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Impact of the Glucosinolate Sinigrin on Bacterial Communities in *Pieris rapae*

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Abstract

Dynamics in animal-associated microbiota can be difficult to study due to community complexity. Previous work showed that microbial communities in the midguts of *Pieris rapae* larvae contain relatively few members. In this study, we used *P. rapae* to test hypotheses related to how diet impacts gastrointestinal microbiota. More specifically, we investigated how the concentration of sinigrin, a glucosinolate in the natural diet of this insect, alters microbial community structure. Larvae were fed either sterile wheat germ diet alone or amended with 3.0 mg/ml, 6.0 mg/ml, or 9.0 mg/ml of sinigrin. In order to determine shifts in the gut microbial community, 16S rRNA genes from midguts were subjected to pyrosequencing and analyzed. Sinigrin had a significant impact on microbial communities in fourth instar *P. rapae* larvae, but this was dependent on concentration. The predominant phyla in all treatment groups were Proteobacteria and Firmicutes. Significant difference in beta diversity was typically observed when sinigrin 6 mg/ml and the control treatment groups were compared. The impact of sinigrin on the structure of the midgut microbiota is dependent on concentration, but not in a linear fashion. This may indicate that types and concentrations of glucosinolates have varied impact on midgut microbial community.

Keywords

Microbiota, Glucosinolates, Plant Secondary Metabolites

1. Introduction

In recent years the inherent complexity of microbial communities has become increasingly apparent. Yet, determining fundamental information regarding species richness, membership, and diversity has often remained elusive [1] [2]. Beyond these issues are questions of classical ecological principles, such as community function, mechanisms of assembly, maintenance of structure, stability and resistance, and the like. Association with ani-

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mal hosts adds yet another layer of complexity for understanding these dynamic assemblages. Some of these questions can be hard to address simply because of high community richness, but external factors such as host physiology can also present a challenge.

While the application of classical ecological principles gives the microbial ecologist a framework on which to build, model systems are also needed [1]-[3]. These models are necessary in order to learn about the complex interactions between hosts and their microbes, as well as the diverse interactions that occur among community members. Insects, like other animals, depend on their microbial communities for a number of functions related to host health and propagation [4]. Many insects have been shown to contain gut microbiota that are relatively simple in composition and exhibit lower diversity than mammalian systems [4]-[6].

Microorganisms inhabiting insect gastrointestinal tracts have been shown to benefit their host via a number of different functions, with several being attributed to the processing of dietary components [7]. For example, studies in termites have revealed that gut microbes aid in the degradation of plant material, while studies in cockroaches revealed that the adaptive abilities of its microbiota in response to changes in diet aid in host survival in different environments [8] [9]. These host-microbe interactions can lead to an improved ability to live on suboptimal diets, improved digestion efficiency, acquisition of digestive enzymes, and detoxification of plant allelochemicals [10]-[12]. With help from their microbial communities, insects can adapt to various challenges [13]. For example, it is hypothesized that insects' differing abilities to consume plants containing toxic chemicals may be partially dependent on the structure of their microbial communities [14].

For many insects, the midgut microbial community is acquired through a combination of maternal source, the environment, and the diet. The dominant influence for community structuring in the family Lepidoptera, which is comprised of the moths and butterflies, appears to be diet [15]-[17]. Lepidoptera contain relatively simple microbiota that are suspected to aid in the metabolism of toxic compounds within the insects preferred host diet [16]-[18]. The natural host plants for *P. rapae* include important crop plants from the Brassicaceae family, all containing different types and concentrations of glucosinolates [19]. Recently it has been suggested that phytochemicals influence the midgut microbiota with the observation of foliar defense chemistry in trembling aspen tree genotypes being positively correlated with shifts in the gypsy moth midgut microbiota [20]. As with most plants, secondary chemical compounds act as a deterrent to herbivorous predators by releasing toxic derivatives after the degradation of the phytochemical [21]. In brassicas, upon tissue wounding—such as chewing, myrosinase is released from damaged vacuoles and degrade glucosinolates, which leads to the accumulation of isothiocyanates, a toxin to insects [22]. However, *P. rapae* is a specialist insect and produces nitrile-specifier proteins, which degrade the majority of glucosinolates into less harmful nitriles [21]-[23]. Previously, we showed that sinigrin, a specific type of glucosinolate, played a role in shaping microbiota structure in the *P. rapae* midgut and altered colonization resistance to non-pathogenic invaders [17]. In this study, we investigate whether sinigrin impacts the structure of the midgut microbiota in *Pieris rapae* in a dose dependent manner. This study will lead to a better understanding of the interactions between plant chemical defenses and insect microbiota.

2. Materials and Methods

2.1. Insect Husbandry

Eggs purchased from Carolina Biological Supply (Burlington, NC) were used to establish the Robinson Lab cabbage white butterfly colony. Eggs from the colony were surfaced sterilized as described previously [17]. Treated eggs were then placed in a sterile Petri dish containing diet. Larvae were reared until fourth instar and Petri dishes were only opened to aseptically change food and clean dishes inside a sterile biosafety cabinet.

2.2. Preparation of Diet

Gypsy moth wheat germ diet premixed with agar was obtained from MP Biomedicals (Santa Ana, CA), prepared according to manufacturer's protocol and then autoclaved. Diet was cooled and amended with filter-sterilized sinigrin to a concentration of 3.0 mg/ml, 6.0 mg/ml, or 9.0 mg/ml, or prepared without sinigrin. All diet preparation after autoclaving occurred in a sterile biosafety cabinet.

2.3. Dissection

Fourth instar larvae were starved for 4 h and then individuals from each treatment group were randomly selected

and dissected as described previously [17]. Dissected midguts were placed in sterile 1X PBS and stored at −20˚C until DNA extraction.

2.4. DNA Extraction

DNA was extracted using the Invitrogen PureLink Genomic DNA Kit (Thermofisher Scientific; Grand Island, NY) with modifications to the protocol as follows. Midguts were physically disrupted by pipetting and vortexing. 500 µl of the 1X PBS plus midgut sample was removed and placed into a sterile 1.5 ml microcentrifuge tube. The sample was then centrifuged at 10,000 g for 1 min—followed by removal of 450 µl of the supernatant. The remaining sample was then vortexed and sonicated for 1 min, followed by the addition of 180 µl of Genomic Digestion Buffer and 20 µl of Proteinase K supplied with the kit. The samples were then incubated for 1 h at 55˚C. From this point on, the manufacturer's protocol was followed. DNA was stored at −20˚C.

2.5. Sequencing and Community Analysis

DNA samples were submitted to the Center for Microbial Systems at the University of Michigan on the Illumina MiSeq platform, using the MiSeq Reagent Kit v2 (500-cycle format) for sequencing of the V3 and V4 regions of the 16S rRNA gene as described previously [24]. Due to low biomass, a "touchdown PCR" protocol was followed during the amplification of the 16S rRNA gene: one cycle of 95˚C for 2 min followed by 20 cycles of 95˚C for 20 s, 60˚C for 15 s and 72˚C for 5 min with a temperature decrease of 0.3˚C each cycle, followed by 20 cycles of 95˚C for 20 s, 55˚C for 15 s and 72˚C for 5 min, and a final extension of 72˚C for 10 min [25]. Sequences were then analyzed in mothur v.1.34.4 and the MiSeq SOP [24] [26], http://www.mothur.org/wiki/MiSeq_SOP. Briefly, sequences were aligned using the Silva database for bacterial identification, chimeras were removed and detected using the mothur implementation of u-chime, and then curated sequences were classified using the mothur-formatted version of the RDP training set (v.9) at a cutoff of 0.03. All sequences that classified as chloroplast, mitochondria, eukaryotic, or unknown at the Kingdom taxonomic level were removed. Operational taxonomic units (OTUs) were generated by binning sequences that were at least 97% similar ($OTU_{0.03}$) using the average neighbor method. To avoid sampling intensity errors, all sequences were sub-sampled to a minimum number of sequences.

Mothur was also used to conduct ecological tests measuring alpha and beta diversity. For alpha diversity measurements the following metrics were used: Good's coverage, Chao1 estimated richness, and the inverse Simpson diversity index. Beta diversity was assessed using metastats and principal coordinates analysis (PCoA) plots of θYC values [27]. Metastats was used to determine whether OTU abundances were significantly different between treatment groups; whereas PCoA plots of θYC values followed by AMOVA was used to detect significant clustering of communities due to similarities in OTU affiliation and abundances [28]. Additionally, HOMOVA was used to detect whether there were differences in community variances between treatment groups.

Sequences were submitted to the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA; http://www.ncbi.nlm.nih.gov/sra) and can be accessed via study accession number SRP078163.

3. Results

Good's coverage values of 99% indicated that the sampling effort was sufficient for all treatment groups, while observed richness and estimated richness were indistinguishable between treatment groups (**Table 1**). Diversity, however, was significantly increased in the communities of larvae fed diet containing 6.0 mg/ml and 9.0 mg/ml sinigrin as compared to those in larvae fed diet containing no sinigrin or 3.0 mg/ml sinigrin (**Figure 1**).

Proteobacteria and Firmicutes were the predominant phyla in the midguts of all larvae. However, there was a significantly lower amount of Firmicutes and a significantly higher amount of Proteobacteria in larvae that were fed diet containing 6.0 mg/ml sinigrin than in larvae fed any other diet. The proportion of Bacteriodetes was also increased by feeding larvae diet containing 6.0 mg/ml sinigrin as compared to no sinigrin (**Figure 2**).

PCoA of θYC values and AMOVA revealed a lack of significant community clustering at the species level of samples based on diet, except for in the case of insects fed 6.0 mg/ml sinigrin, which exhibited significant clustering (**Figure 3**). Applying HOMOVA to θYC values revealed that the variance between individual treatment groups was similar, except when larvae were fed diet containing 6.0 mg/ml sinigrin (**Table 2**).

Table 1. Sample data, coverage and richness.

Treatment groups	Number of sequences	Mean Good's coverage (0.03 cutoff)	Mean observed OTUs[a]	Mean Chao 1 estimated richness (C.I.)	Mean inverse Simpson index (C.I.)
Control[b] (n = 12)	1524	99%	30	40.4 (32.6 - 73.2)	2.5 (2.4 - 2.7)
Sin 3.0 mg/ml (n = 15)	1524	99%	31	45.2 (34.7 - 87.2)	3.1 (2.9 - 3.3)
Sin 6.0 mg/ml (n = 18)	1524	99%	38	50.1 (41.4 - 85.6)	6.2 (5.8 - 6.7)
Sin 9.0 mg/ml (n = 16)	1524	99%	29	44.1 (33.3 - 87.2)	4.0 (3.8 - 4.3)

[a]OTU = operational taxonomic unit, binned at 97% similarity. [b]No sinigrin added to the sterile artificial diet.

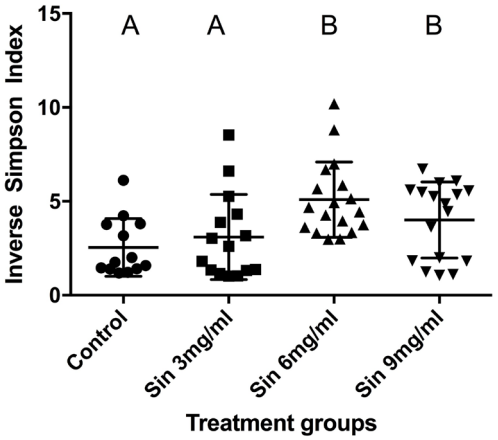

Figure 1. Community diversity across treatment groups. Inverse Simpson index calculated for communities in larvae fed unamended sterile artificial diet (control), and sterile artificial diet amended with 3.0 mg/ml, 6.0 mg/ml, or 9.0 mg/ml sinigrin. Significant differences indicated by different letters (Kruskal-Wallis and Mann-Whitney $p < 0.05$).

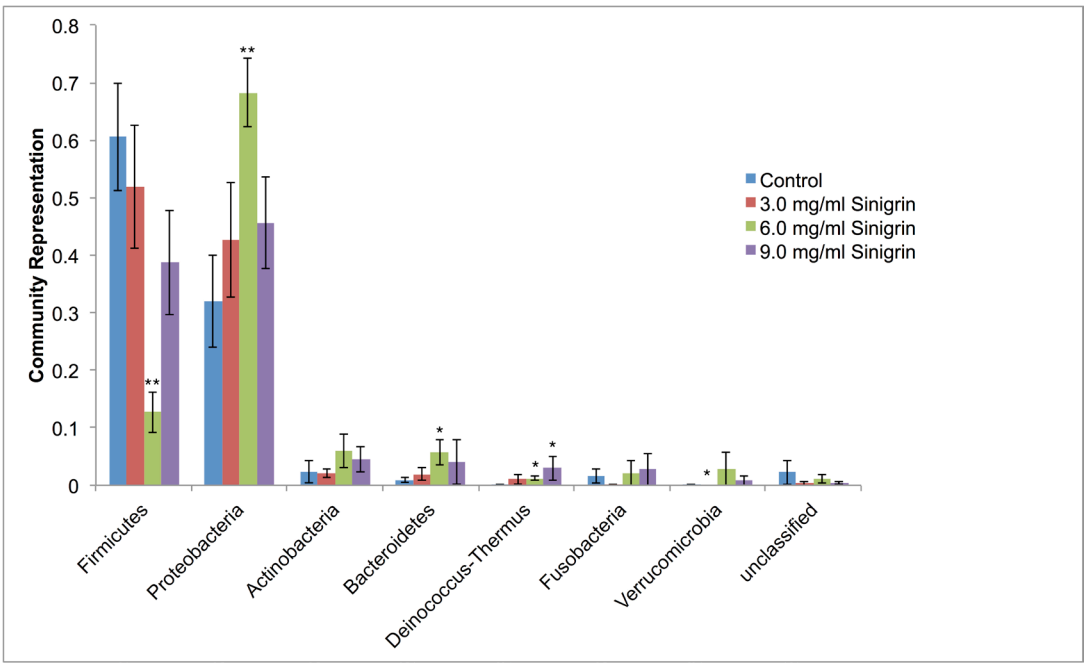

Figure 2. Effects of sinigrin on phylum-level community structure. Population-level analysis showing the abundance of phyla found within each treatment group. Significant major phyla found within each treatment group. Significant differences indicated by asterisks (metastats, $p < 0.05$). *Significant difference between treatment group as compared to control group only; **Significant difference between treatment group as compared to all other groups.

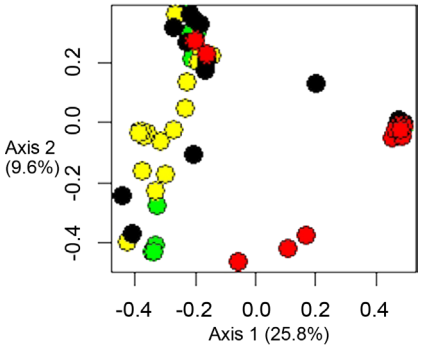

Figure 3. Impact of sinigrin on community structure at the species level. PCoA of θYC values at species-level for communities resulting from different concentrations of sinigrin. Circles indicate differences of communities in individual insects fed an unamended control diet (red), diet amended with 3 mg/ml (green), 6 mg/ml (yellow), or 9 mg/ml sinigrin (black). AMOVA indicated that clustering at the species level was only significant for communities in larvae fed 6 mg/ml of sinigrin ($p < 0.001$).

Table 2. HOMOVA of θYC values at species-level.

Treatment Groups[a]	B Value	P-value
Con-Sin 3-Sin 6-Sin 9	1.490	<0.001*
Con-Sin 3	0.950	0.077
Con-Sin 6	0.963	<0.001*
Con-Sin 9	1.370	0.018
Sin 3-Sin 6	0.002	0.816
Sin 3-Sin 9	0.036	0.484
Sin 6-Sin 9	0.061	0.193

[a]Treatment groups are larvae fed unamended sterile artificial diet (Con), and sterile artificial diet amended with 3.0 mg/ml (Sin 3), 6.0 mg/ml (Sin 6), or 9.0 mg/ml (Sin 9) sinigrin. Significant differences indicated by asterisks (experiment-wise, $p < 0.05$; pair-wise, $p < 0.008$).

4. Discussion

Glucosinolates are naturally found in the preferred diet of *P. rapae* and the goal of this study was to investigate the impact of various concentrations of a single glucosinolate on the microbiota and lay groundwork for understanding its effects on host-microbe dynamics. We determined that sinigrin, a commercially available glucosinolate, shapes community structure and that this influence may be dependent on concentration. Previous work focused on the concentration of sinigrin found naturally in Brussels sprouts (3.0 mg/ml) and its affect on the host gut microbial community [17] [29]. This previous study revealed that communities in larvae fed sinigrin were significantly different from those in larvae fed control sterile artificial diet, but still structurally similar at the phylum level. Likewise, we observed similarities in community structure for larvae fed various concentrations of sinigrin. However, in this study, the effects of sinigrin was most evident in larvae fed a concentration of 6.0 mg/ml sinigrin, while the microbial communities in larvae that fed on 3.0 mg/ml and 9.0 mg/ml sinigrin contained similar structures to that of the control larvae. Interestingly, it was also determined that higher concentrations of sinigrin increased diversity, as measured by the inverse Simpson's index. While larvae fed 6.0 mg/ml and 9.0 mg/ml sinigrin contained structurally different communities, they were similar in the number of types of organisms (defined by $OTU_{0.03}$) and evenness of the distribution of the different types.

The observation that secondary plant metabolites can influence microbiota structure is not specific to glucosinolates or to Lepidoptera. For example, food-derived tannins, compounds found in high concentrations in aquatic angiosperms, were observed to influence the gut microbiota of the herbivorous aquatic moth, *Acentria ephemerella*, whereas the midgut microbiota of *Schistocerca gregaria*, the desert locust, and the termite, have also been influenced by secondary plant compounds [7] [8] [30]. While these specific phytochemicals can create perturbations to the microbiota, a measure of resilience can indicate the influence of plant compounds on the microbial community structure.

Resilience is the rate at which a community returns to its natural state after perturbation [31]. Studies investigating resilience in insect midguts have shown that these communities are able to return to native community structure once removing perturbation factors such as antibiotics or high fat concentrations [17] [32]. In this study, the perturbation factor was not removed and yet the sinigrin 9.0 mg/ml treatment group showed no significant difference from the control treatment group. This suggests that microbial communities within the sinigrin 9.0 mg/ml treatment group may have exhibited higher resilience, than the sinigrin 6.0 mg/ml treatment group. In other words, there may have been an initial structural shift in response to the higher concentration of sinigrin, followed by a return to baseline structure that was not observed in the 6.0 mg/ml treatment group. However, because there was no temporal component of this study, the dynamics of the community in response to higher concentrations of sinigrin remain to be seen.

The impact of the various concentrations of sinigrin on community structure could be the result of multiple factors. Related to resilience, similarities found between larvae feeding on 9.0 mg/ml of sinigrin and the control diet may be attributed to the host's physiological response or a direct effect of higher concentrations of glucosinolates on the microbiota in order to return the microbial community to its core structure [20]. In the study by Mason *et al*. [20], differences in gypsy moth gut microbiota structure was influenced by various concentrations of tannins and phenolic glycosides (secondary plant compounds in trembling aspen genotypes). The study suggested that shifts in the microbial community could be attributed to either direct or indirect factors. Direct effects would include the microbial community shifting due to the antimicrobial nature of the phytochemicals, while indirect effects would include physiological changes in the host due to damaging levels of a toxic phytochemical that would in turn alter community structure. In our system, although *P. rapae* produces nitrile-specifier proteins, which shift glucosinolate degradation towards the production of less harmful nitriles, toxic isothiocyanates are still produced at low levels [22] [33]. Therefore, it is possible that when larvae were fed diets containing higher concentrations of sinigrin, higher amounts of isothiocyanates could have had antimicrobial effects on newly dominant members of the microbiota such they that resulted in a structure that resembled the control community. Additionally or alternatively, these higher concentrations of glucosinolate could have resulted in physiological changes to the host that in turn had impacts on microbiota structure. Further defining these observations and the underlying mechanisms will be the focus of future work.

Similarly, since a change in community structure was observed in larvae fed 6 mg/ml of sinigrin it was expected that larvae fed a higher concentration such as, 9.0 mg/ml of sinigrin, would have also shown a shift in structure as compared to the control group. An early study observing the effects of glucosinolate levels and pest control found that higher levels of glucosinolates in oilseed rape resulted in increased incidence of *P. rapae* [34]; suggesting a beneficial relationship between higher concentrations of glucosinolates and the insects. However, that study also showed that the incidence of insects was influenced by the different amino acid derivatives and/or side chains of the glucosinolates. Future work will help reveal the impact of high concentrations of various types of glucosinolates in addition to sinigrin on midgut community structure. In the case of the Arctiid moth, *Parasemia plantaginis*, feeding on its host plant species containing plant secondary metabolites not only increased its growth rate, but also the ability to effectively encapsulate foreign objects as an immune defense [35]. In a study investigating the impact of host genetic variation as related to glucosinolate production (type of glucosinolate and amounts of glucosinolates) on various aspects of *P. rapae* health when feeding on populations of wild cabbage, sinigrin, an aliphatic glucosinolate, did not affect larval development [36]. However, *P. rapae* larvae were found to develop poorly on high levels of indole glucosinolates such as, neoglucobrassicin [36]. Conversely, another study observed that *P. rapae* developed more poorly on plant types containing a high concentration of aliphatic glucosinolates [23]. It was suggested that higher concentrations of glucosinolates enhanced expression of the nitrile-specifier protein, thus leading to increased energy costs for *P. rapae*. However, it was acknowledged that the opposing findings of the two studies may have been due to differences in strains of insects used as well as plant species [23]. Anecdotal observations during our study indicated that larvae feeding on diet containing sinigrin 6.0 mg/ml appeared to grow at an increased rate with fewer signs of melanization (which plays a role in immune response to pathogens and wound healing) when compared to all other treatment groups. This suggests that there are physiological effects of sinigrin and that there might be an optimal concentration or amount and type of dietary glucosinolates for *P. rapae* as it relates to overall host health.

Whether the impact of plant chemical defenses on insect hosts is tied to alterations to the microbiota structure and/or to the host immune system or other host physiology is an area for future experimentation. Given the diversity of phytochemicals, the insects that are exposed to them, and insect-associated microbial communities, it

is clear that studies like this one have the potential to elucidate many unknown, biologically significant interactions that could have wide reaching implications for understanding plant-insect and host-microbe interactions.

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