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Impact of Manure Storage Time and Temperature on Microbial Composition and Stable Fly (Diptera: Muscidae) Development

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Abstract

Samples are often frozen for preservation until needed for use. It has been a common practice to store fresh dairy manure in the freezer until needed for fly development studies. However, conflicting data have suggested that freezer temperature and duration of manure may impact fly development studies, and it is likely due to the change in microbial comminutes due to the freezer conditions. In this study manure storage conditions were assessed to ascertain how temperatures impact stable fly, *Stomoxys calcitrans* L., survival to pupation and determine which bacterial populations impacted fly development using massively-parallel sequencing and 16S metagenomic analysis. Stable fly survival to pupation was greater in manure that was stored warm (27˚C) or frozen (−20˚C or −80˚C) for 24 days as compared to fresh manure samples. Refrigeration (4˚C) of the manure for 24 days did not affect fly development and slightly decreased the pupal weights. Over 80 bacterial families were detected by sequencing allowing for a more thorough assessment of changes in bacterial populations. Only minor shifts were observed in bacterial family composition in the manure when refrigerated or frozen for 24 days, but significant population changes were observed when the manure was incubated for 24 days at 27˚C. Since it is the temperature and incubation time that yielded the greatest pupation rate, it is hypothesized that the manure microbial community impacts the growth and development of stable flies. This study has determined suggested freezer conditions for the best storage of manure samples to maintain bacterial diversity and retain the closest bacterial populations to freshly collected manure. Although untouched, aged (20 days) manure is best to use to assess fly development, it is not always feasible in la-

boratory experimentations. This study demonstrates the importance of preservation techniques on manure samples, which could also confer to storage of other biological specimens that contain resident microbes.

Keywords

Stomoxys calcitrans L., Manure Microbiota, 16S Metagenomics

1. Introduction

Stable flies, *Stomoxys calcitrans* L., are blood-sucking arthropods that can cause serious health issues for cattle and economic loss for cattle ranchers [1] [2]. They reduce productivity, lead to defense behaviors and bunching, and carry pathogens of cattle, which make stable flies a serious pest [2]. It is estimated that 139 kg of milk is lost per annum due to stable fly infestations resulting in an estimated economic loss of $360 million each year for United States dairy cattle farmers [2]. It was originally thought that stable flies were only a pest problem for confined cattle, but more recent evidence indicates that stable flies can also impact pastured cattle [3]. The impact on pastured cattle is attributed to the use of hay bale feeders during the winter months, and more importantly, the manure accumulation around those feeders [4]. Due to insecticide costs and concern over insecticide-resistance, adequate pasture control of pest flies has been unattainable [1], which makes their development and control an important avenue of research.

Stable flies have shown a preference for aged manure (20 days) in which to oviposit, and larvae are rarely seen in fresh samples [5] [6]. There are many physicochemical features that may change between fresh and aged manure such as moisture content, temperature, pH, and organic matter [5]. These parameter changes will have an effect on microbial composition, which has been demonstrated to impact stable fly larval development [1] [7]. Most studies examining bacterial communities in manure have relied on traditional plating methods, which can be unreliable since there are strict anaerobic bacteria, slow-growing bacteria, and bacteria that are easily out-competed by larger bacterial populations in the manure. Newer DNA sequencing technologies have enabled genomic analyses of bacterial population composition, which can include all organisms, regardless of growth parameters [8]. A study on horse manure used DNA pyrosequencing to assess changes in bacterial community composition and determined a decrease in overall species diversity with manure age and a shift from anaerobic to aerobic bacteria over time [6]. This shift in the microbial population could be contributing to the preferential environment that stable flies require in aged manure. It is, however, often challenging for researchers to store manure adequately, for the precise 18 - 20 days window for stable fly development studies. Therefore researchers have tried to utilize freezer conditions to collect large volumes and just extract samples when needed [9] [10] [11]. Here

we demonstrated why freezing manure may complicate the consensus in fly development, and how studies performed on "ambient temperature" manure in various climates (warm or cold) may have drastic differences in results.

This study utilized a comparison of freshly collected dairy cattle manure to manure aged in a variety of temperatures at 1, 7, and 24 d post-collection, to determine the optimal storage conditions for stable fly development. The abundance of eggs developing to the pupal stage was compared to the bacterial composition, both using live culture-based assays and 16S metagenomics, in order to determine factors that are important for the development of stable flies. The differences in storage conditions impact the bacteria microflora of the manure, which in turn will affect the development of stable flies. This data suggests that research on fly development and prevention needs to take storage into consideration and use ambient methods as much as possible to preserve the natural microbiome.

2. Materials and Methods

2.1. Manure Collection

Fresh bovine manure was collected from five random dairy cattle at the Southwest Regional Dairy Center in Stephenville, TX in October (average temperature 25˚C/14˚C (high/low)). The manure was placed into a 19-L plastic bucket, thoroughly mixed and then separated into seventy-five 100 gram aliquots for the fly development studies and fifteen 10 g aliquots for the bacterial survey. Fifteen of the 100 g samples and three of the 10 g samples were placed into each of the following storage methods: outdoors (27˚C) covered with fly screen to prevent oviposition but allow for air movement, refrigeration (4˚C), freezer (−20˚C), and deep freeze (−80˚C) for a time period of 1, 7, or 24 d. These storage conditions and time points were compared to manure that was processed the day of collection (fresh).

2.2. Bacterial Population Survey

On day 0, using fresh manure, or days 1, 7, and 24 d old using manure stored at 27˚C (covered), 27˚C (uncovered), 4˚C, −20˚C, or −80˚C, a 10-g aliquot was removed and 5 g were used to make five 1% solutions in sterile phosphate-buffered saline (PBS). The treatment solutions were sonicated in a bath sonicator for 5 min. and were then vortexed for 1 min. to distribute the bacterial populations evenly. Total prokaryotic cell counts were obtained by taking 100 µl of each solution and adding it to 100 µl of trypan blue and then manually counting using an inverted microscope and a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA). Two replications from each manure solution were counted by two separate individuals for confirmation of accuracy. Following the counting, three replicates of each of the five solutions were plated onto eosin-methylene blue (EMB) agar for the determination of *Escherichia coli* concentrations in the manure samples. Lastly, Deoxyribonucleic acids (DNA) were

extracted from 200 µl of the manure solution as previously described [12] for massively parallel sequencing and 16S metagenomic analysis of bacterial populations. DNA concentration was determined using a Qubit® 2.0 fluorometer with a Qubit® dsDNA HS Assay Kit according to manufacturer's instructions (Life Technologies, Carlsbad, CA). The DNA from each sample was normalized to a concentration of 5 ng/µl before subsequent analysis.

2.3. Massively-Parallel Sequencing

Bacterial 16S amplicons were generated using primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3') that amplify the V3 and V4 regions [13] [14]. PCR amplification was accomplished through denaturation at 95 degrees Celsius for 3 minutes, followed by 35 cycles of 95 degrees Celsius for 10 seconds, 55 degrees Celsius for 30 seconds, and 72 degrees Celsius for 30 seconds. Barcodes were constructed with the same PCR protocol. Paired-end sequence data were generated on an Illumina MiSeq instrument using v3 600 cycle kits (Illumina, San Diego, CA), with dual 6 basepair index sequences attached to each amplicon during indexing PCR as described in the Illumina 16S Metagenomic Sequencing Library Preparation protocol [15].

The raw sequencing reads were processed with a combination of QIIME [16], USEARCH [17] and FASTX-Toolkit [18] software packages, as well as a series of custom python scripts. Individual sequence tags were compared to the Greengenes 13.8 reference sequence database [19] using UCLUST [17] in order to pick referenced-based Operational Taxonomic Units (OTUs) at 97% similarity. The sequencing dataset was rarified to an equal sequencing depth of 860 sequences per sample by randomly subsampling sequences from each sample without replacement in order to provide even measures of bacterial alpha- and beta-diversity and to have equal sequencing depth for the production of all figures, tables, and statistical analyses.

2.4. Larvae Development

Fly eggs were obtained from the Knipling-Bushland U. S. Livestock Insect Research Lab (Kerrville, TX). Manure was removed from each storage condition, or used fresh, and 100 g of the substrate was placed into 510-g plastic cups (Hefty®, Pactiv Corporation, Lake Forest, IL) (n = 5). The manure was allowed to acclimate to room temperature and 50 stable fly eggs were added to each cup. The cups were placed into an incubator set at 27˚C, 86 % RH at a photoperiod length of 14:10 L:D and the larvae were allowed to develop for eight days until eclosion. Following this incubation period, the pupae were enumerated and weighed.

2.5. Statistical Analysis

For the 16S metagenomic analyses, unweighted UniFrac distance matrices were used in the calculation of diversity measures [20]. In order to determine if mi-

crobial community composition was significantly different between samples, PERMANOVA and bootstrapped Mann-Whitney U tests were conducted using the QIIME package [21] [22]. Differences in microbial taxa between different storage times and temperatures were determined with NCSS statistical package (NCSS, Kaysville UT). Analysis of variance (ANOVA) tests were used to determine significant differences between the treatment groups for the fly and bacterial assays.

Stable fly percentage data was normalized using arcsine transformation before analysis. Percent stable fly development to the pupal stage and pupal weights were analyzed using Statistix 10 and a Least Significant Difference test (LSD) was used to spate multiple means where appropriate. Analysis of results was considered significant at $P < 0.05$ so specific P values are not presented.

3. Results

3.1. Viable Cell Counts

To determine the impact of various storage temperatures and times on the number of viable prokaryotic cells, cells were microscopically counted using trypan blue, which does not permeate the membranes of live cells, and compared to the fresh extraction of manure on day 0. A significant decline in viable cell populations was evident following 20 days of storage using any of the storage conditions (**Table 1**). Growth at 27˚C demonstrated a significant increase in viable cell numbers over the first week, which was predominantly prokaryotic when the storage vial was left uncovered (**Table 1**), but predominantly eukaryotic (fungi) when the storage vial was sealed (data not shown). For this reason, and the noticeable impact on fly development (**Figure 4**), the covered 27˚C treatment group was omitted. Following refrigeration, the total viable prokaryotic cell counts decreased, but not significantly from control, except after 24 days of storage (**Table 1**). The freezer temperatures did not seem to impact viable cells much in the first week of storage, and did not seem to vary much from each other, although the −20˚C freezer did decline more rapidly, but they both did retain viable cells for the entire 24 day trial (**Table 1**). Due to the high prevalence of *E. coli* in manure samples, it was hypothesized that *E. coli* levels may impact the hatching of pupae and development of adult flies, thus *E. coli* viable cell

Table 1. Total Viable Prokaryotic Cell Count (log_{10} cells/ml) ± SEM. Microscopic cell counts using trypan blue stain to differentiate between live and dead cells, with only viable cells included. *Statistically significant ($P < 0.05$) from control (fresh manure).

	Day 0	Day 1	Day 7	Day 24
Fresh	8.19 (0.24)			
27˚C		9.13 (0.95)*	9.14 (0.07)*	6.99 (0.01)*
4˚C		7.41 (0.44)	7.78 (0.61)	6.69 (0.19)*
−20˚C		8.16 (0.76)	7.06 (0.26)*	6.38 (0.07)*
−80˚C		8.01 (0.50)	7.47 (0.31)	6.31 (0.02)*

counts were also measured using EMB agar. At 27˚C (uncovered) the *E. coli* levels significantly increased by day 7, and although a decline was observed by day 24, there was still a significantly higher level of viable *E. coli* cells in this treatment group as compared to control manure (Table 2). Refrigeration in manure appears to have little effect on the viability of *E. coli* as it remains unchanged throughout the entire time course (Table 2). A slight decline in *E. coli* populations was observed following deep freeze (−80˚C) and a significant decline was observed using a standard freezer (−20˚C), but neither appeared to be impacted by duration of freezing (Table 2).

3.2. Sequencing Metrics

Following quality filtering and joining of the paired-end reads, there were 538,249 16S DNA sequence tags for the 39 samples with an average length of 447 bp and an average Q score of 37.94. The lowest number of sequences per sample was 863. For all figures and statistical analyses of the sequencing data, the sequence dataset was randomly subsampled to an even depth of 863 sequences per sample.

3.3. Bacterial Richness

Shannon diversity metrics were used to analyze alpha diversity within the microbial populations comparing duration of storage (Figure 1(a)), temperature of storage (Figure 1(b)), or a combination of the parameters (Figure 1(c)). The rarefaction measure of species richness indicates that temperature (Figure 1(b)) has a greater impact than time (Figure 1(a)) on the number of species present. A comparison of the collective temperatures at each of the time points demonstrates very little change in species number indicating that the duration of time may not be a critical parameter of manure storage and prokaryotic species richness (Figure 1(a)). Temperature did however appear to impact species richness, especially at 27 degrees Celsius (Figure 1(b)). The 27 degree temperature storage decreased the species richness (Figure 1(b)) with a significant impact observed after 24 days of storage (purple line; Figure 1(c)).

3.4. Bacterial Sample Variability

Beta diversity analysis was used to compare the amount of variability within the

Table 2. *E. coli* Cell Count ($\log_{10}$ cfu/ml) ± SEM. *E. coli* counts from EMB plates. *Statistically significant ($P < 0.05$) from control (fresh manure).

	Day 0	Day 1	Day 7	Day 24
Fresh	3.29 (0.44)			
27˚C		3.65 (0.38)	5.12 (0.91)*	4.73 (0.32)*
4˚C		3.59 (0.70)	3.56 (0.17)	3.68 (0.15)
−20˚C		2.52 (0.3.6)*	2.34 (0.43)*	2.30 (0.28)*
−80˚C		2.88 (0.21)	3.03 (0.78)	2.92 (0.06)

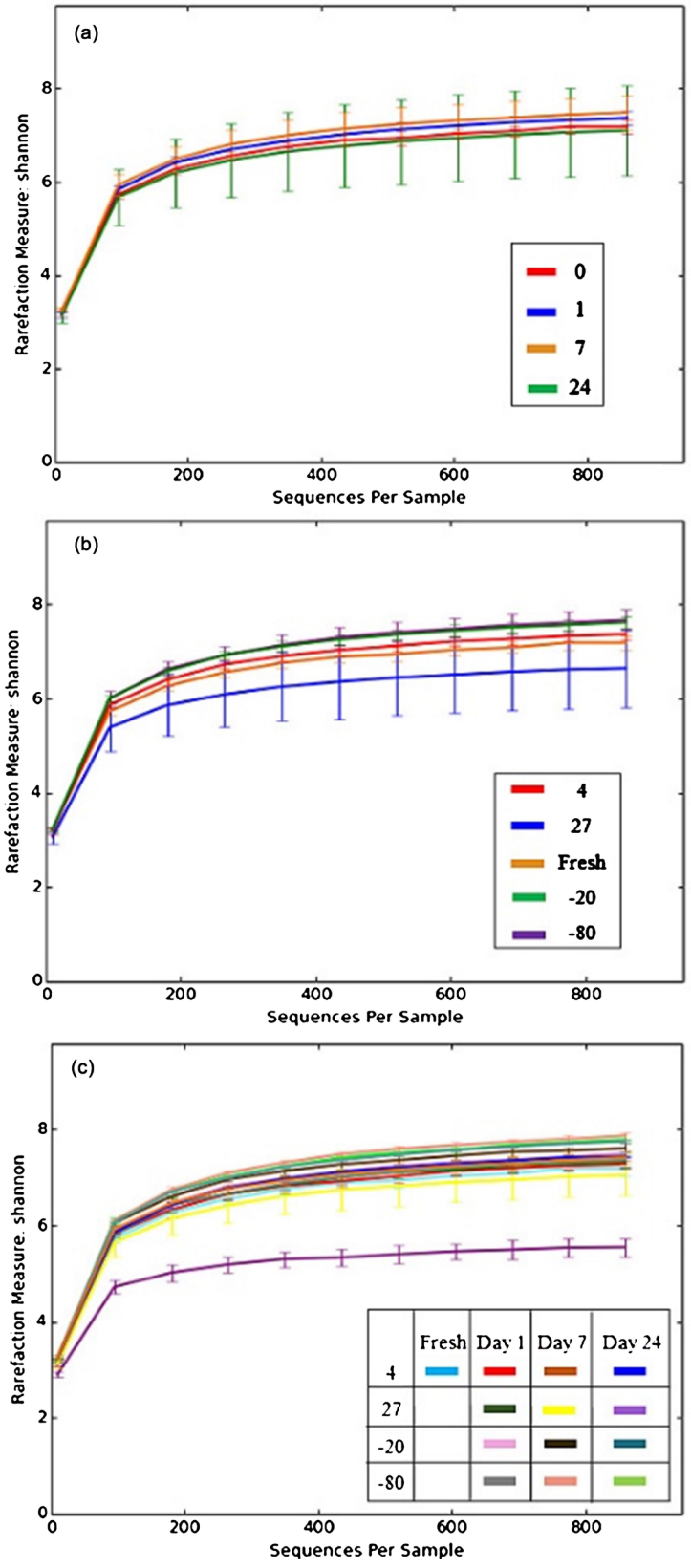

Figure 1. Alpha diversity plots obtained using Shannon diversity metrics. A comparison of duration (a), temperature (b), or combined duration and temperature parameters (c) was used to compare species richness within the samples.

sample set and to examine the similarity between treatment groups. Principle coordinate analysis plots were used to demonstrate the variation present within the dataset to confirm consistent replicates and demonstrate effects of experimental parameters. For all storage temperatures and durations analyzed, there are no observable outliers from any of the three replicates, which is indicated by the close clustering of spheres of similar colors. With only one day of incubation (Figure 2(a)), there is still close clustering of the samples from various storage temperatures to the microbial DNA amplified from the freshly-obtained manure samples (red), suggesting that this short of an incubation time may not have a dramatic effect on bacterial species diversity. However, the frozen samples (blue −20 and orange −80) are a greater distance from the 27˚C (purple) and refrigerated (green) samples, suggesting that even a short freeze may influence bacterial populations (Figure 2(a)). By day 7 of storage, most storage conditions have separated from the fresh manure samples (Figure 2(b)) indicating that the effects of temperature are starting to cause variability in the bacterial populations in the manure samples. The refrigerated samples still remain the most closely associated with the fresh manure after one week (Figure 2(b)). Following 24 days of storage, only the refrigerated samples remain in close proximity to the fresh manure (Figure 2(c)), suggesting that refrigeration of manure will not dramatically change the prokaryotic cell population. The bacterial populations in the 27˚C stored manure exhibited much more variation from the fresh manure samples following 24 days of storage, confirming the change in species richness observed in the alpha diversity plots (Figure 1(c) and Figure 2(c)). The frozen samples remained dissimilar from the fresh manure after 24 days of storage, but there appears to be little variation between freezing at −20 degrees Celsius or −80 degrees Celsius (Figure 2(c)).

3.5. Bacterial Taxa Summary

Although the primers used in this study are designed to selectively amplify bacterial 16S sequences, a small number of 16S sequence tags were generated from archaea. The archaea sequence tags were treated as contaminants for the purpose of this work, and are not discussed. Taxonomic changes of bacteria at the family level were examined between freshly obtained manure and manure stored at

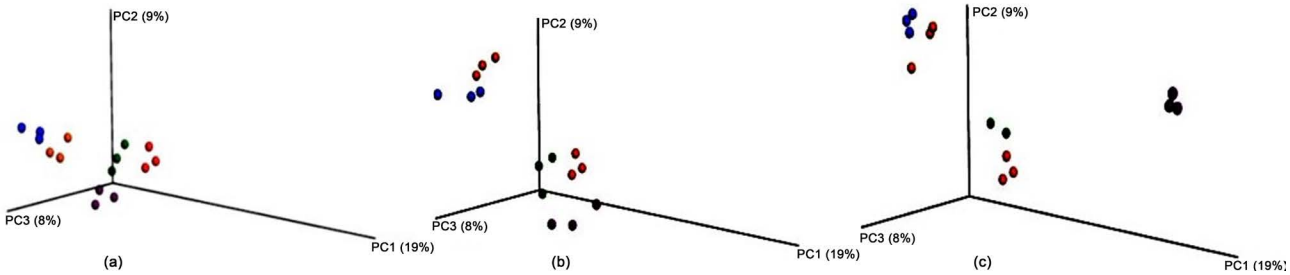

Figure 2. Unweighted UniFrac Beta Diversity Plots. Beta diversity plots comparing temperatures at 1 (a), 7 (b), and 24 (c) days of storage to freshly obtained manure (red spheres). The frozen manure is indicated by the blue (−20˚C) and orange (−80˚C) spheres. The 27˚C stored manure (27˚C) is indicated by purple spheres. The refrigerated manure (4˚C) is indicated by green spheres.

−20˚C, −80˚C, 4˚C, and 27˚C (Figure S1). The only consistent change observed following 24 days in all storage conditions was in the *Succinivibrionaceae* family (Figure 3), which declined 9.8%, 10.3%, 5%, and 8%, respectively (Figure S1). Besides the *Succinivibrionaceae* family, there were no other changes between the fresh manure and the manure stored in the refrigerator for 24 days, and the *Succinivibrionaceae* were not quite statistically significant (Figure 3). More changes in population composition of various families were observed between the frozen manure (−20˚C and −80˚C) with little difference between the two temperatures, but the only large changes were observable increases in the *Clostridiales* order, although they were not statistically significant, and decreases in the family *Bacteroidaceae*, also not statistically significant (Figure 3). Decreases were also observed in percentage of *Campylobacteraceae* and *Desulfovibrionaceae* that were minor, but statistically significant. In the *Clostridiales* order, families "other" and "undefined" doubling in percent of composition, *Clostridiaceae* increasing from 1.4% in fresh manure to 3.3% (−20˚C) or 4.0% (−80˚C), *Lachnospiraceae* increasing from 7.1% in fresh manure to 8.8% (−20˚C) or 10.6% (−80˚C), and *Ruminococcaceae* increasing from 1.7% in fresh manure to 26.1% (−20˚C) or

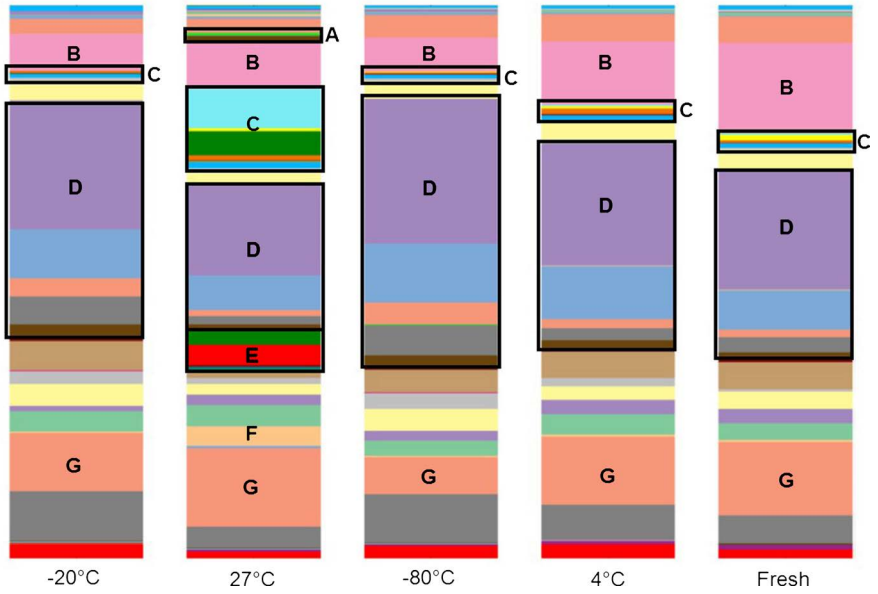

Figure 3. Taxa summary bar graphs at the level of family. The percentage of prokaryote OTU representation of each family is compared at 24 days incubation between the various storage temperatures and a fresh manure control. The pink sections in the box labeled "A" depicts changes in *Moraxellaceae* (brown) and *Xanthomonadaceae* (green). The section labeled "B" depict *Succinivibrionaceae*. The box around sections labeled "C" includes the *Campylobacterales* (light blue), *Desulfovibrionaceae* (yellow), *Comamonadaceae* (green), *Alcaligenaceae* (orange), and *Erysipelotrichaceae* (dark blue) families. The box labeled "D" includes the families in *Clostridiales* order (families *Ruminococcacae*—purple, *Lachnospiraceae* (blue), *Peptococcaceae*—peach, undefined—grey, *Christensenellaceae*—green, other—brown). The box labeled "E" includes the *Paraprevotellaceae* (tan), *Bacteroidales* p-2534-18B5 (brown), *Fibrobacteraceae* (red), and *Planococcaceae* (green) families. The designations "F" and "G" are for the *Porphyromonadaceae* and *Bacteroidaceae* families, respectively.

23.3%2 (−80˚C) (Figure 3; Figure S1). The most significant prokaryotic family shifts from the freshly obtained manure were observed in the manure stored at 27˚C for 24 days (Figure 3). The percentage of OTU sequences from the family *Paraprevotellaceae* (P = 0.055) decreased from 4.9% to 1.2% following a 24 day incubation at 27 degrees (Figure S1), and large increases were observed in the families *Bacteroidales* P-2534-18B5 (P = 0.055), *Porphyromonadaceae* (P = 0.054), *Fibrobacteraceae* (P < 0.05), *Planococcaceae* (P = 0.051), *Comamonadaceae* (P < 0.05), *Campylobacteraceae* (P = 0.050), and *Moraxellaceae* (P < 0.05) (Figure 3; Figure S1).

3.6. Fly Development

Fresh manure extracted from dairy cattle and then inoculated with stable fly eggs resulted in an average of 9.5 percent developing to the pupal stage (Figure 4(a)). The average weight from these control pupae was 0.036 g (Figure 4(b)). Manure stored at −80˚C for 24 days had an increase of about double the number pupae produced (20.1) and nearly double the average pupal size (0.068 g) (Figure 4(a) & Figure 4(b)). There was little difference in pupation rate or average pupae size between manure frozen at −20˚C (data not shown) or −80˚C, so the latter was used for all subsequent studies. When the manure was stored at 27˚C uncovered for 24 days, there was a significant increase of over 3.5-fold for the number of eggs hatched (36.7 eggs) and a 2.7-fold significant increase for pupae weight (0.098 grams) (Figure 4(a) & Figure 4(b)). Manure stored at 4˚C appeared to have no impact on the number of eggs that hatched (Figure 4(a)), but the pupae that hatched were on average smaller (Figure 4(b)). The manure that was sealed and stored at 27˚C had decreases in pupae number (Figure 4(a)) and weight (Figure 4(b)), but these manure samples had an overgrowth of fungi, which

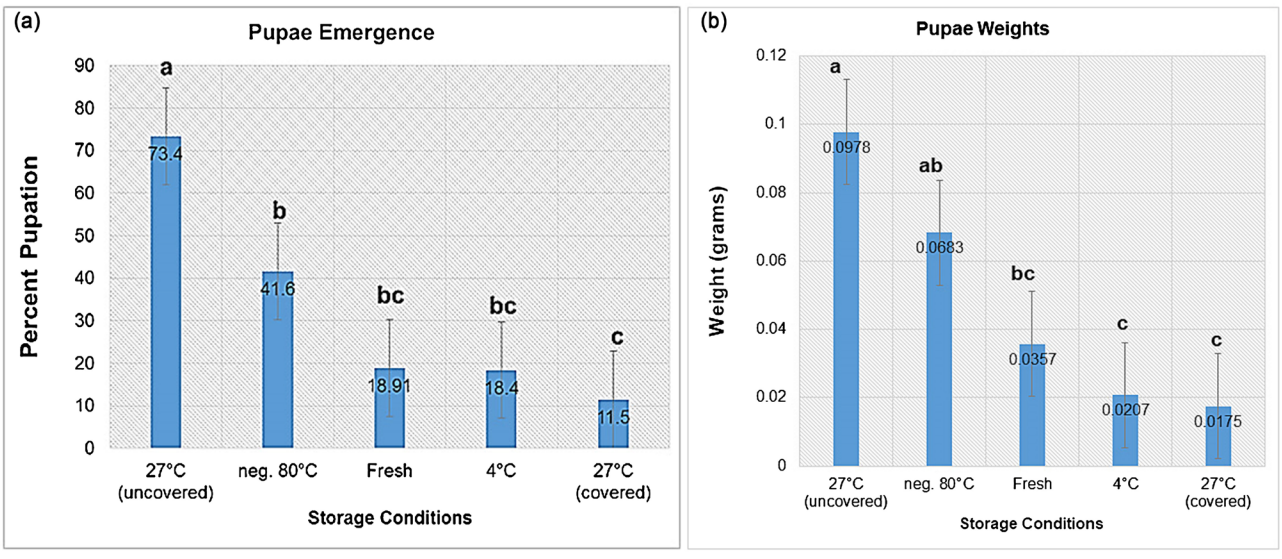

Figure 4. Pupae development. A comparison of pupae emergence from eggs (a) and the weight in grams of the pupae that emerged (b) was made following manure storage for 24 days at the various temperatures. Statistical significance (P < 0.05) is denoted by different letters.

likely impacted the development of stable flies.

4. Discussion

Due to the economic and medical importance of stable flies, much effort has been ongoing to attempt to better understand their breeding and development habits in order to reduce the fly populations around cattle and other livestock. As with many research objectives, there are many parameters that must be assessed to develop a full understanding of adequate methodology to obtain laboratory goals. The major question addressed in this research was does manure storage method and age impact bacterial growth, and in turn, stable fly larvae development? Often it is not feasible or practical to work with fresh manure samples, and thus researchers rely on storage of the manure until the protocols can proceed. Therefore we utilized common storage conditions, outdoors (27˚C), typical refrigerator setting (4˚C), a standard freezer (−20˚C), and a deep freeze (−80˚C), and compared manure samples stored in these conditions for 1 day, 1 week, and 24 days to determine how storage impacted fly larvae development and the bacterial populations in the manure.

The data from the 1 day storage was largely omitted from this manuscript, because it appeared to only be long-term storage that affected manure composition and quality. Although an overall viable cell number decrease was observed following refrigeration for 24 hours (**Table 1**), the overall percent composition of the microbes did not significantly change in this time period as compared with fresh manure (**Figure 2**). Conversely, an increase in total viable cell counts was measured following 24 hours at 27˚C (**Table 1**), but again the overall species diversity was not significantly affected. There was an increase in species diversity and richness following 7 days of incubation, but since the data trended towards what was observed following 20 days of incubation, the data presented here focused mainly on the 24 day results.

Bacterial compositions have been implicated in the ability of stable fly larvae to develop, since sterilized media does not allow for any larval development [1] [6]. However, there are still many inconsistencies as to which bacteria are most important for fly development. Previous studies have relied on assessments of which bacteria grew best from manure, which most likely leads to the inconsistencies [1] [6] [23] [24]. Ascertaining community populations using standard agar plating techniques can be nearly impossible for bacteria since the growth parameters vary dramatically between organisms. Some bacteria require oxygen, while others do not. The optimal temperature of bacteria varies among species. Some species grow quickly (24 - 48 hours), while others take weeks to grow. Bacteria can out-compete other species for nutrients on agar plates. Most importantly however, is the fact that majority of bacteria in the gastrointestinal tract just do not grow in culture [25]. A recent advance in agar based assays was able to culture 88% of the human gut microbiota, which is far greater than any previous attempt, but is still lacking what could be important species [26]. This

research began with examining *E. coli* levels since it was the largest constituent grown from the fresh manure cultures obtained, with 32% of all colonies on the agar plate representing *E. coli* (data not shown). However, further genomic analyses indicated that the prevalence of *E. coli* using sequence-based determination was less than 0.0002% (data not shown) of the bacterial population of the manure, confirming that standard plate techniques can be misleading. Metagenomic analysis provides a more thorough examination of the gut microbiota, and was used here to determine how changes in bacterial populations can impact stable fly larvae development.

The average pupation rate using fresh manure was 20%, and this rate doubled in frozen manure, and was greater than 70% with manure stored at 27˚C for 24 days (**Figure 4(a)**). Pupae size followed a similar trend with 27˚C incubation for 24 days resulting in the heaviest pupae, which were slightly heavier than from manure that was frozen for 24 days, and both of these stored samples produced heavier pupae than fresh manure (**Figure 4(b)**). Refrigeration of the manure had little effect on pupation rate compared to fresh, but did result in pupae of smaller size. After determination of how temperatures and durations impact stable fly development, the next question addressed was what changed in the 27˚C or frozen manure to allow for increased pupation rate and pupae size?

The number of viable prokaryotic cells increased during the first week of incubation at 27˚C, but the cells eventually died off during the 24 day incubation, resulting in a significant decrease in total viable cells from the fresh sample (**Table 1**). The number of viable cells declined over time in both freezer temperatures resulting in a significant decrease in viable prokaryotic cells following 24 days of storage similar to the amount observed following 24 days of incubation at 27˚C (**Table 1**). If this cell death trend is a necessary factor in stable fly development, it may indicate the need for secondary metabolites produced and released during the stationary and death phases of bacterial growth as opposed to the bacteria themselves. However, a similar trend of cell death was also observed with manure refrigerated for 24 days (**Table 1**), which resulted in no increase in pupation rate, and a decrease in average pupae weight, suggesting that perhaps specific species of bacteria are indeed needed for stable fly growth. *Escherichia coli* was selected for further study since *E. coli* growth far outnumbered other bacterial species when fresh manure was plated on Mannitol Salt Agar and Eosin Methylene Blue agar and incubated overnight at 37˚C, resulting in 32% of all observed colonies on the two plates (data not shown). The *E. coli* populations increased over the first week of incubation at 27˚C, but declined a little after 24 days (**Table 2**). However, even after the slight decline at the 24 day time point, the *E. coli* levels were still significantly higher than fresh manure *E. coli* levels, suggesting that this organism may be contributing to the increase in pupae observed after 24 days of incubation at 27˚C. After determination of the *E. coli* levels following 24 days of refrigeration or freezing, this hypothesis was refuted since refrigerated manure has greater levels of *E. coli* than fresh manure, but re-

sulted in comparable fly pupation, and frozen manure had significantly less *E. coli*, but had greater pupation than fresh manure (**Table 2**; **Figure 4**).

Previous studies have attempted to elucidate bacteria that are important for stable fly development, but they too relied on culture methods, which can be misleading [1] [6]. Both of these previous studies cultured bacteria from manure in aerobic conditions, but analysis of the bacterial phyla in ruminant animals indicates that a majority of the fecal microbiome consists of species from the phyla *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, all three of which contain mostly anaerobic species [27]. All families that had significant changes resulting from the various storage conditions tested here belonged to one of these three phyla, indicating that aerobic plating methods may not have allowed for easy depiction of the importance of these bacterial families to stable fly development. It is hypothesized that changes in population diversity among the phyla *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* had the greatest contribution to increased stable fly pupation.

An approximate doubling of average eggs hatched and pupae size was observed when the manure was stored for 24 days at −80˚C compared to fresh manure, although due to sample variation it was not a significant increase (**Figure 4**). The frozen samples (−20˚C or −80˚C) had an increase in prokaryotic species richness (**Figure 2(b)**) and species variation (**Figure 2(c)**) over the fresh manure control (**Figure 1(b)**), suggesting a good preservation of the microbial population. The manure stored at 27˚C for 24 days allowed for a nearly 4-fold increase in hatch number and 2.7-fold increase in pupae size, both of which were statistically significant, over fresh manure pupation rates and growth (**Figure 4**). However, this manure actually demonstrated a dramatic decrease in bacterial species diversity at 24 days with even a slight, but noticeable decline at 7 days incubation (**Figure 1(c)**). The beta diversity analysis also indicated an increase in species variation, suggesting that the incubation at this temperature promoted the growth of certain bacterial species, while inhibiting the growth (or killing off) others (**Figure 2** and **Figure 3**). All observed changes in bacterial families originated in the phyla *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, but no discernable trend can be established to suggest that one of these phyla contributed greatly to the abundance of stable flies.

For the manure incubated at −20˚C or −80˚C, the only increase was observed in families of the *Clostridiales* order; *Firmicutes* phylum (families *Clostridiaceae*, *Lachnospiraceae*, and *Ruminococcaceae*) (**Figure 3**; **Figure S1**). Species of the *Clostridiales* order are anaerobic bacteria found in the gastrointestinal tracts of most mammals that form endospores, which are dormant, protective structures of bacteria, and this may have contributed to their survivability in the freezer temperatures. *Clostridiaceae* are rapidly able to assimilate glucose to glycogen for energy, or dissimilate it into many metabolites (acetate, butyrate, lactate, ethanol, dihydrogen, and carbon dioxide) [28]. *Ruminococcaceae* ferment cellulose and produce ethanol as a waste product [29]. Stable flies have been shown to

be attracted to a combination of acetic acid and ethanol, thus this glucose dissimilation by *Clostridiaceae* may be producing a chemoattractant for the flies [30]. *Lachnospiraceae* have been implicated in the ability to stimulate host epithelial cell growth due to the production of butyric acid [31].

The manure stored at 27˚C for 24 days had many observable changes in bacterial families, all from the phyla *Bacteroidetes*, *Firmicutes*, and *Proteobacteria.* No information was found regarding the unclassified *Bacteroidetes* family *Bacteroidales* p2534-18B5, but the other family with increases in species composition, *Porphyromonadaceae* and a closely-related family, *Fibrobacteraceae*, that used to be linked to the phylum, are both beneficial bacteria that aid in digestion of complex carbohydrates [32] [33]. *Porphyromonadaceae* digest fiber and other carbohydrates into short-chain fatty acids, such as butyrate, which is more digestible and has been shown to aid in healthy digestion and colon health [33]. *Fibrobacteraceae* are highly fibrolytic and cellulytic bacteria that aid in digestion and produce storage carbohydrates glycogen and cellodextrins [32]. Although the *Firmicutes* displayed large variation in manure stored at −20˚C and −80˚C, when stored at 27˚C only one family had any change, which were the *Planococcaceae* which have been found in the rumen by other analyses [34], but whose function in digestion remains unknown. The remaining family diversity changes were observed in the phylum *Proteobacteria*, the largest and most diverse phylum of the bacteria. Significant increases were observed in the families *Comamonadaceae*, denitrifying bacteria, the *Campylobacteraceae*, chemoorganotrophs that reduce nitrate, and *Moraxellaceae*, which have been implicated in infectious bovine keratoconjunctivitis (IBK), but have not been reported in the rumen or fecal material [35] [36] [37].

Although a specific pattern was not formed to identify a particular family or species of bacteria implicated in the growth and development of stable flies, this study was able to assess the impact of fastidious, anaerobic bacterial families that are likely impacting stable fly pupation. Previous studies have relied on laboratory growth of bacteria in aerobic conditions, which limits some of the most prevalent species of gut microorganisms from assessment. Increases in bacteria that aid ruminants in digestion of complex carbohydrates resulting in the production of fermentation products of ethanol or butyrate (or butyric acid) or storage carbohydrates such as glycogen and cellodextrins, may be providing a food source for the flies. Alternatively, a need for a more bioavailable form of nitrogen might be a requirement for stable fly pupation, and the *Proteobacteria* may be providing a nitrogen source through their nitrogen catabolism pathways. Growth at 27˚C, more accurately mimics a likely scenario of what is occurring in the cattle fields, thereby further studies in bacterial families observed to increase here may elucidate the bacterial composition, and catabolism products, required for stable fly growth and development. Regardless, it is imperative that researchers in the fields of manure microbiome research and fly development take into account the temperature and duration of storage of the manure and know that

these parameters could drastically impact the results obtained. This knowledge is not only important for manure studies, but for any culture preservation that involves preserving a microbiome.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplement

Taxon	-20	27	-80	4	Fresh
None;Other;Other;Other;Other	2.5%	1.1%	2.3%	2.6%	1.6%
k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae	0.1%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;Other;Other;Other;Other	0.2%	0.4%	0.2%	0.5%	0.8%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae	0.1%	0.1%	0.1%	0.0%	0.0%
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae	0.1%	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;Other	0.1%	0.1%	0.1%	0.1%	0.3%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__	9.0%	3.8%	8.8%	6.4%	5.0%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__BS11	0.1%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae	10.5%	14.2%	6.7%	12.3%	13.2%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Marinilabiaceae	0.0%	0.4%	0.0%	0.0%	0.0%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae	0.3%	3.6%	0.3%	0.4%	0.5%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	3.7%	3.8%	2.6%	3.7%	2.9%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__RF16	0.9%	2.0%	1.9%	2.6%	2.6%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae	4.0%	1.8%	4.0%	2.5%	3.1%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7	2.4%	1.1%	2.8%	1.4%	0.5%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Barnesiellaceae]	0.1%	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae]	0.0%	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae]	5.4%	1.2%	4.1%	4.8%	4.9%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__p-2534-18B5	0.0%	0.3%	0.0%	0.0%	0.0%
k__Bacteria;p__Chlorobi;c__OPB56;o__;f__	0.0%	0.5%	0.0%	0.0%	0.0%
k__Bacteria;p__Cyanobacteria;c__4C0d-2;o__YS2;f__	0.4%	0.3%	0.2%	0.3%	0.5%
k__Bacteria;p__Fibrobacteres;c__Fibrobacteria;o__Fibrobacterales;f__Fibrobacteraceae	0.0%	3.6%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae	0.0%	0.0%	0.0%	0.1%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae	0.0%	2.6%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae	0.0%	0.0%	0.0%	0.0%	0.1%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other	2.4%	1.1%	2.3%	1.6%	1.2%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__	5.1%	1.5%	5.5%	2.2%	2.7%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae	3.3%	1.1%	4.0%	1.6%	1.4%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	8.8%	6.2%	10.6%	9.6%	7.1%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae	0.0%	0.0%	0.1%	0.1%	0.1%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	23.3%	16.2%	26.1%	22.0%	21.7%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	3.5%	2.5%	3.2%	3.7%	3.7%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae]	0.5%	0.6%	0.6%	0.6%	0.3%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae]	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	0.7%	1.1%	0.7%	0.9%	0.7%
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];o__Victivallales;f__Victivallaceae	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__RF32;f__	0.1%	0.0%	0.1%	0.1%	0.1%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__	0.1%	0.1%	0.0%	0.1%	0.0%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae	0.3%	0.9%	0.2%	1.1%	0.5%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae	0.0%	4.3%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Rhodocyclales;f__Rhodocyclaceae	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae	0.0%	0.4%	0.1%	0.4%	0.9%
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae	0.0%	7.2%	0.0%	0.2%	0.3%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Succinivibrionaceae	6.6%	8.4%	6.1%	11.4%	16.4%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae	0.1%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae	0.0%	1.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae	0.0%	0.4%	0.0%	0.0%	0.0%
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae	2.7%	2.6%	4.0%	5.0%	4.8%
k__Bacteria;p__TM7;c__TM7-3;o__CW040;f__F16	0.6%	0.3%	0.5%	0.2%	0.0%
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Acholeplasmatales;f__Acholeplasmataceae	0.0%	0.5%	0.0%	0.0%	0.0%
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;f__Anaeroplasmataceae	0.2%	0.4%	0.1%	0.3%	0.5%
k__Bacteria;p__Tenericutes;c__Mollicutes;o__RF39;f__	0.6%	0.1%	0.6%	0.2%	0.3%
k__Bacteria;p__Tenericutes;c__RF3;o__ML615J-28;f__	0.0%	0.1%	0.0%	0.1%	0.2%
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__[Cerasicoccales];f__[Cerasicoccaceae]	0.0%	0.0%	0.0%	0.0%	0.1%
k__Bacteria;p__Verrucomicrobia;c__Verruco-5;o__WCHB1-41;f__RFP12	0.0%	0.2%	0.0%	0.0%	0.0%
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae	1.0%	0.6%	0.5%	0.6%	0.7%

Figure S1. Percentage of prokaryotic taxa changes at the family level. Prokaryotic families detected in the manure were assessed for their percent composition of the whole population (detectable range ≥ 0.1%). The percentages were compared to fresh manure and increases were denoted in green, decreases in red, and no change in yellow.