A Need for Stronger Regulation: Commercially Sold Probiotics for Honey Bees Do Not Live Up to Their Claims

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Key words

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Abstract

Antibiotic use in apiculture is often necessary to ensure the survival of honey bee colonies. However, beekeepers are faced with the dilemma of needing to combat bacterial brood infections while knowing that antibiotics also kill beneficial bacteria important for bee health. In recent years, bee probiotics have become increasingly purchased by beekeepers because of product claims like, being able to “replenish the microbes lost due to agricultural modifications of honey bees' environment” or "promote optimal gut health." Unfortunately, these products have little scientific evidence to support their efficacy, and previous lab experiments have refuted some of their claims. Here, we performed hive-level field experiments to test the effectiveness of SuperDFM-HoneyBee™, the most commonly purchased honey bee probiotic in the United States, on restoring the honey bee gut microbiome after antibiotic treatment. Our results indicated that bees administered the probiotic after antibiotic treatment did not recover their microbiota faster than bees only given the antibiotic treatment. Moreover, the microorganisms in the probiotic supplement were never found in the guts of the worker bee samples, even during active treatment. These results highlight that more basic research is needed to test the efficacy and outcomes of currently available commercial honey bee probiotic supplements

Introduction

Honey bees are an essential part of the global economy. They play a crucial role in the pollination of crops and contribute over 1.8 billion dollars in revenue to crop production in the United States alone [1]. During critical pollination periods, honey bees are often treated with antibiotics as a preventative measure against bacterial infections. Antibiotics have been used in apiculture for over 60 years in the United States and they are also utilized in apiculture in several other countries. The most commonly applied antibiotic by beekeepers in the United States is oxytetracycline, sold as Terramicin or TerraPro, which is primarily used to treat and control *Melissococcus plutonius* and

Paenibacillus larvae, the causative agents of European and American Foulbrood (EFB and AFB), respectively [2, 3]. AFB is particularly threatening because *P. larvae* is a spore-forming bacterium. Thus, antibiotics can only stop the growth of the disease-causing form of *P. larvae* but do not impact the spores, which can remain in the hive for up to 50 years or more. The bacterium that causes EFB, *M. plutonius*, is not a spore-forming bacterium, so it can be more easily eradicated from infected hives through antibiotic treatment [2]. Both diseases can be spread from one hive to another when bees drift from an infected hive to an uninfected one, when bees rob an infected hive, or when beekeepers exchange or reuse infected equipment. Due to the challenges of eradicating *P. larvae* spores, it is recommended that infected hives be destroyed via burning and that all surrounding hives be treated with antibiotics. In large commercial operations where hundreds to thousands of hives are maintained and transported across the United States for pollination services, the threat of disease spread is more severe, which could ultimately result in the loss of hundreds of hives. Additionally, inspecting and diagnosing thousands of hives regularly is almost impossible. Therefore, in the case of large commercial operations, it is common practice to continuously treat (metaphylactically) with antibiotics in the spring and fall and/or before and after entering a highly concentrated pollination site to prevent EFB and AFB diseases.

One major issue with antibiotics is that they target not only pathogens but also beneficial microbes [4]. The honey bee gut microbiome plays an essential role in honey bee health, including immune priming, nutrition and metabolism, growth and development, and protection against pathogens [4–8]. Several studies have shown that exposure to chemicals commonly used in apiculture and agriculture, such as antibiotics and pesticides, disrupt the gut microbiome and lead to decreased honey bee survival [9–11]. Specifically, the antibiotic oxytetracycline has been shown to have detrimental effects on the size and composition of the gut microbiome, resulting in decreased survival and increased susceptibility to opportunistic infections [11–14]. Antibiotic treatment has also been linked to weakened honey bee immune responses, indicated by reduced expression of antimicrobial peptides [12]. Reduced antimicrobial peptide expression has been shown to result in increased infection by the fungal parasite

Nosema and higher titers of Deformed Wing Virus (DWV) and Israeli Acute Paralysis Virus (IAPV) in honey bees [15]. Antibiotic treatment has also been associated with nutritional deficiencies in honey bees, which are likely caused by perturbation of the gut microbiota [12].

While the use of antibiotics can have negative consequences on bee health, there are currently few alternatives for the treatment and prevention of bacterial infections. In December 2022, an American biotechnology company released a study showcasing the first-ever oral vaccine for *P. larvae* [16]. However, the vaccine is still in the early stages of research and development, has not been thoroughly tested in the field, and only targets American Foulbrood's causative agent. Thus, it is safe to assume that antibiotic use will continue for years to come in commercial beekeeping operations in countries where it is legal. Probiotics, defined as live microorganisms intended to have health benefits when consumed or applied to the body, have risen as a viable, cost-effective option to improve animal health. Although, no current evidence shows that probiotics provide health benefits to healthy hosts with a “normal” gut microbiome, probiotic application has been shown to effectively restore microbiota members and function following antibiotic treatment [17, 18]. Therefore, the use of probiotics could help lessen the adverse effects of antibiotic treatment on honey bees. Currently, there are only a few companies commercially selling probiotics for honey bees (i.e., Fat Bee Probiotics, Durvet, SCD Probiotics, and Strong Microbials), none of which contain native honey bee bacteria. The most popular amongst beekeepers in the United States is Strong Microbials SuperDFM®-HoneyBee™ and it is marketed as a product able to “replenish the microbes lost due to agricultural modifications of honey bees' environment.” Yet, all the products mentioned above are exclusively made of microbes isolated from mammals or the environment that have never been identified in the honey bee gut, and to date, none have been scientifically proven to restore the native honey bee gut microbiome.

Here we tested the effectiveness of SuperDFM®-HoneyBee™ (herein referred to as DFM), the most common marketed honey bee probiotic in the United States, on

restoring the honey bee gut microbiome after treatment with TerraPro (oxytetracycline). We hypothesized that the gut microbiome of honey bees would not be restored by DFM following exposure to the antibiotics due to the lack of native bee gut or hive-associated microbes present in the probiotic supplement. Our results show that not only did the probiotics fail to restore the bee gut microbiome to its natural state, but the microorganisms present in the probiotic supplement were never identified in the guts of sampled bees. These results highlight that more basic research is needed to test the efficacy and outcomes of currently available commercial honey bee probiotic supplements, not only for the sake of honey bees but also beekeepers and the environment.

Methods

Sample Collection

Nine hives of Apis mellifera (subspecies - Italian honey bees), were created from established colonies in early Spring 2019 and kept in an apiary in Dallas, North Carolina. Experimental hives had never been treated with antibiotics prior to this experiment, but the hives were treated with the acaricide, Apivar®, in Summer 2018, and with oxalic acid treatment in Winter 2018. No additional treatments for varroa mites were used during the experiment. Additionally, all new hive materials were used (e.g., boxes, frames, and foundations) to reduce any prior contamination. Hives were also distanced from one another to reduce bees drifting into neighboring colonies.

Each hive was comprised of three five-frame deep nucs of approximately equal population size. The nine hives were divided into three experimental groups in June 2019: 1) Control (n=3), 2) TerraPro treatment only (n=3), and 3) TerraPro treatment followed by DFM probiotic treatment (n=3). Five bees were sampled from each hive before the experiment started (baseline – samples taken prior to the experiment starting, day-0). The treatment hives (TerraPro-Only and TerraPro+DFM) were given three applications of TerraPro (DC-560, Mann Lake) at 4-5 day intervals following the

manufacturer's instructions. One week after the TerraPro application period ended (week-0), 15 bees per hive were sampled (135 bees total) and group three was given SuperDFM®-HoneyBeeTM, following the manufacturer's instructions. On week-1 and week-2 after probiotic treatment, 15 bees per hive (control, TerraPro only, and TerraPro+DFM) were sampled per time point (270 bees total). The guts from all sampled bees were extracted immediately upon collection using sterile forceps and preserved in 100% ethanol at 4°C until they were processed. In addition to processing the bee gut samples, DNA was also directly extracted from the SuperDFM-HoneyBeeTM powder (in duplicate) using the same methods as above. Although exact hive metrics were not recorded during this study, no overall differences in population size or hive health were noted between treatment and control groups, with the exception of some increased open brood mortality observed in the TerraPro-Only and TerraPro+DFM hives.

DNA Extraction and Sequencing

Individual bee gut samples were homogenized and DNA was extracted from the bee guts and DFM probiotic powder using the phenol-chloroform with bead beating extraction protocol described by [19]. For the extracted DNA, a two-step 16S rRNA library preparation was performed. The first step consisted of PCR amplification of the V4 region of the 16S rRNA gene using the 515F and 806R primers containing Illumina platform-specific sequence adaptors: Hyb515F_rRNA: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTA -3′ and Hyb806R_rRNA: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3′. The PCR cycling conditions were as follows, 98°C for 30s followed by 20 cycles of 98°C (10s), 58°C (30s), 72°C (30s), with a final extension at 72°C for 7m and a hold at 4°C. The PCR product was cleaned using the AxygenTM AxyPrep Mag™ PCR Clean-up Kit. Samples were indexed using the Illumina Nextera XT Index kit v2 sets A and D. The PCR cycling conditions were 98°C for 2m followed by 15 cycles of 98°C (10s), 55°C (30s), 72°C (30s), with a final extension at 72°C for 7m and a hold at 4°C. The indexed

product was cleaned using the AxygenTM AxyPrep Mag™ PCR Clean-up Kit, quantified with a Qubit3.0 (Life Technologies) with the Qubit dsDNA BR Assay kit, and pooled in equal concentrations for sequencing. A 30% PhiX spike-in was included in the pooling library before sequencing to increase the diversity of the run. The amplicon sequencing was completed at UNC Greensboro using an Illumina iSeq100 with 2×150 paired-end reads. Of the 450 samples collected in this study, 395, including a negative control, were successfully sequenced.

Sequence Analysis

The forward and reverse reads were merged using FLASH [20] with a minimum overlap of 5 bp, which resulted in a total of 6,048,186 total reads. Joined reads were quality filtered in Qiime2 using the DADA2 pipeline, resulting in 4,180,969 reads. The data was filtered to remove all sequences corresponding to mitochondria, chloroplast, and unassigned taxa as well as reads present at less than 1% frequency. After quality-filtering a total of 3,353,951 reads were retained, with a mean frequency of 3,488 per sample corresponding to 328 amplicon sequence variants (ASVs). Our negative control only contained 92 reads and was removed from further analysis. Downstream analyses were performed in Qiime2 at a sampling depth of 1,000 reads per sample. This sampling depth was chosen to maximize the number of samples included in the analysis while still maintaining enough reads per sample to capture the richness of the dataset. Rarefying to 1,000 reads per sample resulted in retaining 337 samples, including the two DFM powder controls.

Quantitative PCR

We amplified total copies of the 16S rRNA using the primers EUB338F and EUB518R (Table 1) on an Applied Biosystems QuantStudio 6 Real-Time PCR system. Reactions were completed in triplicate using 5 µL of universal SYBR Green (Bio-Rad, Inc.), 2 µL of molecular grade water, 1 µL (each) of 3 nM primers, and 1 µL of template DNA. The PCR cycle was 95°C (3 min) followed by 40 cycles of 95°C (3 s) and 60 °C (20 s). The

average Ct (cycle threshold) was determined for each sample. A gBlocks™ gene fragment (Integrated DNA Technologies) was used to create a standard curve (Table 1) of known copy number. We then estimated absolute copy number by interpolating the Ct value into standard curves of known copy numbers, from 10^2–10^8 copies.

Table 1. Primers and gBlocks fragment used for qPCR.

Primers + gBlock	Sequence (5′-3′)
EUB338F Forward Primer	ACTCCTACGGGAGGCAGCAG
EUB518R Reverse Primer	ACTCCTACGGGAGGCAGCAG
16S gBlocks fragment	GTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAG CCGGTGCTTATTCGTTAGATACCGTCATAATCTTCTCTAACAAAAGGAGT TTACAATCCTAAAACCTTCATCCTCCACGCGGCGTTGCTGCTTCAGGCT TTCGCCCATTGAGCAATATTCCCTACTGCTGCCTCCCGTAGGAGTCTGG ACCGTGTCTCAGTT

Statistical analysis and data visualization

The script "qiime diversity core-metrics-phylogenetic" was used to perform Alpha and Beta diversity analyses [21]. The taxonomy of the representative sequences was determined using “qiime feature-classifer classify-sklearn” [21] using a classifier trained on the Silva 16S (release 138) reference database [22] and based on the specific primers that were used for amplification. Taxonomic assignments for poorly classified ASVs were confirmed through manual verification using NCBI blastn [23]. The analysis of taxonomic diversity was carried out at both the genus and ASV level. Alpha diversity analyses were performed in Qiime2 and statistically tested using the Kruskal-Wallis test with Benjamini-Hochberg FDR correction, and the results were plotted in R [24]. Beta diversity analyses were done in Qiime2 using weighted UniFrac [21] and statistically analyzed using PERMANNOVA (999 permutations) with Benjamini-Hochberg FDR correction. The PCoA plots with 95% confidence intervals (stat_ellipse) were generated using Qiime2R [25]. Differences in absolute abundance based on qPCR were

determined using the Kruskal-Wallis test with Benjamini-Hochberg FDR correction, and the graph was made in GraphPad Prism.

Results

In this study, nine honey bee hives (three-story, five-frame deep nucs) were split into three groups (control, TerraPro only, and TerraPro+DFM) and a total of 455 bees were sampled (Figure 1). Using 16S rRNA sequencing, we profiled the guts of worker honey bees at each sampling point within the three groups (Figure 1). Finally, we used qPCR to measure the absolute abundance of bacteria to determine if the overall bacterial load differed following antibiotic treatment (TerraPro) or after the application of the probiotic supplement (TerraPro+DFM).

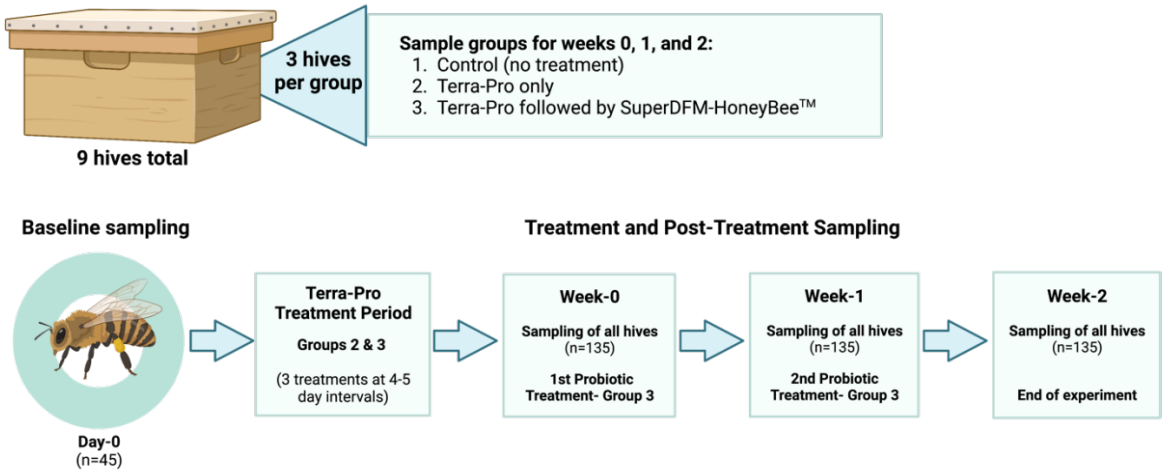

Figure 1. Schematic of experimental setup and sampling timeline. Created with BioRender.com.

After hives were treated with antibiotics but before being given the probiotic supplement (week-0), differences in ASVs (amplicon sequence variants) were observed between the baseline, control, and TerraPro-Only bees, with TerraPro-Only bees containing the highest number of ASVs (Figure 2A; $P<0.05$). The gut microbiota of the baseline control bees presented a significantly lower amount of phylogenetic diversity than the week-0 TerraPro-Only group bees (Figure 2B). At week-1 post-antibiotic treatment, the number of ASVs between the control and TerraPro-Only bees was no different. However, at week-1, the TerraPro+DFM bees displayed a significantly lower

number of ASVs than the control (*P*=0.0044) and TerraPro-Only bees (*P*=0.0329), and a lower amount of phylogenetic diversity than the control bees (*P*= 0.0121) (Figure 2A–B).

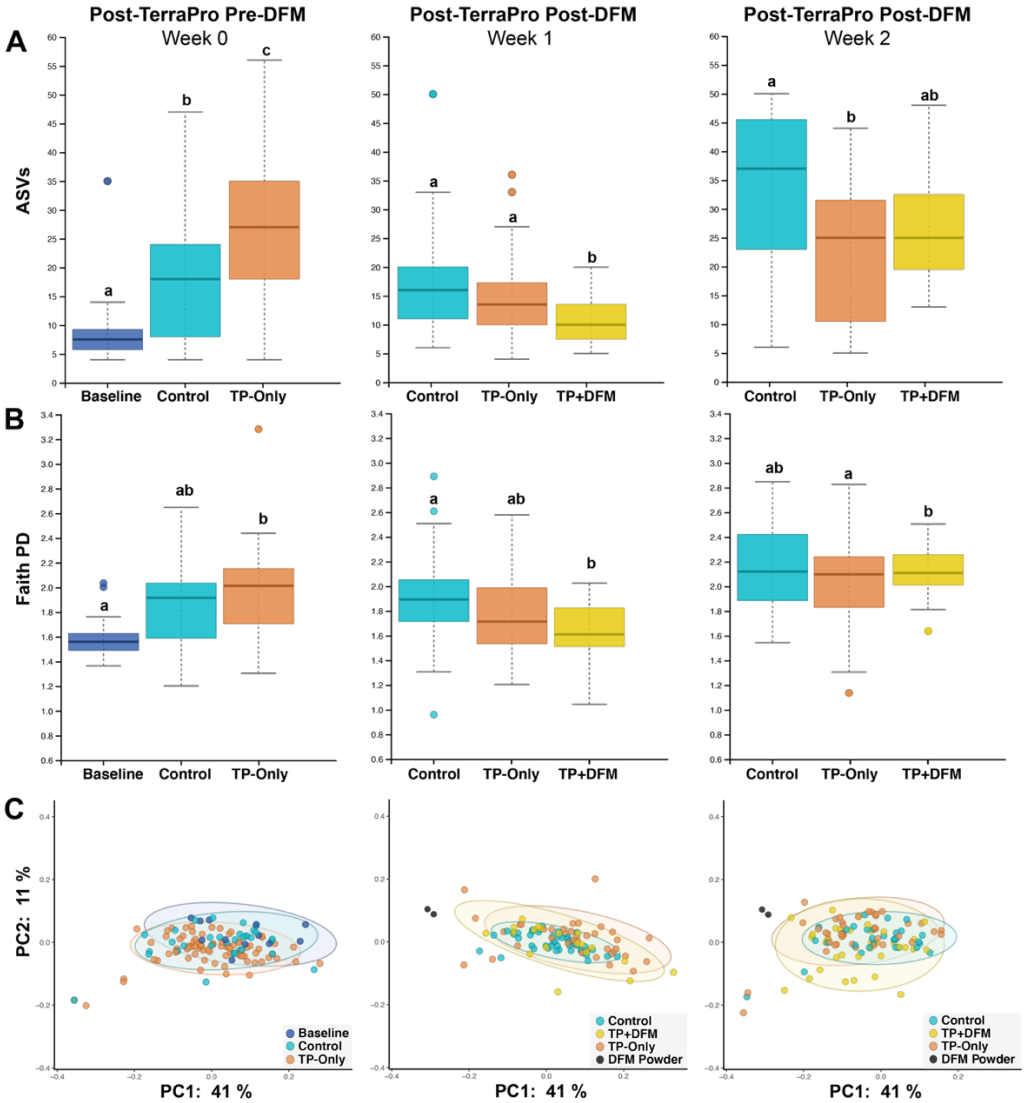

Figure 2. Alpha and Beta diversity comparisons of the gut microbiomes of (left column) post-TerraPro treatment pre-DFM week zero, (center column) post-TerraPro treatment post-DFM week one, and (right column) post-TerraPro treatment post-DFM week two based on (A) richness (# of ASVs) and (B) phylogenetic diversity (Faith's phylogenetic diversity index). (C) Principal coordinate analysis plots based on weighted UniFrac. For Alpha diversity comparisons (A–B), significance was determined using the Kruskall-Wallis test with Benjamini-Hochberg FDR correction. Significance (*P*<0.05) is represented bydiffering letters. For Beta diversity comparisons, (C), significance was tested using PERMANOVA with 999 permutations followed by Benjamini-Hochberg FDR correction. Ellipses represent the 95% confidence interval.

Two weeks after TerraPro treatment (week-2), the TerraPro-Only bees had fewer bacterial ASVs when compared to the control bees (*P*= 0.0164). In contrast, the TerraPro+DFM bees were no different from the control or TerraPro-Only bees in the average number of ASVs (*P*= 0.3065) or phylogenetic diversity (*P*= 0.4193) (Figure 2A–B). No significant differences were identified when comparing taxon evenness in the gut microbiota of bees across experimental groups (Figure S1). Overall, the alpha diversity results did not indicate a strong trend according to treatment group.

When assessing the beta diversity, or the microbiota similarity between experimental groups, significant differences were found between the baseline (*P*=0.0178), week-0 control bees (*P*=0.037125) and the week-0 TerraPro-Only bees (Figure 2C). Week-1 control samples had different microbiome compositions than the TerraPro-Only bees (Figure 2C; *P*=0.007). In contrast at week-1, the TerraPro+DFM bees did not show any significant difference when compared to the control bees (Figure 2C; *P*=0.5). Two weeks (week-2) post-antibiotic treatment, the only significant difference in microbiome composition was between the TerraPro-Only and TerraPro+DFM bees (Figure 2C) *P*=0.006). The DFM powder was always significantly different from all experimental groups (Figure 2C; *P*<0.05). In general, the TerraPro-Only and TerraPro+DFM bees displayed more variation between individuals than the control bees at week-1 and week-2 post treatment.

To access variation across hives within experimental groups, we compared the microbiome diversity, phylogenetic relatedness, and evenness of the microbiota of bees from different hives within the same group at week-1 and week-2. Within the control group, bees from hives one (H1) and two (H2) had a higher number of ASVs in week-2 than in week-1, whereas bees from hive three (H3) showed a dramatic reduction in ASVs in week-2 when compared to all bees in all hives in the control group at week-1 and bees from H1 and H2 in week-2 (Figure 3A; *P*<0.01). Bees from the TerraPro-Only hives also differed significantly in week-2, with bees from hives H4 and H6 displaying an increase in ASVs and bees from H8 having a significantly reduced number of ASVs when compared to all bees in all TerraPro-Only hives at week-1 and week-2 (Figure 3A;

P<0.02). Amongst hives in the TerraPro+DFM group, variation in the number of ASVs was also observed, with bees from H5 at week-1 having fewer ASVs and bees from H5 and H9 at week-2 having significantly more ASVs than bees in all other TerraPro+DFM hives at both timepoints (Figure 3A; *P*<0.01). Overall, we observed a large degree of variation in the number of ASVs across hives from the same treatment group.

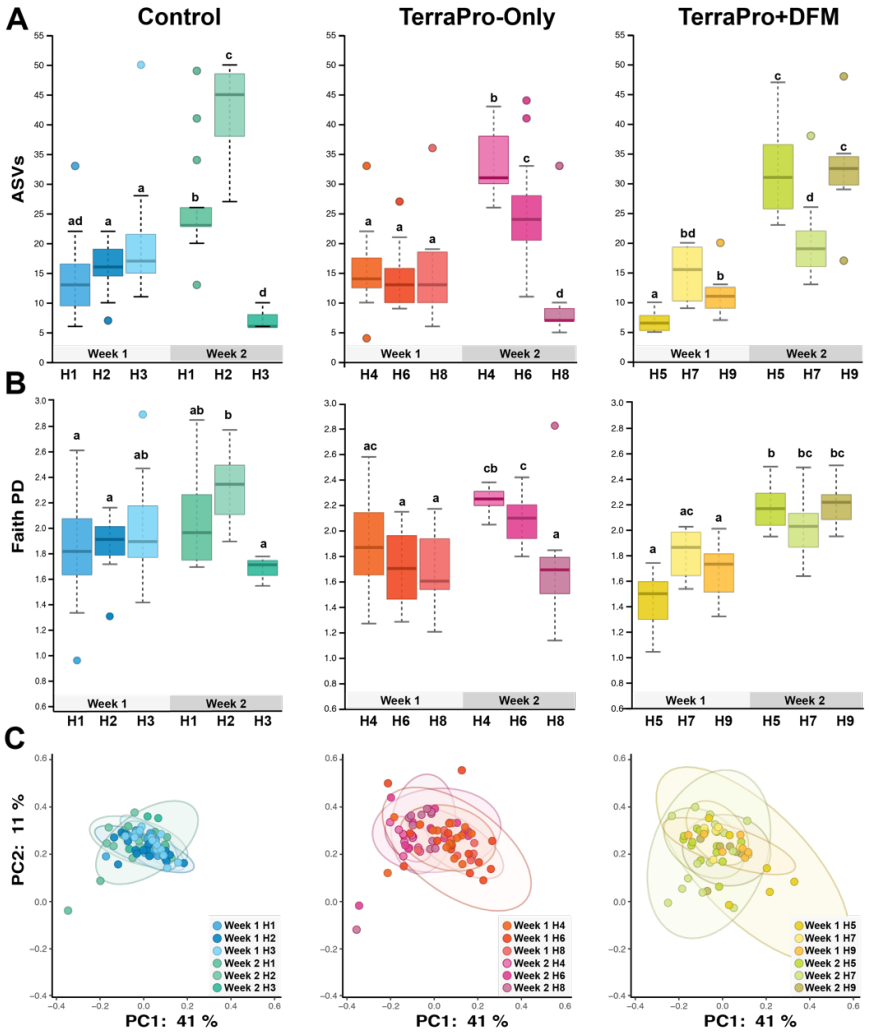

Figure 3. Alpha and Beta diversity comparisons of the gut microbiomes of (left) control, (center) TerraPro-Only, and (right) TerraPro+DFM group hives from weeks-1 and -2 based on (A) richness (# of ASVs), (B) phylogenetic diversity (Faith's phylogenetic diversity index), and (C) principal coordinate analysis plots based on weighted UniFrac. For Alpha diversity (A–B), significance was determined using the Kruskall-Wallis test with Benjamini-Hochberg FDR correction. Significance (*P*<0.05) is represented by differing letters. For Beta diversity comparisons (C), significance was tested using PERMANOVA with 999 permutations followed by Benjamini-Hochberg FDR correction. Ellipses represent the 95% confidence interval.

The phylogenetic diversity in gut microbiota of bees from different hives within each experimental group also varied. At week-2 control H2 bees possessed higher microbial phylogenetic diversity in their microbiota than bees in H1 and H2 at week-1 (Figure 3B; $P<0.005$). At week-2, bees from TerraPro-Only hives H4 and H6 displayed higher phylogenetic diversity than bees from H4 and H8 at week-1 and bees from H8 at week-2 (Figure 3B; $P<0.05$). In general, phylogenetic diversity increased in bees from TerraPro+DFM hives at week-2 when compared to week-1 (Figure 3B; $P<0.02$). Consistent with the analysis of bees from different experimental groups (Figure S1), few differences were seen in terms of microbiota evenness in bees from different hives within the same experimental group (Figure S2).

We also compared beta diversity, or microbial community similarity, between the three hives within the same experimental group at different timepoints. Overall bees from control hives did not differ in microbiota composition across timepoints, with the exception of week-1 control H3 which was significantly different from week-2 control H2 (Figure 3C, $P=0.0431$). Significant differences in microbiota composition were observed within the TerraPro-Only group between week-1 and week-2 within H4 ($P=0.018$) and H8 ($P=0.018$), but H6 did not differ across any timepoints (Figure 3C). In week-1, the composition of TerraPro-Only H6 was significantly different from both H4 ($P=0.03$) and H8 ($P=0.05$, Figure 3C). For the TerraPro+DFM hives, within H5, the microbiota composition differed significantly from week-1 to week-2 (Figure 3C, $P=0.039$). Although the data points are more scattered in the TerraPro+DFM PCoA plot (Figure 3C), suggesting they are overall less similar to each other than the control and TerraPro-Only hives, no other significant differences were observed across the TerraPro+DFM hives.

We quantified the absolute abundance of bacteria based on 16S rRNA gene copy number within individual bees from each experimental group at weeks -0, -1, and -2 (Figure 4). No significant difference in 16S rRNA gene copy was observed between the control and TerraPro-Only bees at week-0 (Figure 4). However, at week-1 post-antibiotic treatment, there was a significant increase in 16S rRNA copy number between the control and TerraPro-Only bees ($P=0.0056$), but the TerraPro+DFM bees did not

significantly differ from either the control or TerraPro-Only bees (Figure 4). In contrast, at week-2, the TerraPro-Only bees had a higher bacterial abundance than the TerraPro+DFM bees (*P*=0.0025), whereas control bees did not differ from TerraPro-Only or TerraPro-DFM bees in terms of 16S rRNA copy number (Figure 4). Overall, no obvious treatment-specific trends were observed regarding bacterial load across treatment groups or timepoints.

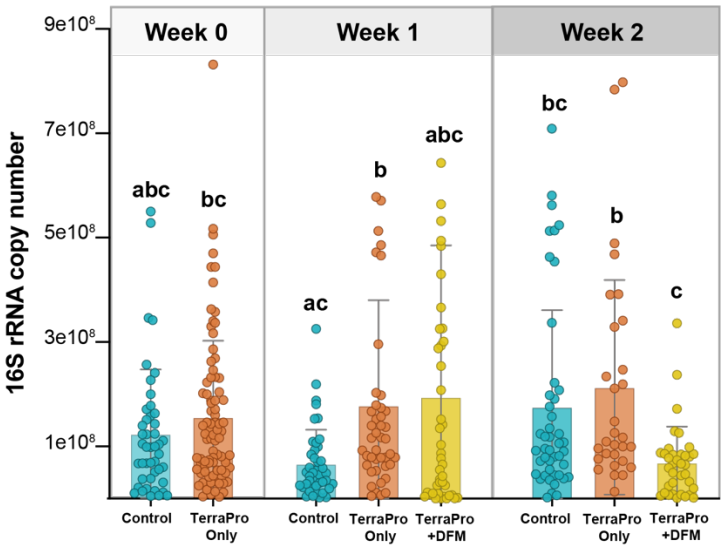

Figure 4. Total bacterial 16S rRNA gene copies estimated by qPCR in control, TerraPro-Only, and TerraPro+DFM bees at week-0, -1, and -2. Box plots show median values with standard deviation and each point represents an individual bee. Significance was tested using the Kruskal Wallis Test with Benjamini-Hochberg FDR correction and is indicated by differing letters.

When comparing bacterial abundance within bees from the same experimental group across timepoints, week-1 control bees had less 16S rRNA copies than week-2 control bees (*P*=0. 0116). However, within the TerraPro-Only and TerraPro+DFM groups, bees did not significantly differ in bacterial load at any timepoint (Figure 4, *P*>0.05). Comparison of bees from different groups across timepoints revealed that week-1 TerraPro-Only bees had a significantly higher number of 16S copies than week-2 TerraPro+DFM bees (*P*=0.0135), and week-1 control bees had a significantly lower abundance of 16S copies than the week-2 TerraPro-Only group (Figure 4, *P*=0.0004).

To better visualize the changes in microbiome composition across experimental groups and sampling weeks, we averaged the relative abundance of taxa present in all bees from each timepoint and experimental group (Figure 5; see Figure S3 for individual bee data). Regardless of treatment group, all honey bees contained the five core honey bee gut microbiome taxonomic groups, *Lactobacillus* Firm4 and Firm5, *Bifidobacteria*, *Gilliamella*, and *Snodgrassella* (Figure 5). However, shifts in relative abundance were seen amongst all five core species members and in other less abundant native bee gut species like *Frischella perrara* within and across all experimental groups at all timepoints (Figure 5). Some notable differences in relative abundance between the controls and treatment groups were an increase in "other" taxa in both the TerraPro-Only and TerraPro+DFM at week-1, a decrease in *Snodgrassella* the TerraPro-Only and TerraPro+DFM bees at week-2, and an increase in *Frischella* in the TerraPro+DFM bees at week-2 (Figure 5). It is important to note that the SuperDFM-HoneybeeTM probiotic bacteria were never found in any of the bee gut samples, even during active treatment with the probiotic powder (Figure 5, Figure S3).

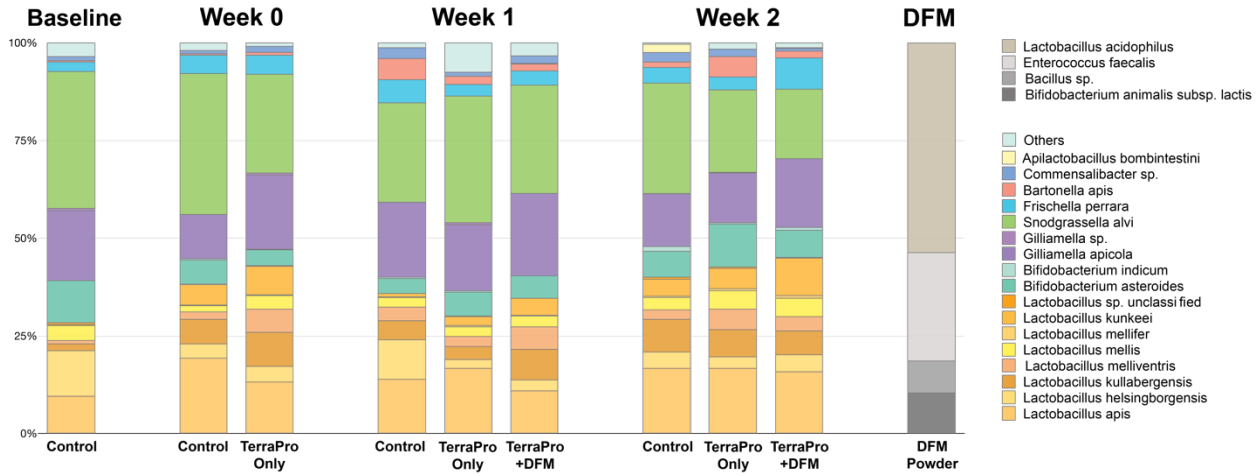

Figure 5. Bar plots representing the average relative abundance of taxa found in the bee gut samples within each experimental group at each sampling timepoint. The DFM powder (probiotic) was also sequenced as a positive control. See Figure S3 for data on individual bees.

Discussion

Despite promising claims, there is no scientific evidence demonstrating the effectiveness of current commercially available probiotics in restoring the honey bee gut microbiome after a perturbation. Additionally, none of the current commercially available probiotics contain native bee microbes. One of the most popular honey bee probiotics is SuperDFM-HoneyBee™, made by the company Strong Microbials. This product is advertised as a probiotic that can “replenish the microbes lost due to agricultural modifications of honey bees' environment” and “Promotes optimal gut health”. Therefore, we sought to determine whether this probiotic can restore the honey bee gut microbiome after a routine treatment with the antibiotic oxytetracycline (TerraPro). We found that bees from hives given the probiotic following TerraPro treatment were not any more similar to bees from the control hives than bees from hives only given TerraPro treatment. Moreover, we never detected the microbes present in the probiotic in the guts of bees during or following supplementation. Although we did not specifically evaluate any other hive health metrics aside from microbiota composition and overall colony size in this study, our findings, at the very least, demonstrate that the honey bee probiotic supplement SuperDFM-HoneyBee™ does not restore the bee’s gut microbiota after treatment with the TerraPro (oxytetracycline) and does not colonize honey bees. These results are consistent with a previous study that evaluated an unnamed commercial probiotic and revealed that it never colonized the honey bee gut (even of “microbiota-free” bees) and that the probiotic led to an increase in the abundance of opportunistic pathogens [26]. Thus, further studies are needed not only to test if the currently available probiotics provide any health benefits but also if they are safe for honey bees.

Although we saw significant differences between control hives and hives treated with TerraPro-Only and TerraPro+DFM, the effects of the antibiotic treatment were much less severe than expected. This might sound like a promising result for bees and beekeepers, but we are skeptical our study accurately reflects the impacts of antibiotic treatment on the honey bee microbiome, especially because these results contradict several published studies. For example, previous hybrid lab and field based studies

found that both oxytetracycline (the active ingredient in TerraPro) and tylosin (the active ingredient in Tylan) severely perturbed the gut microbiome of bees by reducing the size and composition of bacteria present in the community, resulted in increased susceptibility to opportunistic pathogens, and reduced survivorship [11, 13]. We hypothesize that several factors limited our ability to capture the full effects of TerraPro on the honey bee microbiome in our study: 1) bees were only sampled from brood frames at random so we are unsure how long they were exposed to the antibiotic and some could have been newly emerged (lacking the characteristic microbiome), 2) all hives were treated with the acaricide Apivar (active ingredient amitraz) prior to the experiment, which could have impacted their microbiomes [27], and 3) we observed a large amount of variation between individual bees and across hives within the same group. Therefore, we argue that more controlled hive-level studies are required to make a definitive statement about the effects of in hive TerraPro treatment on the honey bee microbiome and health.

Recently, several research groups have been investigating other probiotic treatments [28–31], as reviewed in [26], which are not yet commercially available to beekeepers. One probiotic that is currently being tested is the The BioPatty™, which is made of a mixture of three *Lactobacillus* strains, including *Apilactobacillus kunkeei* (a hive-associated bacterium that is often found in the honey bee gut) [29, 30]. The BioPatty™ has been shown to increase the expression of bee immunity genes, reduce infection my *P. larvae*, and help restore the microbiome following antibiotic perturbation [29]. Another research group has been exploring the use of native bee gut strains as a potential probiotic therapy [13, 26]. This native bee gut cocktail has been demonstrated to successfully colonize and persist in germ-free bees and reduce the ability for opportunist bacteria to proliferate [13, 26]. Given that all honey bees possess a highly conserved microbiome composed for five core bacterial taxa that are functionally important for be health [4], a probiotic comprised of native honey bee bacteria would increase the chance restoration of the community and eliminate the risk of introducing foreign microbes into the hive ecosystem [13, 26]. The continued development and scientific testing of probiotic blends containing native honey bee gut bacteria will

hopefully lead to an effective and safe commercially available supplement that can help improve bee and hive health.

Conclusion

Probiotics, in theory and concept, are a promising solution to enhance bee health, but the current market available products for beekeepers are making claims that far outreach the ability of their products [32]. However, research into proprietary blends of native bee gut bacteria shows promise and holds great potential for revitalizing honey bee health. Along with scientific research, there is a need for policies and regulations that better reflect the growing research on the potentially hazardous impacts of probiotics on honey bee health. Currently, probiotics are regulated as food, which circumvents the responsibility of companies to provide data to the FDA on the product's long-term safety for the targeted organism and whether the claims made about the product are accurate [33, 34]. This loophole in regulation has allowed companies to market useless or even harmful feed supplements as probiotics. To address this issue, the FDA's Center for Veterinary Medicine (CVM) should revise its policy on Animal Foods with Drug Claims to regulate honey bee probiotics as a nutritional ingredient (which is regulated as a drug) rather than an animal feed (which is regulated as food) [34, 35]. This policy change will save farmers and beekeepers from purchasing ineffective products and help safeguard honey bee health for future generations.

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Author contributions

M. E. D. performed molecular experiments, collected molecular data, analyzed the data, and wrote and edited the manuscript. D. G. performed molecular experiments and helped edit the manuscript. B. B. helped conceive and organize the project, established honey bee colonies, performed all fieldwork, and collected samples. K.R. helped conceive and organize the project, funded the project, analyzed data, created the figures, and wrote and edited the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Statements and Declarations

Conflict of Interest: The authors declare no conflict of interest.

Data availability statement

All raw sequencing reads are deposited in NCBI Sequence Read Bioproject under accession number PRJNA971198. All other data generated are included in the supplemental material files.

References

1. Keel CC, United States Department of Agriculture (2022) The Buzz About Pollinators. https://www.usda.gov/media/blog/2022/06/22/buzz-about-pollinators. Accessed 12 Feb 2023
2. Mueller CM, Ashley N. Mortensen, Cameron J. Jack, Jamie Ellis Identification and Treatment of European Foulbrood in Honey Bee Colonies
3. Genersch E (2010) American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. Journal of Invertebrate Pathology 103:S10–S19. https://doi.org/10.1016/j.jip.2009.06.015
4. Raymann K, Moran NA (2018) The role of the gut microbiome in health and disease of adult honey bee workers. Current Opinion in Insect Science 26:97–104. https://doi.org/10.1016/j.cois.2018.02.012
5. Bonilla-Rosso G, Engel P (2018) Functional roles and metabolic niches in the honey bee gut microbiota. Current Opinion in Microbiology 43:69–76. https://doi.org/10.1016/j.mib.2017.12.009
6. Kwong WK, Mancenido AL, Moran NA (2017) Immune system stimulation by the native gut microbiota of honey bees. Royal Society Open Science 4:170003. https://doi.org/10.1098/rsos.170003
7. Zheng H, Nishida A, Kwong WK, et al (2016) Metabolism of Toxic Sugars by Strains of the Bee Gut Symbiont Gilliamella apicola. mBio 7:. https://doi.org/10.1128/mBio.01326-16
8. Zheng H, Powell JE, Steele MI, et al (2017) Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. PNAS 114:4775–4780. https://doi.org/10.1073/pnas.1701819114
9. Kakumanu ML, Reeves AM, Anderson TD, et al (2016) Honey Bee Gut Microbiome Is Altered by In-Hive Pesticide Exposures. Front Microbiol 7:. https://doi.org/10.3389/fmicb.2016.01255
10. Motta EVS, Moran NA (2020) Impact of Glyphosate on the Honey Bee Gut Microbiota: Effects of Intensity, Duration, and Timing of Exposure. mSystems 5:. https://doi.org/10.1128/mSystems.00268-20
11. Raymann K, Shaffer Z, Moran NA (2017) Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. PLOS Biology 15:e2001861. https://doi.org/10.1371/journal.pbio.2001861
12. Duan X, Zhao B, Jin X, et al (2021) Antibiotic Treatment Decrease the Fitness of Honeybee (Apis mellifera) Larvae. Insects 12:301. https://doi.org/10.3390/insects12040301
13. Powell JE, Carver Z, Leonard SP, Moran NA (2021) Field-Realistic Tylosin Exposure Impacts Honey Bee Microbiota and Pathogen Susceptibility, Which Is Ameliorated by Native Gut Probiotics. Microbiol Spectr 9:e0010321. https://doi.org/10.1128/Spectrum.00103-21

14. Raymann K, Bobay L-M, Moran NA (2018) Antibiotics reduce genetic diversity of core species in the honeybee gut microbiome. Molecular Ecology 27:2057–2066. https://doi.org/10.1111/mec.14434
15. Deng Y, Yang S, Zhao H, et al (2022) Antibiotics-induced changes in intestinal bacteria result in the sensitivity of honey bee to virus. Environmental Pollution 314:120278. https://doi.org/10.1016/j.envpol.2022.120278
16. Dickel F, Bos NMP, Hughes H, et al (2022) The oral vaccination with Paenibacillus larvae bacterin can decrease susceptibility to American Foulbrood infection in honey bees—A safety and efficacy study. Frontiers in Veterinary Science 9:
17. Beck LC, Masi AC, Young GR, et al (2022) Strain-specific impacts of probiotics are a significant driver of gut microbiome development in very preterm infants. Nat Microbiol 7:1525–1535. https://doi.org/10.1038/s41564-022-01213-w
18. Korpela K, Salonen A, Vepsäläinen O, et al (2018) Probiotic supplementation restores normal microbiota composition and function in antibiotic-treated and in caesarean-born infants. Microbiome 6:182. https://doi.org/10.1186/s40168-018-0567-4
19. Powell E (2014) CTAB genomic DNA extraction from single bee guts preserved in alcohol
20. Magoč T, Salzberg SL (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27:2957–2963. https://doi.org/10.1093/bioinformatics/btr507
21. Bolyen E, Rideout JR, Dillon MR, et al (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37:852–857. https://doi.org/10.1038/s41587-019-0209-9
22. Xue Z, Xing'an L, Qinzhi S, et al (2019) A curated 16S rRNA reference database for the classification of honeybee and bumblebee gut microbiota. Biodiversity Science 27:557. https://doi.org/10.17520/biods.2019021
23. Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461. https://doi.org/10.1093/bioinformatics/btq461
24. R Core Team (2022) R: The R Project for Statistical Computing. In: 1.13 Citing R. https://www.r-project.org/. Accessed 12 Feb 2023
25. Bokulich NA, Kaehler BD, Rideout JR, et al (2018) Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6:90. https://doi.org/10.1186/s40168-018-0470-z
26. Motta EVS, Powell JE, Leonard SP, Moran NA (2022) Prospects for probiotics in social bees. Philosophical Transactions of the Royal Society B: Biological Sciences 377:20210156. https://doi.org/10.1098/rstb.2021.0156
27. Żebracka A, Chmielowiec-Korzeniowska A, Nowakowicz-Dębek B, et al (2022) Intestinal Microbiota of Honey Bees () Treated with Amitraz. Journal of Apicultural Science 66:199–207. https://doi.org/10.2478/jas-2022-0015
28. Borges D, Guzman-Novoa E, Goodwin PH (2021) Effects of Prebiotics and Probiotics on Honey Bees (Apis mellifera) Infected with the Microsporidian Parasite

Nosema ceranae. Microorganisms 9:481. https://doi.org/10.3390/microorganisms9030481

29. Daisley BA, Pitek AP, Chmiel JA, et al (2019) Novel probiotic approach to counter Paenibacillus larvae infection in honey bees. ISME J 1–16. https://doi.org/10.1038/s41396-019-0541-6
30. Daisley BA, Pitek AP, Chmiel JA, et al (2020) Lactobacillus spp. attenuate antibiotic-induced immune and microbiota dysregulation in honey bees. Commun Biol 3:534. https://doi.org/10.1038/s42003-020-01259-8
31. Tejerina MR, Benítez-Ahrendts MR, Audisio MC (2020) Lactobacillus salivarius A3iob Reduces the Incidence of Varroa destructor and Nosema Spp. in Commercial Apiaries Located in the Northwest of Argentina. Probiotics & Antimicro Prot 12:1360–1369. https://doi.org/10.1007/s12602-020-09638-7
32. Raymann K (2021) Honey Bee Microbiota and the Physiology of Antimicrobial Resistance. In: Honey Bee Medicine for the Veterinary Practitioner. John Wiley & Sons, Ltd, pp 125–134
33. Bodie A, Corby-Edwards A, Cornell A (2021) Regulation of Dietary Supplements: Background and Issues for Congress. Congressional Research Service
34. Damico ME (2022) Comment to the food and drug administration's center for veterinary medicine. Federation of American Scientists
35. Damico ME (2022) Regulating Probiotic Use and Improving Veterinary Care to Bolster Honeybee Health. In: Day One Project. https://www.dayoneproject.org/ideas/regulating-probiotic-use-and-improving-veterinary-care-to-bolster-honeybee-health/. Accessed 20 Apr 2022

Supplementary Material

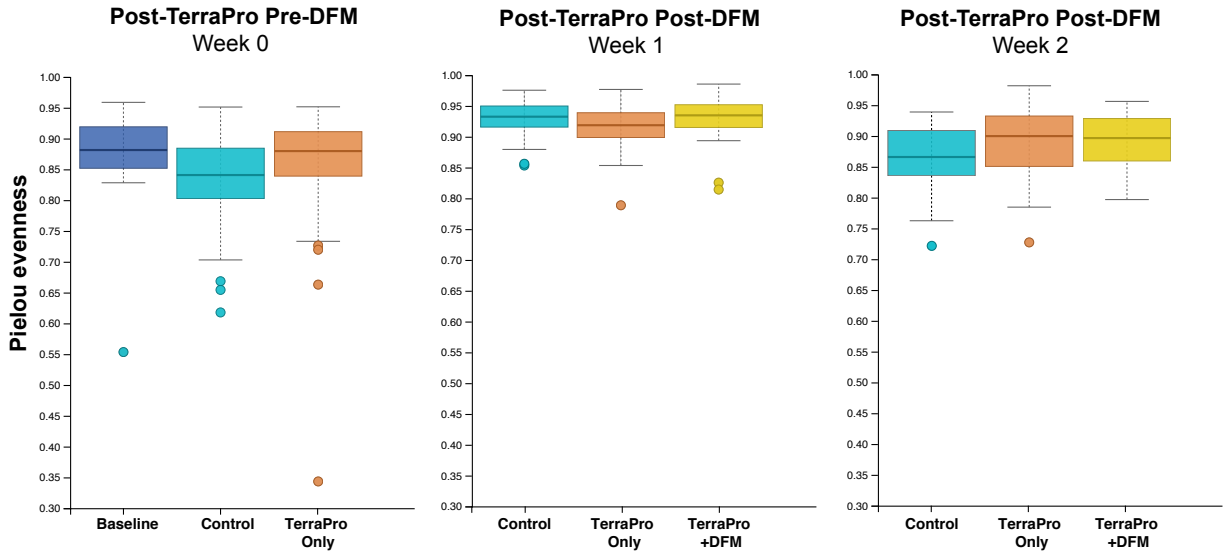

Figure S1. Gut bacterial community evenness of 1) post-TerraPro treatment pre-DFM week zero, 2) post-TerraPro treatment post-DFM week one, and 3) post-TerraPro treatment post-DFM week two based on Pielou's evenness index. Significance was determined using the Kruskall-Wallis test with Benjamini-Hochberg FDR correction. Significance (P<0.05) is represented by differing letters.

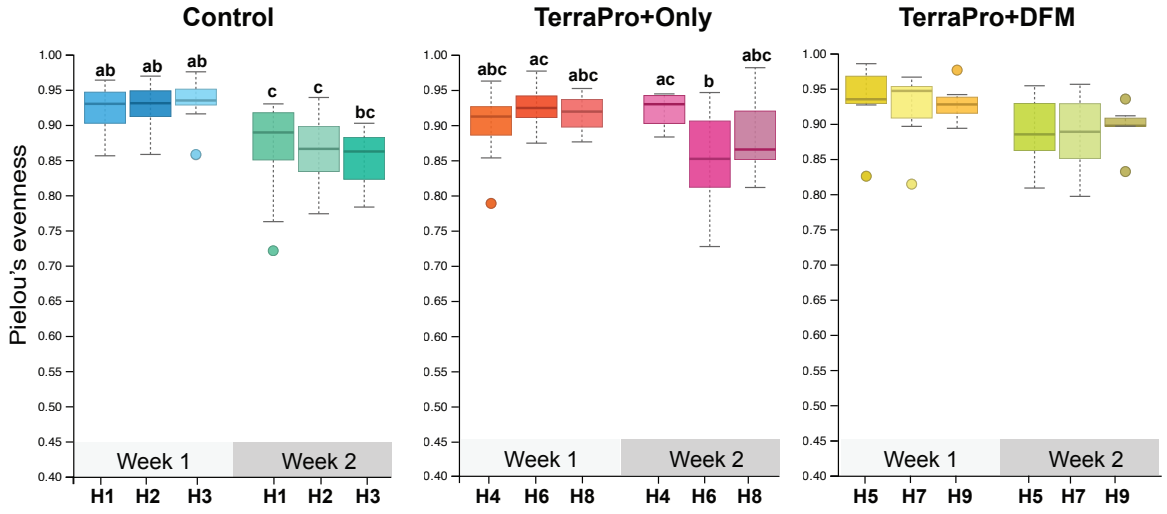

Figure S2. Gut bacterial community evenness of of 1) control, 2) TerraPro-Only, and 3) TerraPro+DFM group hives from weeks-1 and -2 based on Pielou's evenness index. Significance was determined using the Kruskall-Wallis test with Benjamini-Hochberg FDR correction. Significance (P<0.05) is represented by differing letters.

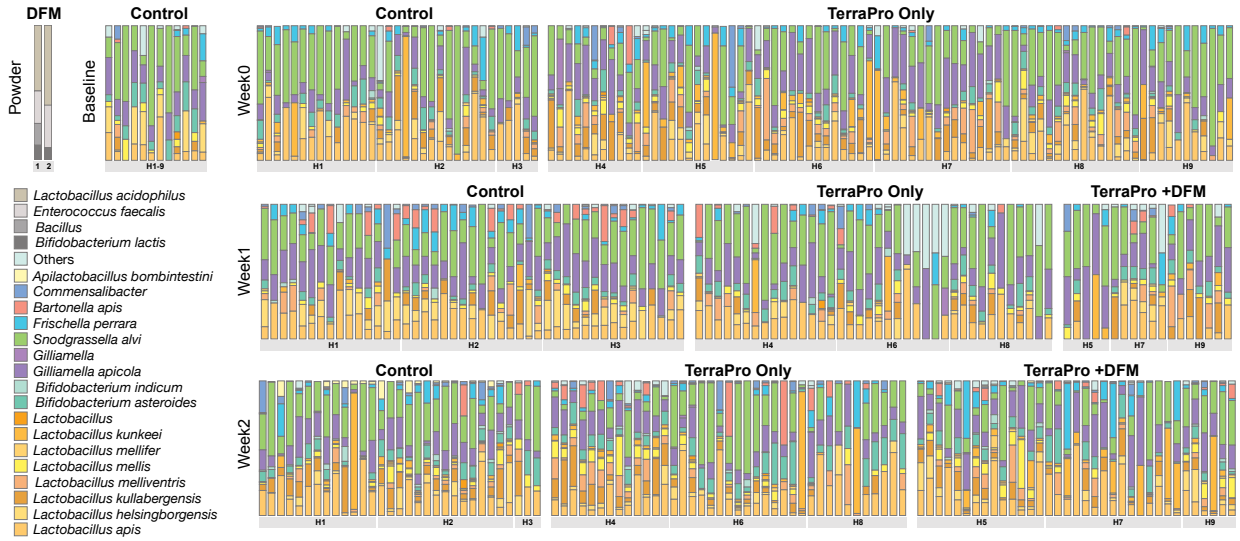

Figure S3. Bar plots representing the relative abundance of bacterial taxa found in individual bee samples from each experimental group, hive and sampling timepoint. The DFM powder (probiotic) was also sequenced as a positive control.