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Influence of Microbes in Mediating Sorghum Resistance to Sugarcane Aphids

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Abstract: Gut microbiomes profoundly influence insect health and mediate interactions between plant hosts and their environments. Insects, including aphids, harbour diverse obligate symbionts that synthesize essential nutrients and facultative symbionts that enhance host fitness in specific ecological contexts. Sorghum (*Sorghum bicolor*) is a significant cereal crop cultivated worldwide that has been negatively affected by the presence of an invasive piercing-sucking insect pest, the sugarcane aphid (SCA; *Melanaphis sacchari*). We previously identified SC265 and SC1345 as the resistant and susceptible sorghum lines, respectively, among the founder nested association mapping (NAM) population. Here, using these resistant and susceptible lines, we explored variations in the SCA gut microbiome when they feed on two different sorghum lines with varied resistance levels. Analyses after excluding the obligate endosymbiont *Buchnera aphidicola* from the dataset showed a significant difference in microbial diversity and composition between resistant and susceptible sorghum lines 7- and 14 days post aphid infestation. Our results indicate that the SCA fed on susceptible and resistant sorghum lines had *Pseudomonadaceae* and *Rhizobiaceae*, respectively, as the most abundant bacterial families. Differences in gut microbial community composition were underscored by alpha diversity metrics and beta diversity compositional analyses. These findings contribute to our understanding of the intricate interplay between plant and aphid microbiomes, shedding light on potential avenues to bolster sorghum resistance to SCA.

Keywords: *Buchnera aphidicola*; sorghum; sugarcane aphids; microbiome; plant defense; *Pseudomonadaceae*; *Rhizobiaceae*

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1. Introduction

Insects possess diverse symbiotic microbes that play significant roles in their evolution. Many insects depend on obligate microbial symbionts (bacteria and fungi) to synthesize nutrients that are absent from their hosts [1]. In addition to these obligate symbioses, insects can harbour facultative symbionts that are not essential for host survival but may increase host fitness under specific ecological conditions. Sugarcane aphids (SCA; *Melanaphis sacchari*) are considered a major threat to sorghum (*Sorghum bicolor*) production because it severely damages the plant by sucking sap from leaves that results in considerable yield loss [2–4]. SCA, like other insects, have a diverse microbial community as well as harbouring an obligate endosymbiont [5]. Recently, it was shown that antibiotic treatment of the SCA population resulted in a decreased survival rate [6], suggesting that SCA microbiota plays a crucial role in insect development.

The symbiotic relationship between aphids and their primary endosymbiont, *Buchnera aphidicola* (*B. aphidicola*), plays a crucial role in the success of aphids as phloem-feeders. Phloem sap, aphid's primary nutrient source, lacks essential components necessary for

aphid growth and reproduction. *B. aphidicola* addresses this nutritional deficit by providing amino acids, thereby completing the aphid diet [7]. This endosymbiont is pivotal, as evidenced by studies involving its elimination or suppression. Antibiotic treatment or genetic manipulation leading to the removal of *B. aphidicola* results in decreased fitness, impaired reproduction, and altered feeding behaviour in SCA, emphasizing its significance in supporting optimal aphid performance. Moreover, *B. aphidicola* influences the manipulation of plant physiology by aphid feeding. The *Buchnera*-derived proteins in aphid saliva modulate plant defences, potentially suppressing the host's ability to mount an effective response against aphid infestation [8]. Research indicates that *B. aphidicola* contributes to the induction of plant defences, affecting the overall susceptibility of host plants to aphid feeding [9]. Therefore, the absence of *B. aphidicola* can lead to nutritional deficiencies in the aphids, affecting their growth, reproduction, and overall fitness [10].

In a broader context, research utilizing deep sequencing of bacterial 16S rRNA genes in various insect samples explored the factors influencing microbial community structure in aphids [11–13]. These studies delve into the role of taxonomy, revealing greater microbiome similarity within aphid species than between species. Additionally, the authors establish correlations between microbiome structure and the genetic distance between hosts. It was also shown that aphid species sharing the same host plant exhibit more similar microbiomes than expected based on phylogeny [14].

Considering the plant perspective, the plant microbiome is integral to plant defence mechanisms, employing diverse strategies such as immune responses, induced systemic resistance (ISR), and callose deposition to fend off pathogen attacks [15,16]. Microbial communities contribute to plant protection by producing antimicrobial compounds and exhibiting inhibitory effects on pathogens through competition for nutrients and space [15,17]. The complex and dynamic interactions between plants and microbiomes are greatly influenced by the host, microbes, and the environment, forming a trio complex that determines their overall outcome [18,19].

Sorghum is an economic cereal grown for its numerous uses in humans, animals, and manufacturing and agricultural industries, and is a food source for approximately 500 million people in 30 countries in Africa and Asia [20]. Sorghum can also be a biofuel energy source and a healthy food crop [21]. In the United States, in addition to corn, sorghum grain is also used in ethanol production [22]. Unfortunately, sorghum production is negatively affected by insect pests. In 2013, a massive outbreak of SCA occurred in the U.S. Similar to other aphids, SCA is a phloem-feeding insect that feeds on the plant sap by inserting its slender stylet, allowing them to feed continuously from phloem tissues [23]. Previously, we have identified SC265 and SC1345 plants as SCA-resistant and susceptible sorghum lines, respectively [24–26], which are part of the founder nested association mapping (NAM) population [27]. In this study, we investigated the role of SCA gut microbiomes that potentially modulate sorghum defences by comparing the gut microbiomes of aphids fed on SC265 (SCA-resistant) and SC1345 (SCA-susceptible) sorghum lines [24]. We anticipated differences between the gut microbiomes of aphids fed on the SCA-resistant and susceptible sorghum lines to be underscored by differences in the mechanism of resistance in the sorghum lines.

2. Materials and Methods

2.1. Plants and Insects

Two sorghum lines that were previously identified as SCA-resistant (SC265) and SCA-susceptible (SC1345) that are part of the founder NAM population [24] were used in the present study. The seeds were grown in a mixture of vermiculite and perlite (PRO-MIX BX BIOFUNGICIDE + MYCORRHIZAE; Premier Tech Horticulture Ltd., Quakertown, PA, USA) in SC 10 cone-trainers. Plants were raised under a 16:8 h light-dark photoperiod and watered regularly. Two-week-old plants (three-leaf stage) were used in the experiments. The aphid colony was maintained on the BCK60 sorghum plant, which is highly susceptible

to SCA, under a 16:8 h light-dark photoperiod at 26 °C. New plants were introduced into the colony every week to provide a continuous supply of aphids.

2.2. Aphid Bioassay, DNA Extraction, and Microbiome Sequencing

Two-week-old sorghum SC265 and SC1345 plants were infested with SCA. Each plant was infested with five adult apterous aphids and covered with clear tubular plastic cages. The cages were ventilated with organdy fabric on the sides and top for proper aeration. All the plants were randomly arranged and infested with aphids. After seven days of infestation, the cages were removed, and 100–150 aphids were collected from the plants in tubes for the resistant and susceptible lines, respectively. The cages were returned, and a similar aphid collection procedure was repeated on day 14. The collected aphids were further used for DNA analysis and quantification.

DNA extraction was performed using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) following the standard protocol recommended by the manufacturer. The concentration and quality of extracted DNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The extracted plant and aphid DNA samples were submitted for high-throughput paired-end Illumina MiSeq library preparation and sequencing was done at the University of Nebraska Medical Center Genomics Core Facility. Briefly, a limited cycle PCR reaction was performed on each sample to create a single amplicon, including the V4 (515-F) and V5 (907-R) variable region [28]. The resulting libraries were validated using the Agilent BioAnalyzer 2100 DNA 1000 chip (Agilent, Santa Clara, CA, USA), and DNA was quantified using Qubit 3.0 (Qubit™, Thermo Fisher Scientific Inc., Waltham, MA, USA). A pool of libraries was loaded into the Illumina MiSeq at 10 pM. The pool was spiked with 25% PhiX (a bacteriophage) at 10 pM for MiSeq run quality as an internal control [29] to generate 300 bp paired ends with the 600-cycle kit (version 3).

2.3. Processing Data and Statistical Analyses

Bioinformatics analyses were performed following the Bioconductor workflow for microbiome data analysis using R studio (version 4.2) [30] and Bioconductor variant. For denoising, the R package DADA2 (version 1.26.0) was used to process fastq primer-trimmed MiSeq paired-end reads obtained from the sequencing centre [31]. Forward reads were truncated to 280 base pairs and reverse reads to 250 base pairs, and truncation was done to keep median quality scores above 30 across samples. The reads were merged, and chimeras were filtered out. To construct the taxonomy table, a naive Bayes taxonomy classifier was employed to classify each amplicon sequence variant (ASVs) against the SILVA 138.1 reference database [32]. Sequences identified as chloroplasts, mitochondria, or eukaryotes as well as all ASVs with an abundance of less than 0.0005% were filtered [33]. All samples were rarefied to 191,000 sequences per sample and 49 reads per sample for sequences without the primary endosymbiont bacteria, *Buchnera*, before proceeding with the downstream analysis, and the rationale for rarefying has been explained previously [34].

Statistical analyses of the rarefied sequences were performed in R using R Studio (version 4.2) and the Bioconductor variant utilizing the vegan package. The observed ASVs, and the Chao1 index, which is a richness estimate index that determines estimated ASV based on observed ASV from sequence data [35], were used to quantify and estimate population diversity for alpha diversity studies and comparisons among groups and categories. A Bray-Curtis distance matrix community distance matrix was generated for beta diversity studies and used to generate non-metric multidimensional scaling (NMDS) visualizations using the vegan package 'metaMDS' function in R. This was followed by permutational multivariate analysis of variance (PERMANOVA) test of differences between groups and categories. The indicator species analysis method was utilized in R, employing the indicspecies package and test interval to discover indicator species underscoring differences in community composition. The statistical significance of each

sample was determined using 999 permutations, and only samples with ASVs ($p < 0.05$) were regarded as good indicators for constructing a relative abundance plot.

3. Results

This study revealed the gut microbiome diversity and composition of SCA that fed on the foliage of SCA-resistant and susceptible sorghum lines for 7 and 14 days. To prevent overestimation of bacterial diversity, we eliminated chloroplast reads, yielding a total of 12,382,727 million reads. Filtering, merging, chimera removal, and additional curation of the generated merged count and taxonomy table yielded 460 ASVs across 23 samples (mean reads per sample: 374,103.086; Minimum: 191,249.000, Maximum: 1,585,631.000). Data rarefaction resulted in a read count of 191000 per sample (Figure 1) and 49 per sample when we removed endosymbiont bacteria, *Buchnera* (Figure 2). We first analyzed the data including *Buchnera* (Figure 1; Supplemental Table S1) and then without *Buchnera* (Figure 2; Supplemental Table S2).

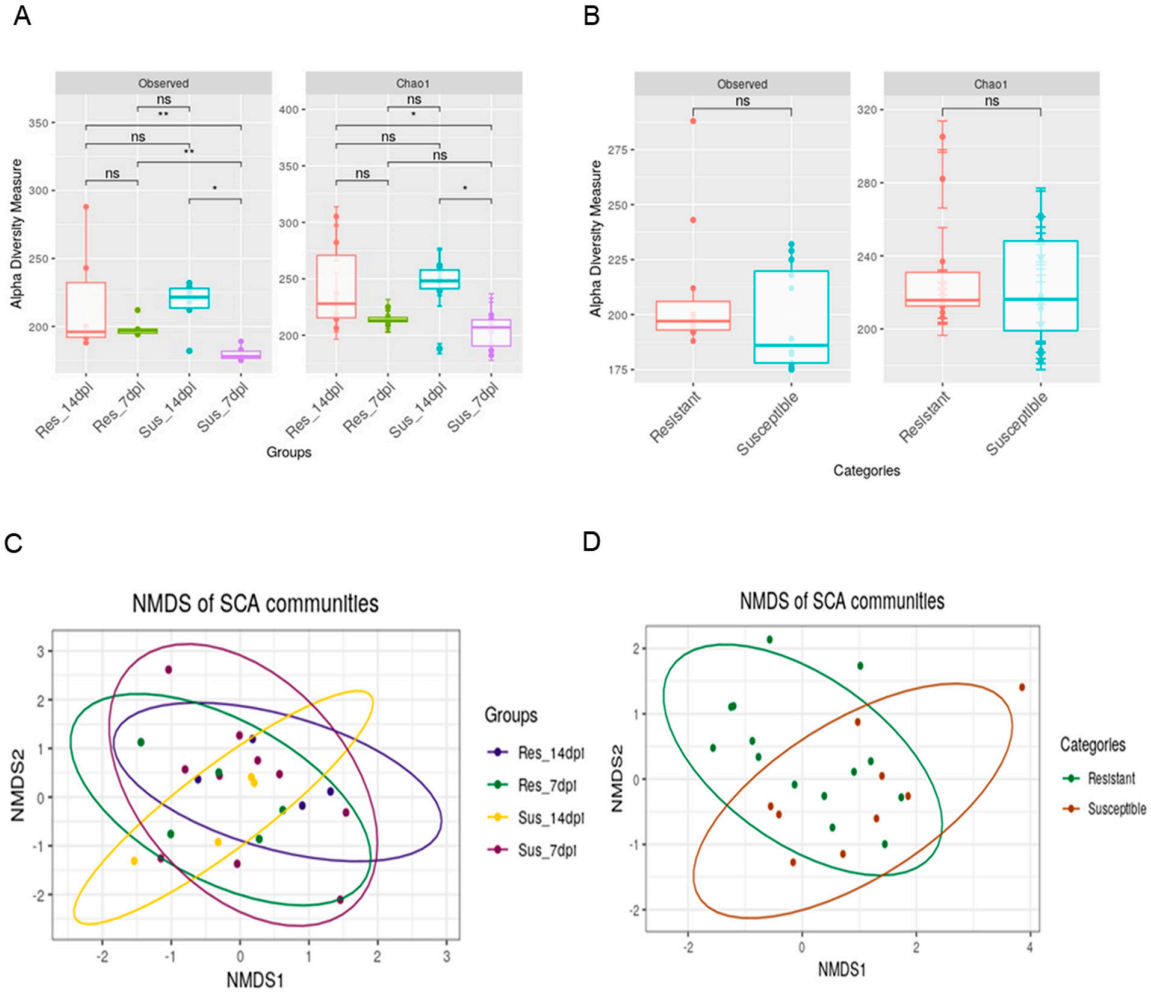

Figure 1. Alpha diversity of bacterial communities in sugarcane aphids (SCA) subjected to different days of infestation in resistant and susceptible sorghum lines. Diversity was measured using observed operational taxonomic units (ASVs) and the Chao1 index. Boxplots of bacterial richness and diversity indices within infestation groups (**A**), and by plant categories (**B**). Non-metric multidimensional scaling (NMDS) plots show no variations in microbiome composition among groups (**C**) and categories (**D**). The colours used in Figure 1 are not intended to represent the same object. All samples were rarefied to a sequence depth of 19,100 reads before being subjected to diversity analyses. *p*-value (0.001 < ** < 0.01; 0.01 < * < 0.05). Res: resistant; Sus; susceptible; ns: no significance; dpi: days post infestation.

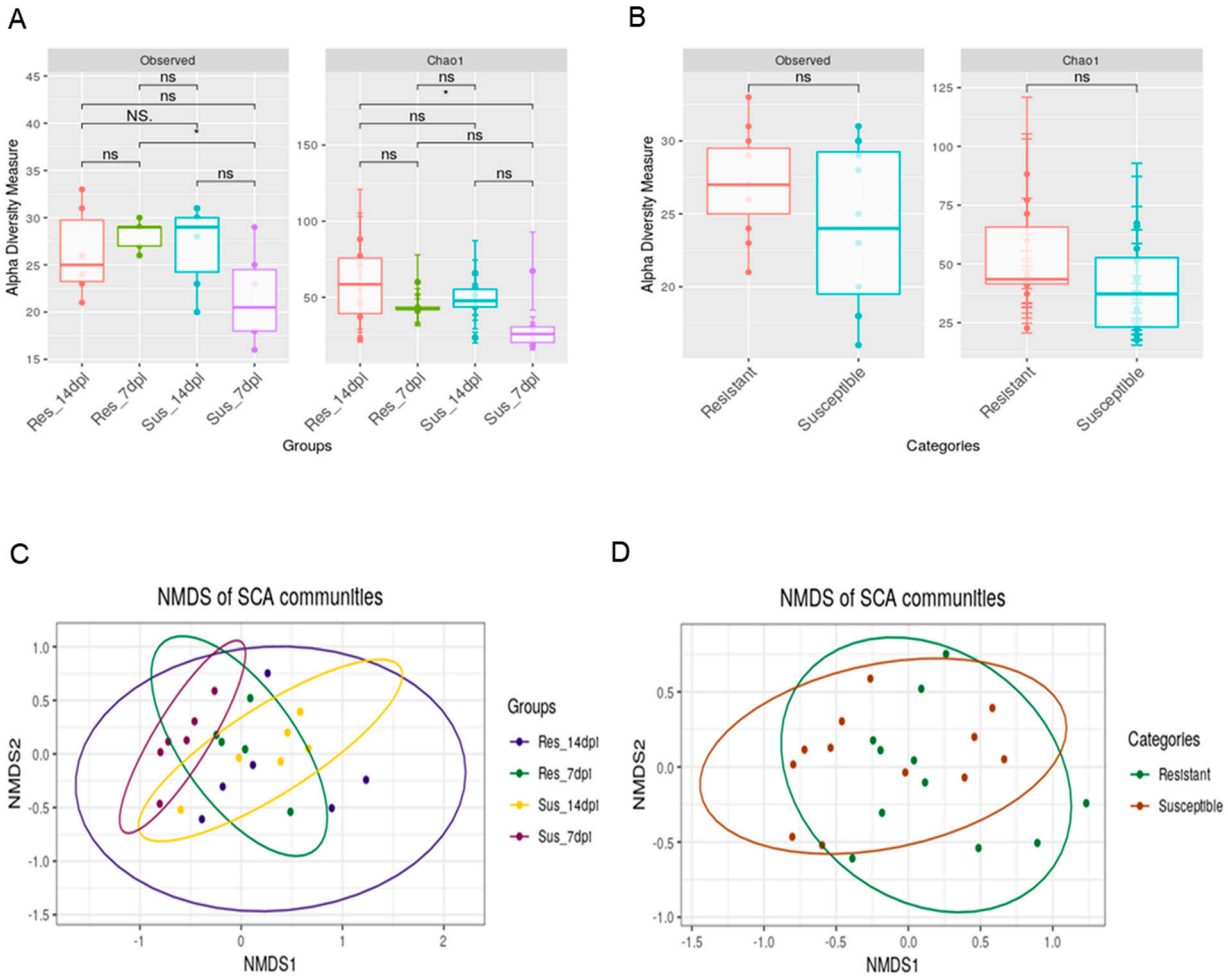

Figure 2. Alpha diversity of the gut microbiome communities in sugarcane aphids (SCA) subjected to different days of infestation on resistant and susceptible sorghum plant lines without endosymbiont bacteria, *Buchnera aphidicola*. Diversity was assessed using observed operational taxonomic units (ASVs), and Chao1. Boxplots of bacteria richness and diversity indices within infestation groups (**A**) and by plant categories (**B**). Non-metric multidimensional scaling (NMDS) plots showed variations in microbiome composition among groups (**C**) and no statistical difference among categories (**D**). The colours used in Figure 2 are not meant to represent the same thing. After removing the primary endosymbiont bacteria, *B. aphidicola*, all samples were rarefied to a sequence depth of 49 reads before being subjected to diversity analysis. *p*-value (0.01 < * < 0.05). Res: resistant; Sus; susceptible; ns: no significance; dpi: days post infestation.

With *Buchnera*, there were significant differences in alpha diversity among groups ($p < 0.05$). There were significant differences in observed ASVs and Choa1 estimates sample categories (Res_7 dpi, Sus_7 dpi Res_14 dpi, and Sus_7 dpi groups) (Figure 1A). There were no significant differences (Figure 1B) among categories (aphids fed on resistant and susceptible sorghum lines). Examination of compositional differences among the gut microbiomes of SCA revealed no distinct clustering of samples according to groups (days post infestation) or categories (resistant vs. susceptible) based on Bray–Curtis dissimilarities (Figure 1C,D). Overall, the variances did not differ significantly among samples for groups (PERMANOVA: F = 0.5, $p = 0.8$) and categories (PERMANOVA: F = 0.9, $p = 0.6$).

Without *Buchnera*, alpha diversity differed significantly among Res_7 dpi, Sus_7 dpi Res_14 dpi, and Sus_7 dpi groups, only for the Chao1 index (Figure 2A). Similarly, there were no significant differences among categories for both diversity estimates (Figure 2B). However, significant differences (Figure 2C) were found among group comparisons (PER-

MANOVA: F = 1.59, $p = 0.02$), whereas no significant differences were observed between the resistant and susceptible categories (Figure 2D) of the sorghum plant lines (PPERMANOVA: F = 0.58, $p = 0.92$) when the primary endosymbiont *Buchnera* bacteria were removed from the sequencing data. Significant differences were also seen in the alpha diversity of groups ($p < 0.05$), specifically in Res_7 dpi and Sus_7 dpi for observed ASVs and Res_14 dpi and Sus_7 dpi for Chao1 (Figure 2A). There were no significant differences among categories (resistant and susceptible) of plants (Figure 2B).

An analysis of microbial taxa driving differences in microbiome composition among the analyzed groups revealed four bacterial families that contributed to the dissimilarity between the groups (Figure 3A) and 14 bacterial families that varied between sample categories (Figure 3B). *Pseudomonadaceae* was the most abundant (94.5%) in Sus_7 dpi, followed by Sus_14 dpi (65.1%), Res_7dpi (36.5%) and Res_14 dpi (30.4%). *Rhizobiaceae* was more abundant in Res_14 dpi (53.2%) and Sus_14 dpi (6.3%) than in Sus_7 dpi (5.5%). The presence of *Pseudomonadaceae* and *Rhizobiaceae* in these orders emphasizes the distinct grouping of Sus_7 dpi compared to the other groups (Figure 3A). Furthermore, the abundance of *Methylophilaceae* at Res_14 dpi (51.9%), Res_7 dpi (16.4%), and Sus_14 dpi (25.4%) distinguished Sus_7 dpi. *Sphingobacteriaceae* was the least abundant bacterial family across all sample groups and was only distributed in Res_7 dpi and Sus_7 dpi.

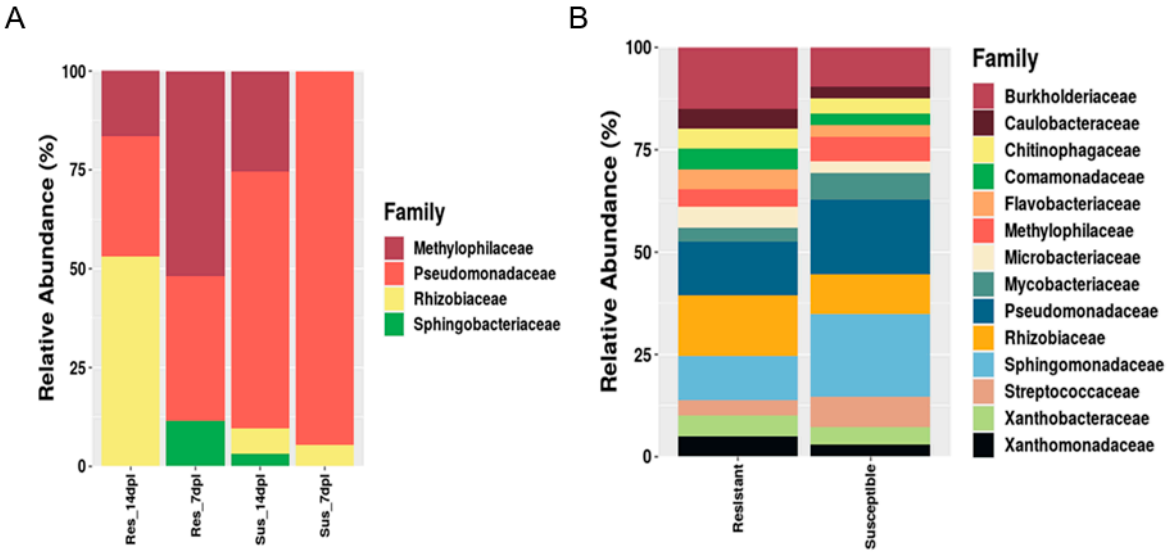

Figure 3. Microbiota differences across gut sugarcane aphids (SCA) subjected to different days of infestation on resistant and susceptible sorghum lines. Differential abundance of family microbial communities among groups (**A**), and categories (**B**). Res: resistant; Sus; susceptible; dpi: days post infestation.

4. Discussion

Plants and animals are surrounded by different microbiomes, inside their systems or outside, where bacteria and fungi are the most abundant [36]. The endosymbiotic bacterium, *Buchnera*, is an already established obligate endosymbiont living inside insect hemocoel in specialized cells called bacteriocytes, synthesizing essential amino acids [37]. The bacteria are maternally transmitted to the progeny (vertical transmission). Sap-sucking insects like aphids have a heavy dependence on endosymbiont bacteria *B. aphidicola* for the synthesis of amino acids, which are deficient in phloem sap [7]. Failure to access essential amino acids like tryptophan and phenylalanine may have adverse effects on aphid growth and reproduction [38].

Previously, it was shown that the *B. aphidicola*-derived protein, the chaperonin GroEL, triggered plant defence responses and reduced susceptibility to aphid infestation [8]. Additionally, *Buchnera* proteins were detected in aphid saliva, suggesting a key role for this

endosymbiont in aphid–plant interactions [8]. Because of the abundance of *B. aphidicola* in the SCA gut microbiome, there was no significant difference in the microbial community and diversity analysis (Figure 1). However, when *B. aphidicola* was removed from the analysis (Figure 2A,B), there was a significant difference in the microbial community, diversity similarity and dissimilarity in the aphids collected 7 days post infestation on the susceptible line, and those from 14 days post infestation on the resistant sorghum line. Recently, it was also demonstrated that the pea aphid (*Acyrthosiphon pisum*) facultative endosymbiont, *Serratia symbiotica*, operates in concert with an aphid effector that benefits aphid colonization on host plants [39]. The *S. symbiotica* in pea aphids upregulated the effector in the salivary glands and suppressed the plant defences, which facilitated the colonization of aphids on host plants [39]. Similarly, symbiotic bacteria present in the mealybug (*Phenacoccus solenopsis*) saliva modulated phytohormone levels, thereby enhancing mealybug colonization on host plants [40]. Whether SCA endosymbionts utilize a similar or different mechanism(s) to manipulate host defences is yet to be determined.

Previous studies have shown that the flagellin derived from one of the symbiotic bacteria, *Pseudomonas sp*. of the Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) larvae, was shown to induce salicylic acid (SA) signalling. Negative crosstalk between the jasmonic acid (JA) and SA signalling pathways, the flagellin suppressed the JA signalling pathway, thereby reducing resistance against the CPB larvae [41]. Similarly, the family *Rhizobiaceae* plays a crucial role in plant-insect interactions by influencing plant growth and defence mechanisms through nitrogen fixation and the production of signalling molecules. This symbiotic relationship can impact insect herbivores and their interactions with host plants [42]. The *B. aphidicola* endosymbiont plays a pivotal role in the intricate interaction between SCA and their host plant, such as sorghum. This symbiotic association has co-evolved over 200 million years, resulting in a mutualistic relationship that significantly influences the fitness, nutrition, and ecological success of both the aphid and its host plant [10].

We have previously shown that SC265 deters SCA feeding from sieve elements, and SCA feeding at early and later times on SC265 plants induced higher levels of SA compared to the RTx430 plants [25]. However, there was no information on the role of microbes in the interaction between SCA and sorghum lines. When the endosymbiont *B. aphidicola* was removed from the analysis, we observed a significant abundance difference in *Pseudomonadaceae* in SCA when they were fed on susceptible sorghum line at 7- and 14-days post-infestation (Figure 3A), which indicates the prominence of these bacteria in sorghum-aphid interactions.

Known for versatile metabolic capabilities, *Pseudomonadaceae*, especially through ISR, significantly contribute to enhancing plant defence mechanisms [43,44]. Their role as keystone microbes not only impacts the fitness of host plants but also influences the behaviour and performance of insects [45]. Similarly, the *Rhizobiaceae* family emerges as a crucial player in plant-insect interactions [46,47]. Through symbiotic relationships with plants, *Rhizobiaceae* influences plant defence mechanisms by modulating hormone levels, affecting the attractiveness of plants to insect herbivores and influencing their feeding behaviour [48]. Previously, it was shown that SCA feeding significantly enhances the SA levels compared to the RTx430 plants [25]. However, the role of *Rhizobiaceae* in modulating sorghum phytohormone levels after SCA feeding remains to be determined. It is also possible that differences in sorghum genotypes (i.e., aphid diets) may influence the gut microbiota of SCA and these microbial changes potentially contribute to differences in the feeding behaviour of the aphids on host plants. Collectively, the potential influence of *Rhizobiaceae* on the rhizosphere and phyllosphere microbiomes underscores its role in the intricate dynamics of insect-microbe-plant interactions.

Our study highlights the intricate relationships between plant and aphid microbiomes, unravelling their impact on sorghum resistance to SCA. Despite dissimilar microbial community abundances in the susceptible (SC1345) and resistant (SC265) lines, no statistically significant differences emerged, underscoring the limited influence of different genotypes

on diverse microbial communities in plant tissues. It is equally possible that the differences in the aphid gut microbial community may be due to the aphid feeding duration than the plant genotype. However, how aphid feeding time influences the microbial community remains to be determined. These observations align with previous studies emphasizing the potential dominance of symbionts over genotypic factors in host resistance [49]. However, the proposition that host genotype can shape keystone microbes introduces a layer of complexity, suggesting a nuanced interplay between host genetics and microbiome dynamics [45].

Conclusively, identifying specific microbial families associated with resistance or susceptibility, exemplified by *Pseudomonadaceae* and *Rhizobiaceae*, offers promising pathways for developing sustainable agricultural strategies. The intricate dynamics explored in this study contribute valuable insights to our understanding of plant-insect-microbe interactions, laying the foundation for targeted interventions in agriculture. The observed differences in the NMDS of microbial communities in the SCA highlight the multifaceted nature of these interactions, encouraging further exploration and refinement of strategies for sustainable agriculture.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16020085/s1, Supplemental Table S1: Differential abundance of the bacterial taxa (including *Buchnera*) in SCA after feeding on two different sorghum lines. Supplemental Table S2: Differential abundance of the bacterial taxa (without *Buchnera*) in SCA after feeding on two different sorghum lines.

Author Contributions: J.L. conceived the research and designed experiments with P.A., E.I. conducted greenhouse experiments. E.I. and P.A. extracted DNA and sent it for sequencing. S.C. analyzed the data. E.I. and S.C. wrote the first draft of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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