Synthetic diets containing a single polysaccharide disrupt gut microbial community structure and microbial interaction networks in the American cockroach

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Running Head: Synthetic Diets Modify Cockroach Microbiome

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Abstract

Achieving and maintaining a healthy gut microbiome has numerous benefits for the host. Host diet plays a key role in shaping the gut microbial community, and understanding how diet composition influences gut microbiome structure and stability is key to developing effective interventions to treat gut microbiome dysbiosis. We use the American cockroach (*Periplaneta americana*) as a model system to dissect the response of gut microbes to host diet modification. Here, we designed synthetic diets from lab-grade, purified ingredients to identify how the cockroach gut community responds to different carbohydrate components (chitin, methylcellulose, microcrystalline cellulose, pectin, starch, xylan) in otherwise balanced diets. Using 16S rRNA gene sequencing, we show that synthetic diets produce replicable shifts in the cockroach gut community diversity and phylogenetic composition, with xylan-fed insects displaying the largest alterations. Comparison with cockroaches fed whole-food diets reveal that, rather than introducing new microbes, synthetic diets alter microbiome composition by inducing blooms among taxa present basally within the cockroach gut community. Synthetic diets are also associated with less-robust, more fragmentary microbial co-occurrence networks compared to cockroaches fed whole-food diets. Our results highlight the utility of lab-grade artificial diets in microbiome research and shed light on how purified polysaccharides may exert more influence over a stable gut community to generate noticeable change than whole food-derived fibers.

Importance

Cockroaches are unusual but effective model organisms for understanding how the gut microbiome reacts to dietary changes. Previously, research in the cockroach gut microbiome demonstrated surprisingly robust stability following extreme dietary shifts using whole foods, but our work uncovers the potential of synthetic diets to induce change in these stable communities. The observation that diets enriched in purified polysaccharides reduced gut microbiome stability and undermined microbial interaction networks has important implications for the use of prebiotics to induce targeted changes in gut microbiome composition and structure.

Introduction

The gut microbiome is a key player in host metabolism and homeostasis; it extracts energy from recalcitrant dietary components, provisions essential nutrients, and stimulates the host's immune system to protect against pathogens and toxins [1-4]. Microbiome research seeks to identify specific microbes and microbial community configurations that influence host wellbeing, as well as techniques to maintain a desirable community once achieved [5, 6]. Key drivers of gut microbiome community structure include host genetics [7, 8], environment, and most notably, diet [9, 10]. Diet has gained particular attention as the most easily manipulated of these factors, and a clear relationship exists between microbially derived metabolic products from the gut microbiome and overall host health [11, 12]. However, the complex nutritional needs of mammalian models limit the extent of dietary manipulation possible, while well-known insect models such as *Drosophila* have limited dietary range. To address this challenge, we are developing the omnivorous American cockroach (*Periplaneta americana*) as a model of microbiome dynamics that extends our understanding of human-relevant bacteria while offering the benefits of invertebrate research.

Omnivorous cockroaches such as *P. americana* are colonized by a complex hindgut microbiome that is taxonomically similar to the human colonic flora, consisting of many shared lineages of microbes, including diverse Bacteroidota, Firmicutes (now Bacillota), and Proteobacteria (now Pseudomonadota) [13-15]. The hindgut microbiota also plays an analogous role in host nutrition, with hindgut bacteria fermenting otherwise indigestible dietary components into volatile fatty acids that can be utilized as an energy supplement by their hosts or influence host development [16-19]. Further, the extreme dietary flexibility conferred by *Blattabacterium* endosymbionts and the unique ability for cockroaches to store nitrogen as uric acid for future use

facilitates extensive dietary manipulation [20-22]. Studies of cockroach gut microbiome responses to diet have generated contrasting responses, with multiple large-scale studies finding that the gut microbiome is highly stable following dietary shifts [14, 23, 24], while others have demonstrated shifts in response to select diets [25-27]. Currently, there is no consensus on why these studies produced differing results and comparison is difficult due to inconsistent use of synthetic, natural, or whole food diets across studies. Whole food or natural diets may obscure bacterial responses to nutritional contents given their complexity, while synthetic diets are more amenable to precise dietary changes, thus controlling for more variables [28-31]. Artificial diets have been successfully developed and used as replacement for natural diets in insects with far more specialized dietary needs than cockroaches, implying that cockroaches are an ideal candidate for use with lab-synthesized diets [32-35].

In order to facilitate precise manipulation of dietary composition in cockroaches, we have developed a series of synthetic cockroach diets based on the work of early entomologists [36-38]. These artificial diets serve as a nutritionally complete base to precisely evaluate the influence of specific dietary components on the *P. americana* hindgut microbiome, which as mentioned above had previously been shown to be strongly resistant to dietary manipulation using whole food diets [14]. Using these synthetic diets as a base, we tested a spectrum of polysaccharides as the primary carbon and energy source to identify if the hindgut microbiome responds to specific fibers. We found that these diets resulted in much stronger impacts on gut microbiome composition than even highly divergent whole food diets. In seeking to understand these divergent responses, our results will facilitate future studies of gut microbiome responses to diet in the cockroach and shed light on how gut microbiome stability fluctuates when challenged with synthetic diets compared to complex whole food diets.

Results

Impacts of synthetic diets on gut microbiome diversity and community composition

We formulated a series of synthetic diets composed of a fixed base of 25% protein amended with dietary salts, vitamins, and cholesterol while differing only in complex carbohydrate type. Initial experiments utilized five alternative polysaccharide sources: chitin, methylcellulose, microcrystalline cellulose (MCC), pectin, or xylan. Following initial analysis of these results, we tested an additional starch-based diet. The prepared diets were readily consumed by the cockroaches in all cases.

To evaluate the impact of these diets on the gut community, each diet was fed to adult cockroaches for a period of 14 consecutive days, after which the insects were sacrificed, and their hindgut dissected out for 16S rRNA gene library sequencing. Following library preparation and sequencing, we used DADA2 to obtain 2,321,848 quality-controlled, assembled sequences assigned to 3308 amplicon sequence variants (ASVs) after removal of endosymbiont (*Blattabacterium* sp.) and mitochondrial sequences [39].

At the phylum level, at least 80% of each sample was dominated by Bacteroidota, Firmicutes, and Desulfobacterota, in agreement with previous studies on the cockroach gut microbiome (**Figure 1A**) [14, 40, 41]. The relative abundances of these three phyla were similar across all samples excluding xylan-fed cockroaches; these insects hosted notably more Firmicutes and less Desulfobacterota than cockroaches fed other diets (**Figure 1A**).

Alpha diversity, as measured by Shannon index, evenness, and community richness significantly differed across diet treatments (**Figure 1D-F;** Kruskal Wallis $p < 0.001$ for each). Pairwise analyses found that chitin-fed insects possessed higher Shannon index values ($p < 0.05$)

and community evenness ($p < 0.01$) than that of MCC- and starch-fed insects, while the xylan diet resulted in lower alpha diversity measures than all other diets ($p < 0.05$ for each).

Beta diversity analyses using weighted and unweighted Bray Curtis dissimilarity revealed significant impacts of our synthetic diets on gut microbiome composition. On average, between-diet variation was greater than within-diet variation, with xylan-fed communities showing the greatest dissimilarity (**Figure 1G and 1H**). Ordination analyses using non-metric multidimensional scaling (NMDS) and PERMANOVA analysis showed that samples clustered based on diet composition in both weighted (**Figure 1B;** PERMANOVA: R^2=0.42; p<0.001) and unweighted (**Figure 1C;** PERMANOVA: R^2=0.329; p<0.001) Bray-Curtis metrics, with especially clear separation of the xylan-based diet from other synthetic diets. Removing xylan-fed samples from diversity calculations did not eliminate diet-based clustering for weighted (**Figure S1A;** PERMANOVA: R^2=0.343; p<0.001) or unweighted (**Figure S1B;** PERMANOVA: R^2=0.247; p<0.001) measures, suggesting that each carbohydrate source enriched for a unique community composition. Interestingly, all samples produced significantly larger weighted distances than unweighted from others within their diets, except for xylan-fed samples (**Figure S2**; xylan vs xylan: $p = 0.1266$), suggesting that variation in microbial abundance rather than microbial presence/absence may be driving much of the microbiome response to diet.

We used DESeq2 to identify 76 microbes that exhibited significantly higher abundance in a single synthetic diet across pairwise comparisons against all other treatments, which we termed "diet-characteristic taxa" (**Figure S3**) [42]. Diet-characteristic ASVs were primarily assigned to Firmicutes (n=48) and Bacteroidota (n=20); other phyla with diet-responsive taxa include Fusobacteriota, Deferribacterota, Desulfobacterota, Fibrobacterota, and Spirochaetota. We found

that the chitin and methylcellulose diets were not associated with any diet-characteristic taxa by this definition, while diets made with xylan, MCC, starch, and pectin enriched for 45, 10, 13, and 8 ASVs respectively.

Cohort effects on diet-driven differences in gut microbiome composition

To evaluate cohort effects on gut microbiome responses to diet composition, we conducted a replicate experiment utilizing the MCC- and xylan-based diets. These diets were selected for replication and follow-up experiments due to both their contrasting molecular structure and the dissimilarity they generated in Bray-Curtis analyses (**Figure 1B-C**). Data from the first and second experiments exhibited similar alpha diversity measurements (**Figure 2C-E**), with replicates maintaining the significant shifts in alpha diversity ($p < 0.001$) observed in the initial experiment between these two diets while showing no difference between replicates.

Beta diversity analysis showed that samples clustered by both replicate and diet (**Figure 2A-B**). Diet had large effects on both weighted (PERMANOVA: R^2=0.34; $p<0.001$) and unweighted (PERMANOVA: R^2=0.20; $p<0.001$) Bray-Curtis dissimilarity, while replicate had a much smaller effect significant for unweighted (PERMANOVA: R^2=0.058; $p<0.01$) but not weighted (PERMANOVA: R^2=0.037; $p=0.1$) measures. Replicate cohorts differed primarily in the representation of rare ASVs, with 27% of ASVs present uniquely in cohort 1 and 20.2% in cohort 2 samples (**Figure 2F**). However, 66.7% and 61% of these taxa appeared only in one sample (**Figure S4**), and overall represented 1.6-1.7% of total sequences obtained for each cohort. Direct pairwise comparison of cohorts separated by diet treatment showed similar patterns, with rare taxa contributing few reads despite comprising 19.7-33.7% of unique ASVs

(**Figure 2F**). Altogether, these results show that synthetic diets reproducibly alter the gut microbiome composition in cockroaches.

Microbiome diversity comparison with whole food diets

The changes in community composition triggered by our synthetic diets were unexpected given that previous experiments examining the impact of whole food diets with strongly differing macronutrient profiles did not induce substantial change in gut microbiome composition [14]. Therefore, we compared the samples from this current study ("synthetic" diet type) to samples from the previous study ("whole food" diet type) of cockroaches fed butter, tuna, honey, white flour, or whole wheat flour [14].

We found that gut microbiome samples from cockroaches fed synthetic diets exhibited higher ASV richness ($p<0.01$) but lower evenness ($p<0.001$) and Shannon index ($p<0.01$) than those from insects fed whole foods (**Figure 3C-E**). Synthetic and whole food diets produced distinct diet type clusters in NMDS ordinations (**Figure 3A-B**) for weighted (PERMANOVA: $R^2=0.105$; $p<0.001$) and unweighted (PERMANOVA: $R^2=0.152$; $p<0.001$) Bray-Curtis dissimilarities. When we analyzed the samples by diet, we found diet explained more variation in NMDS ordination than diet type as interpreted from PERMANOVA R^2 values (weighted: $R^2=0.393$; unweighted: $R^2=0.369$).

Beta dispersion analysis of variation within diet types showed that, together, the gut microbiota of cockroaches fed synthetic diets was more variable than that observed among whole food fed cockroaches (**Figure S5A-B**; Tukey's HSD: $p<0.001$). However, when diets were analyzed individually, they were equally dispersed in weighted Bray-Curtis dissimilarity (**Figure S5A)** but not unweighted measures **(Figure S5B;** ANOVA: $p<0.001$). Xylan-fed cockroaches

exhibited significantly greater within-group variability than all ten other diets (Tukey's HSD range: $p = 0.037 – 3.17e^{-06}$), with no significant differences observed in pairwise comparisons of all other diets. When synthetic diets were compared to whole food diets without including xylan-fed samples, we observed no significant differences in unweighted beta dispersion (**Figure S5D**) or Shannon index values (**Figure 3D, red boxes**). However, significant differences remained in weighted Bray-Curtis dispersion (**Figure S5C**), richness and evenness (**Figure 3C, 3F**), and in both weighted and unweighted Bray-Curtis ordination analysis **(Figure S6A-B)**, highlighting that the altered microbiomes produced by the synthetic diet type were not solely due to biases produced by xylan-fed samples.

Core taxa differences between synthetic and whole food diets

Given these strong differences in community structure, we utilized the R package UpSetR to determine how the ASVs in different diet types overlap [43]. UpSet plots are akin to Venn diagrams, considering only presence/absence of an ASV. Rarefied count tables were aggregated by diet and ASVs were marked as either present or absent per diet. ASVs present in the same subset of diets were grouped into "Sets", with the phylum-level composition per set depicted as stacked bar charts labelled with the number of included ASVs (**Figure 4A**). We supplemented the UpSet plot with pie charts illustrating the relative abundance (calculated as the fraction of total reads recovered from the collapsed treatment group) of reads assigned to ASVs within each set (**Figure 4A**). For simplicity's sake, **Figure 4** shows only the five largest intersecting sets as well as all single-diet sets; additional sets are presented in **Figure S7**.

A total of 492 ASVs ("Set 1") were shared across all diet treatments (**Figure 4A**). These ASVs made up over half of the sequences recovered for all diets except the MCC diet, for which

they represented 49% of sequences. Only 43 ASVs ("Set 4") were exclusive to the whole food diets, contributing between 0.9% and 1.65% of reads in these diet sets. The 57 ASVs ("Set 3") identified as exclusive to synthetic diets made up 1.6-3.4% of the reads recovered from starch-, pectin-, chitin-, and methylcellulose-fed cockroaches, and 7% of xylan- and 13% of MCC- fed cockroaches. Together, these results argue that the synthetic diets did not eliminate core taxa present in the guts of cockroaches fed whole foods, nor did they result in hindgut colonization by a large new set of microbial taxa.

Similarly, individual synthetic diets were not associated with hindgut colonization by large groups of unique microbes. In general, taxa that were unique to individual synthetic or whole food diets represented a very small proportion of sequences recovered (**Figure 4A**). ASVs unique to the MCC diet formed the second largest set overall, with 160 diet-unique taxa, yet they only represented 0.4% of total recovered sequences (**Figure 4A**). A xylan-based diet, which repeatedly produced the largest community dissimilarities (**Figure 3A-B; Figure S5**), was associated with 90 diet-specific taxa comprising only 0.63% of reads. Interestingly, our analysis revealed that a xylan-based diet did result in the loss of 136 taxa that were present in all other diets in abundances ranging from 4.78%-10.14% ("Set 2" in **Figure 4A**). However, other sets that excluded individual diets were substantially smaller (**Figure S7A**) suggesting that this was not a common mechanism underlying the diet-driven changes in gut microbiome composition. Instead, synthetic diet-driven changes in gut microbiome composition were primarily associated with changes in the relative abundance of individual taxa that were consistently present in the cockroach gut regardless of dietary treatment.

To follow up on these observations, we used DESeq2 to assess enrichment of individual ASVs between synthetic vs whole food fed cockroaches. Of the 492 ASVs present in at least one

sample belonging to each diet (Set 1), 95 ASVs were significantly enriched in cockroaches fed synthetic diets, while 38 ASVs were enriched in cockroaches fed whole food diets (**Figure 4B**; padj < 0.001). Set 1 ASVs were present in all diets, often at high abundances, and shifts across diet type were modest (log fold change <5). Bacteroidota, Desulfobacterota, Proteobacteria and other phyla showed similar enrichment distributions to each other, but more Firmicutes were enriched by the synthetic diets.

We also examined enrichment of ASVs that fell outside of Set 1 but were both abundant (baseMean > 1) and present in at least 5 samples (**Figure 4C**). These 1270 ASVs generally had smaller abundances than members of Set 1, but greater log fold changes between diet types (**Figure 4C**). The ASVs *Ruminococcus_NA* and *Fusobacterium ulcerans* were exceptions, being both highly abundant (baseMean = 1020 and 651, respectively) and unique to the synthetic diets. In contrast, the *Christensenellaceae* ASV (R7_NA.68) unique to whole food diets had high log-fold change, but a baseMean of only 4.29. Apart from a few highly abundant ASVs in the synthetic diets, most abundant, diet-enriched taxa were shared by all diets, supporting that change is driven by common gut bacteria restructuring the community rather than interloping bacteria disrupting the community.

Differential diet-based fluctuations of abundant Firmicutes and Bacteroidota ASVs

To explore the impact of diet on microbial taxa associated with fiber degradation, we evaluated dietary responses of abundant taxa within the Firmicutes and Bacteroidota. For this analysis, we selected the two most abundant representatives of each of these phyla from each diet and examined their abundance across all diets (**Figure 5**). We identified 16 Firmicutes and 11 Bacteroidota as most or second-most abundant in at least one diet.

The most abundant taxa from both Firmicutes and Bacteroidota represented a small fraction of reads across the whole foods diets, consistent with the higher observed Shannon diversity and evenness of the gut microbiome for whole food-fed cockroaches (**Figure 3D, E**). In contrast, the synthetic diets produced strong 'blooms' of individual ASVs, particularly among the Firmicutes (**Figure 5A**). Several abundant Firmicutes were both present in Set 1 (**Figure 4B**) and enriched in individual synthetic diets (**Figure S3**), namely *Lachnoclostridium_NA* (xylan), *Lachnoclostridium_NA.1* (xylan), *Enterococcus_NA* (xylan), *Enterococcaceae_NA* (xylan), and *ChristensenellaceaeR7_NA* (pectin). In contrast, Firmicutes identified as enriched in the MCC and Starch diets were not typically found across all diets: *Ruminococcus_NA* (MCC), *Lachnospiraceae_NA.5* (starch), and *Ruminococcaceae_NA* (starch). Among the Bacteroidota (**Figure 5B**), we observed greater overlap in the most abundant taxa present in each diet group, with all but one (*3M1PL1.52termite_NA*) of the abundant Bacteroidota ASVs belonging to Set 1, and only two classified as diet-characteristic: *3M1PL1.52termite_NA* (MCC) and *Bacteroides_NA.12* (pectin).

Analysis of Microbial Co-Correlation Networks

As synthetic diets appeared to reduce gut microbiome compositional stability and induce blooms of individual taxa, we constructed co-correlation networks with SparCC to examine the community structure underlying synthetic and whole food microbiome data sets [44]. To filter out noise and reduce spurious correlations, datasets were filtered to include ASVs present in at least 25% of samples for each diet type, resulting in 976 ASVs for whole food diets and 700 ASVs for synthetic diets. Networks were further pruned to contain edge weights with absolute values larger than 0.4, removing 75 and 168 ASVs from whole food and synthetic networks

respectively. Both positive and negative edges were retained for network layout formation (**Figure S8**), but for the rest of analyses only positive edges were considered (**Figure 6, Figure S9**). After negative edge removal, the whole food network contained 875 ASVs with 9515 edges forming two connected components (**Figure 6A**), while the synthetic diet network contained 497 ASVs with 2536 edges that formed six connected components (**Figure 6B**).

Gut microbiota from whole food-fed cockroaches formed an extensive and dense interaction network (**Figure 6A**), with higher edge counts (**Figure 6C**), node counts (**Figure 6D**), network strength (**Figure S9**), and betweenness scores (**Figure S9**) than synthetic diets at most levels of filtering based off SparCC correlation values. High-degree nodes, or ASVs with large numbers of neighbors, were present throughout the network structure, while in the synthetic diets two primary clusters of ASVs appeared with fewer connecting ASVS. When a range of inclusion cutoffs were considered, the synthetic diet network degraded more quickly than the whole food network into separate connected components (**Figure 6E)** displaying greater fragility and tendency to fragment. These overall network structures suggest that the synthetic diets disrupt the stability of the cockroach microbiome.

Discussion

The core finding of this work is that synthetic diets dominated by a single carbohydrate type induced marked changes in hindgut microbial community composition in omnivorous cockroaches. Diets featuring the hemicellulose xylan induced the largest shifts in alpha diversity, inter-individual variability, and overall community composition (**Figure 1**). Xylan is a common structural polysaccharide containing a xylose backbone with branching arabinose, glucuronic acid, and/or acetyl groups that has been extensively investigated for prebiotic potential both in livestock and humans [45-48]. Research investigating xylan degradation ability in gut microbiota mainly focuses on *Bacteroides* [49-54] although select Firmicutes such as *Roseburia intestinalis* have been identified as key butyrate-producing xylan fermenters [55-57]. Interestingly, we found that in cockroaches, xylan-based diets decreased the relative abundance of Bacteroidota, while increasing Firmicutes (**Figure 1A**). Concurrent with this enrichment of specific taxa, xylan decreased the overall diversity of the gut community (**Figure 1**). This result contrasts with studies of gut microbiome composition changes in chickens [58] and mice [59] fed diets supplemented with xylooligosaccharides where gut diversity increased, although both studies reflected the increase in *Lachnospiraceae* observed in our experiments.

However, while xylan-based diets induced the largest change in hindgut community composition, samples clustered by diet treatment even when the xylan treatment group was excluded (**Supplement 1**). Most of these diets were associated with ‘blooms’ of specific Bacteroidetes and Firmicutes, excluding those made with methylcellulose and chitin, which were not associated with enrichment of any single taxa in pairwise comparisons with the other diets. This suggests that while the disproportionate impact on community structure due to xylan

warrants its own research, synthetic diets in general appear to reduce hindgut microbiome stability compared to whole food-based diets.

These results stand in stark contrast to previous work from multiple investigators, who found minimal to no change in hindgut microbial community composition in response to diet alterations [14, 23, 24]. A commonality between these experiments is that the investigators utilized complex, minimally processed or whole food diets that were highly biased in macromolecular composition. On the other hand, a few investigators have previously observed substantial influence of diet on the gut microbiome composition [25-27]. These experiments all utilized synthetic diets that contained purified carbohydrate and protein sources. For example, in experiments using *B. germanica*, Pérez-Cobas et al [26] prepared synthetic diets with a dextrin and micronutrient base amended with either 50% cellulose or 50% casein while Zhu et al [27] used diets composed of a cellulose and micronutrient base with supplemented with 40% by mass purified starch, casein, or sesame oil. In *P. americana*, Bertino-Grimaldi et al [25] utilized purified cellulose to compare with sugarcane bagasse, a complex dietary substrate.

A disproportionate influence of synthetic diets with highly purified components on gut microbial composition has been observed beyond cockroach research as well. Termites, a close relative to cockroaches, responded to single carbohydrate source diets with larger alterations in gut community composition than termites fed mixed-carbohydrate diets [60]. Other insect model systems produced similar results, such as in silkworms [61], ladybugs [62], waxworms [63], and honeybees [64]. Among mammals, dogs provided a purified diet also exhibited reduced alpha diversity compared to those fed a complex diet [65], wild-caught mice transitioned from natural diets to laboratory diets lost large portions of native gut microbes [66], and humans given meal replacement shakes showed loss of biodiversity in their microbiome compositions [67].

Comparison of our dataset with data recovered in previous whole food-based dietary experiments suggest that synthetic diets altered the gut community by inducing overgrowth of microbes already present in the cockroach gut microbiome (**Figure 4).** Taxa that were unique to individual diets represented <1% of sequence reads in all diets but starch, of which they made up 2.84% (**Figure 4**). In contrast, 15 out of 20 highly abundant Firmicutes and Bacteroidota associated with one or more diets were shared across all diet types, while the remaining 5 were found sporadically in other diets (**Figure 5)**.

We hypothesize that highly purified synthetic diets enabled microbial 'specialists' to bloom beyond their former constraints in the whole food diets. While the whole food diets used in Tinker and Ottesen [14] were highly biased in macromolecular composition, as natural foods they may have had a more diverse nutritional profile according to the "eye" of a bacterium [51, 68, 69]. Both whole and white wheat flour, for example, are composed predominantly of endosperm-derived starch, which physically impacts microbial adhesion due to variance in particle size following milling [70, 71]. The bran, germ, and, to a lesser degree, endosperm fractions of wheat contain structural polysaccharides and bioactive phytochemicals that are targeted by gut bacteria and influence health parameters of the host [28, 72-74], while honey contains complex mixtures of sugars (glucose, fructose, disaccharides) and fructooligosaccharides in addition to organic acids, nitrogenous compounds, vitamins, and bee-derived enzymes [75]. Tuna and butter are similarly high-complexity substrates that offer resident gut microbes diverse metabolizable compounds that are lost in purified dietary components such as those utilized in our study. The purified components used in our synthetic diets are not entirely homogeneous but are far more processed than the 'natural' whole foods used in our comparison sets and lack bioactive compounds present basally in these diets [68]. As

a result, the less complex dietary structures of our synthetic diets may not require as much cooperative metabolism to digest while the overall loss in nutritional diversity edges out bacteria that are unable to keep pace with specialist polysaccharide fermenters.

This conclusion is supported by network analysis of the diet types (**Figure 6**), where the whole food network is highly interconnected with numerous paths from one ASV to the next, while the synthetic diet network is easily fragmented into modules of microbes that are weakly or negatively associated with the other network members (**Figure S9**). Hallmarks of a stable and resilient gut microbiome often include metabolic redundancy, where many microbes are capable of filling different niches and are rate limited by their partner activity but can adjust to shifts in diet without extreme disruption [76, 77]. Synthetic diets may weaken the cooperative potential of the gut microbiome, narrowing the niches that can flourish to fewer bacteria, and allow drastic shifts such as those observed in the xylan diet that would otherwise not occur.

A key limitation of this study is the fact that the comparison group of "whole food" fed cockroaches were from an earlier experiment and we lack 'contemporaneous' controls fed whole food diets. However, an examination of cohort effects suggest that observed responses to synthetic diets were highly conserved across cohorts in experiments conducted one year apart (**Figure 2**). Further, despite the large time gap between the whole food and synthetic diet experiments, a set of 492 ASVs (14% of total ASVs) shared by all treatment groups comprised approximately 68% of the reads recovered (**Figure 4**), suggesting minimal drift in overall gut microbiome composition due to time alone.

Conclusions

Overall, this study showed that synthetic diets that were highly enriched in a single carbohydrate type could induce large shifts in gut microbiome composition in the American cockroach, which has previously been shown to be highly resistant to diet-induced shifts in gut microbiome composition [14]. Changes in gut microbiome composition in response to synthetic diets were primarily associated with 'blooms' of diet responsive ASVs already present at lower levels in the gut rather than introduction new taxa. This alteration in gut microbiome composition is associated with fragmentation of gut microbiome interaction networks and increased inter-individual variability. Together, these results suggest that overconsumption of a single, purified class of polysaccharides can have destabilizing effects on the gut microbiota. This has major implications for the potential for prebiotics to induce changes in gut microbiome composition even in highly stable systems, as well as potential implications regarding the impact of highly processed foods on gut microbiome homeostasis. Future work will explore the functional and metabolic basis of observed shifts in microbial community composition.

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Data Availability

Data associated with this study are available from the NCBI short-read database under BioProjects PRJNA1096047 and PRJNA1105088. Data associated with the earlier study of the impact of whole food diets on cockroaches is available under BioProject accession PRJNA320546.

Materials and Methods

Insects and Experimental Conditions

Our *Periplaneta americana* colony has been maintained in captivity at the University of Georgia for over a decade. Mixed age and sex stock insects are maintained at room temperature in glass aquarium tanks with wood chip bedding and cardboard tubes for shelter in a 12:12 light:dark cycle. Water via cellulose sponge fit to a Tupperware reservoir and dog chow (Purina ONE chicken & rice formula, approximately 26% protein, 16% fat, and 3% fiber) are provided to stock colonies *ad libitum*.

Synthetic Diets

The synthetic diets created for dietary testing were designed to provide balanced nutrition while remaining malleable to component manipulation. Diets contained Vanderzant vitamin mix (MP Biomedicals, Irvine, CA, USA), Wesson salt mix (MP Biomedicals, Irvine, CA, USA), peptone (Bacto Peptone) and casein (Sigma-Aldrich, St. Louis, MO, USA). The peptone and casein utilized were high purity mixes with greater than 90% total protein content. Cholesterol (VWR International, Radnor, PA, USA) was added since insects are unable to synthesize sterols themselves. The dry ingredients were combined and suspended in minimal volumes of H_2O to create a batter or dough, formed into pellets, then dehydrated at 65°C until they were sufficiently dry to maintain shape. Food pellets were stored at -20°C until use.

In most experiments, the only component changed was the carbohydrate source. Polysaccharides used include microcrystalline cellulose (MCC; Sigma-Aldrich, St. Louis, MO, USA), methylcellulose (Sigma-Aldrich, St. Louis, MO, USA), xylan from corn core (TCI

Chemicals, Portland, OR, USA), pectin from apple (Sigma-Aldrich, St. Louis, MO, USA), starch (Sigma-Aldrich, St. Louis, MO, USA), and chitin (Alfa Aesar, Ward Hill, MA, USA).

Experimental Design

Experimental conditions were prepared as described in [14]. Briefly, mixed-sex healthy adult insects were transferred from the stock colony to plastic tanks containing pebbles and bleached polyvinyl chloride tubes for footing and shelter, respectively. Food and water were provided *ad libitum* in rigid plastic or glass dishes following two days of food restriction and habituation. Dietary treatment lasted two weeks, during which debris, oothecae, and lethargic insects were removed daily.

Upon completion of dietary treatments, each insect was isolated in a sterile culture plate and placed on ice until torpid. Sternites were removed with sterile forceps to expose the intact gut and fat body tissue was cleaned away. The cleaned gut was frozen on a sterile aluminum dish on dry ice and divided into foregut, midgut, and hindgut sections for collection in 500-800µL phosphate-buffered saline (1X PBS). Gut contents and tissue-attached bacteria were disrupted with a sterile pestle, and the samples stored at –20°C until DNA extraction. For this study, only the hindgut community was analyzed due to higher microbial density and activity than in other gut regions.

DNA Extraction

DNA was extracted from 200µL aliquots of individual samples using the EZNA Bacterial DNA Kit (Omega Biotek, Norcross, GA, USA) with some modifications. Sample aliquots were centrifuged at 5000g for 10min, with the resulting pellet resuspended in 100µL TE buffer plus

10µL lysozyme (50mg/mL) and incubated for 30 min at 37°C. Following incubation, samples were vortexed with glass beads (25mg, Omega Biotek) for 5 min at 3000rpm, then incubated at 55°C for one hour with 100µL TL buffer, 20µL proteinase K and continuous 600rpm shaking. The kit protocol was followed for additional incubations with BL buffer and DNA isolation using the provided column. DNA was eluted into 50µL of provided Elution Buffer and quantified using either a Nanodrop Lite spectrophotometer (Thermo Scientific) or the Take3 plate for BioTek plate readers (Agilent).

16S rRNA Gene Library Preparation and Sequencing

The V4 region of the 16S rRNA gene was amplified via 2-step polymerase chain reaction (PCR) from individual hindgut lumen samples as previously described in [14, 41, 78]. Both PCR reactions used 0.02U/L Q5 Hot Start high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA) with 200M dNTPs and 0.5M forward and reverse primers in 1M Q5 reaction buffer. The first 10µL reaction containing 3ng DNA and primers targeting the V4 region (**515F**: GTGCCAGCMGCCGCGGTAA; **806R**: GGACTACHVGGGTWTCTAAT) was performed under the following conditions: activation at 98°C for 30s; 15 cycles of 98°C for 10s, 52°C for 30s, and 72°C for 30s; final extension at 72°C for 2 min. Immediately following amplification, 9µL of the first reaction was added to 21µL of Q5 reaction mix containing barcoded primers with adaptor sequences for Illumina sequencing [79]. Cycling was performed as follows: activation at 98°C for 30s; 4 cycles of 98°C for 10s, 52°C for 10s, and 72°C for 30s; 6 cycles of 98°C for 10s and 72°C for 1 min; final extension at 72°C for 2 min.

After product size verification via gel electrophoresis, samples were cleaned as instructed in Omega Biotek's Cycle Pure kit, quantified, and pooled for equimolar representation of each

sample. Prepared libraries were sent to the Georgia Genomics and Bioinformatics Core at the University of Georgia for 250 base pair paired-end Illumina MiSeq sequencing.

DNA Sequence Processing

Each dataset collected in this study was processed separately in R (version 4.2.1) by sequencing run using R package DADA2 (version 1.24.0), with the cumulative Amplicon Sequence Variants (ASVs) generated input as a priors table for each successive run [39, 80]. To allow for comparison with this dataset, raw data from previous research in the Ottesen lab were reprocessed to generate ASVs following the same procedures as in this current study [14]. All sequence tables produced by these datasets were combined by ASV sequence prior to taxonomy assignment to ensure continuity in naming. Taxonomy was assigned using DADA2 and the ARB Silva v138 classifier to the species level, uniquely numbered, and filtered to remove sequences matching eukaryotic (chloroplast, mitochondria) or endosymbiotic *Blattabacterium* DNA [39, 81].

Community analysis

Alpha and beta diversity analyses were performed via the R package vegan (version 2.6-4) [82]. Samples were rarefied prior to diversity analysis to 7924 reads for comparisons between synthetic and/or whole food diets, and 9685 reads for analysis of repeat xylan and MCC diet experiments. Alpha diversity was measured via Shannon index, the count of ASVs observed in rarefied samples, and Pielou's evenness (calculated as Shannon/log(Observed)). Weighted and unweighted Bray-Curtis dissimilarities were calculated, assessed for dispersion, and plotted using the vegan functions vegdist, betadisper, and metaMDS. Statistics for alpha diversity

indices were calculated with the Wilcoxon rank sum test (pairwise comparisons) and Kruskal-Wallis test (multi-group comparisons). The significance of community composition differences observed in beta diversity measures was assessed using PERMANOVA. Beta dispersion was further examined through the Tukey's HSD test for pairwise comparisons and ANOVA for multi-group comparisons.

Differential abundance analysis was conducted using DESeq2 (version 1.36) [42]. For identification of 'diet-characteristic taxa", raw count data for the synthetic diet set (n=66) was filtered to exclude ASVs present in less than 5 samples and run through the 'DESeq' command with parameters 'fitType = "local"' and 'design = ~ Diet'. Pairwise result tables were obtained for all diet comparisons and filtered for significant data, defined as having an adjusted p-value smaller than 0.05 and a baseMean larger than 10. ASVs significantly upregulated for one diet vs the other five diets were identified as diet-characteristic (n=76) and used to generate the heatmap in **Supplement 3**. For comparison of "synthetic vs whole", raw count data for both diet sets (n=125) were combined and filtered to exclude ASVs that appeared in fewer than 5 samples. DESeq was run with parameters 'fitType = "local"' and 'design = ~ Diet_Type' to identify differentially abundant ASVs between the diet types. The resulting baseMean and log2 fold change were used to generate the MA plots in **Figure 4**.

UpSetR (version 1.4) was employed to visualize intersecting sets of taxa, providing insights into the distribution of taxonomic features across samples [43]. For UpSet analysis, samples were rarefied to 7924 reads then collapsed together to obtain total counts per diet. Both a presence/absence table and a proportion table were generated from these data, with the presence/absence table used for UpSet graph generation. Relative abundance of each set was

calculated using the proportion table with ASVs collapsed per set and visualized as pie charts within the UpSet graph.

Co-correlation analysis was conducted to evaluate the impact of synthetic diets on microbial interaction networks using the SparCC procedure [44]. Networks were constructed separately for the synthetic diet group and the whole food diet group using sequence count tables that were filtered to only include ASVs with at least 5 representatives present in 25% of samples (synthetic: 17 samples; whole food: 15 samples), preventing spurious correlations from rare taxa. SparCC was implemented in R with standard parameters, and the resultant networks were characterized and analyzed with the igraph R package (version 1.5.1) [83, 84]. Networks were pruned to contain only edges with a correlation absolute value of at least 0.4 and exported into Cytoscape for visualization using the edge-weighted spring embedded layout method [85].

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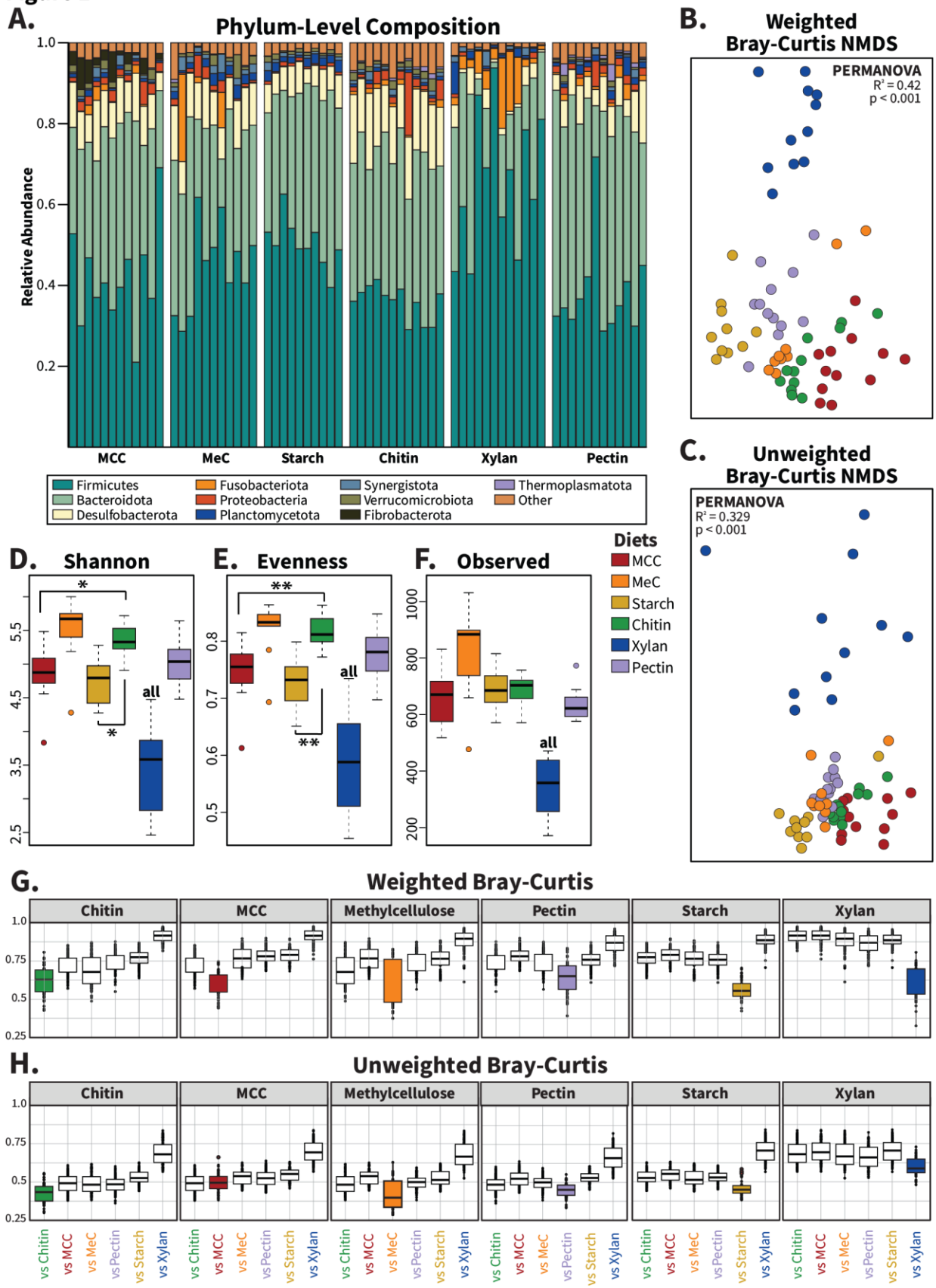
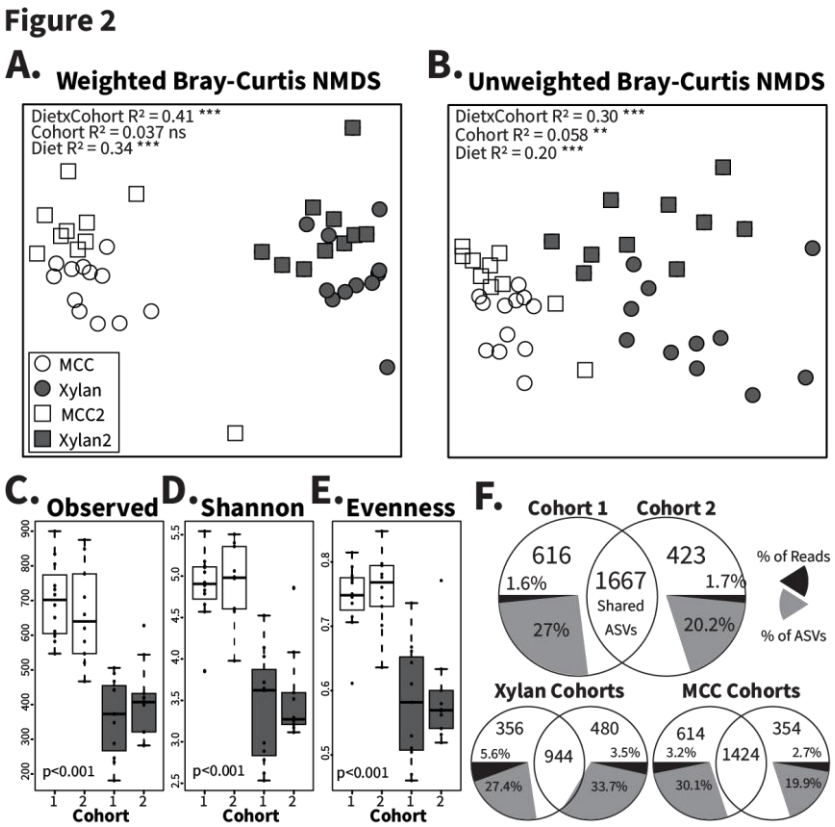
Figure 1


A.
Phylum-Level Composition
Relative Abundance
1.0
0.8
0.6
0.4
0.2
MCC
MeC
Starch
Chitin
Xylan
Pectin
Firmicutes
Bacteroidota
Desulfobacterota
Fusobacteriota
Proteobacteria
Planctomycetota
Synergistota
Verrucomicrobiota
Fibrobacterota
Thermoplasmatota
Other
B.
Weighted Bray-Curtis NMDS
PERMANOVA
R² = 0.42
p < 0.001
C.
Unweighted Bray-Curtis NMDS
PERMANOVA
R² = 0.329
p < 0.001
D.
Shannon
E.
Evenness
F.
Observed
all
Diets
MCC
MeC
Starch
Chitin
Xylan
Pectin
G.
Weighted Bray-Curtis
H.
Unweighted Bray-Curtis
Chitin
MCC
Methylcellulose
Pectin
Starch
Xylan
vs Chitin
vs MCC
vs MeC
vs Pectin
vs Starch
vs Xylan

727

Figure 1: Composition of gut microbiomes from cockroaches fed synthetic diets. (**A**) Barplot showing the relative abundance of phyla across samples for each of the synthetic carbohydrate diets. Bars represent individual hindgut samples, clustered and labeled by diet polysaccharide source. Phyla present at an abundance greater than 1% in at least one sample are plotted. Non-metric multidimensional scaling (NMDS) was used to plot **(B)** weighted and **(C)** unweighted Bray-Curtis dissimilarity. The alpha diversity measures **(D)** Shannon index, **(E)** Pielou's evenness, and **(F)** number of observed taxa were plotted. Boxplots of **(G)** weighted and **(H)** unweighted Bray-Curtis display each diet vs self (colored boxes) and the other five synthetic diets (white boxes). Samples were rarefied to a constant depth of 7924 sequences for alpha and beta diversity calculations. For alpha diversity measures, pairwise statistics were calculated with Wilcoxon rank-sum tests and multivariate analysis was performed using Kruskal-Wallis tests. PERMANOVA was used to generate statistics for ordination analyses. "all" indicates $p<0.05$ vs all other diets; * = $p<0.05$; ** = $p<0.01$



741

742 **Figure 2: Analysis of cohort effects on gut microbiome responses to MCC and Xylan diets.**

743 NMDS ordinations of **(A)** weighted and **(B)** unweighted Bray-Curtis dissimilarity**.** R^2 and p-

744 values calculated by PERMANOVA for diet ("MCC" and "Xylan") and cohort ("Cohort 1" and

745 "Cohort 2") separately and for interactions. Boxplots show observed **(C)** ASVs, **(D)** Shannon

746 index**,** and **(E)** Pielou's evenness with Kruskal-Wallis p-values. The last panel **(F)** contains Venn

747 diagrams of shared and unique ASVs between cohorts for both diets together as well as

748 separately, constructed using rarefied count tables. Grey pie slices represent the percent of ASVs

749 observed that are cohort-unique, while black pie slices represent the percentage of sequence

750 reads assigned to the indicated unique ASVs. Xylan and MCC-fed samples from replicate

751 experiments were rarefied to 9685 ASVs for alpha and beta diversity assessment. ** $p<0.01$

752 ***$p<0.001$

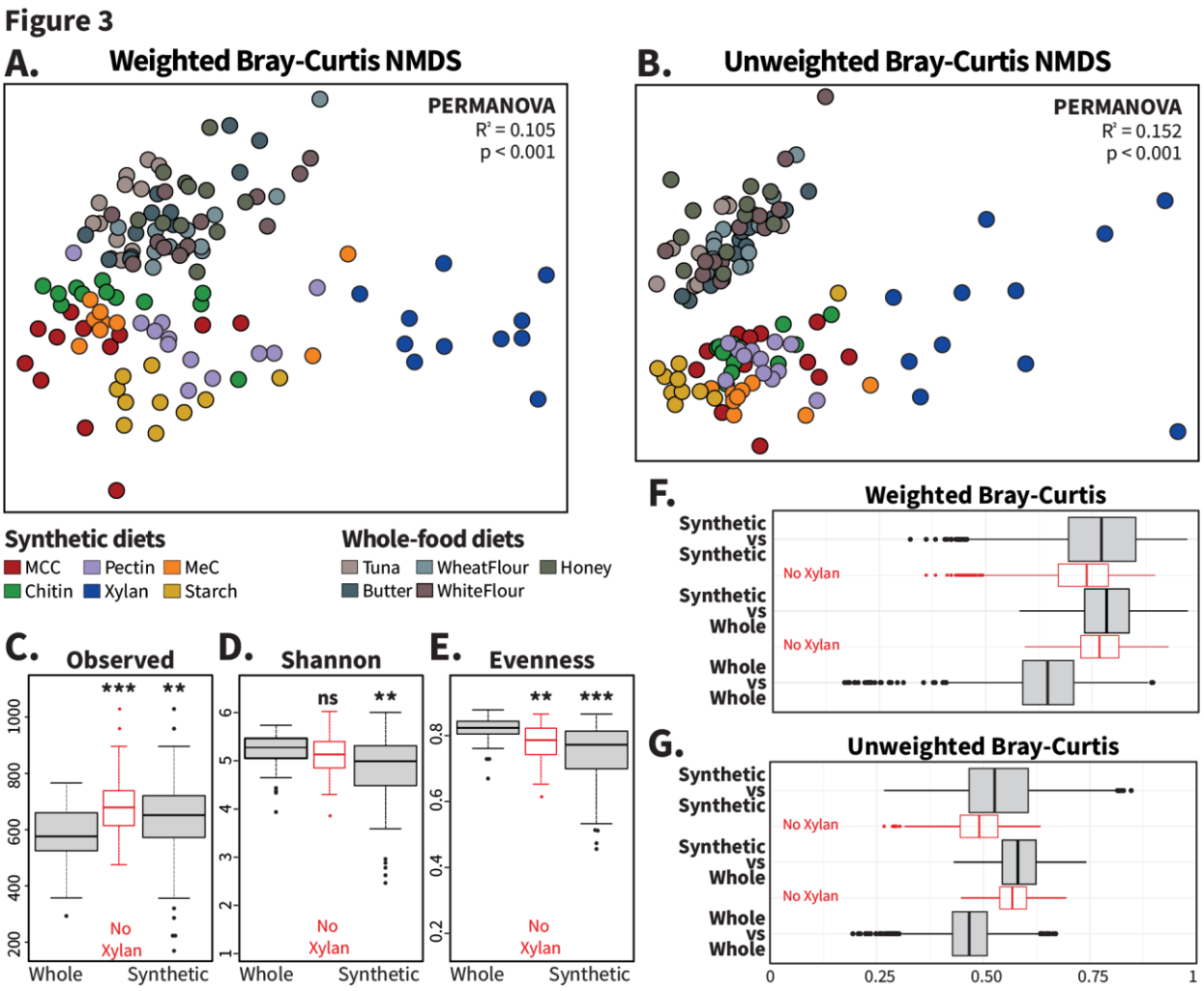


Figure 3: Whole food and synthetic diets differ in alpha and beta diversity measures. NMDS ordinations of **(A)** weighted and **(B)** unweighted Bray-Curtis distances between cockroach hindgut samples from this study ("Synthetic") and data from Tinker and Ottesen (2016) for cockroaches fed tuna, butter, honey, wheat flour, and white flour ("Whole Food Diets"). R^2 and p-values for diet type comparisons were calculated using PERMANOVA. Boxplots show **(C)** observed ASVs, **(D)** Shannon index**,** and **(E)** Pielou's evenness for each diet type, with red boxes representing the synthetic diets minus xylan-fed samples. Wilcoxon rank-sum test was used for pairwise statistical analysis between diet types. The final panels display boxplots of **(F)** weighted and **(G)** unweighted Bray-Curtis dissimilarity, with red boxes

representing synthetic diets excluding xylan. Raw sequence data from Tinker and Ottesen (2016) were reprocessed using the methods in this experiment to generate comparable ASVs. All samples were rarefied to 7924 ASVs for alpha and beta diversity analysis. ** =p <0.01; *** =p<0.001, ns =no significance

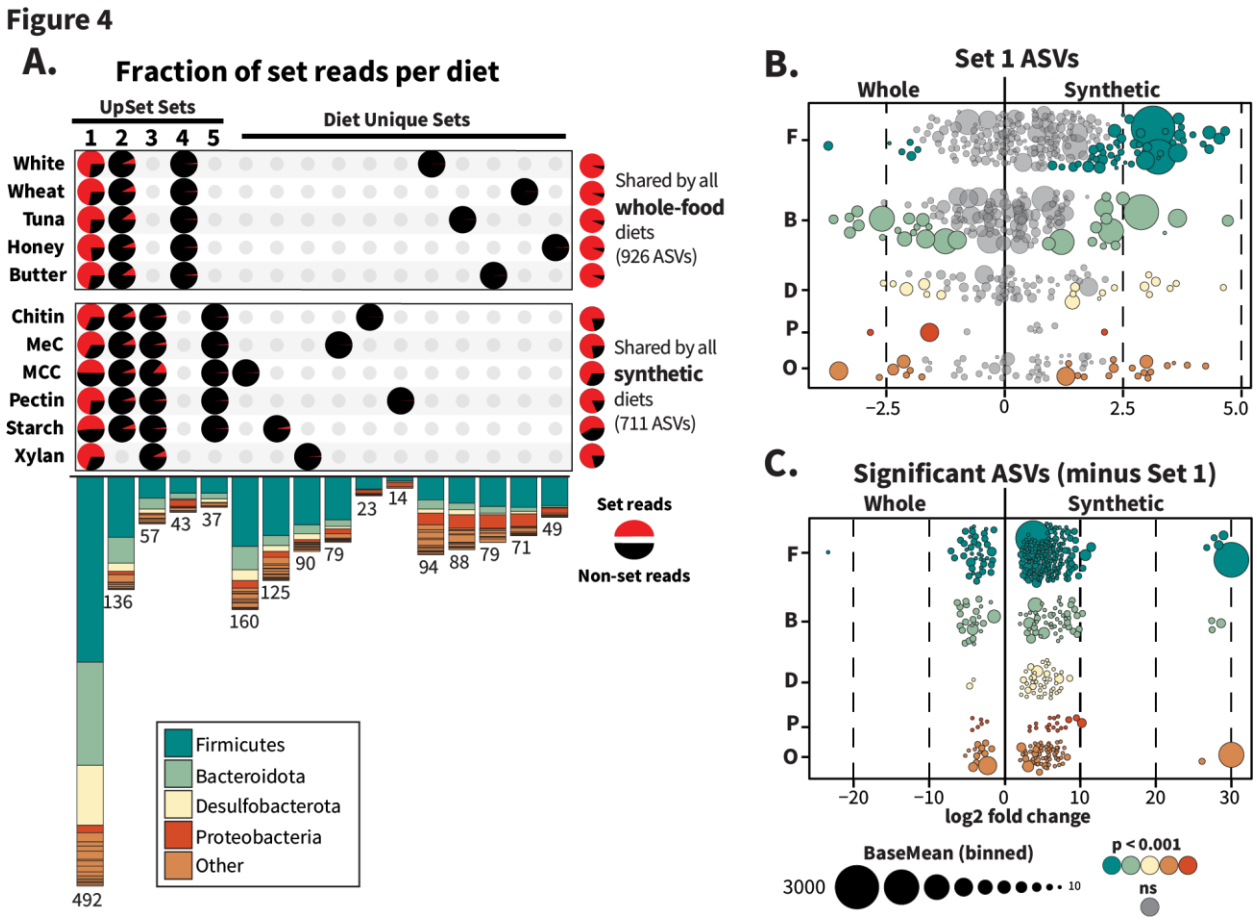


Figure 4: Whole-food diets share more ASVs than synthetic diets. The rarefied samples from Figure 3 were used to generate an UpSet plot of **(A)** ASVs present in at least one sample per diet; the five largest intersections (Sets 1-5) and diet-unique sets are displayed. Pie charts represent the percent of reads within a diet that originate from each set (red slices), and the bar charts are colored to display the phylum-level distribution of ASVs assigned to each set. Pie charts to the right of the UpSet plot represent the reads from ASVs that are shared by all diets in a diet type, regardless of presence in the other diet type. For the MA plots in panels **(B)** and **(C)**, raw sequence count tables were filtered to include ASVs present in at least 5 samples (out of 125 total) and analyzed using DESeq2 with diet type as the design factor. Panel **(B)** includes only ASVs from Set 1, while panel **(C)** displays the ASVs, excluding Set 1, with an adjusted p-value

smaller than 0.001. The ASVs in both plots are scaled to the same baseMean size and colored based on phylum. Note that the plots differ in their x-axis scale ($\log_2$ fold change).

Figure 5

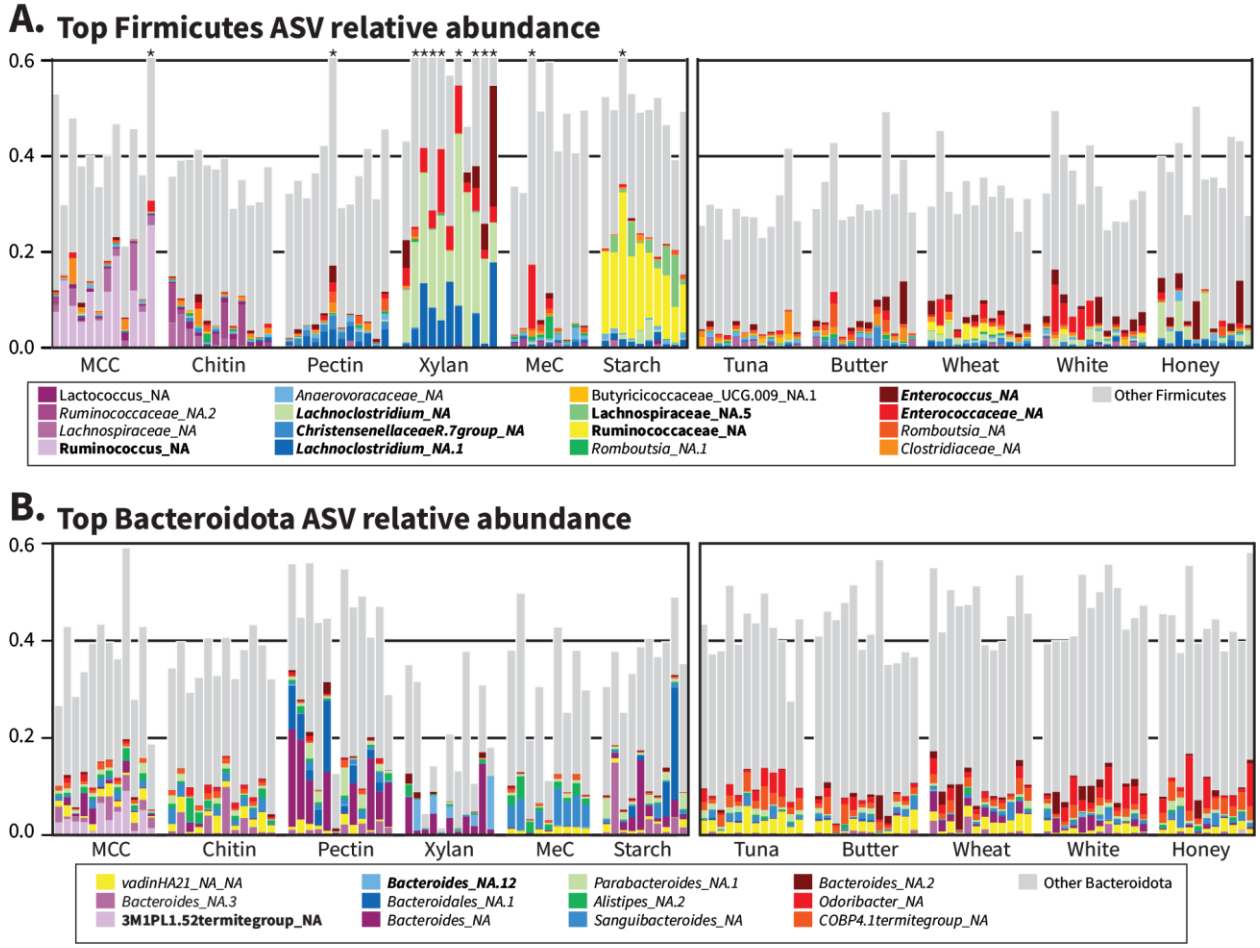


Figure 5. Individual ASVs explain large differences between synthetic but not whole-food diets. Variance-stabilized count data from DESeq2 were used to determine the top two ASVs for every diet belonging to **(A)** Firmicutes and **(B)** Bacteroidota, and the relative abundances of ASVs belonging to the combined 'top ASV' set were plotted for all samples. Grey bars include all Firmicutes or Bacteroidota not named in the key. * indicates "Other Firmicutes" extends beyond 60% relative abundance; please refer to **Figure 1A** for full values. Bolded names indicate diet-characteristic ASVs from **Supplement 3**, and italics indicate Set 1 ASVs from **Figure 4**.

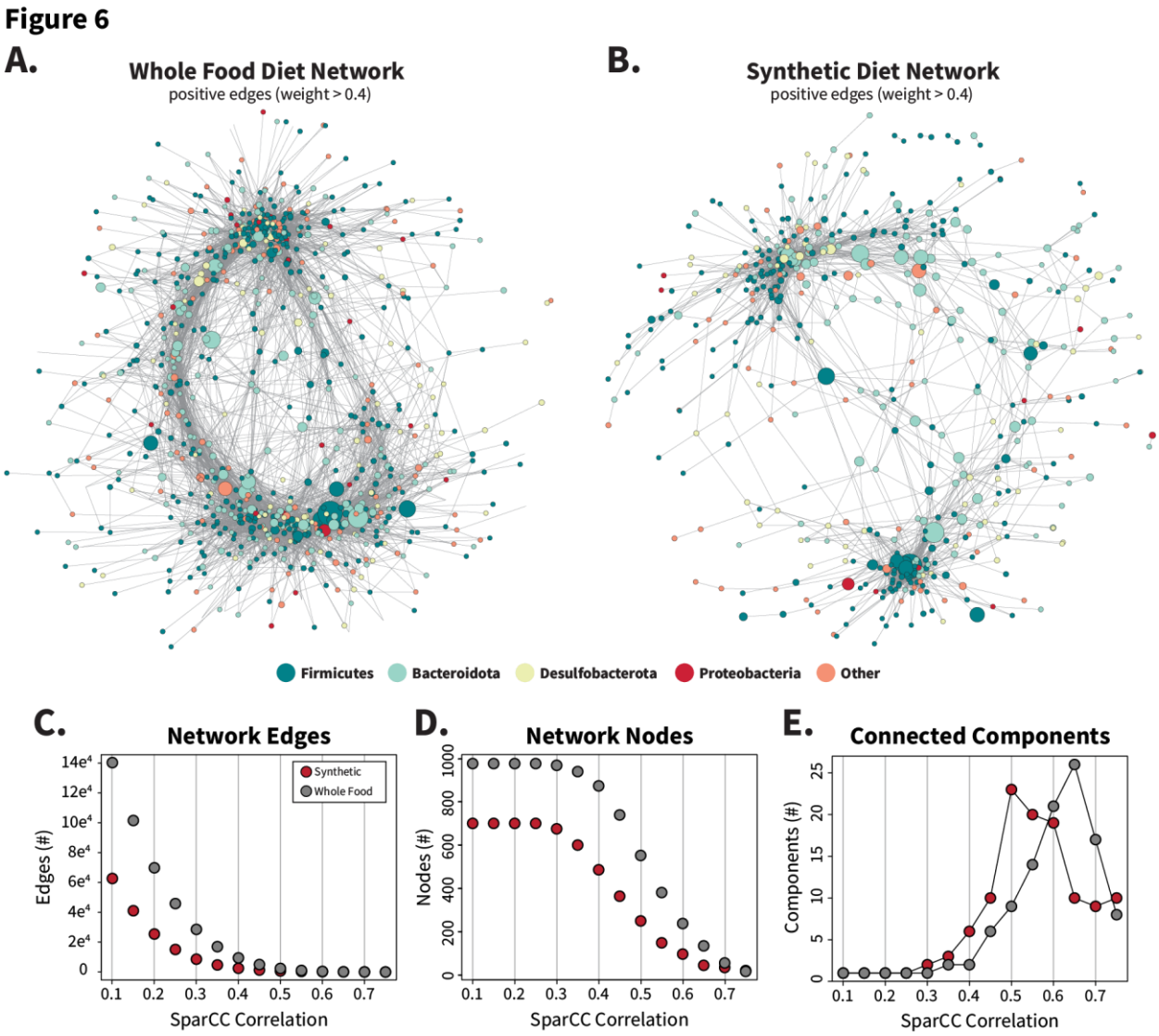


Figure 6: Synthetic diet correlation networks are smaller and less interconnected than whole food diet correlations networks. Networks were calculated by SparCC from filtered count tables (ASVs present in > 25% of samples per diet set) for synthetic and whole food diets separately to create two distinct networks containing 976 (whole food) or 700 (synthetic) nodes. Networks were imported into Cytoscape and edges with absolute values < 0.4 were removed to generate panels **(A)** and **(B)**. Negative edges were included during initial layout generation with the edge-weighted spring embedded layout method and are displayed in **Supplement 8.** The

number of nodes, edges, and connected components that remain when the networks are filtered by increasing correlation values are charted in **C**, **D,** and **E** respectively.