculated for each Mormon cricket and those that comprised less than 1% of the sequences on average were discarded. Statistical significance of the terms in the db-RDA model were determined by 999 permutations of the distance matrix as implemented in vegan, restricting the permutations to within each individual to retain the nested structure of the data.

We assessed difference in the abundance of specific OTUs identified in the db-RDA analysis in univariate analyses by fitting their abundance to a negative binomial generalized linear mixed model using the lme4 package (Bates *et al.*, 2013), specifying the individual ID as the random effect and the tissue type and animal source (field vs. lab) as fixed effects. Likelihood ratio tests were used to determine the statistical significance of each factor using the MASS package (Venables and Ripley, 2002). Goodness-of-fit was assessed by comparing the fit of the data to a negative binomial distribution with a Chi-square test (Faraway, 2006), and homoscedasticity was assessed by examination of residual plots. P-values were adjusted for multiple tests using the false discovery rate (Benjamini and Hochberg, 1995).

## RESULTS

*Spatial structure of the gut microbiome*

We recovered 11 dominant OTUs from field and lab-raised individuals, with the remaining 749 OTUs comprising <1% of the sequences from a given Mormon cricket (Fig. 2). Field and laboratory-raised individuals shared 7 of the 11 OTUS, including the most abundant *Pediococcus acidilactici* phylotype that varied with mating status in a previous study (*P. acidilactici* 102222; Smith *et al.*, 2016). The remaining five shared OTUs were two Lactobacilliaceae (*Lactobacillus sp* and *P. acidilactici* 2), two Enterobacteriaceae (*Pantoea agglomerans* and a *Klebsiella sp*) and one Streptococcaceae (*Lactococcus garvieae*). Field Mormon crickets had three OTUS that were not shared with laboratory-raised individuals, while lab-raised individuals had two OTUs that were not shared with field individuals (Fig. 2). Guts from two laboratory individuals were almost completely comprised of the enterobacterium *Pantoea agglomerans* (99.3% and 80.8% of reads respectively), so we conducted our analysis with and without these individuals.

Species richness and diversity differed among gut regions and was higher in field compared to lab-raised animals (Table 1, Fig. 3). There was no significant interaction between collection source and tissue type (Table 1), indicating that differences in alpha diversity among tissue types were shared between lab and field caught animals. We found that the midgut was the most diverse part of the gut with two of the three measures of alpha diversity (species richness and the Chao1 diversity estimator), while the hindgut and foregut had similar levels of richness and diversity. The third metric (Shannon-Weiner) also found the foregut to be the least diverse region, but differed in that the midgut and hindgut had similar levels of species diversity (Table 1, Fig 3).

The db-RDA analysis revealed that the structure of the gut microbiome also varied among gut regions and between field and laboratory animals (Table 2, Fig. 4). The non-significant interaction in this analysis indicates that the community structure among tissue types differed in similar ways between field and laboratory individuals (Table 2). To determine which members of the gut microbiome varied among gut regions, we ordinated the OTU scores from db-RDA analyses of field and laboratory Mormon crickets (Fig. 5). Three groups of bacteria appeared to separate along the gut axis: a *Lactobacillus sp.* lactic-acid bacterium associated with in the foregut, *Pediococcus* lactic-acid bacteria were associated with the midgut, and *Pantoea agglomerans*, an enterobacterium, was found in association with the hindgut. Inspection of the plots from laboratory animals indicate that *Pantoea agglomerans* is more abundant in the rectum, with the composition of the ileum, which is separated from the rectum by the colon, closely resembling that of the midgut (Fig. 5b).

Univariate analyses of these three groups largely confirmed the pattern in the ordination (Table 3, Fig. 6). The interaction between tissue type and source was not significant in any of the

analyses and dropped to estimate the differences in abundance between tissue types using the coefficients from the generalized linear mixed models. *Lactobacillus sp*. was 3 and 7 times more common in the foregut than in the midgut ($\beta$=1.4 $\pm$ 0.50, p=0.02) and hindgut ($\beta$=2.0 $\pm$ 0.51, p<0.001) respectively, *Pediococcus* was similar in abundance in the midgut and hindgut but 4.7 times more common in these areas than the foregut ($\beta$=1.1 $\pm$ 0.36, p=0.006), and *Pantoea agglomerans* was 209 and 12 times more abundant in the hindgut compared to the foregut ($\beta$=3.8 $\pm$ 0.87, p<0.001) and midgut ($\beta$=2.5 $\pm$ 0.82, p=0.007) respectively.

*Bacterial density*

The number of copies of bacterial 16s rRNA genes was significantly different among tissue types, as indicated by the significant interaction between tissue type and the source of the Mormon crickets (Analysis of deviance: Source, $F_{1,14}$=25.9, p<0.001; tissue type, $F_{3,161}$=7.8, p<0.001; Interaction, $F_{3,161}$=2.8, p=0.04, Fig. 7). We decomposed the interaction to determine how the total number of 16s rRNA copies differed among tissue types within field and laboratory-raised animals. The major difference between the two sources was that in wild Mormon crickets, the midgut had the lowest abundance of all gut regions, while in laboratory-raised individuals, both the midgut and the ileum had the lowest abundance of bacterial 16s rRNA genes (Table 4, Fig. 7).

*Culturing*

Ten bacterial phylotypes were recovered from the Mormon cricket gut based on 99% sequence similarity of their near full-length 16s rRNA genes (mean $\pm$ sd: 1406 $\pm$ 30bp). Two of the phylotypes were lactic-acid bacteria (Lactobacillaceae) and eight were enteric bacteria (Enterobacteriaceae).

The lactic-acid bacteria fell into two clades in our phylogenetic analysis (Figure 8). The first clade was comprised of *Pediococcus* isolates derived from environmental sources, such as plants and various human foodstuffs, as well as strains from the human gut. Similarity to sequences from the BLAST search was high (>99.5%) and branch lengths were short, indicating *Pediococcus acidilactici* H11 from the Mormon cricket gut is not highly derived from its relatives, as has been found for *Lactobacillus* species isolated from bees (Fig. 8; McFrederick *et al.*, 2013). Our search for *Pediococcus* sequences from insect guts in Genbank isolates recovered from the termites *Macrotermes bellicosus* and *M. subhyalinus*, which formed their own well-supported clade exclusive of the other *Pediococcus* sequences, including those from Mormon crickets. *P. acidilactici* H11 shared 100% sequence identity in the V4 region with the *P. acidilactici 1* phylotype sequenced using the Illumina platform in this study and with the *P. acidilactici* (102222) phylotype associated with variation in mating status in Mormon crickets (Smith *et al.*, 2016). Morphologically, *P. acidilactici* H11 is nonmotile and spherical (0.8 – 1.0 μm), often dividing to form pairs as described for other *Pediococcus.* As other members of the genus, the *P. acidilactici* H11 is gram-positive, non-motile, faculatively anaerobic, grows at low pH, and produces lactic acid from lactose (Table S1).

The second clade of lactic-acid bacteria was comprised primarily of plant-associated *Lactobacillus.* Unlike *P. acidilactici* H11, *Lactobacillus* H09 formed a distinct clade with high branch support, indicating it is genetically distinct enough at the 16s rRNA locus to distinguish itself from other clades in the phylogeny. Similar to *P. acidilacitici* H11, *Lactobacillus* H09 had high sequence similarity (>99.5%) to other members of the clade and a short branch length, indicating that while it is distinct enough to form its own clade, it is not highly derived from its relatives at the 16s rRNA locus. Our Genbank search for *Lactobacillus* isolated from insect guts

found sequences from ants, bees, and termites, and fruit flies, all of which fell into a different clade than *Lactobacillus* H09. *Lactobacillus* from these taxa thus appear to have a different evolutionary history than *Lactobacillus* H09. *Lactobacillus* H09 shared 100% sequence identity in the V4 region with the Lactobacillaceae 2 phylotype sequenced using the Illumina platform in this study. Morphologically, *Lactobacillus* H09 appear as non-motile straight rods, approximately 1.3-2 μm in length and 0.8-1.0 μm wide. *Lactobacillus* H09 is gram-positive, non-motile, faculatively anaerobic, grows at low pH, and produces lactic acid from lactose (Table S1).

The eight Enterobacteriaceae strains were most similar to *Enterobacter* strains in our BLAST search, which recovered sequences from a variety of plant and animal sources (sequence similarity=98.7-99.8%). Our survey of Genbank found *Enterobacter* from alimentary tracts of a diverse group of insects, including termites, cockroaches, flies, beetles, stink bugs, bees, ants, and moths. Like other studies (Brenner *et al.*, 2005), however, the 16s rRNA gene did not have enough signal to resolve relationships among *Enterobacter* and its relatives (data not shown) so we present a simpler phylogeny with the Mormon cricket isolates and type strains from the family (Figure 9). We found that our Mormon cricket isolates formed their own clade with moderate statistical support. A multilocus sequencing approach, however, is needed to improve the inference (Brenner *et al.*, 2005). All five strains isolated from Mormon crickets had 100% identity at the V4 region with the *Klebsiella* phylotype sequenced on the Illumina platform, however the phylogenetic (Fig. 9) and phenotypic data (Table S2) suggests that it is unlikely to be a correct taxonomic assignment. Morphologically, all isolates were straight rods, approximately 0.8-1.0 μm in length and 0.6-0.8 μm wide. Unlike most *Klebsiella*, these strains

were motile, which is typical of *Enterobacter* and other Enterobacteriaceae (Brenner *et al.*, 2005). Strains were gram-negative, and facultatively anaerobic (Table S2).

## DISCUSSION

We found striking differences in the diversity and structure of the gut microbiome in the Mormon cricket *Anabrus simplex*. While most OTUs were represented in the foregut, midgut and hindgut, there were dramatic differences in abundance within the Lactobacillaceae and between the Lactobacillaceae and Enterobacteriaceae, the main families recovered in our culture and culture-independent studies. Our phylogenetic analysis of cultured isolates found that Mormon cricket gut bacteria are not highly derived from related bacteria associated with plants or the guts of other animals, suggesting that gut bacteria are either acquired from the environment in each generation or have not been restricted to Mormon crickets over appreciable periods of evolutionary time. Our findings have important implications for our understanding of the ecological and evolutionary processes that influence the assembly and function of gut microbial communities in orthopterans and other insects, as it suggests that host-microbe and microbe-microbe interactions shape the abundance and distribution of the microbiome.

Our finding that the density of bacteria is lower in the midgut is in agreement with reports from orthopterans (Hunt and Charnley, 1981; Ulrich *et al.*, 1981) and other insects (Köhler *et al.*, 2012), and has been attributed to characteristics that make the midgut less hospitable to bacteria than other regions of the alimentary tract (Douglas, 2015). The midgut in insects secretes a host of digestive enzymes, is immunologically active, and lined by the peritrophic membrane, which acts as a protective barrier that restricts microbes to the lumen and protects the epithelium (Douglas, 2015). In the two orthopterans that have been studied in detail, bacteria are found in the midgut lumen but not in association with the epithelium (Hunt and Charnley, 1981; Mead *et*

*al.*, 1988). As a consequence, midgut bacteria might need to be continually replenished from ingested food (Blum *et al.*, 2013) because the peritrophic membrane is continually shed into the hindgut. In some insects, specialized midgut crypts provide niches for microbes to colonize (Kikuchi *et al.*, 2005; Bistolas *et al.*, 2014), however we did not observe analogous structures in Mormon crickets (Fig. 1).

The midgut is particularly vulnerable to pathogens because the lack of an endocuticle leaves the epithelium exposed once the peritrophic membrane is penetrated (Lehane and Billingsley, 1996; Copping and Menn, 2000; Ruud A. de Maagd *et al.*, 2003; Nehme *et al.*, 2007). The Mormon cricket midgut was populated by lactic-acid bacteria, with *Pediococcus* specifically exhibiting greater abundance in the midgut (and hindgut) than in the foregut. Lactic-acid bacteria are known for their beneficial effects in insects, increasing resistance to parasites in bees (Forsgren *et al.*, 2010) and promoting development in fruit flies by enhancing proteolytic activity (Erkosar *et al.*, 2015) and upregulating host ecdysone and insulin-like peptides (Storelli *et al.*, 2011). Lactic-acid bacteria are also known to suppress pathogenic bacteria by reducing pH and producing a number of antimicrobial compounds, such as hydrogen peroxide and bacteriocins (Cintas *et al.*, 2001).

A previous study found that sexual interactions in Mormon crickets influences the abundance of three *Pediococcus* phylotypes (Smith *et al.*, 2016), however spatial information on where in the gut *Pediococcus* is located has been unavailable until now. *Pediococcus* in the midgut suggests could provide immunological or nutritional benefits to Mormon crickets, as has been shown for *P. acidilactici* in other animals (Castex *et al.*, 2009, 2009). The culured isolates of *P. acidilactici* we obtained from Mormon crickets in this study will enable future experimental and comparative genomic approaches to evaluate these hypotheses.

Lactic-acid bacteria were also common in the foregut, which was dominated by a *Lactobacillus sp.* that averaged 73.9% of the sequences recovered from this region. Bignell (1984) noted that the foregut of insects tends to be the most acidic compartment, however studies that measure the physiochemical environment and characterize microbiome composition of the foregut are rare (but see Köhler *et al.*, 2012). This is because the endocuticle, lack of differentiated cells for absorption of nutrients, and frequent purging of consumed material into the midgut provides little opportunity for foregut microbes to contribute to host nutrition The large differences in community structure between the foregut and the rest of the alimentary tract in our study does illustrate the dramatic transition in microbial communities between what is ingested and what can colonize the more distal regions of the gut.

In contrast to the foregut and midgut, the hindgut was characterized by a dramatic increase in enteric bacteria (Enterobacteriaceae). Ordination of the laboratory Mormon cricket samples indicated that the rectum, not the ileum, was primarily responsible for the difference in community structure in the hindgut. Enterobacteriaceae comprised 83.5% of the sequences from the rectum compared to 57.5% from the ileum, which was more similar to the midgut in community structure (Fig. 5). This distinction is potentially important because higher digestive efficiency in conventional compared to germ-free crickets has been attributed to microbial colonization of the ileum in the orthopteran *Achetus domesticus* (Kaufman and Klug, 1991). Detailed taxonomic information on the gut microbiota of *A. domesticus* or are not yet available for comparison to our study (but see Santo Domingo *et al.*, 1998).

Of the three enteric bacteria represented in this study, *Pantoea agglomerans* was common to both field and lab individuals and increased in abundance in the hindgut. *Pantoea* are known plant pathogens and have been associated with a variety of medical conditions in humans

(Walterson and Stavrinides, 2015). In insects, however, *Pantoea* have been shown to have mutualistic associations with their host. They are required for the completion of development in stinkbugs (Hosokawa *et al.*, 2016; but see Dillon and Charnley, 2002), produce compounds that attract insects to their host plants in flies (Robacker *et al.*, 2004; Maccollom *et al.*, 2009), and in the orthopteran *Schistocerca gregaria*, produce a key component of the locust aggregation pheromone (Dillon *et al.*, 2000, 2002) and reduce susceptibility to entomopathogens (Dillon and Charnley, 1986, 1995). *P. agglomerans* similarly occurs at its highest abundance in the hindgut of *S. gregaria*, with histological surveys showing that enterobacteria colonize the cuticle within crevices formed by the rectal papillae (Hunt and Charnley, 1981). Whether *P. agglomerans* similarly protects Mormon crickets from its own fungal entomopathogens (MacVean and Capinera, 1991) or influences its aggregation behavior (Wakeland, 1959; MacVean, 1987) is an important direction for future research.

*Conclusions*

Variation in morphology and physiology is thought to differentiate niches within the gut that influence the organization of the microbiome. Our study describes at high resolution how bacterial communities vary among gut regions, and suggests that host-microbe and/or microbe-microbe interactions have a role in how microbial communities are assembled and maintained. While the taxonomic information gleaned from our study suggests that some of these bacteria might benefit Mormon cricket nutrition, immunity, and perhaps even modulate social behavior, experiments are needed to evaluate this possibility. Our establishment of methods for culturing Mormon cricket gut bacteria will enable experimental and comparative genomic approaches in the future to infer the ecological and evolutionary consequences of host-microbe symbiosis.

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Table 1. Analysis of deviance comparing alpha diversity between source populations (wild or laboratory) and among tissue types (foregut, midgut, and hindgut). Values represent the F-statistic (p-value) for each term. Statistically significant terms ($p<0.05$) are indicated in bold. Degrees of freedom were estimated using the Kenward-Rogers approximation.

| | **Reduced Dataset** | | | **Full Dataset** | | |
|---|---|---|---|---|---|---|
| | Species Richness | Chao1 | Shannon-Weiner | Species Richness | Chao1 | Shannon-Weiner |
| Source | **13.9 (0.003)** | **11.0 (0.007)** | **9.17(0.01)** | **14.0(0.002)** | **10.5 (0.006)** | **8.22 (0.013)** |
| Tissue type | **5.85 (0.010)** | **4.79 (0.02)** | **7.07 (0.005)** | **6.77 (0.004)** | **5.68 (0.008)** | **8.44 (0.001)** |
| Interaction | 0.51 (0.61) | 1.02 (0.38) | 0.98 (0.39) | 0.66 (0.53) | 1.15 (0.33) | 1.28 (0.29) |

Table 2. Permutation test from distance-based redundancy analysis comparing Bray-Curtis distance between source populations (wild or laboratory) and among tissue types (foregut, midgut and hindgut).

| | **Reduced Dataset** | | **Full Dataset** | |
|---|---|---|---|---|
| | *F* | *p* | *F* | *P* |
| Source | **8.99** | **<0.001** | **7.75** | **<0.001** |
| Tissue type | **9.85** | **<0.001** | **5.49** | **<0.001** |
| Interaction | 0.26 | 0.47 | 0.52 | 0.84 |

Table 3. Likelihood ratio tests from GLMMs fitting the abundance of OTUs to source population (wild or laboratory) and tissue type (foregut, midgut or hindgut). Values are Chi-square (p-value). LAC1=*Lactobacillus sp.*, PED=*Pediococcus*, PAG=*Pantoea agglomerans*.

| | **Reduced Dataset** | | | **Full Dataset** | | |
|---|---|---|---|---|---|---|
| | LAC1 | PED | PAG | LAC1 | PED | PAG |
| Source | 1.32 (0.25) | 0.80 (0.37) | 2.36 (0.12) | 0.18 (0.66) | 0.51 (0.48) | 0.01 (0.96) |
| Tissue type | **16.3 (<0.001)** | **9.25 (0.10)** | **20.7 (<0.001)** | **12.7 (0.002)** | **16.1 (<0.001)** | **41.9 (<0.001)** |
| Interaction | 0.36 (0.84) | 0.84 (0.66) | 3.86 (0.15) | 0.3 (0.86) | 0.95 (0.62) | 2.67 (0.26) |

Table 4. Posthoc Tukey tests comparing the total number of 16s RNA copies between tissue types in wild and laboratory-raised Mormon crickets. Values are the test statistic with the significance of the test indicated with an asterisk. Comparisons within field-caught individuals are on the bottom diagonal and comparisons within laboratory-raised individuals are on the upper diagonal. FG=foregut; MG=midgut; ILE=ileum; REC=rectum.

| | FG | MG | ILE | REC |
|---|---|---|---|---|
| FG | | 2.38 | 2.76* | 0.21 |
| MG | 3.82*** | | 0.39 | 2.60* |
| ILE | 1.26 | 2.61* | | 2.98** |
| REC | 0.64 | 3.28** | 0.66 | |

* p=<0.05, ** p<0.01, *** p<0.001

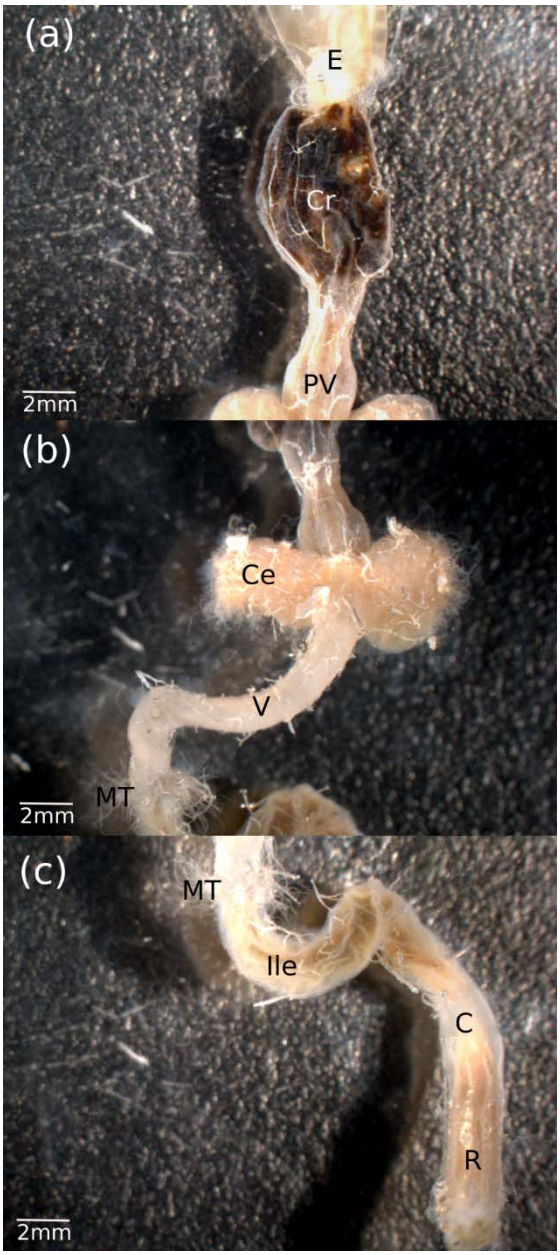

Figure 1. External morphology of the (a) foregut, (b) midgut, and (c) hindgut in the Mormon cricket. E=esophagus, Cr=crop, PV=proventriculus, Ce=cecum, V=ventriculus, MT=Malphigian tubules, Ile=ileum, C=colon, R=rectum. Malphigian tubules have been trimmed to illustrate their entry point into the hindgut.

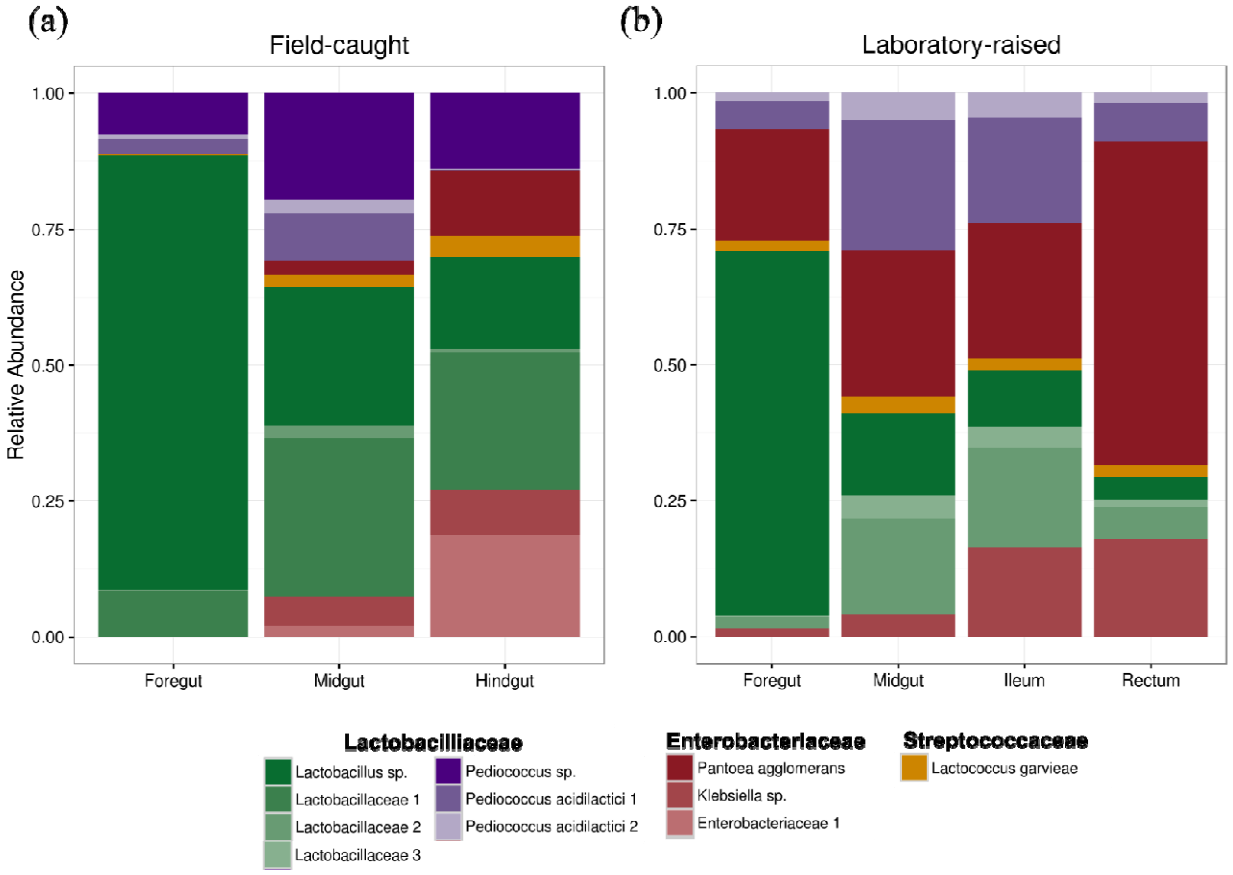


Figure 2. Mean relative abundance of the eleven dominant OTUs from (a) field-caught and (b) laboratory-raised Mormon crickets from 16s rRNA Illumina sequencing.

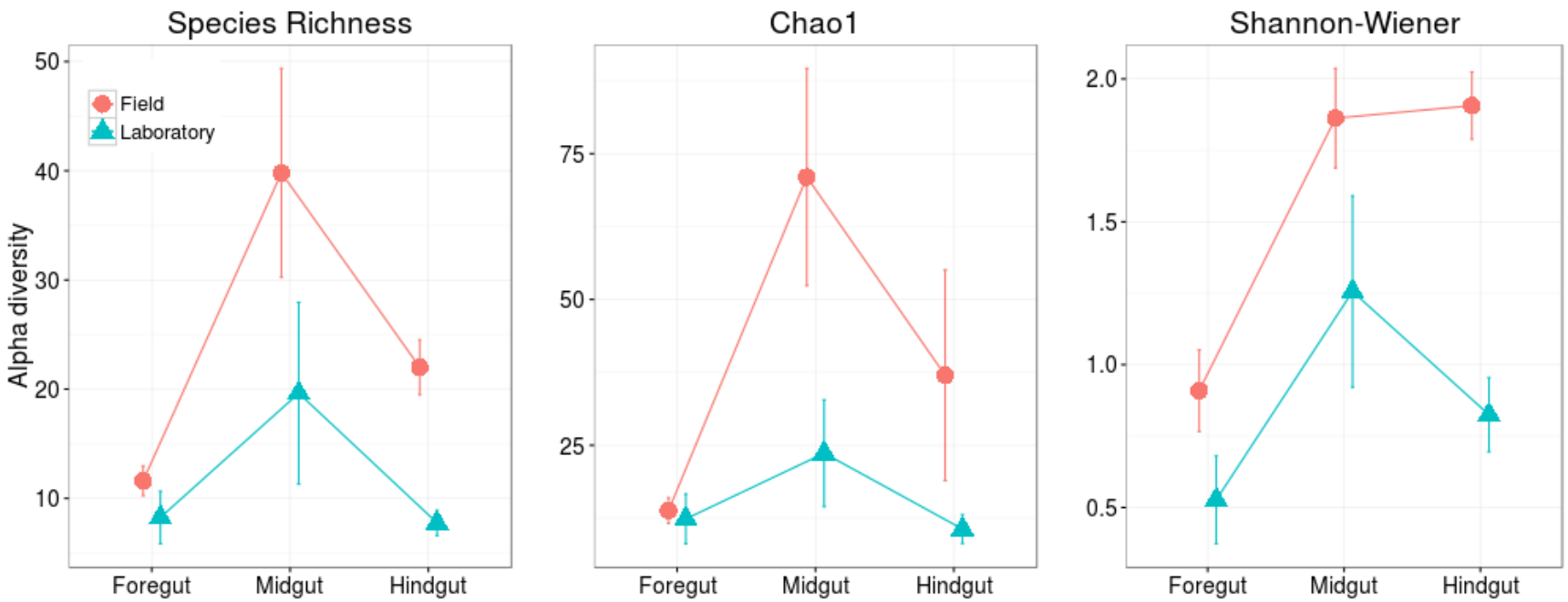


Figure 3. Alpha diversity in field and laboratory-raised Mormon crickets. Means ± SE are depicted for each region of the gut.

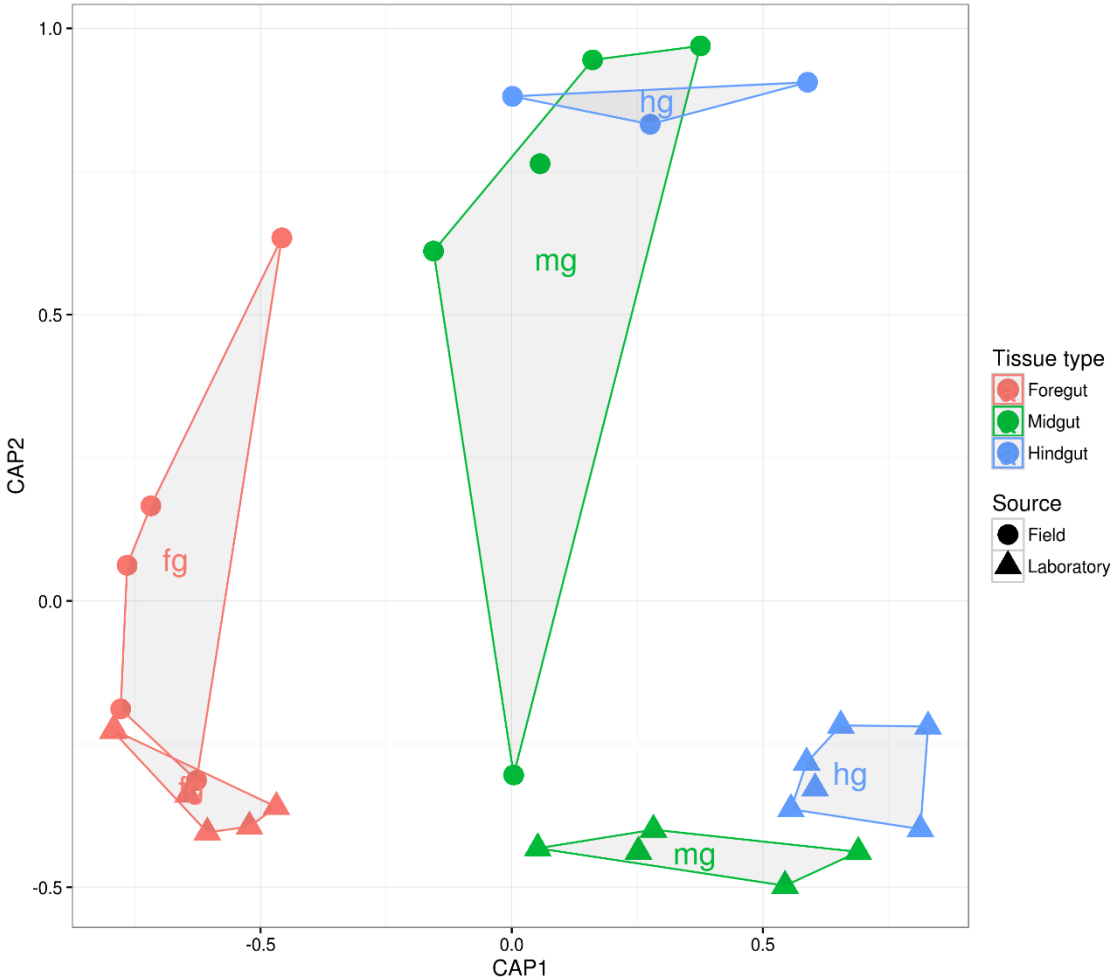


Figure 4. Ordination of sample scores from the db-RDA of the reduced dataset.

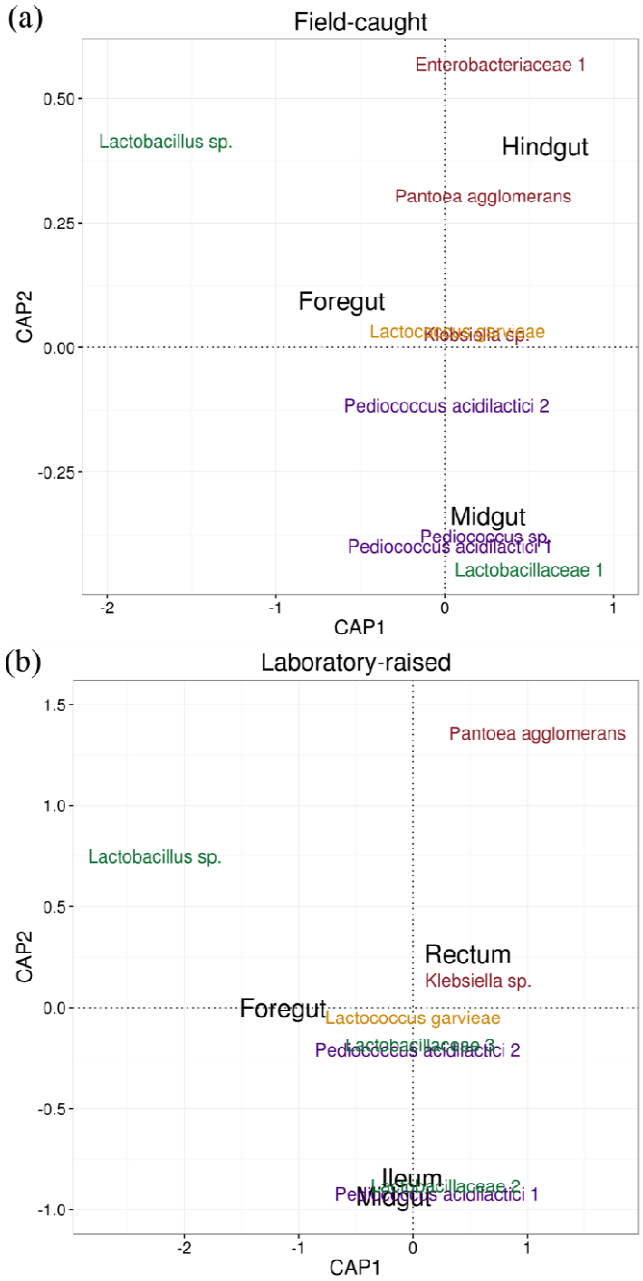


Figure 5. Ordination of OTU scores from db-RDA of (a) field-caught and (b) laboratory-raised (reduced dataset) Mormon crickets. Means of sample scores for each tissue type are indicated. OTUs are colored to represent taxonomic groups as in Figure 1.

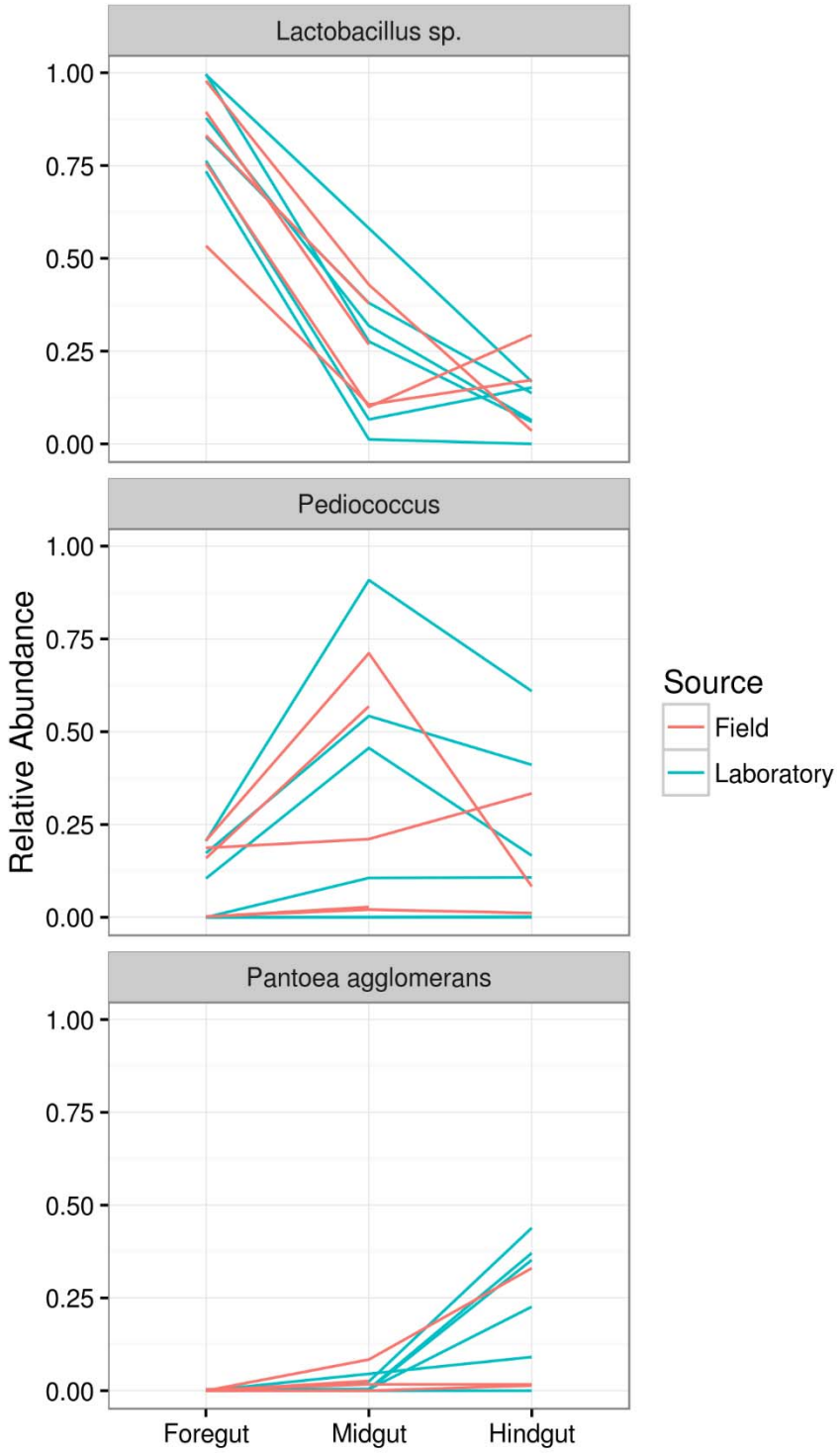


Figure 6. Relative abundance of *Lactobacillus sp*., *Pediococcus*, and *Pantoea agglomerans* from the field and laboratory (reduced dataset) identified in the ordination (see Fig. 5) as associated with different gut regions. Each line represents an individual Mormon cricket.

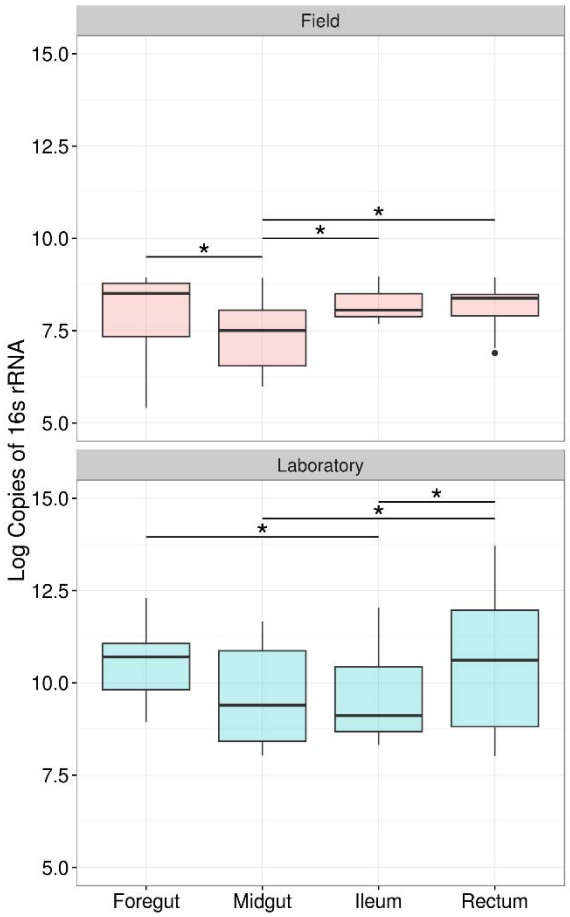


Figure 7. Abundance of bacterial 16s rRNA genes in the Mormon crickets gut. Bars indicate significant differences between regions (* $p<0.05$).

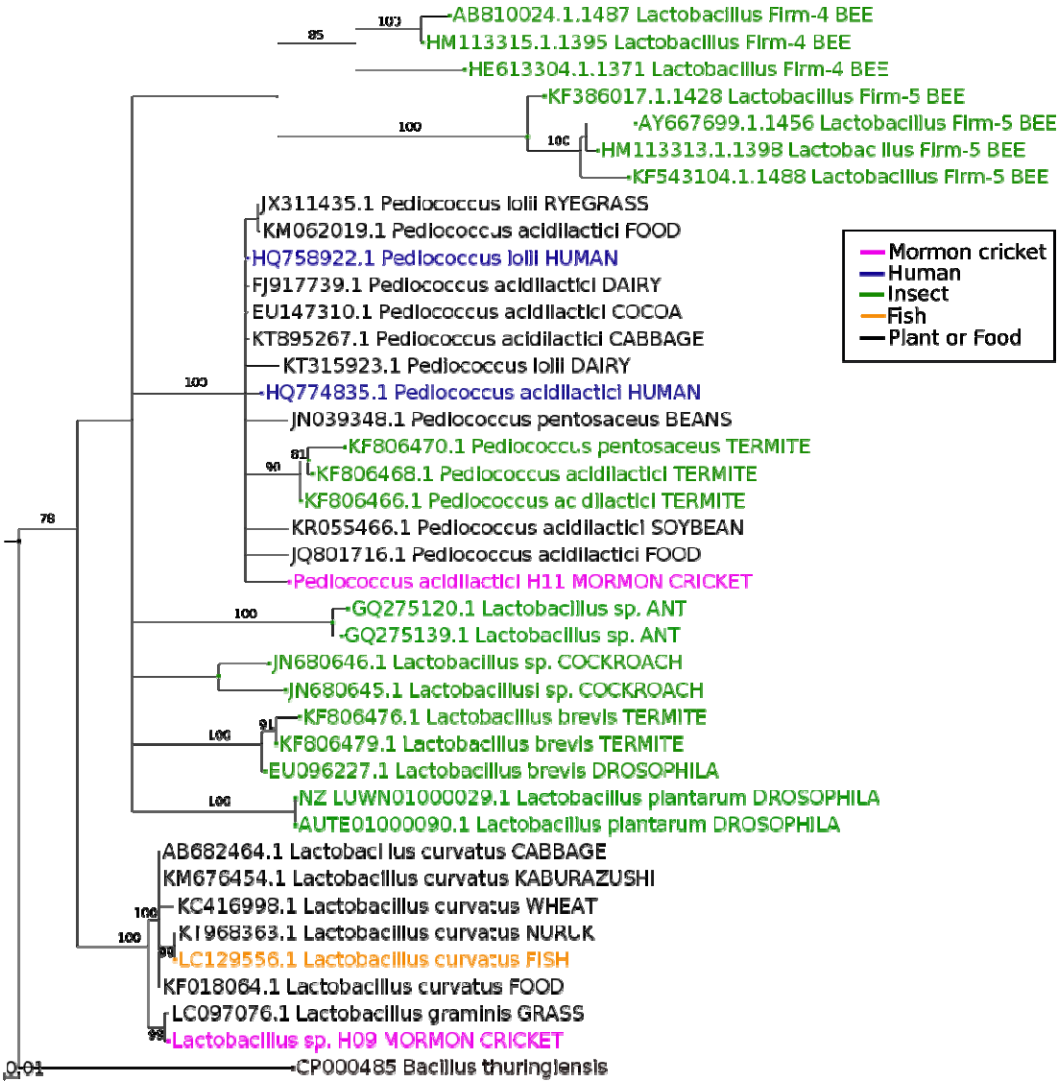


Fig 8. Maximum likelihood estimation of phylogenetic relationships among lactic-acid bacteria 16S rRNA sequences from Mormon cricket gut isolates and their relatives. Branches with bootstrap support <75% are collapsed.

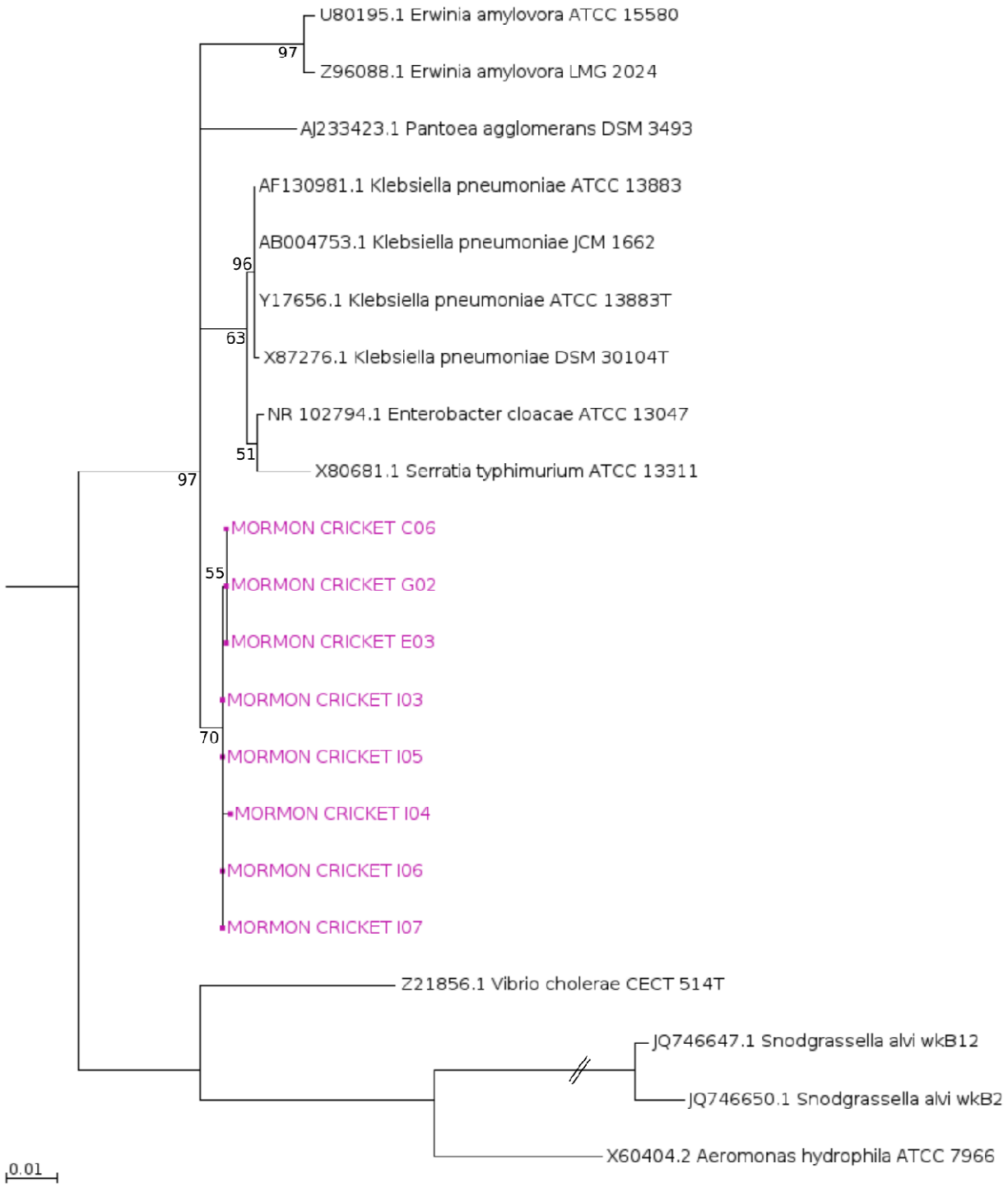


Figure 9. Maximum likelihood estimation of phylogenetic relationships among enteric bacteria 16S rRNA sequences from Mormon cricket gut isolates and type strains. Branches with bootstrap support <50% are collapsed